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Behavioral Audiometry Testing in Drosophila melanogaster

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Behavioral Audiometry Testing in *Drosophila melanogaster*

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Health and Behavioral Studies
James Madison University

by Amanda Nicole Cascio

May 2018

Accepted by the faculty of the Department of Communication Sciences and Disorders, James Madison University, in partial fulfillment of the requirements for the Honors College.

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Abstract

The genetic make up of *Drosophila melanogaster* aligns closely enough to humans for them to function as models for the study of hearing loss and disorders (Albert & Göpfert, 2015; Duyk *et al.*, 1997). The purpose of this project was to design a computer automated program capable of quickly assessing the hearing of flies based on their suppression of courtship behaviors in the presence of an audible stimulus. We were unable to document the male courtship song due to low frequency noise present in our sound attenuating booth. We continued the experiment using a spectrum of fly noise unassociated with courtship. When our program was triggered by fly noise, it responded by playing a synthetic pulse tone at a variety of frequencies and intensities. It then measured the interval of time between the tone and the next fly trigger, known as the inter-buzz interval. We considered length of the inter-buzz interval as an indicator of fly perception. Our data was originally highly skewed, with more than 75% below the mean length. After disregarding the longest 25% of intervals as extreme values, we produced a significant positive trend between intensity level and inter-buzz interval length. The correlation indicated that the louder the tone was, the longer the flies ceased their activity. While we have begun the programming process, more success would likely be found by further calibrating the trigger criteria and documenting the suppression of the courtship song, rather than fly noise.
Introduction

Biologists have long used *Drosophila melanogaster* (fruit flies) as a model organism for genetic studies due to their high percentage of conserved genes with humans, rapid generation time, and ease in rearing (Pandey & Nichols, 2011). Fruit flies were the first complex organism to be genetically mapped and have been at the forefront of many other major scientific discoveries (Adams et al., 2000; Pandey & Nichols, 2011). Additionally, flies are particularly useful in modeling disease in humans due to the countless shared biological pathways (Pandey & Nichols, 2011). It is our hope to extend that relevance from biology/genetics to the field of Communication Sciences and Disorders, by taking advantage of the genetic homologies in the mechanosensory systems of vertebrates and flies (Kamikouchi & Ishikawa, 2016). The flies’ mechanosensory systems, which includes the auditory system, has allowed them to serve as model organisms for the study genetic and environmentally induced hearing disorders (Albert & Göpfert, 2015; Duyk et al., 1997). Our goal was to exploit the flies’ courtship behavior in order to design a computer program capable of quickly and efficiently testing the hearing of the flies.

*D. melanogaster* sense air-borne sound via a small, specialized organ housed within each antenna. This organ, known as the Johnston’s organ, consists of hundreds of auditory neurons which activate upon stimulation of mechanical sound vibration (Kamikouchi & Ishikawa, 2016). This system is similar to the inner hair cells in the human cochlea whose movement causes the auditory nerve to fire. In contrast to humans, however, who can hear sound varying in frequency between 20 and 20,000 Hz, *D. melanogaster* can perceive only a limited range of low frequencies, from about 100 to 300 Hz (Albert & Göpfert, 2015). There are no known sexual dimorphisms in the mechanosensory systems of *D. melanogaster*, but auditory input nonetheless
has an important role in the flies’ sexual behavior (Albert & Göpfert, 2015; Kamikouchi & Ishikawa, 2016). The mating process of D. melanogaster relies on primarily auditory and tactile stimulation, with very little use of visual cues (Spieth, 1974). The courtship song is a crucial, species specific, cue for males to effectively court females, evidenced by decreased receptivity in deafened females by removal of an antenna (Mayr, 1950). Similarly, when male flies’ wings are removed they experience reduced success in their mating attempts. When supplemented with an artificial courtship song playing from a speaker, however, it increases again (Brussel et al., 2014).

