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Acoustic stimulus influences ovipositioning in *Drosophila melanogaster*

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Abstract

Evidence indicates that *Drosophila melanogaster* Meigen (Diptera Drosophilidae) utilize numerous environmental factors to assess the available resources while simultaneously evaluating protection for their offspring in determining ovipositioning. While a number of physical stimuli have been examined, auditory stimuli has not. If *D. melanogaster* incorporate sound into their determining schema, it is unknown if such a tone would be perceived as beneficial or threatening. In order to assess their tone preference and to confirm previous findings, 120 female *D. melanogaster* were tested. Their ovipositioning preferences were measured by counting the amount of eggs laid in areas categorized as On, Near, and Off relative to tone location. A 250 Hz tone was presented either to one side of a forced choice chamber, presented on both sides, or there was no tone. Also within the study, substrates (sucrose, caffeine, and none) were varied. It was found that *D. melanogaster* laid significantly more eggs under the tone ($p < 0.001$) compared to controls. When given the option to lay their eggs under tone or on sucrose (a previously determined preference), they chose tone at a significant higher rate compared to sucrose ($p < 0.001$). This study demonstrates that female *D. melanogaster* may positively associate 250 Hz tone. The evolutionary implications of such behavior are investigated.

Key words: *Drosophila melanogaster*, ovipositioning, sucrose, caffeine, acoustic stimulus.

Introduction

Drosophila melanogaster Meigen (Diptera Drosophilidae) has surprisingly complex cognitive capabilities. They encode, store, and recall information at rates that are comparable to some vertebrates (Kasuya *et al.*, 2009; Bellen *et al.*, 2010; Ofstad *et al.*, 2011; Aso *et al.*, 2014). Research indicates their cognition enables a weighing of the costs and benefits associated with the surrounding possibilities (e.g., environmental cues) to decide the best course of action (Hammond and O'Shea, 2007; Card and Dickinson, 2008; Dickson, 2008; Maimon *et al.*, 2008; Herberholz and Marquart, 2012). One of the most intricate and interesting behaviors they exhibit is oviposition discretion (Yang *et al.*, 2008; Miller *et al.*, 2011; Schwartz *et al.*, 2012). Though various factors attributing to their discretion have been examined (Dahanukar *et al.*, 2001; Scott *et al.*, 2001; Chyb *et al.*, 2003; Amrein and Thorne, 2005; Satralkar *et al.*, 2007; Slone *et al.*, 2007; Yang *et al.*, 2008; Joseph *et al.*, 2009; Sarin and Dukas, 2009; Battesti *et al.*, 2012; Kannan *et al.*, 2012; Lefèvre *et al.*, 2012; Schwartz *et al.*, 2012; Dweck *et al.*, 2013; Abed-Vieillard *et al.*, 2014; Zhu *et al.*, 2014; Wu *et al.*, 2015), auditory cues have not been explored.

D. melanogaster have a well-defined auditory pathway (Boekhoff-Falk, 2005; Eberl and Boekhoff-Falk, 2007; Hammond and O'Shea, 2007; Maimon *et al.*, 2008; Card and Dickinson, 2008; Dickson, 2008; Herberholz and Marquart, 2012; Morley *et al.*, 2012; Kamikouchi, 2013). Their auditory system consists of a Johnston's hearing organ and antennal receptors. The antennal receptors can sense near-field sound, gravity, and wind (Boekhoff-Falk, 2005; Eberl and Boekhoff-Falk, 2007). It is assumed such mechanisms evolved because the female *D. melanogaster* needed to be capable of hearing the courting male to determine his species and sense his direction and positioning (Murthy, 2010). Their mating ritual involves several components (e.g., Villella and Hall, 2008). The male *D. melanogaster* utilizes olfactory cues, initiates a courtship dance, vibrates his wings creating a sound similar to a sine tone and a pulse sound, and positions himself for copulation if the mate accepts (Ewing, 1964; Villella and Hall, 2008; Shirangi *et al.*, 2013). The pulse sound has been found to increase female receptivity and it has a higher level of intensity than the sine tone (von Schilcher, 1976). Typically, *D. melanogaster* perceive sound between the ranges of 100 Hz to 300 Hz (Dickson, 2008). Though auditory signals are known to affect mating behaviors it is unknown whether they are incorporated into egg laying decisions.

