The Aromatic Scents of Four Plants in Learning and Memory of *Drosophila melanogaster*

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ABSTRACT

Introduction. Folkloric claims have surrounded essential oils, including their enhancement of learning and memory through inhalational exposure. Few studies in humans have shown a benefit in cognition, albeit incremental. However, this benefit may not be entirely attributable to the essential oil aroma but may be confounded by psychological associations. We investigated rosemary, peppermint, lemon, and coffee aromas in a learning and memory model of *Drosophila melanogaster* to eliminate this confounder.

Methods. We screened for concentrations of the four treatments that are non-stimulatory for altered locomotory behavior in the flies. At these concentrations, we determined if they were chemoneutral (i.e., neither chemoattractant nor chemorepellent) to the flies. Learning and memory of the flies exposed to these aromas were determined using an Aversive Phototaxis Suppression (APS) assay.

Results. The aromas of rosemary, peppermint, and lemon that did not elicit altered mobility in the flies were from dilute essential oil solutions that ranged from 0.2 to 0.5% v/v; whereas for the aroma in coffee, it was at a higher concentration of 7.5% m/v. At these concentrations, the aromas used were found to be chemoneutral towards the flies. We observed no improvement in both learning and memory in the four aromas tested. While a significant reduction ($p < 0.05$) in learning was observed when flies were treated with the aromas of rosemary, peppermint, and coffee, a significant reduction ($p < 0.05$) in memory was only observed in the peppermint aroma treatment.

Conclusion. This study demonstrated that in the absence of psychological association, the four aromas do not enhance learning and memory.

Keywords: Drosophila melanogaster, learning, memory, APS assay, Rosmarinus officinalis, Mentha x piperita, Citrus limon, Coffea robusta, Coffea arabica

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INTRODUCTION

In the contemporary era, numerous folkloric claims have surrounded plant essential oils, which led to their widespread use in aromatherapy.[1] One of these claims studied in previous literature have shown that learning and memory in humans can be enhanced by exposure to scents of plant essential oils.[1,2] Four of these plant essential oils associated with learning and memory are rosemary, peppermint, lemon, and coffee.

The evergreen shrub rosemary (*Rosmarinus officinalis*) has been previously tested for its effect on cognitive function. Exposure to this aroma during the performance of cognitive tasks was correlated with better speed and accuracy measures.[3] Another study has shown it to improve overall memory, albeit with an impairment in speed of memory recall.[4] Peppermint (*Mentha x piperita*), an herbaceous rhizomatous plant, is a hybrid mint between spearmint and watermint. A previous

study has shown that its aroma can improve the accuracy of memory, not at the cost of speed of recall.[5] However, this is contradictory to another study's result which had shown an enhancement in attention only and not in memory.[6] Lemon essential oil is derived from the lemon plant (*Citrus limon*), a plant commonly used in culinary arts. A study looking upon chronic exposure of the lemon aroma attributes an improvement in learning of rats due to improved attention.[7] Rosemary, peppermint, and lemon essential oils are rich in terpenes and terpenoids and are hypothesized to be the main bioactive components of their respective aromas.[8] Coffee (*Coffea robusta/Coffea arabica*) has known wake-promoting effects due to its caffeine content. The composition of the aroma of coffee is highly-variable and dependent on plant origin and processing.[9] It is mainly composed of furans, pyrans, pyrazines, and pyrroles among others.[9] Habitual consumption has been epidemiologically linked to decrease the risk of neurodegenerative disease.[10] Although not conventionally used in aromatherapy, coffee volatiles were found to exert anxiolytic activity in mice.[11] A study in humans had found coffee aroma to enhance working memory and stimulate alertness although without modulation of autonomic stress responses.[12]

Most of the aforementioned studies on the four plant aromas posit that the volatile compounds of these plant essential oils exert direct pharmacological effects on cognition via the olfactory or respiratory system and then the nervous system. However, these studies have been done mostly on humans that are capable of higher order cognitive function which enables them to have psychological associations to these scents. The scents may thus trigger preformed emotional experiences or changes in mood or mental state, that in turn, influence performance on cognitive tasks.[1,13] These intermediary psychological mechanisms may be partially responsible for the beneficial effects observed in these previous studies. It is unclear if aromatic scents can enhance learning and memory without intermediary psychological mechanisms. To be able to attribute direct effects of aromatic scents alone on learning and memory, it is necessary to uncouple the psychological associations that are linked to the scents. One way to address this issue is by utilizing scent-naïve animal models in learning and memory assays.