Male D. melanogaster produce two different types of song, called pulse and sine, by a single wing vibration (Shirangi et al. 2016). The sinusoidal song is produced at low frequencies within the range of fly hearing, close to 160Hz (Menda et al. 2011). The pulse song consists of a series of pulses each generated for approximately 3ms, 34-35ms apart (Bennet-Clark & Ewing, 1969; Duyk et al., 1997; Von Schilcher, 1976). Kamikouchi & Ishikawa (2016) describe the pulse song to be close to 170Hz. The particle vibration amplitude of melanogaster is said to be large in comparison to other species of Drosophila, and thus should be easier to record with a microphone during experimentation (Spieth, 1974). While the males generally alternate between pulse and sine songs, the pulse song is thought to be more sexually stimulating to both sexes. This difference is evidence that flies are able to differentiate acoustic signals (Kamikouchi & Ishikawa, 2016).

Male flies become sexually mature at a much faster rate than females, approximately six hours after eclosion, or hatching (Tyler, 2000). While male flies may begin courting just hours after eclosion, females will not be sexually mature until twenty-four to forty-eight hours, and most will not demonstrate receptive behaviors until at least forty-eight hours have passed. The
receptive behaviors of female flies can also be influenced by other factors, such as previous mating experience (Spieth, 1974). While male flies cannot distinguish virgin female flies from non-virgins, some experimentation has revealed that females who have previously mated produced cues via smell that inhibited male courting, thus it was the virgin flies who are more strongly courted (Siegel & Hall, 1979). Female flies respond to courtship in two distinct patterns depending on her receptivity to mating with the specific male. She will signal various acceptance behaviors including, but not limited to, extending her wings outward that will prompt the initiation of the mating process. In contrast, the female flies can also exhibit rejection behaviors such as removing herself from contact with the male, fluttering her wings (distinct from the extending behavior), and kicking her legs (Spieth, 1974). Finally, the courtship song along with other cues induces increased pausing on behalf of the female to also display receptivity to mating (Bussell et al., 2014; Von Schilcher, 1976). Many of these behaviors related to courtship have the potential to be utilized in hearing assessments.

Past studies have assessed fly hearing mainly by subjectively scoring their locomotor activity and behavior. Von Schilcher (1976) recorded courtship songs using a ribbon microphone and then synthesized both songs at 105dB. The researchers played to songs at varying frequencies and intensities and subjectively scored male movement seen on a video recording as an indication of hearing. Duyk et al. (1997) also created audiograms by observing and subjectively scoring video recordings of fly behavior in response to a computer generated pulse song. The researchers scored the number of males participating in courtship behaviors, such as following one another in a chain. Arthur et al. (2011) systematically conditioned flies to expect a food reward after the playing of an auditory stimulus. Therefore, the flies would expectantly extend their proboscis (in order to feed) after a tone. The researchers were able to document the
flies’ response to different frequencies and levels of tones to determine thresholds. This design allows for the equal testing of both males and females, but also requires a large time commitment for conditioning and testing the flies.

Our experiment aimed to utilize the naturally occurring courtship and mating behavior of D. melanogaster in order to behaviorally determine their frequency and decibel threshold of hearing, but through an automated program. The purpose of the experiment was to design a method to reliably and behaviorally find fly thresholds, without the need to spend time conditioning the flies or manually scoring video recordings. Our first objective was to reliably record the courtship song via microphone using a small combination of young virgin males and females. Following the hypothesis that the male flies will cease their songs in the presence of an unknown audible tone, the second objective was to induce and document suppression of the courtship song in response to various amplitudes of said tone to obtain the threshold. The final objective was to design a computerized method to automatically test and record fly response. Considering the biological and genetic relevance of the species Drosophila melanogaster, the success of this project would contribute to efficiency in future studies of effects of environmental agents such as noise exposure or mutagens on the thresholds of this species. These effects could potentially model those that could in humans due to the homology between their auditory systems.
Methodology

Obtaining Virgin Flies:

The first step is the collection and separation of male and female virgin flies. This process can be started once darkening pupae begin to appear on the sides of the vial. At this point, all the adult flies are transferred to a second tube, leaving only the maturing larvae and pupae in the agar of the first tube. Before six hours pass, the mature pupae emerge as adult flies, but will not yet be sexually mature (Tyler, 2000). If more than six hours pass, then it is possible that the flies mated and the process should be restarted from the beginning. Once there are newly emerged virgin flies, they are separated by sex as soon as possible. After placing the tube of young flies into a typical freezer for approximately two minutes, they are alive but unconscious. Then they are identified as male or female under a microscope and separated into properly labeled vials: one for only males and one for only females. Females are typically larger in body size and have little pigmentation down their ventral abdomen (Image 1). Males have dark pigmentation surrounding their anal plate on the inferior ventral abdomen as well as tiny, dark “sex combs” on their forelegs (Doran et al., 2010). Testing virgin flies ensures that all trial flies are equal in age and sexual experience and is thought to elicit courtship more strongly (Siegel & Hall, 1979).