D. melanogaster have a specific procedure for egg laying. They will search a given local area, probe the environment with their proboscis and ovipositor to evaluate the virility of a site, and then either accept or reject the medium (Yang *et al.*, 2008). The search time varies from a few seconds to a few minutes (Yang *et al.*, 2008). *D. melanogaster* reject sites by withholding their egg and continuing their search behavior (Richmond and Gerking, 1979; Takamura and Fuyama, 1980; Allemand and Boulétreau-Merle, 1989; Eisses, 1997; van Delden and Kamping, 1990; Spradling, 1993; Yang *et al.*, 2008). Acceptance entails a bending at the abdomen into the substrate, followed by forward and backward motions for approximately 6 seconds (Yang *et al.*, 2008). After the egg is deposited, they typically clean themselves and rest. It is assumed that egg laying is a costly decision. While *D. melanogaster* can lay thousands of eggs within their lifetime, they can only lay one egg at every oviposition, thus utilizing energy and time more efficiently is essential (Yang *et al.*, 2008).

D. melanogaster dorsal paired medial neurons appear to either enhance or inhibit egg-laying preference (Azanchi *et al.*, 2013; Wu *et al.*, 2015). The behavior seems to be moderated by a neuropeptide and a distinct subset of dopaminergic neurons (AMN) (Azanchi *et al.*, 2013; Wu *et al.*, 2015). A group of insulin-like peptide 7 producing neurons communicate when to release the egg (Yang *et al.*, 2008).

Typically, *D. melanogaster* prefer to lay eggs directly on sucrose as opposed to bitter or salty mediums (Dahanukar *et al.*, 2001; Scott *et al.*, 2001; Chyb *et al.*, 2003; Amrein and Thorne, 2005; Slone *et al.*, 2007; Schwartz *et al.*, 2012). However, most *D.* genotypes will lay their eggs close to, but not directly on sucrose if the area is unusually small (Yang *et al.*, 2008; Miller *et al.*, 2011). This preference may be due to microbial decomposition avoidance, predator avoidance, and/or dietary balance for emerging larva (Yang *et al.*, 2008; Miller *et al.*, 2011). Typically, *D. melanogaster* lay eggs in rotting fruit (Lutz, 1914; Evans, 1916; Jaenike, 1983; Miller *et al.*, 2011). Miller and colleagues (2011) observed *D. melanogaster* laying eggs in the stem cavity of a fruit, instead of directly on the food source, after it began decomposing, consistent with Yang and colleagues (2008) laboratory findings. However, if the perceived foraging costs are high, then the *D. melanogaster* will reject any other site besides sucrose to avoid potential starvation (Yang *et al.*, 2008).

Site selection based on substrate is moderated by past experience (Miller *et al.*, 2011; Abed-Vieillard *et al.*, 2014). When faced with food mixed with menthol and food media, *D. melanogaster* will lay eggs on both substances. However, when *D. melanogaster* are only exposed to food mixed with menthol, they avidly avoid ovipositioning on this substance. If *D. melanogaster* are given a choice in consuming food media and food media with menthol then there is a decreased aversion to menthol, even attraction in some flies (Abed-Vieillard *et al.*, 2014). This behavior may be brought about in order to ensure future generations retain optimal adaptation capacity (Abed-Vieillard *et al.*, 2014).

Although substrate type and quantity largely influence female *D. melanogaster* decisions, they do not provide enough information about the potential well-being of their offspring (Yang *et al.*, 2008; Sarin and Dukas, 2009; Battesti *et al.*, 2012; Kannan *et al.*, 2012; Lefèvre *et al.*, 2012; Schwartz *et al.*, 2012; Zhu *et al.*, 2014; Wu *et al.*, 2015). Edges (Schwartz *et al.*, 2012), temperature (Kannan *et al.*, 2012), light (Satralkar *et al.*, 2007; Zhu *et al.*, 2014), social cues (Sarin and Dukas, 2009; Battesti *et al.*, 2012), and presence of a threat (Miller *et al.*, 2011; Lefèvre *et al.*, 2012; Kacsoh *et al.*, 2013) are factors that females appear to employ. Edges may provide some protective element against wind and/or predators (Schwartz *et al.*, 2012). Mid-range temperatures can protect eggs from the deleterious effects of extreme temperatures on the physiology, ecology, and fitness of *Drosophila* (Kannan *et al.*, 2012). Light may serve as an indirect cue for temperature (Satralkar *et al.*, 2007). *D. melanogaster* avoid several predatory threats by avoiding ovipositing near wasps, potential bacteria sites, and microorganisms (Miller *et al.*, 2011; Lefèvre *et al.*, 2012; Kacsoh *et al.*, 2013).

