Linear and non-linear summation of responses to visual and olfactory cues in male *Drosophila melanogaster*

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**SUMMARY**

The decision-making and navigating abilities of animals assist them in surviving in an environment with multimodal stimuli, but how the nervous system integrates multisensory information remains unknown. Thus, we chose to study summation, a main mode of integration in *Drosophila melanogaster*. We investigated whether summation of the responses to visual and olfactory cues was linear or non-linear and explored the significance of both patterns. By a population assay, we quantitatively characterized male *Drosophila*’s spatial steering behavior in response to visual and olfactory cues. Based on a pioneer study from previous research, we hypothesized that the response to concurrent presentation of visual and olfactory cues would be a simple linear summation of cues presented separately. The visual stimulus in our study was 250 lux white light, and the five attractive odorants were 1-hexanol, acetoin, ethyl acetate, 2,3-butanedione, and isopentyl acetate. The results showed that the performance of 1-hexanol, ethyl acetate, and isopentyl acetate supported the hypothesis. These phenomena indicate that visual and olfactory information are relayed to descending neurons in the ventral nerve cord to direct behavior separately, suggesting independent sensorimotor pathways between these odorants and light. However, acetoin demonstrated inhibition, and 2,3-butanedione demonstrated synergy with light. These phenomena indicate that information first be relayed to some higher site in brain for multisensory integration and computation then be relayed to descending neurons in the ventral nerve cord to direct behavior. Therefore, the results indicate that the integration pattern of phototaxis and odorotaxis may depend on the specific odorant. In addition, our data suggest that the integration pattern may not depend on odorant representation by glomeruli in the antennal lobes. Instead, integration pattern may depend on some higher order of odorant processing center, where information could be more efficiently and accurately processed. Our experiment contributes to understanding neural circuits and information processing in insects and more complex species.

**INTRODUCTION**

Living in a world with a variety of stimuli, many animals could integrate different sensory modalities to make robust behavioral decisions, such as finding food and mate and escaping from threats. To achieve this goal, they must first perceive different kinds of stimuli, such as odor, light, wind, temperature, and humidity, then represent them in the brain, integrate the multisensory information, and compute the information to guide behavior. Among different behaviors, spatial navigation provides an excellent model for studying multisensory integration in the brain due to the 3 features: 1. It is stereotyped to some degree; 2. It is undertaken by a variety of species, from insects to fish, birds and mammals; 3. It is influenced by nearly every sensory modality. Here we studied multisensory integration in navigation of *Drosophila*, an important model organism in neuroscience. This small creature has provided information about many issues of the nervous system. The findings in *Drosophila* have influenced research in more complex vertebrate neuroscience, such as memory, learning, and sleeping (1).

So far, four distinct modes of sensory integration have been observed in navigation of *Drosophila*: suppression, gating, summation, and learned association (2). Suppression occurs when the addition of a cue changes original behavioral state, such as from navigation to feeding. Gating is complementary to suppression. Association occurs when a cue that promotes innate response is paired with an innocuous cue from another modality. Summation occurs when two cues, such as odor and light, influence the same navigation parameter, such as velocity, wingbeat frequency, and amplitude, as well as turn rate. Our study focused on summation, which is believed to represent the main mode of integration (2).

Summation could be either linear or non-linear. The two patterns of summation can indicate how *Drosophila* fit the environment. Linear summation indicates that two sensory inputs are preserved as they are with less processing so *Drosophila* can make a quick but rough decision. Non-linear summation indicates that the two sensory inputs converge somewhere in the nervous system and are processed so that *Drosophila* can make a slow but wise decision. In addition, the two patterns of summation contribute to the depiction of neural circuits and computation. Linear summation indicates the separate sensorimotor pathways while non-linear summation indicates that the two neural circuits converge somewhere in the brain and are processed.