Experiment I.

Our first experiment was conducted in an effort to record the male courtship song. We then planned to use this understanding of the song to document its suppression in the presence of stimuli audible to the flies. All experiments occurred in an Industrial Acoustics Inc (city), Model 1202 double walled, double floored sound attenuating booth. The analog output of a Bruel and Kjaire model 2235 sound level meter set to 40-110 dB equipped with a Bruel and Kjaire model 4176 one-inch microphone was sent to an Agilent model dynamic signal analyzer. The equipment was calibrated using a known 94 dB tone which gave 163.875 mVRMS. The microphone was placed over a cylindrical, plastic 1.6cm³ container for the flies. The small size of the chamber ensured that the microphone was as close as possible due to the faint amplitudes of the courtship song. The spectrum analyzer was set to 100 lines for the faintest speed and peak track. A Dell computer ran a local written Matlab program to constantly query the spectrum analyzer for the frequency and intensity of the peak. Flies were tested overnight for
approximately 16 hours or 30,000 peaks, whichever came first. The run time of each trial was also determined by how long the equipment was able to function successfully. In order to identify the proper frequency and decibels of the courtship song, we compared frequency and intensity spectra from trials with no flies to those with different combinations of males and females. In trials with both males and females, only virgin females were tested because the literature documents stronger courting of virgin females by male flies.

Based on previous literature documenting the low frequency (between 100 and 300Hz) nature of courtship song, we filtered out frequencies greater than 500Hz (Albert & Göpfert, 2015). We ran our control trial first with an empty chamber for two hours. We then ran one test trial with a fly combination of one male and two females for two hours. We ran a third test in which males outnumbered females in a combination of three males and two females. This trial ran for three hours. Finally, we ran trials of solely females to provide comparisons because females do not create song, and thus any suspected courtship signals of the combination trials should be absent in these groups. The recorded signals produced by only female flies can be assumed to be the basic fly sounds associated with movement and flight. This trial had the longest run time of eighteen hours. For each trial, we excluded signals with the greatest 0.1% of decibel levels.
Experiment II.

As we were unable to reliably document the male courtship song, we tried to stimulate a group of flies with a sound and then measure whether they ceased creating sounds unrelated to courtship (Figure 4). First, we conducted a no-fly control in which we ran the same Matlab program we would use for the treatment (Appendix A) for the same amount of time, but on an empty tube. In the treatment group, ten mixed sex flies were recorded for twelve hours. We used a half-inch microphone to pick up signals from the fly tube and deliver them to the sound level meter and dynamic signal analyzer. The smaller microphone was used because of its ability to better capture the high frequency fly noise. We created a trigger system in which fly emissions within particular criteria triggered the Matlab program to play a two second pulse tone. The program searched for buzzes continuously. A “buzz” was defined as a signal between 500 and 3000Hz with an intensity level greater than 200 microvolts (or 35.7dB SPL). For a buzz to trigger the program, two additional criteria must have been met. First, the program looked for two signals meeting the frequency and intensity requirements within a three second interval. Additionally, the two subsequent signals could not have been exactly equivalent to one another. When two buzzes within the above criteria fell within three seconds of each other and were not perfect matches, the speaker emitted one of thirty different stimuli.