The fruit fly, *Drosophila melanogaster*, is a standard model system for evaluating learning and memory using the Aversive Phototaxis Suppression (APS) assay, as described by Ali et al.[14,15] In contrast to higher-order organisms wherein mood, perception, and mental state may mediate effects on cognitive performance, the fly mainly relies on instinct. Nonetheless, Zhuravlev et al. showed the presence of short and long-term memory, associative and non-associative learning, evolutionarily-conserved genes in learning and memory, and centers for sensation, association, and motor functions that provide validation for the use of *D. melanogaster* in cognitive research.[16] In addition, the olfactory system of the fly has been well characterized in existing literature. As reviewed by Martin et al., the fruit fly's olfactory system is inherently similar to human; odorant molecules bind to receptors of bipolar olfactory receptor neurons, which send out axons to make connections with the second-order neurons of the antennal lobe of insects (a homolog of the olfactory bulb).[17]

In this study, we utilize the fruit fly, a model organism for cognitive research with a comparable olfactory system, to test the direct pharmacological effects of four plant aromas on learning and memory.

MATERIALS AND METHODS

Sample preparation and dilution

Organic essential oils (Quality Assurance International certified) of rosemary, peppermint, and lemon were obtained (Aura Cacia, Frontier Co-op, Norway). These were added to 60% DMSO (vehicle) to make aqueous solutions of diluted essential oils. In higher concentrations, flies display hyperactive behavior, an indicator of stress, which may interfere with learning and memory. To determine the concentrations of essential oils to be used for the assays, flies were exposed to lowering concentrations of essential oil solutions (Table 1) and were observed for altered locomotory behavior. Flies either demonstrated hyperactivity or normal mobility. Pre-test has shown that distilled water alone and vehicle alone do not elicit hyperactivity in *D. melanogaster*. The highest concentration of each essential oil solution that did not alter the normal locomotory behavior of *D. melanogaster* (Figure 1A) was set to be the "HI" concentration for further experimentation. The "LO" concentration was set to be 50% of the HI concentration. For each set-up, a total of 15 flies were used.

Compounds that are chemoattractant or chemorepellent are unsuitable for testing in an APS assay as they would influence the phototaxis of the fly. The high concentration of each aroma was tested in a set-up as shown in Figure 1B. If the substance was a chemoattractant, the flies would cluster near the cotton bud tip with the treatment; if it was a chemorepellent, the flies would move and distribute themselves away from the cotton bud tip. For each set-up, a total of 15 flies were used.

Since coffee essential oil was unavailable, we prepared a comparably similar solution for coffee. In brief, 1.5 g of medium-roasted ground coffee beans of *Coffea robusta* (Benguet variety) was immersed in 20 mL of 60% DMSO, vortexed for 2 minutes and warmed for 15 minutes at 70°C in a water bath. The mixture was left standing overnight in a screw-cap container. The next day, the mixture was vortexed for 2 minutes and warmed for 15 minutes at 70°C in a water bath. The mixture was centrifuged for 5 minutes at 10000 RPM and the supernatant was collected. Finally, the supernatant containing the aroma was tested for its effects on locomotory behavior in the flies, as previously described (Figures 1A and 1B).
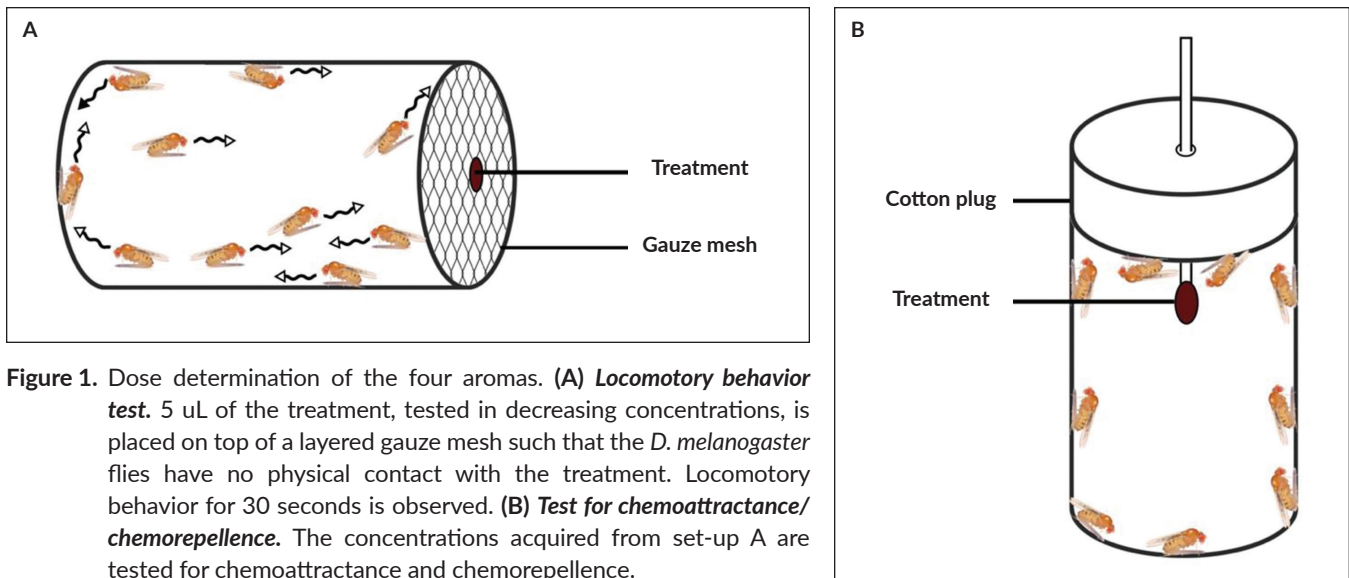
Figure 1. Dose determination of the four aromas. **(A)** *Locomotory behavior test.* 5 uL of the treatment, tested in decreasing concentrations, is placed on top of a layered gauze mesh such that the *D. melanogaster* flies have no physical contact with the treatment. Locomotory behavior for 30 seconds is observed. **(B)** *Test for chemoattractance/chemorepellence.* The concentrations acquired from set-up A are tested for chemoattractance and chemorepellence.