Our study focused on olfactory stimulus and visual stimulus on *Drosophila* navigation since they display chemotaxis and phototaxis. Previously, the peripheral olfactory and visual systems of *Drosophila* have been studied in great detail (3, 4). In terms of the olfactory system, *Drosophila*’s antennae and maxillary palps contain olfactory receptor neurons (ORN). Typically, each ORN expresses a single type of olfactory receptor. ORNs send axons to the primary olfactory process-
ing center, the antennal lobe (Figure 1), which is subdivided into about 40 glomeruli (3). Axons from ORNs that express the same type of olfactory receptor generally converge onto a specific antennal lobe glomerulus (3). Since many odorant molecules can stimulate multiple olfactory receptors, an odorant is represented by a combination of glomeruli in the antennal lobe. In glomeruli, projection neurons receive synaptic inputs from ORNs and then relay information from the antennal lobe to the secondary olfactory processing centers: the mushroom bodies and lateral horns in the brain. These sites may either assign preference to an odorant and relay information to descending neurons in the ventral nerve cord to direct behavior or relay information to some higher site for multisensory integration and computation. However, it remains unclear which of these mechanisms occurs or dominates (6-8).

*Figure 1: An overview of the stereotypic connections in the insect olfactory system. Odorant molecules bind to ORNs which then descend to other higher processing regions like mushroom bodies and lateral horns. The processed signal passes through the ventral nerve cord (not shown in the figure) to coordinate movements.*

*Drosophila*’s visual system is composed of compound eyes and the optic lobes of the brain. Photoreceptors in the compound eyes detect light and project it to the optic lobes. Visual information will then be relayed to higher centers such as the central complex for multisensory information integration and processing. Then information would be relayed to descending neurons in the ventral nerve cord to direct behavior (9). Interestingly, *Drosophila*’s visual system is similar to the olfactory system in terms of neural circuits: photoreceptors in the compound eyes have a similar function to ORNs in antennae and the maxillary palps, the optic lobes are equivalent to antennal lobes, and the central complex is similar to the mushroom bodies and lateral horns. At last, both optic and olfactory information conduct through ventral nerve cord to direct various activities. Though, ventral nerve cord plays a less important role in the integration of various clues, only coordinating the output of various signals (10).

Nowadays, effort shifts from study individual sensory system to the integration mechanism of multiple sensory signals. Previous studies show that visual and olfactory cues simply sum linearly in many circumstances. These studies indicate that visual and olfactory information are relayed to descending neurons in the ventral nerve cord to direct behavior separately, suggesting independent sensorimotor pathways. In one study, turns evoked by aversive visual and attractive olfactory cues were well-described as a linear sum of the responses to each individual stimulus (11). In another study of larval flies, aversive visual cue and attractive olfactory cue were found to both drive taxis by modulating turn rate and the turn probability reflected a linear sum of the responses to the two individual cues (12).

In our study, we investigated the relationship between chemotaxis and phototaxis with a quantitative population assay. The visual stimulus was 250 lux white light, and the five attractive odorant stimuli were 1-hexanol, acetoin, ethyl acetate, 2,3-butanedione, and isopentyl acetate at a concentration of 0.1% (v/v). We measured the attraction power (AP) of light (AP_L) and of each odorant (AP_O) and then each odorant in combination with light (AP_O+L). We found that for three of the odorants, *Drosophila* performed linear summation of visual and olfactory inputs (i.e., AP_O+AP_L=AP_O+L), while they used non-linear summation for the other two odorants (inhibition: AP_O+AP_L>AP_O+L; synergy: AP_O+AP_L<AP_O+L).

**RESULTS**

To find the integration pattern of the nerve circuit, we adopted a macroscopic approach: we described the sensory inputs and behavioral outputs quantitatively, and we considered the central nervous system processing to be a “black box” (Figure 2A). We took this larger question apart and designed three small experiments (Figure 2B). With the variables of sensory input and behavioral output, experiment 1 and experiment 2 focused on the effect of visual and olfactory inputs on behavioral outputs, respectively, while Experiment 3 incorporated visual and olfactory inputs together. Then we calculated the AP_L, AP_O of each odorant, AP_O+L of each odorant in combination with light to see if AP_O+AP_L=AP_O+L.