Each stimulus was a pulse song that was programmed as closely as possible to the description by Schilcher (1976) but at a variety of frequencies and intensities (Image 2). A stimulus was a two second pulse song one of five frequencies (120, 160, 180, 250, or 1000Hz) and one of six intensities (0, 50, 60, 70, 80, or 90dB SPL). The pulse songs consisted of fifty 10ms pulses that were each 40ms apart. Each 10ms pulse consisted of 5ms $\cos^2$ rise/fall times.
Songs were delivered through a Tucker-Davis Technologies (Alachua, FL) MF1 closed 2.5cm³ tube into a B&K coupler. After playing the two second stimulus, the program waited an additional 2.2 seconds before resuming the collection of fly buzzes to ensure that there were no residual signals following the pulse tone. Then, the program documented the amount of time (in seconds) following the stimulus that the flies failed to again trigger the program. This time period was known as the “inter-buzz interval”, and was based on the idea that animals will cease their own noise production when they perceive an auditory stimulus. Thus, the length of the “inter-buzz interval” was used to determine whether the flies were able to hear the stimulus. We conducted an ANOVA test of regression using an alpha level of 0.05.

**Image 2.** Oscilloscope trace of three (of the fifty) pulses within one two second pulse song. Each individual pulse was 10 milliseconds (ms) long with 5 ms cos² rise/fall times.
**Image 3.** Instrumental set up for experiment II. Tubing was used to physically connect the Tucker-Davis speaker (left) to the B&K fly chamber, and the ½ inch microphone was inserted into the open end of the fly chamber (right).
Results

Experiment I.

Our control trial (empty tube) resulted in a spectrum of low frequency, low amplitude noise to which we compared our results for tubes with flies (Figure 1). The majority of signals clustered between 0 and 200Hz and under 0.2 Volts RMS. The first fly test containing one male and two virgin females resulted in fewer total signals for the same amount of time as the no fly control (Figure 2). Nearly all the signals were below 200Hz and 0.1 Volts RMS. Similarly, our results for the test containing three males and two females also had the majority of signals fall below 200Hz and 0.1 Volts RMS (Figure 3). The female only test, however, resulted in a greater number of triggers at higher frequencies. The majority of frequencies fell between 250 and 500Hz (Figure 4), but the entire distribution of frequencies included lower frequencies as well (Figure 5). The intensities were very low (0 Volts RMS) throughout the trial. The female only test accumulated a far larger number of triggers, but also ran over fifteen hours longer.
Figure 1. No fly control recorded over a two-hour period plotted for the number of triggers at each frequency (Hz) and intensity (Volts RMS) level.
Figure 2. Fly test run for a combination of two virgin females and one male over a two-hour period. Signals are plotted for number of triggers at each frequency (Hz) and intensity level (Volts RMS).
Figure 3. Fly test run for a combination of two virgin females and three males over a three-hour period. Signals are plotted for the number of triggers at each frequency (Hz) and intensity level (Volts RMS).
Figure 4. Fly test run on five females and no males over an eighteen-hour period. Signals are plotted for the number of triggers at each frequency (Hz) and intensity level (Volts RMS).
**Figure 5.** Female only test represented by the number of triggers per hour (top left), the number of triggers at each intensity level (bottom left), the intensity level plotted against frequency (top right), and the distribution of triggers at each frequency (bottom right)

**Experiment II:**

We continued into our second experiment using the data supplied by our female only test (Figure 4 & 5). We made the assumption that any signals produced by female flies only would constitute typical noise generated by flies in the absence of courtship behavior. Therefore, we continued with our original methodology which involved documenting the suppression of fly-generated noise in the presence of an acoustic signal. We compared the data from the no fly control (empty chamber) to the treatment with flies to control for random noise present in the room. Signals fell within the frequency and intensity criteria 767 times for the empty chamber and 5084 times for the fly chamber. The test with flies successfully met the trigger criteria 88 times, while the no fly control only triggered a stimulus once (Table 1).
There was also a large amount of data that did not fit the trigger criteria. The treatment with flies produced 1574 signal pairs that were disqualified as triggers because they were equivalent to the previous or following signal (Table 2). The no fly control only produced one duplicated trigger pair. 780 signal pairs for the treatment and 381 signals pairs for the control fell outside the three second interval that would qualify them as triggers. The average frequencies were nearly equivalent for the disqualified data, but the average intensity was much higher for the control.