Drosophila melanogaster cultures

Wild type *D. melanogaster* (Oregon-R strain) were grown in fly culture bottles. A study by Tantengco et al. found that wild type female flies were more amenable to improvement in both learning and memory than male flies.[18] Hence, 2- to 3-day old female *D. melanogaster*, naïve to the four aromas in this study, were used for the purposes of this experiment.

Aversive Phototaxis Suppression (APS) Assay

Female flies, aged 2-3 days old, were collected with the aid of a stereoscope and a flypad that slowly releases carbon dioxide gas as an anesthetic. Flies were individually placed in plastic tubes and kept in the dark. The flies were given an hour to recover from the anesthesia.

The young female flies collected were screened individually for phototactic behavior before conducting the APS assay. Using a T-maze, each fly was placed in the dark chamber while the opposite chamber was lighted. If the fly moved towards the lighted chamber within one minute for two trials, this fly was accepted for the learning and memory assay (Figure 2A); if not, it was excluded from the experiment.

With the T-maze gate closed, the phototactic *D. melanogaster* is then transferred into a new plastic tube containing the aroma to be tested. Prior to adding the fly in the tube, 5 uL of the diluted essential oil was transferred on one side of a cotton ball using a pipette and inserted in the tube until it reached the base, with the wet side facing the pointed closed end (Figure 2B, left side). The tube was screwed onto the T-maze. A quinine-soaked filter paper, serving as an aversive tactile stimulus for the fruit fly, was placed in the middle of the other chamber (Figure 2B, right side).

The learning phase of the assay begins as the gate is opened and the light is shone at the end of the chamber with quinine. This should attract the fruit fly towards the light stimulus but its tarsal gustatory receptors make contact with the aversive stimulus on its way to the end of the lighted chamber. The fly is given one minute to cross the open gate. Afterwards, the fly is tapped back towards the dark chamber and allowed to rest for one minute before repeating. This was done on the same fly for 14 more repetitions. The pass rate was computed for each fly tested. Afterwards, the fly is put in a fly vial with media and food source. A range of 10 to 13 flies were used for each treatment tested in the APS assay.

Six hours after, the tested *D. melanogaster* were subjected to the memory phase of the assay. The process is essentially the same as in the learning phase; shining light on the chamber with quinine and counting the pass rate for 15 repetitions. The test stimulus was still presented in the dark chamber.