There are two possible outcomes if *D. melanogaster* discriminate their egg laying site utilizing auditory signals: either they find the tone advantageous or dangerous. Tone could resemble several representations including the presence of another *D. melanogaster*. If the tone is perceived as another female in the area, then they may find it advantageous. Typically, the social cues of other females are transmitted via olfactory signal (Wertheim *et al.*, 2002; Lefèvre *et al.*, 2012). Since females typically share resources (Chen *et al.*, 2002), and younger *D. melanogaster* use more experienced female *Drosophila* choices as guidance (Lefèvre *et al.*, 2012; Wu e*t al.*, 2015), they may move towards the tone in hopes of receiving further cues. *D. melanogaster* lay eggs where they believe other eggs have been laid due to larva's ability to enrich an area with their gut bacteria (Venu *et al.*, 2014). In general, the other's presence may indicate resource availability. If *D. melanogaster* avoid tone, then they may perceive the auditory signal as a threat possibly originating from a male or another insect; to avoid resource competition and potential parasites they withhold their egg (Wertheim *et al.*, 2002).

The purpose of the current study was twofold. First we wished to further test the notion that oviposition serves as an indicator of evolved preferences in *D. melanogaster*. We expected to replicate the hypotheses that sucrose is a positive substrate and caffeine is a negative substrate when foraging costs are high. Secondly, we wanted to determine how sound factors into their preferences. We believed that if sound is utilized as a deciding factor it could be either perceived as positive or negative. We have previously employed similar methods (i.e, paring tone and substrate) with some success (D'Elia *et al.*, 2015). We employed a tone that was somewhat ambiguous (250 Hz) as it is not a typical mating/courtship song though it has elements that could potentially be associated positively (i.e, it is on the upper range of the pulse-song frequency; Morely *et al.*, 2018). It is worth noting that this study is not designed to discern between vibrational and auditory perception though it is safe to assume that both modes are at work in this examination (Fabre *et al.*, 2012; Mazzoni *et al.*, 2013).

Materials and methods

Subjects

There were 120 female *D. melanogaster*, Carolina Biological, standard stock collected and randomly assigned to 12 conditions (see below). There were 10 females per condition. *D. melanogaster* who produced less than five eggs during the 48 hour egg laying session were replaced.

Husbandry

D. melanogaster were kept in standard 95 mm (height) by 25 mm (diameter) vials. The vials were prepared utilizing dehydrated starch with 13 ml of ionized H_2O (D'Elia *et al.*, 2015). Twenty yeast pellets were added to the top of the mixture along with plastic netting. Vials were kept in an incubator which had an average temperature of 27 °C and a twelve hour light/dark cycle was employed.

In order to control for age, adults were eradicated from the vials. Only larva and pupa remained. The emerging adults were separated by sex every 24 hours insuring the youth and sexual vitality of the subjects.

Procedure

There were two independent variables, one with 3 levels (Petri Dish Substrate: Agar on both sides; Agar on one side/Sucrose on the other side; and Agar on one side/Caffeine on the other side) and one with 4 levels (Tone: Tone was played on both sides; Tone was not played; Tone was played above the substrate; Tone was played opposite to the substrate). This made for a total of 12 conditions (e.g., one condition had a Petri dish with caffeine and agar with a tone played above the substrate). The dependent variable was egg laying preference which was determined via egg count and location. Eggs were categorized into On, Off, and Near zones relative to tone location. If the tone was playing over the area, the eggs were laid this was considered On, if the eggs were laid in the opposite chamber, this was considered Off, and if the eggs were laid in the same chamber but outside of the On zone, it was considered Near. The substrate was sucrose, caffeine, or agar in the On zone (depending on the condition; see below and figure 1).

Tones were created using Wavtones software (wavtones.com) at 250 Hz, 5 seconds repeating sine function (44.1 kHz sampling) recorded at −6db on PC based hardware running Sonar (www.cakewalk.com). Prior to the experiment, tones were measured for db (86db at 1 cm) and recorded (Shure 57/58 microphones at 1 cm: www.shure.com) and reanalyzed through Sonar conversion to Matlab to confirm Sine function (www.mathworks.com).