For the signals that did trigger the pulse stimulus, we calculated the average inter-buzz interval (IBI) at each intensity level. Our original data was highly skewed. We took the log of the data in an attempt to approach a normal distribution (transformation of data found in Appendix B). The majority of the data fell below the mean. We first filtered out the longest 5% of IBIs and began to see a slight trend (Figure 6). Then, using the same data, we chose to consider the longest 25% of the data to be extreme values because of the skew. After filtering out the top 25%, we saw a positive correlation between intensity level and IBI (Figure 7). Our data within the lesser 75% of IBIs resulted in a p-value of regression of 0.051.

**Table 1.** Comparison of signals that met the trigger criteria on an empty chamber and a chamber with 10 flies.

<table>
<thead>
<tr>
<th></th>
<th>Empty Chamber</th>
<th>10 flies (mixed sex)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># Defined as Buzz</strong></td>
<td>767</td>
<td>5084</td>
</tr>
<tr>
<td><strong># Stimuli Delivered</strong></td>
<td>1</td>
<td>88</td>
</tr>
</tbody>
</table>
Table 2. Comparison of disqualified data collected on an empty chamber and a chamber with 10 flies.

<table>
<thead>
<tr>
<th></th>
<th>Empty Chamber</th>
<th>10 flies (mixed sex)</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td># Duplicated Signals</td>
<td>1</td>
<td>1574</td>
<td>Significantly more</td>
</tr>
<tr>
<td>Mean Frequency</td>
<td>548</td>
<td>549</td>
<td>Same</td>
</tr>
<tr>
<td>Mean Intensity</td>
<td>0.1042</td>
<td>0.0063</td>
<td>Softer</td>
</tr>
<tr>
<td># Signals &gt; 3s</td>
<td>381</td>
<td>780</td>
<td>More</td>
</tr>
</tbody>
</table>
Figure 6. Average inter-buzz interval or IBI (seconds) as a function of intensity level over the twelve-hour test period. Five different frequencies (120, 160, 180, 250, and 1000Hz) were played at each of the six decibel levels (0, 50, 60, 70, 80, 90). The longest 5% of intervals were removed from this data set as extreme values.
Figure 7. Average inter-buzz interval (seconds) as a function of intensity level over the twelve-hour test period. Five frequencies were played at each of the six intensity levels. The longest 25% of intervals were removed from this data set as extreme values.
Discussion

An automated method of testing hearing in *Drosophila melanogaster* would save a tremendous amount of time for researchers studying the effects of experimentally-induced trauma or various mutations on fly hearing. We aimed to design a program able to determine when the courtship song is produced, play a tone, and document whether the song is suppressed. Despite having more modern technology than Schilcher (1976), we were unable to reliably record the male sinusoidal song using our designated frequency cut-offs that we inferred from the literature. One factor influencing our results was the low frequency noise present even in the sound attenuating booth that overlapped the expected frequencies of the courtship song. This noise made it difficult to determine which signals were coming from the flies and which were coming from the room. Due to these difficulties, we continued our experiment using noise spectra collected from female flies, under the assumption that the higher frequency signals found in this test were due to noise associated with movement and buzzing.

We still recorded many more signals for the tube with flies that were not considered triggers because they were either too similar to a surrounding signal or outside of the three second window. The average intensity of the control’s disqualified data was unusually high, but this was most likely due to one very loud random noise that was picked up by the computer (e.g. something falling). There were substantially less disqualified signals in the control than in our fly treatment. While it is possible that the trigger criteria we created was too stringent and caused us to miss some fly generated emissions, it also means that the triggers that we did collect were likely all due to the flies rather than background noise. Additionally, however, strict criteria meant that we had a relatively small sample of data to work with.
Again, we had a small sample of data so any outliers greatly influenced the mean and trend of the data. Over 75% of our inter-buzz interval data fell below the mean (Appendix A), implying that our mean interval was largely skewed by extreme high values. Such long periods between triggers were likely due to random bouts of inactivity, not actually a response to a stimulus. For that reason, we disregarded the top 25% of data which then produced a trend with an p-value lower than our designated alpha level. This significant positive correlation between intensity level and the length of the inter-buzz interval is consistent with our hypothesis that flies would suppress their activity and noise levels when stimulated by an unidentified tone they can hear. Thus, we have begun the process of designing a computer program to document a psychometric function from *Drosophila melanogaster*, but there is still work to be done. The suppression of the courtship song (the original goal), rather than random noises, would be a much clearer indicator of hearing. Also, using a pulse tone may have been too stimulating to document noise suppression, and future studies may have better results using tones with no relation to courtship. Future research could also focus on calibrating precisely the right criteria to distinguish fly noise from random noise to be able to maximally utilize signals produced by flies. All of these alterations would aid in meeting the ultimate goal of this project, which was to find a psychometric function based on both frequency and intensity through automated means.
Bibliography