Pass Rate Computation and Statistical Analyses

As described by Ali et al., if a *D. melanogaster* stays in the dark chamber throughout the duration of one minute, it is recorded as a "pass" (Figure 2B).[14] If it passes through the open gate and travels towards the lighted chamber within the test duration, it is recorded as a "fail" (Figure 2B).[14]

For the learning phase, the number of "pass" instances over 15 (the total number of repetitions for each fly) is the learning pass rate for each fly, expressed in percentage. Computing for the mean of all of the fly learning pass rates in a treatment, a **l**earning **a**verage **p**ass **r**ate (LAPR) was produced. This was compared to the LAPR of the 60% DMSO (negative control) treatment. Another pass rate was computed for the memory phase of each fly. The mean was also computed, in order to get a **m**emory **a**verage **p**ass **r**ate (MAPR). This was compared to the MAPR of the 60% DMSO treatment. Statistical analyses on the average pass rates were done via Kruskal-Wallis analysis with Dunn's post-hoc test.
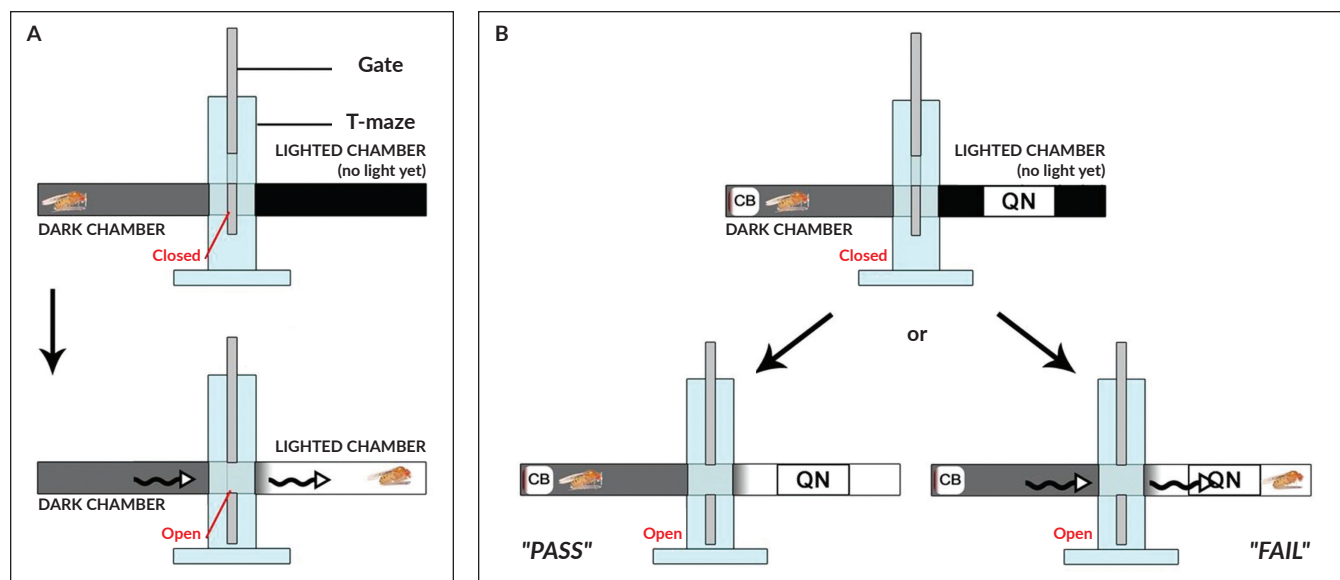
Figure 2. The APS assay **(A)** *Test for phototaxis.* After acclimatization to the dark, the *D. melanogaster flies* are screened for photo-tactic behavior. To qualify as phototactic, the fly must have gone to the lighted chamber within one minute in at least 2 out of the 3 times it was tested. **(B)** *Learning assay.* The T-maze apparatus starts with the gate in the closed position. Dropping the gate to the open position exposes the fly to the light source at the opposite end of the chamber where a filter paper with quinine, serving as aversive tactile stimulus, is situated. The fly is given one minute to decide whether to remain in the dark chamber or cross the gate towards the light source. Should it cross, it is recorded as a fail; should it stay in the dark chamber, it is recorded as a pass. This is done for 15 repetitions in the learning phase and another 15 repetitions in the memory phase, 6 hours after the former. The pass rate of a fly is the number of passes over the number of repetitions, in percentage.

Abbreviations: CB, cotton ball; QN, quinine.