**Figure 2: Experiment Design Overview.** a. Experimental design is based on “black-box” testing. b. Three separate experiments are designed based on “black-box” testing.
Light and three out of the five odorants (1-hexanol, acetoin, and ethyl acetate) significantly attracted *Drosophila* (p-value < 0.05, two-tailed t-test, Figure 3), while 2,3-butanedione and isopentyl acetate did not significantly attract them (p-value > 0.05, two-tailed t-test, Figure 3).

After looking at the APs of the variables, we found that 1-hexanol, ethyl acetate, and isopentyl acetate showed a linear summation of visual and olfactory inputs. For 1-hexanol, AP_O+L/L = 0.662 and AP_O/L = 0.634. Thus, AP_O+L is almost equal to AP_O/L suggesting a simple linear summation of visual and olfactory inputs (Figure 4). For ethyl acetate, AP_O+L/L = 0.600 and AP_O/L = 0.587. Again, AP_O+L is almost equal to AP_O/L (Figure 4). Although isopentyl acetate failed to attract *Drosophila* (AP_O = 0), AP_O+L = 0.523 and AP_O/L = 0.522. Again, AP_O+L is almost equal to AP_O/L (Figure 4). Nevertheless, for acetoin, the AP_O+L is only 0.378, which is about half of AP_O+L value, 0.674, suggesting inhibition between the two stimuli (AP_O+L < AP_O/L; Figure 4).

For 2,3-butanedione, the AP_O/L is 0.723, which is about 40% greater than AP_O+L values, 0.507, suggesting a synergistic effect between the two stimuli (AP_O+L < AP_O/L; Figure 4). A big standard deviation of AP_O+L is likely due to great variance between the replicates. To confirm whether there is an interaction between each odorant and light, we next performed a two-way ANOVA. The two-way ANOVA will indicate whether there are interactions between variables. The results show that there is no combined effect between light and each odorant of 1-hexanol, ethyl acetate, and isopentyl acetate (p-value > 0.05, two-way ANOVA, Table 1).

However, there is combined effect between light and each odorant of acetoin and 2,3-butanedione (p-value < 0.05, two-way ANOVA, Table 1).

We found that how the light and odorant is summed has little to do with glomeruli in the antennal lobe. The odorant is first represented by glomerulli combination in the antennal lobe, the primary olfactory processing center. We hypothesized that the summation pattern of odor and visual input might relate to odor representation by glomeruli in the antennal lobe. If so, ethyl acetate should have the most similar representation to 1-hexanol since they are both attractive and have linear summation with visual input. However, by observing the glomerulli activation pattern in the heat map, it was difficult to rank the similarity between each odorant pair (Table 2). Thus, we then quantified the similarity by cosine similarity. The similarity between ethyl acetate and 1-hexanol was only 0.35, ranking eighth out of ten pairs, which fails to support our hypothesis (Table 3). To our surprise, ethyl acetate had the most similar representation to 2,3-butanedione, which showed non-linear summation (Table 3).

**DISCUSSION**

Our experiment was designed to study multisensory integration in male *D. melanogaster*, focusing on linear and non-linear summation of the responses to visual and olfactory cues. Prior research has shown *Drosophila* use linear summation, indicating that independent circuits mediate the ‘decision’ portion of each navigational algorithm and only converge at the motor output level (10). Our study indicated that some odorants follow this rule while other odorants fail to.

Here, we studied five attractive odorants for *Drosophila* as cues to guide locomotion. The odorants, 1-hexanol, acetoin, ethyl acetate, 2,3-butanedione, and isopentyl acetate, are components of fruits, the food source of *Drosophila*. However, 2,3-butanedione and isopentyl acetate failed to attract *Drosophila* in our experiment, possibly due to low concentration.

For three odorants, 1-hexanol, ethyl acetate, and isopentyl acetate, the similarity between each odorant pair was calculated (Table 3). The table shows the p-values of each odorant and light combination. (n = 5). Two-way ANOVA.