Female *D. melanogaster* were placed in a freezer for four minutes to immobilize them. While immobilized they were separated and placed in the middle of the forced choice chambers. *D. melanogaster* were free to explore the forced choice chamber for a period of 48 hours to provide them with enough time to lay a substantial amount of eggs regardless of fertilization (*Drosophila* will lay unfertilized eggs). Preference was measured via egg count after the 48 hour period.

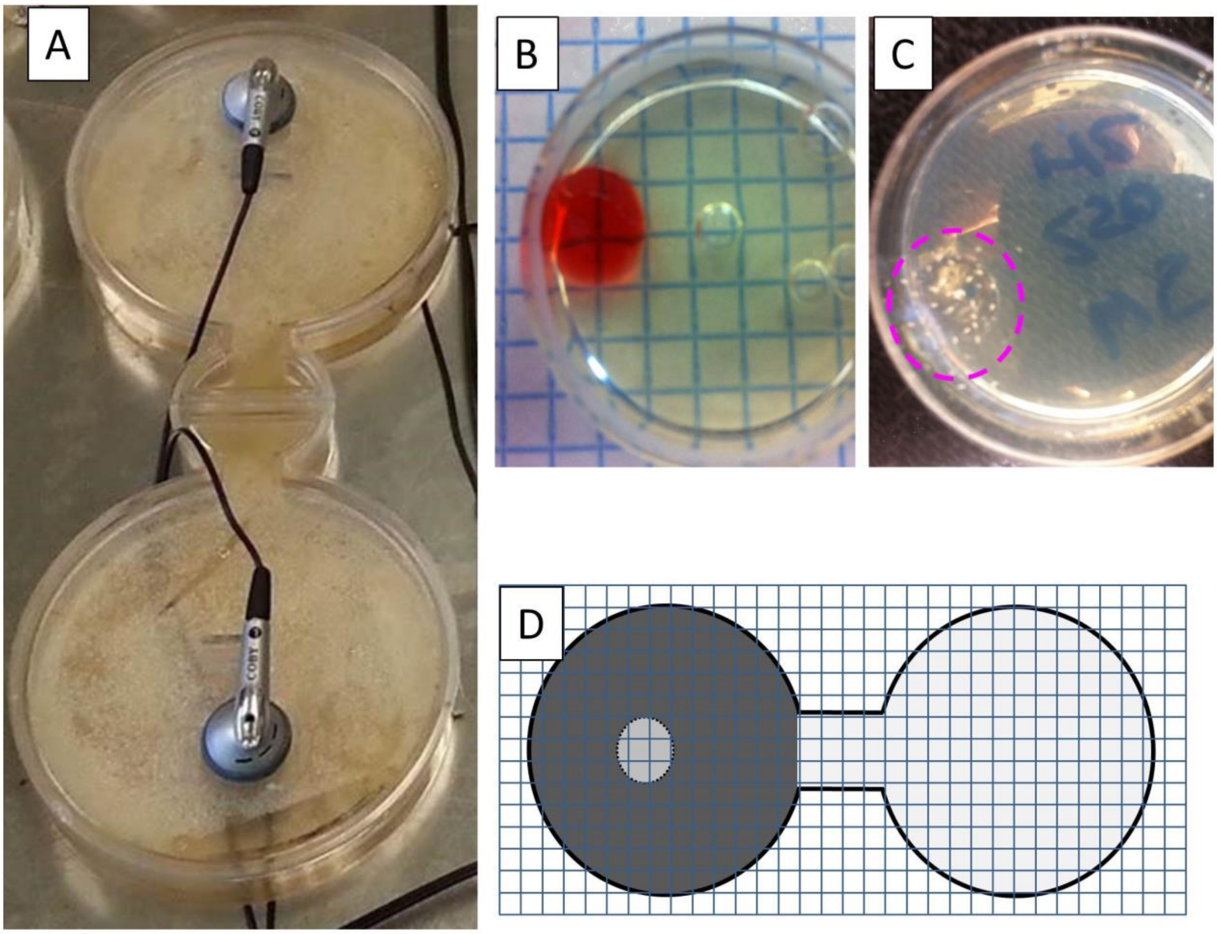

Figure 1. Diagramed are the Forced Choice chambers employed (Carolina Biological). The actual plates used (**A**) had speakers affixed in all condition even when tones were not always presented. To demonstrate the spread and location of the substrate (in this case caffeine) dye was employed (**B**) in a separate series of dishes to confirm the near zone. In actual trials no dye was employed. Actual eggs laid (**C**) and the overall cartooned representation (**D**) of the experiment are displayed.