RESULTS AND DISCUSSION

High and low concentrations of the four aromas were chemoneutral to *D. melanogaster*

As seen in Table 1, flies were exposed to the three essential oils (rosemary, peppermint, and lemon) in different concentrations. Mobility of the treatment flies exposed to the essential oils was compared to the control flies (without exposure). The highest concentration used for rosemary, peppermint, and lemon aroma exposure was set at 1%. For flies exposed to the coffee aroma, 7.5% (g/mL) coffee infusion in 60% DMSO was shown to display locomotory behavior comparable to control. The highest concentration of each aroma, which conferred no observable change in fly mobility (in comparison to the control), was used as the "HI" concentration of that specific aroma (Figure 1A); half of this concentration was regarded as the "LO" concentration.

Both the HI and the LO concentrations of each aroma were tested for their chemoattractant or chemorepellent properties on the flies (Figure 1B), as these may influence the outcome of the APS assay. The flies did not cluster around the source of the scent, neither did they distance themselves away from the source of the scent. The flies were evenly distributed inside the fly vial. This suggests that the aromas, at the HI and LO concentrations, are chemoneutral to the flies.

The four aromas did not improve learning in *D. melanogaster*

Each fly went through 15 trials in the gated T-maze during the learning phase of the APS assay. The test aroma

Table 1. Concentrations of Aromas Tested and Corresponding Locomotion of *D. melanogaster*

Treatment	Concentration (% v/v)	*D. melanogaster* locomotion
Rosemary	1	Hyperactivity
	0.5	Hyperactivity
	0.25	No hyperactivity
Peppermint	1	Hyperactivity
	0.5	Hyperactivity
	0.25	Hyperactivity
	0.20	No hyperactivity
Lemon	1	Hyperactivity
	0.5	No hyperactivity
Coffee	7.5*	No hyperactivity
60% DMSO (Vehicle)	—	No hyperactivity

*in g/mL

was allowed to diffuse through the dark chamber. Afterwards, each fly's learning pass rate was computed. The pass rates of all the flies in a treatment were averaged, in order to yield an LAPR (Figure 3). The fly was then put back into its corresponding media container for memory testing six hours later. The vehicle (60% DMSO) was prepared by diluting in distilled water; when tested, pass rates of the vehicle and pure distilled water were comparable (LAPR of distilled water = 56.7%; LAPR of vehicle = 60%; p>0.05).

A study by Rasoolijazi et al. has been done on orally-administered rosemary extract and its potential bioactivity on middle-aged rats; their study concluded no improvement in learning parameters, as tested in a Morris water maze test.[19] Despite the difference in mode of administration, our findings are similar with Rasoolijazi's results. Our results show no beneficial effects on the LAPR of D. melanogaster. In fact, a significant decrease in the LAPR of the high concentration of rosemary (LAPR = 29.1%) has been observed in comparison to the LAPR of the vehicle. It is possible that the high concentration of the rosemary aroma

may disrupt the learning of the flies. A similar effect was also seen in another study wherein the aroma of ylang-ylang impaired processing speed as well as decreased the alertness of human subjects which resulted to lower scores in tests of cognition.[5] However, this effect was not seen in the lower concentration of rosemary aroma as the volatile compounds present may not be in a sufficient concentration to exert the disruptive effect in learning. A similar effect of cognitive impairment, only in high doses, was seen in a trial on elderly individuals consuming rosemary extract.[20]