<table>
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<th>Odorant</th>
<th>p-value</th>
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<td>ethyl acetate</td>
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*Table 1: The interaction between each odorant and light. The table shows the p-values of each odorant and light combination. (n = 5). Two-way ANOVA.*
navigation. With the support of rough navigation by common brain which confer odorant and light integrate in higher processing centers in rough direction of the fruit area. As for non-linear summation, less precisely in the ventral nerve cord, which only provides a this scenario, the interference for of fruits (14). However, since so many fruits emit such odorant, kinds of fruits, is crucial for the navigation towards many kinds summation with light. This common odorant, emitted by many but rough decision. In our study, 1-hexanol showed linear simple linear summation in this case.

Next we explore how the linear and non-linear summation between this odorant and light in the brain. By contrast, the integration happening in the ventral nerve cord results in non-linear summation (13). By hypothesized that the three odorants with linear summation were set at 0.1% instead of varying concentrations. It is worth investigating the effects of more kinds of odorants on multisensory integration of both male and female Drosophila and trying to find the possible corresponding specialized sensory modalities integration sites in future experiments. Consequently, we think that the brain region which determine the summation pattern could be some higher-order processing centers, including the lateral horn, and the mushroom body which needs further research.

It should be noted that all the concentrations of odorants included. odors, these non-linear summation odorants can help Drosophila navigate the precise location of a specific kind of food in a wide fruit area (15). By the same token, the speed for linear integration is much faster than for non-linear integration due to direct integration in the ventral nerve cord, making it faster for Drosophila’s rough navigation and slower for precise navigation: there is trade-off between rate and preciseness (16). In summary, the linear summation may serve for a quick but rough orientation and non-linear summation for a slow but precise orientation; jointly, these integration patterns assist Drosophila’s finding of food and survival to a large extent. However, the significance of the integration pattern of acetoin (attractive itself but showing inhibition with light) cannot be well explained from our study.

Next, we explored which brain region determines whether the summation should be linear or non-linear. Sensory information goes through the primary and higher orders of the processing center, ultimately be relayed to descending neurons in ventral nerve cord to a behavioral response to the stimulus. For the olfactory input, it is known that each odorant is first represented by about 40 glomeruli in the antenna lobe. One specific odorant could activate certain glomeruli in the antennal lobe. One specific odorant could activate certain glomeruli while they fail to do so for the rest. Thus, the antenna lobe is considered the primary processing center for olfaction. We hypothesized that the three odorants with linear summation might be represented similarly by glomeruli. However, the heat map and cosine similarity seem to reject our hypothesis. Consequently, we think that the brain region which determine this summation pattern could be some higher-order processing centers, including the lateral horn, and the mushroom body which needs further research.

It should be noted that all the concentrations of odorants were set at 0.1% instead of varying concentrations. It is possible that Drosophila behave differently in response to the five odorants at higher or lower concentrations, manifesting other kinds of integration of visual and olfactory inputs for each odorant. Additionally, the conclusion we made is only applicable to young male flies. The neural circuits between males and females are not very similar, such as their response to pheromones and their mushroom bodies (16,17). It is worth investigating the effects of more kinds of odorants on multisensory integration of both male and female Drosophila and trying to find the possible corresponding specialized sensory modalities integration sites in future experiments. Then, we could better explain the feature of visual and olfactory interactions behaviorally and physiologically.

In sum, the evidence shows that odorants selectively

### Table 2: Five odorant representations by glomeruli in the antennal lobe

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Table 3: Cosine similarity between glomeruli activation pattern of each odorant pair. 1 indicates identical, while -1 indicates the opposite. A higher value indicates more similar patterns between two odorants. Glomeruli not activated by any of the five odorants are not included.