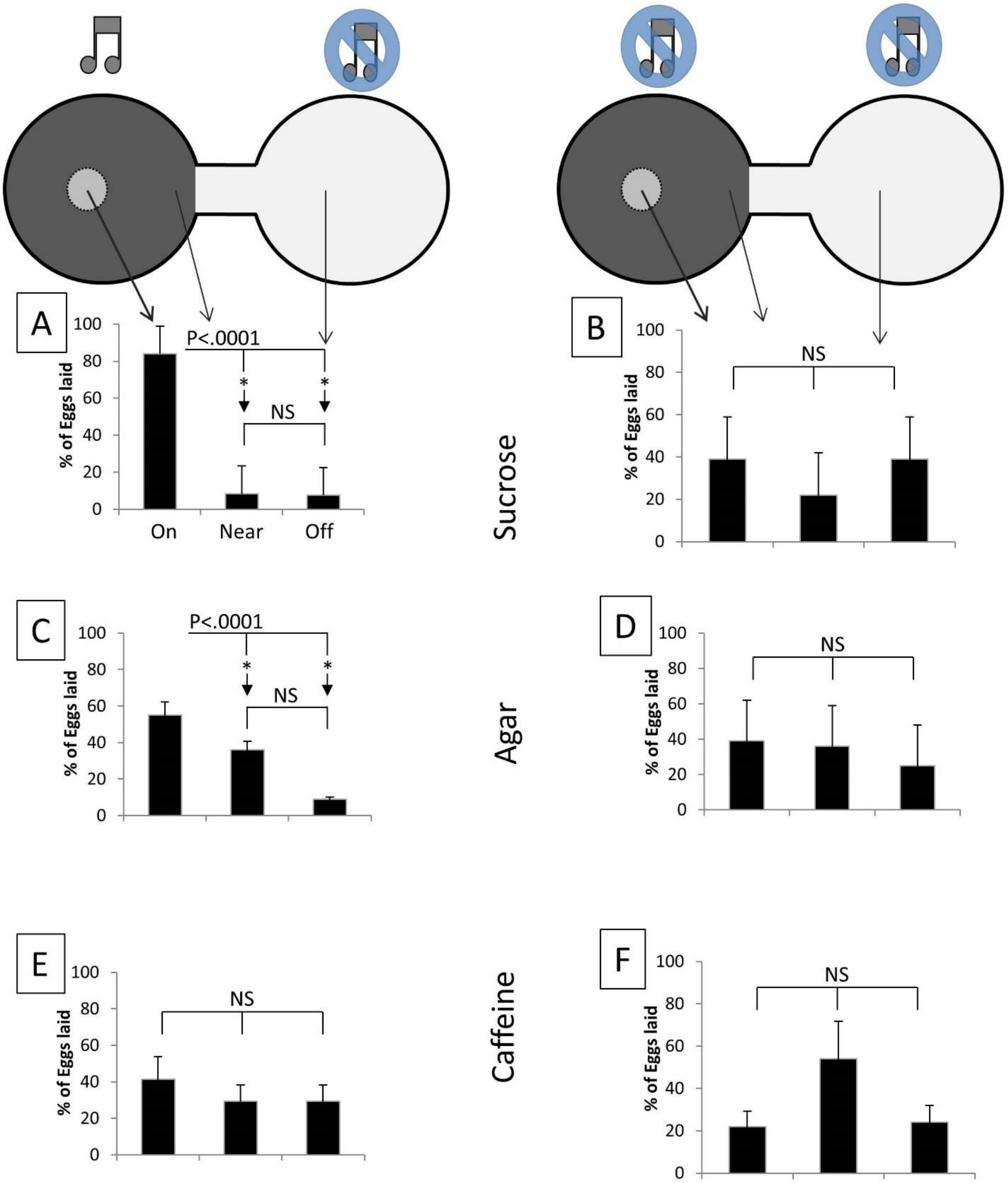

Figure 2. The most dramatic interaction of conditions was when the tone was paired with sucrose. *D. melanogaster* laid significantly more eggs in the On position with this pairing ($p < 0.0001$) compared to the other conditions (**A**). With Agar only, tone also had a significant resulting in more eggs laid under the tone (**C**). In all other conditions (**B**, **D**, **E**, **F**), there were no differences. Not shown are conditions with tones played to both chambers or tones played to the opposite chamber.