There is no statistically significant difference between the LAPR of the high concentration of peppermint (LAPR = 47.7%) in comparison to the vehicle. However, an exposure to a low concentration of peppermint aroma significantly decreased (p<0.05) the LAPR of D. melanogaster (LAPR = 31.7%). Since the aroma is composed of multiple volatile compounds, a possible scenario that may explain this learning inhibition only seen in the low concentration is when another component may predominate and cancel out the inhibitory effect at higher concentrations of the peppermint
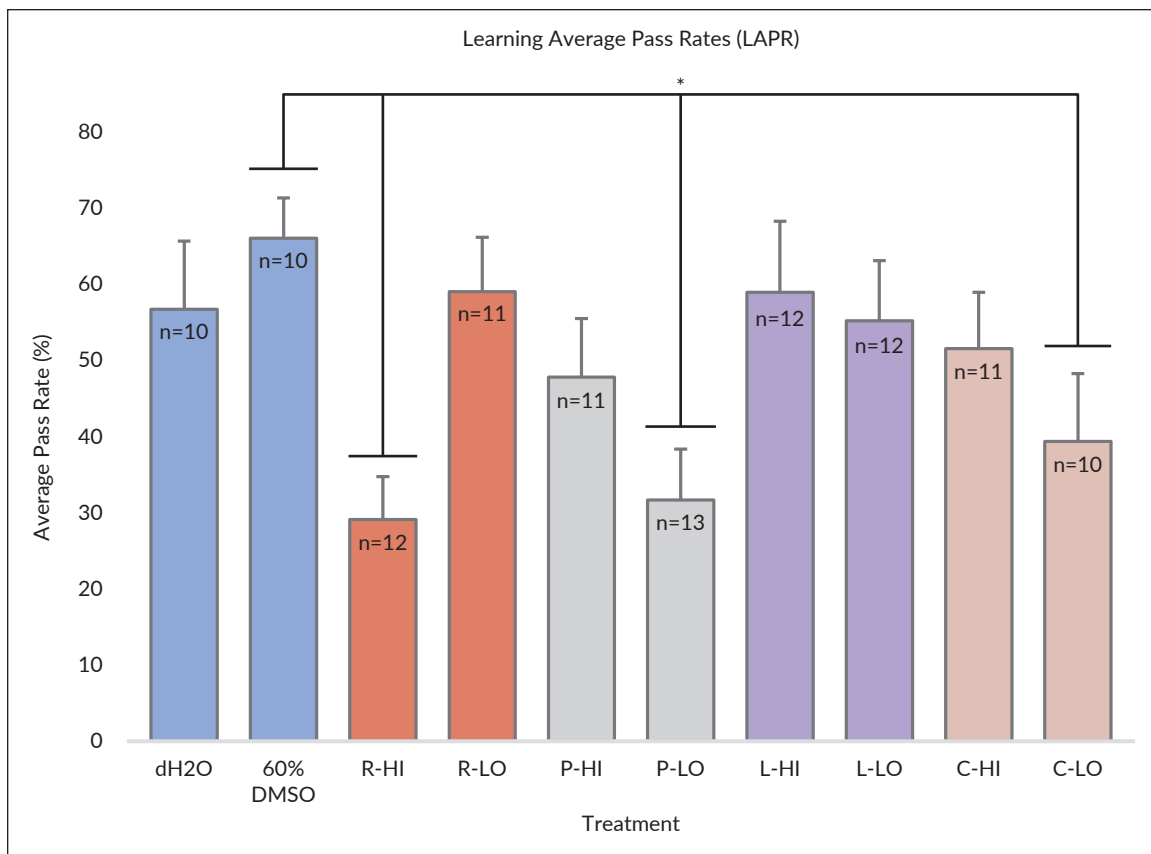

Figure 3. The average pass rates (%) from the learning phase (LAPRs) of the APS assay. The treatments R-HI, P-LO and C-LO each had a lower average pass rate than the vehicle (p<0.05).

Abbreviations: dH2O, distilled water; R-HI, rosemary high concentration; R-LO, rosemary low concentration, P-HI, peppermint high concentration; P-LO, peppermint low concentration; L-HI, lemon high concentration; L-LO, lemon low concentration; C-HI, coffee high concentration; C-LO, coffee low concentration. (*) denotes statistically significant difference among treatment groups.

aroma. Nevertheless, this decrease in pass rate attributed to the peppermint aroma is similar with another study wherein a decreased avoidance rate in a shuttle-type avoidance test was observed in peppermint essential oil-injected mice.[21] Furthermore, exposure to peppermint aroma had previously shown no improvement in the performance of individuals undergoing a visual vigilance task.[22] Within-group analyses of the same study revealed that subjective ratings of the odorants affected task performance.[22] This further supports the hypothesis that psychological associations greatly influence cognitive performance.

A study by Ogeturk et al. demonstrated an improvement in learning of male Wistar rats chronically exposed to lemon aroma, as demonstrated by their finding that less time was needed for these rats to find a target point in a labyrinth maze.[7] Our approach was different wherein lemon aroma was only introduced during the performance of a learning task. Thus, the duration of exposure may be an important factor in conferring beneficial effects on cognition, as our results show no improvement in the learning capabilities of

D. melanogaster, established by the LAPRs of both lemon aroma concentrations (LAPR of Lemon high = 58.9%; LAPR of Lemon low = 55.2%).