<table>
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<tr>
<th>Odorant</th>
<th>1-hexanol</th>
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<th>Isopentyl acetate</th>
<th>2,3-butanedione</th>
<th>Acetoin</th>
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</table>

### Table 2: Five odorant representations by glomeruli in the antennal lobe

Activated glomeruli of each odorant are assigned number 0 and highlighted with yellow. Non-activated glomeruli are assigned number 0 and highlighted with yellow.
caused specific modulation between visual and olfactory pathways in Drosophila, including no interaction (linear summation), inhibition, and synergy. It provides a new perspective on revealing neural circuits and computation in the brain. Due to the homology between the structure of the brain of Drosophila and vertebrates, this information could be used to advance research in the neuroscience field, helping to understand more complicated multisensory integrations of the brains of vertebrates.

MATERIALS AND METHODS

Culture D. melanogaster

The male D. melanogaster strain W1118 used in the experiment was strictly controlled with a standard diet. They were kept at 60% humidity and 25 ± 0.5°C and reared at a 12 h light:12 h dark (LD12:12) photoperiod at age 3 ± 1 days. Double distilled water, agar, corn flour, Methyl p-hydroxybenzoate and ethanol were mixed in proportion (Double distilled water:sucrose:glucose:agar:corn flour:yeast: Methyl p-hydroxybenzoate:ethanol =1750ml:49.42 g;94.8 g:16.8 g:116.55 g:45 g:2.25 g;22.5 mL) and served as the nutrient medium for the culture. Starvation for 6 hours before the experiment was conducted to motivate the Drosophila to seek food. Each breed of Drosophila was only used once in one trial, and in each trial 30±2 Drosophila were used.

Experimental Procedure

To dissect and combine the influence of chemotaxis and phototaxis on Drosophila behavior, we used round glass tanks (diameter: 15 cm; height: 8 cm). Each glass tank was covered with black cardboard all round to prevent the factor of external light. Meanwhile, the top of each glass tank was sealed with white gauze to prevent Drosophila from escaping. To measure AP\textsubscript{o} of each odorant, two glass bottles (5 mL each) were placed opposed to each other at the edges of the round glass tank (Figure 5). The two bottles are approximately 10 cm far. The control bottle was filled with 1 mL double distilled H\textsubscript{2}O while the treatment bottle was filled with one odorant dissolved in ddH\textsubscript{2}O (1mL, 0.1%, v/v). The five odorants were: 1-hexanol (H103420, Aladdin), acetoine (A109410, Aladdin), ethyl acetate (E116142, Aladdin), 2,3-butanedione (B104601, Aladdin), and isopentyl acetate (I112107, Aladdin). To measure the AP\textsubscript{p}, the control bottle was as before while the treatment bottle was filled with 1 mL double distilled H\textsubscript{2}O and illuminated with LED. The LED was a half sphere with radius of 1.65 mm. It was placed under the glass bottle and light intensity was set to 250 lux, matching the average light intensity of local daytime on sunny days. To measure the AP\textsubscript{p,l}, the control bottle was as before while the treatment bottle was filled with one odorant dissolved in ddH\textsubscript{2}O (1mL, 0.1%, v/v) and illuminated with LED of 250 lux. The top of every glass bottles was inserted with a small plastic funnel-shaped tube so once Drosophila fled in, they could hardly escape from bottle.

We used R to perform the two-tailed t-tests. In the glass tank, there were three environments for Drosophila: the control bottle environment, the treatment bottle environment, and the glass tank environment outside the two bottles. The control bottle environment is the space within the control bottle; the treatment bottle environment is the space within the control bottle; the glass tank environment outside the two bottles is the space within the tank but out side of two bottles. We first calculated the AP of each environment to Drosophila: AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{2}}}D} = number of Drosophila in the environment / (number of Drosophila in the environment + number of Drosophila in glass tank environment outside two bottles).