Assay

Tone was played through standard mounted earbuds (www.cobyusa.com) placed over the substrates. A hole was drilled into the top of the Petri dish and the speaker was adhered over the hole. Speakers were on both sides of the forced choice chambers (www.carolinabiological.com) regardless of condition. The forced choice chambers were larger than the Petri dishes used in Yang *et al*. (2008) study. The forced choice chambers employed were essentially two Petri dishes with a connecting smaller circle in the center. The agar substrate was directly under the speakers and consisted of sucrose, caffeine, or agar. Light was consistently on as eggs are laid predominately during the day hours. The area was kept at 30 °C.

Statistical analyses

All statistical analysis was performed on PC based computers. Data were first entered into MS excel and exported to SPSS. Chi-square tests were performed within SPSS and all reported descriptive measures and variances were also calculated through SPSS. The results are presented as mean ± standard deviation.

Results

We first confirmed that *D. melanogaster* prefer laying eggs in sucrose and avoid caffeine. In order to assess this we first looked at caffeine without tone present or with tone on the opposite side of the substrate during egg laying and the same for sucrose. It was found that caffeine is a highly avoided substance. *D. melanogaster* laid an average of 15 eggs (s = 1.7 eggs) on caffeine which was 32 percent of their eggs when they had a choice. Whereas they laid an average of 54 ± 6.5 eggs on sucrose and which was 47 percent of their eggs. Indeed these patterns persisted; *D. melanogaster* on average laid the least amount when caffeine was present 60 ± 1.9 eggs on average, the most when sucrose was present at 133 ± 5.4 eggs on average, and 98 ± 2.7 eggs on average for agar.

We then wanted to see if tone had an effect on egg laying. Since *D. melanogaster* laid the expected average on agar, the percentage of eggs for agar was used as the expected value for the Chi square. *D. melanogaster* tone preference was evident when the agar 250 Hz condition was examined. *D. melanogaster* laid significantly more eggs under the tone (test χ^2 120,2: $\chi^2 = 37$, $p < 0.001$). We found that eggs were laid significantly more on sucrose under the tone as opposed to the Near or Off zone (test χ^2 120,2: $\chi^2 = 87.8$, $p < 0.001$; figure 2). They also laid eggs more often on the caffeine side despite the negative effects when tone was present, however not at a significant rate (test χ^2 120,2: $\chi^2 = 4.14$, $p = 0.1262$). *D. melanogaster* laid eggs the most during the tone same side sucrose condition in comparison to the agar and caffeine conditions (test χ^2 120,2: $\chi^2 = 98.5$, $p < 0.001$). As expected, *D. melanogaster* avoided caffeine laying the least amount of eggs when present, however, they laid their eggs under tone despite caffeine's presence on the opposite side (test χ^2 120,2: $\chi^2 = 27.6$, $p < 0.001$). More surprisingly eggs were laid significantly more underneath the tone when sucrose was on the opposite side (test χ^2 120,2: $\chi^2 = 42.4$, $p < 0.001$).

The combination of these results demonstrate that *D. melanogaster* may prefer to lay their eggs under tone. There were overall more eggs laid in the 250/250 Hz egg laying conditions with an average egg deposit of 12.77 ± 0.4 eggs per individual in comparison to 9.53 ± 0.22 in the no tone condition ($\chi^2(2, N = 120) = 68.2$, $p < 0.001$; figure 3).