Coffee, particularly its caffeine component was found in multiple studies to have no effect on the performance of learning tasks.[23] However, more recent studies show that other bioactive compounds administered to aged rats conferred an improvement in working memory, a component necessary for learning.[24] Our study examines the effect of coffee aroma alone on learning. We found no increase in learning in both high and low concentrations of coffee aroma. In fact, a significant decline ($p < 0.05$) in learning was found for the low concentration (LAPR = 39.3%). We hypothesize that volatile compounds that predominated at low concentrations are able to induce relaxation which may be responsible for the low LAPR. This is in agreement with a transcriptomic study on rat brains which showed that those exposed to the aroma of coffee have an increased expression of anti-stress genes which may explain the inhibition in learning process.[11]
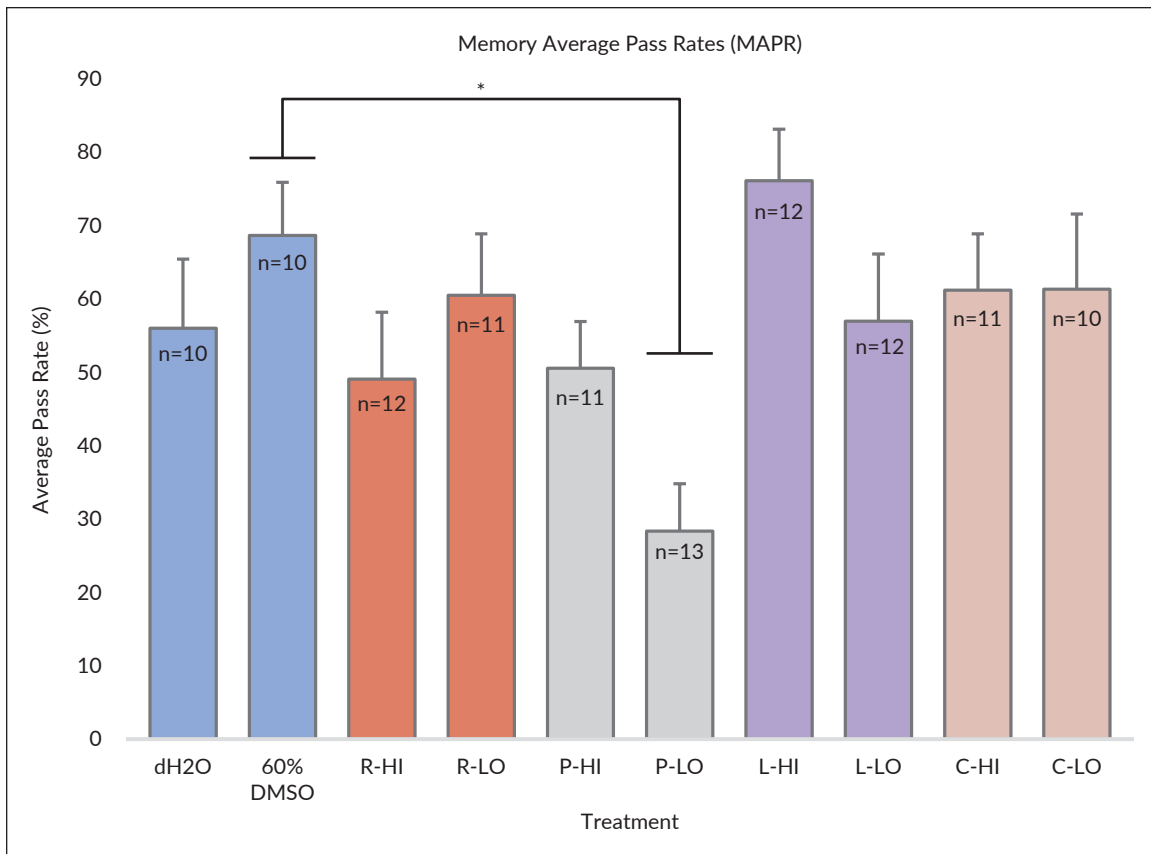

Figure 4. The average pass rates (%) from the memory phase (MAPRs) of the APS assay. Only P-HI treatment showed a decreased memory pass rate compared to the vehicle ($p < 0.05$).

Abbreviations: dH2O, distilled water; R-HI, rosemary high concentration; R-LO, rosemary low concentration, P-HI, peppermint high concentration; P-LO, peppermint low concentration; L-HI, lemon high concentration; L-LO, lemon low concentration; C-HI, coffee high concentration; C-LO, coffee low concentration. (*) denotes statistically significant difference among treatment groups.