i.e. AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{2}}}D} = B/(B+E); AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{1}}}D} = O/(O+E); AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{L}}}D} = L/(L+E); AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{B+O}}}D} = (O+L)/(O+L+E). Then, based on the assumption of independence between bottle and treatment (i.e. light and odorant), we separated the AP of odor, light, and odorant in conjunction with light from the sole bottle. i.e. AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{1}}}D} = 1-((1-AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{2}}}D})/(1- AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{1}}}D}; AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{B}}}D} = 1-((1-AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{3}}}D})/(1- AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{B}}}D}); AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{O+L}}}D} = 1-((1-AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{4}}}D})/(1- AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{O+L}}}D}). Next, we assumed that the odorant and light were independent and calculated the combined effect: AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{2}}}D} = 1-((1-(AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{1}}}D})(1-AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{B}}}D})

We used excel to finish the bar chart and we used R to perform the two-way ANOVA with the inputs of AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{B}}}D}, AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{O}}}D}, and AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{B+O}}}D}.

We made the heat map showing glomeruli activation by each odorant using glomeruli activation data from other studies:

The supplementary table 3 from “Olfactory Coding from the Periphery to Higher Brain Centers in the Drosophila Brain” provided the data for odorant 1-hexanol, ethyl acetate, 2,3-butanedione and isopentyl acetate) of 29 ORN classes to 17 odors (5). Figure 2 from “The Olfactory Coding System of Drosophila” provided data for odorant 1-hexanol, ethyl acetate, and 2,3-butanedione (6). Supplementary Table 1 from “The Olfactory Logic Behind Fruit Odor Preferences in Larval and Adult Drosophila” provided the data for the odorants 1-hexanol, acetoine, ethyl acetate, and isopentyl acetate (18).

Figure 5: Schematic diagram of the experimental setup. Glass tank 1 is set up to study AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{2}}}D}; glass tank 2 is set up to study AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{1}}}D}; glass tank 3 is set up to study AP\textsubscript{\textsuperscript{\textcircled{\textscriptsize{L}}}D}.
“Odor Detection in Insects: Volatile Codes” provided data for odorant acetoin (19).

To quantify the activation for later calculation, we assigned the number 1 to glomeruli activated by a specific odorant and assigned the number 0 to glomeruli not activated by a specific odorant. To tell the difference visually, we highlighted the activated glomeruli in blue and the non-activated in yellow. The other glomeruli not activated by any of the five odorants are not included in the figure.

We then calculated the similarity between the glomeruli activation patterns of each odorant pair. Glomeruli activation pattern similarity of each odorant pair was measured quantitatively by cosine similarity. Cosine similarity is a measure of similarity between two vectors. For example, the 1-hexanol activation pattern could be considered as a vector \((1,1,1,1,1,1,1,1,0,0,0,0,0,0,0)\) and ethyl acetate as \((1,1,1,1,0,0,0,0,0,0,0,0,0,1,1,1,1,1,0,0,0)\). The formula for cosine similarity is

\[
\frac{\sum_{i=1}^{n} A_i B_i}{\sqrt{\sum_{i=1}^{n} A_i^2 \sum_{i=1}^{n} B_i^2}}
\]

where \(A\) and \(B\) are the two vectors. The cosine similarity belongs to the interval \([-1,1]\), where 1 indicates identical vectors and -1 indicates opposite vectors. Here, a value closer to 1 indicates more similar glomeruli activation patterns between two odorants. Note that glomeruli not activated by any of the five odorants were not included in the vector.

**Notations**

- **B**: number of *Drosophila* in the control bottle
- **O**: number of *Drosophila* in a treatment bottle with one odorant
- **L**: Number of *Drosophila* in treatment bottle illuminated by LED
- **OL**: number of *Drosophila* in treatment bottle with one odorant and illuminated by LED
- **E**: number of *Drosophila* in glass tank environment outside two bottles
- **AP**: attraction power
- **APg**: attraction power of control bottle
- **APbO**: attraction power of treatment bottle with one odorant
- **APbL**: attraction power of treatment bottle illuminated by LED
- **APo**: attraction power of 1 odorant
- **dL**: attraction power of light
- **APoL**: attraction power of 1 odorant in conjunction with light
- **APbOL**: attraction power of treatment bottle with one odorant and illuminated by LED

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**REFERENCES**