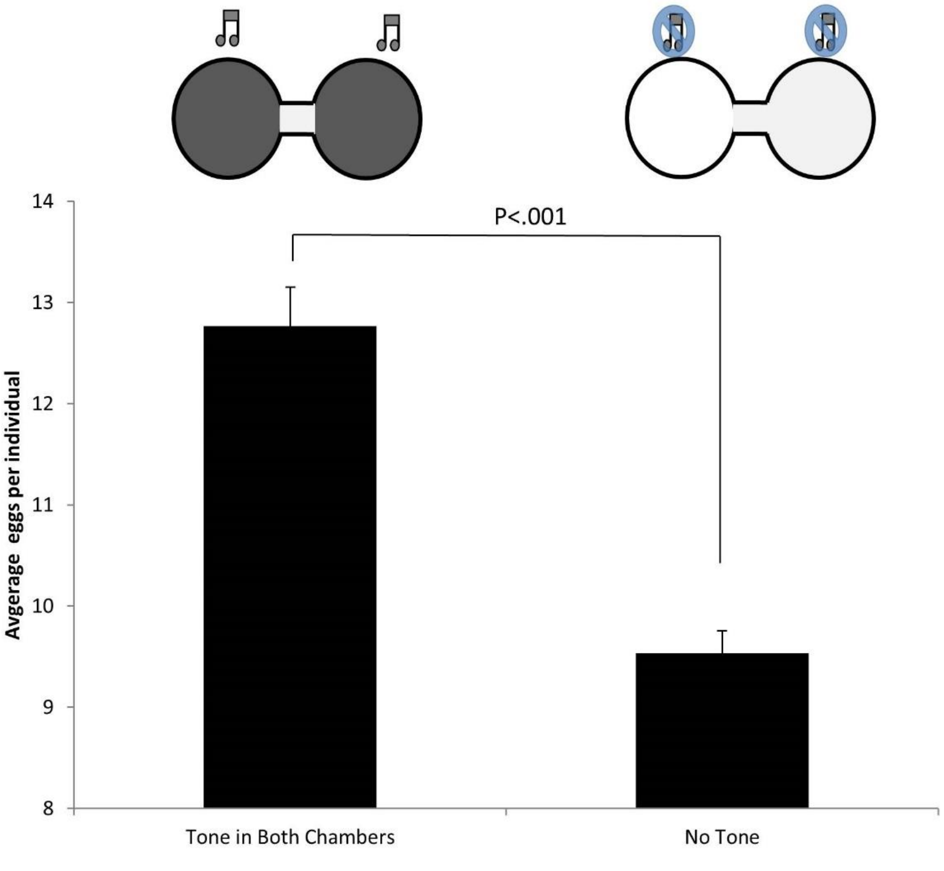

Figure 3. *D. melanogaster* laid the greatest number of eggs when tone was presented to both chambers compared to no tone (p < 0.001). Data are presented across all level of substrate.

Discussion and conclusions

Results from this study indicate *D. melanogaster* prefer to lay eggs under tone. This was demonstrated both through the increased egg laying on the side of the tone in the agar condition and the heightened egg laying during the sucrose tone condition. Further support for tone preference comes from increased eggs under the tone when sucrose was on the opposite side. They viewed tone in this instance as more advantageous than food. Therefore *D. melanogaster* seem to find laying eggs under tone beneficial.

D. melanogaster may associate 250 Hz sine tones with other *D. melanogaster*, though this is speculative. Their presence may indicate resources, protection or possibly competition. Since young female *D. melanogaster* were collected it's possible that there was a positive association due to their youth. Younger *D. melanogaster* seek olfactory cues from experienced females since it is assumed experienced *D. melanogaster* have found success. They also may benefit from having *D. melanogaster* in the general area as larvae's gut bacteria aids emerging larvae (Venu *et al*., 2014). Older females may avidly avoid the tone since they already have egg laying experience and can avoid a potential threatening male (Wertheim *et al*., 2002).

Since egg laying decisions are not impoverished by age (Wu *et al.*, 2015) future directions could explore how heavily young *D. melanogaster* utilize auditory social cues for egg laying in comparison to older females. Furthermore, females' responses to courtship songs could be explored. Pulse tones are typically male mating sounds and may have separate associations from the tone used in this study. Reduced egg laying time would also be a future research interest. Reducing the egg laying time may show how *D. melanogaster* determine the most advantageous positions to first lay their eggs.

Given the increasing ease of examining genetics in *D. melanogaster*, further research could be directed toward discovering the underlying genes responsible for tone preference. Furthermore, it is not known if these differences would be found in all *Drosophila*, or if they are exclusive to *D. melanogaster*. Further research should investigate other species of *Drosophila.*

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