Appendix A

Matlab program used for experiment 2:

hours=12; %.0083=30s, .02=1ish mins %12 hrs maybe safe for batteries.

HFcutoff=3000;
LFcutoff=500;
dBLimit=200e-06; %200uV from quick peak avg.

triglim=3; %need to get two trigs in triglim secs

Hzs=[120 160 180 250 1000];
dBs=[0 50 60 70 80 90];

while (toc<wait && n < maxTrigs)
    n2=n2+1;
    fprintf(SA, 'CALC:MARK:X?');
    pause(0.03);
    buzzHz=str2double(fscanf(SA));
    if (buzzHz > LFcutoff && buzzHz < HFcutoff)
        n3=n3+1;
        fprintf(SA, 'CALC:MARK:Y?');
        pause(0.03);
        buzzLvl=str2double(fscanf(SA));
        if (buzzLvl>dBLimit)
            n4=n4+1;
            switch firstOfTwo
                case 0 %starting to look for two trigs in 8 secs
firstOfTwo=1;
F1=buzzHz; L1=buzzLvl;

**case 1 %a second trig**

twotrigin=toc-prevchirp;

**if** twotrigin < triglim && buzzHz~=F1 && buzzLvl~=L1

firstOfTwo=2;
nCs=mod(n,nSounds);
yT=mod(nCs,6)+1;
xT=floor(nCs/6)+1;
RZ6.SetTagVal('Freq',Hzs(xT));
RZ6.SetTagVal('Amp',10^(-(calLvls(xT)-dBs(yT))/20));
RZ6.SoftTrg(1);

disp([num2str(n) ' playing ' num2str(Hzs(xT)) ' Hz at
   dB = ' num2str(dBs(yT))])

F1=buzzHz; L1=buzzLvl;
pause(2.3) %wait for tone and reveerb to be over

**else**

firstOfTwo=0;

disp(['two trigs in ' num2str(twotrigin) ' s'])

**if** twotrigin<triglim

nDupTrig=nDupTrig+1;
aveDupF1=aveDupF1+buzzHz;
aveDupL1=aveDupL1+buzzLvl;

**else**

n2long=n2long+1;
ave2trigIn=ave2trigIn+twotrigin;
aveF1miss=aveF1miss+buzzHz;
aveL1miss=aveL1miss+buzzLvl;
end
dend
prevchirp=toc;
case 2
if buzzHz~=F1 && buzzLvl~L1 %prevent dup trigs on loud
    long noises
    n=n+1;
time=toc;
data(n,1)=Hzs(xT);
data(n,2)=dBs(yT);
data(n,3)=time-prevchirp; %IBI the critical DV
data(n,4)=time;
data(n,5)=buzzHz;
data(n,6)=buzzLvl; %new in Feb 2018
data(n,7)=twotrigin; %new in Feb 2018
end
pause(2) %ITI new in Feb 2018
firstOfTwo=0;
prevchirp=toc;
end
else
    n5=n5+1; %not loud enough
avedBlow=avedBlow+buzzLvl;

dBlowS=dBlowS+buzzLvl^2;

end

else

n6=n6+1; %outside Hz limits

end

end
Figure 8. Original data collected from experiment 2 (10 flies) based on frequency.

**Large variations present in 1000Hz are likely because this frequency is outside of the flies’ documented threshold and contributed to skew."
Figure 9. Inter-buzz interval histogram before taking the log of the data.
Figure 10. Inter-buzz interval histogram after taking the log of the data.