The four aromas did not improve memory in *D. melanogaster*

Each *D. melanogaster* was reintroduced to the gated T-maze with the test aroma, six hours after the learning phase of the APS assay. Upon conducting 15 trials, the corresponding pass rate of each fly was computed. The pass rates in one treatment were averaged, giving a memory average pass rate (MAPR, Figure 4). Memory average pass rates between distilled water and the vehicle (60% DMSO) did not show a statistically significant difference (MAPR of distilled water = 56.0%; MAPR of vehicle = 68.7%; p>0.05).

Putatively due to the volatile 1,8-cineole component, the aroma of rosemary essential oil improved the memory of healthy human adults as measured by a series of cognitive tests.[3] In our study, we used *D. melanogaster* naïve to any external stimuli, aside from laboratory conditions, which should minimize any bias from any predetermined psychological association with the aromas. No significant differences (p>0.05) have been detected for both high and low concentrations of rosemary (MAPR of Rosemary high = 49.1%; MAPR of Rosemary low = 60.5%), in comparison with the vehicle. Our results also show that despite the impairment in learning at high concentrations of rosemary aroma, memory retrieval is not impaired.

The aroma of peppermint was found to enhance memory and increase alertness in a human study.[5] This enhancement in memory was replicated in another study wherein human subjects were exposed to a non-transdermal patch giving off a low-level exposure of peppermint aroma.[25] Nevertheless, these studies were done on human adults and thus unconscious psychological mechanisms may play a role in the results of these studies. Our model organism, *D. melanogaster*, did not incur any improvements in memory, upon exposure to the high concentration of peppermint aroma (MAPR = 50.6%). On the other hand, the low concentration of peppermint induced a significant decline in the memory of fruit flies (MAPR = 28.3%). This decline in MAPR may be resultant to the impaired learning process as evidenced by the impaired LAPR of this treatment.

Orally-administered components of lemon essential oil, *s*-limonene and *s*-perillyl alcohol, were found to improve memory in scopolamine memory-impaired rats, particularly through dopamine induction and acetylcholinesterase inhibition.[26] Our current research looks at lemon aroma alone and its effects on normal model organisms. Our results reflect a modest 7.4% increase on the MAPR of the high concentration of lemon, although this was not able to reach statistical significance. MAPR of the low concentration of lemon was also comparable to the vehicle (MAPR = 57.0%).

Scopolamine-induced memory impairment in rats was prevented through administration of decaffeinated coffee for two weeks.[27] This was replicated in another study on age-associated cognitive decline wherein an improvement in long-term memory and object recognition was found in rats fed with coffee.[24] Epidemiologically, it was found that higher coffee consumption was associated with better executive function but also a smaller hippocampal volume and worse memory function.[28] While there have been a myriad of studies looking upon coffee consumption and memory, our study focuses on coffee aroma alone and its effect on a model organism. Treatments of both high and low concentrations of our coffee aroma had no effect on memory (MAPR of Coffee high = 61.2%; MAPR of coffee low = 61.3%), as evidenced by no statistically-significant difference with the vehicle (p>0.05). Despite the learning impairment in low concentrations of coffee, our results show that memory retrieval remains unaffected.

CONCLUSION AND RECOMMENDATIONS

Our data shows that plant aromas, without any psychological associations, produce no beneficial effect on the learning and memory of scent-naïve fruit flies. We provide indirect evidence that psychological mechanisms play a large role in the improvements seen in the cognitive function of humans seen in some previous studies. In fact, Villemure and colleagues showed that only odors that participants self-selected as pleasant improved mood and decreased anxiety and pain unpleasantness.[29] A disliked odor worsened mood and the emotional effects of pain.[29] Children and adults exposed to a novel odor while engaged in a frustrating experience later also showed less motivation to complete an unrelated task upon re-exposure to the same odor.[30,31] Our results, however, do not discredit the claim that plant aromas induce beneficial cognitive effects in humans. The chemical nature of the odorant itself possibly plays only a secondary role. It is more likely that the individualized psychological responses, acquired through associative learning, are responsible for the improved cognitive outcomes seen in previous research. These psychological mechanisms may be significant enough that they are able to surpass declines in learning and memory conferred by volatile compounds of plant aromas. Further investigation may shed better light on the pharmacological and psychological pathways by which learning and memory can be improved.

Statement of Authorship

BPDG contributed in the conceptualization, experimental work, analysis of data, and drafting and revising of proposal. PMBM contributed in the conceptualization, guidance on experimental work, analysis of data, and drafting and revising of proposal.

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