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The Exploration of Neuronal Responses to Auditory Stimuli in the Dragonflies, *Anax junius* and *Aeshna Constricta*

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The Exploration of Neuronal Responses to Auditory Stimuli in
the Dragonflies, *Anax junius* and *Aeshna constricta*

By: Brian Shaw

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Submitted in partial fulfillment
of the requirements for
Honors in the Department of Biological Sciences

UNION COLLEGE
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I. Abstract

Shaw, Brian: The Exploration of Neuronal Responses to Auditory Stimuli in the Dragonflies.

Department of Biological Sciences, June, 2018.

Advisor: Professor Robert Olberg

To date there is no published evidence that dragonflies (*Odonata*), have a nervous system equipped to process auditory stimuli. Even with considerable research on these creatures due to their specialized vision and flight mechanics, there is no evidence that dragonflies have ears or even auditory neurons. Last year student Andrew Hamlin and Professor Robert Olberg recorded neuronal responses in the dragonfly to auditory stimuli of 100-2000Hz sounds (Olberg and Hamlin, unpublished). This year our research was aimed at understanding a sensory modality that was previously unknown in dragonflies, the sense of hearing. In order to investigate this question we used behavioral and electrophysiological studies on the Aeshnid dragonfly *Anax junius* and various *Aeshna* species. Behaviorally, dragonflies were loosely tethered to a standing mount allowing free movement while computer-generated sound stimuli were played to the animal and video-recorded. Electrophysiological studies were done by extracellular recording of the ventral nerve cord to detect neuronal activity in response to these computer-generated frequencies (50Hz – 22KHz). This study suggested that sound waves do stimulate an auditory sense through a tympanum or external ear in dragonflies. This is an extremely subtle sense in these highly visual creatures but it is consistent in the far field of a sound wave meaning the response is characteristic of an external ear and not mediated by mechanoreception or sensory hairs.

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A: Dragonflies, the Visual Creature

As entomology research has heightened in past decades investigators are discovering new insights into the most successful phylum on our planet, the insects. Many of these new discoveries are due to changing technologies, especially in the field of neuroscience. Thanks to computer and recording technologies researchers are finding out more about the nervous system than ever before. The study of neuroethology focuses on the neuronal components that underlie animal behavior and exactly how an immensely complicated behavior occurs. The current study investigates a sensory modality that was previously unknown in dragonflies, the sense of hearing.

Dragonflies are insects belonging to the order *Odonata* and the suborder *Anisoptera*. A total of 3012 individual species of dragonflies have been discovered and they constitute 11 families that have a very broad distribution around the world. Most species of dragonflies begin their life as aquatic nymphs and then emerge to their terrestrial adult stage that people see flying around outside. These creatures may have developed this aquatic nymph stage due to selection forces of predation on dragonfly eggs forcing these creatures to adopt protective strategies for laying eggs much like any oviparous animal (Corbet, 1963). Many of these nymphs have developed a burrowing behavior to assist in predator avoidance and thus rely on mechanosensory and tactile stimuli recognition to catch prey and avoid predators (Corbet, 1963). However the really interesting sensory application for these creatures does not come from their mechanoreception or tactile recognition but rather from their visual and flight systems. When someone pictures a dragonfly they see a swift flying insect with abnormally large compound eyes. Even for these aquatic nymphs who live in burrows under or near water for the first half of their life, they mostly hunt with their vision (Corbet, 1963). Humans perceive the world with tri-

chromatic vision meaning we see three different colors (red, blue, and green). A study on dragonfly vision found that different species could see many more different colors in a range of spectral sensitivities (Futahashi, 2015). Furthermore the visual acuity of dragonflies has made them tremendous hunters of other flying insects. A study on dragonfly interception for prey capture found only a single miss out of 38 attempts giving a 97% success rate for a dragonfly to capture its prey with one aerial attack (Olberg, Worthington, and Venator, 2000). Another study found that the latency for this visual reflex involved in prey capture is mediated in approximately 29ms (Olberg, Seaman, Coats, and Henry, 2007). It has been well documented that dragonfly vision and flight mechanics are some of the best examples of what evolutionary biology can produce in the natural world. With these incredible systems it is no wonder that audition has never been looked at in these creatures.

B: Historical Understanding of Insect Audition

The study of insect audition had previously been quite minimal throughout the 20th century. Due to changing technologies and growth in the field of neuroscience it had been found that many insects might not be able to respond to sound. David Yager, a leading neurobiologist in the field stated in one of his articles on insect auditory systems that, “Only a small portion of all insect species can hear” (Yager, 1999, 380). Furthermore an article in The New York Times that interviewed neurobiologists studying dragonflies stated that, “Dragonflies can’t really hear” (Natalie Angier, 2013). If the majority of insects do not have a sense of audition then why look at this sense in dragonflies?

Through various research projects up until 1990 it had been found that only five insect orders had an auditory sense, Orthoptera, Neuroptera, Dictyoptera, Coleoptera, and Lepidoptera

(Yager and Fenton, 1990). This is only a fraction of the insect world, but new discoveries started to show that more insects actually have a nervous system equipped to process auditory stimuli than previously thought. The first studies involving insect audition were done in green lacewings. Extensive behavioral and neurophysiological studies have shown that green lacewings encode ultrasonic sound to escape from echolocating bats. These studies have found that green lacewings turn in the opposite direction from certain frequency ultrasonic waves and have specific flight patterns to escape from an approaching bat (Roeder, 1967). Research with crickets and locusts flying tethered in the laboratory have shown steering responses when exposed to bat like pulses as well (Yager and Fenton, 1990). Another study in 1989 found increased neuronal activity when ultrasonic sound waves were played to flying insects like moths and crickets compared to when they were stationary (Hoy, Nolen, and Brodfuehrer 1989). In 1989 the preying mantis, which was never thought to have had any auditory sense was found to be sensitive to ultrasound as well. Free flying and field experiments proved that the preying mantis responded and had specific avoidance patterns to ultrasonic sound waves much like green lacewings (Yager and Hoy, 1989). Mantids are very visual creatures much like dragonflies so the discovery that these insects were encoding sound was surprising. Evolutionarily it makes sense that insects have a sense of audition to escape from predators or to assist in finding potential mates.

Since insect audition had been discovered, research has shown that air-born acoustic signals can be detected by both tympanic organs like in vertebrates and by receptor hairs that respond to particle displacement (Michelsen and Larsen, 1985). A tympanum ear on the ventral thorax encoded the auditory responses found in the preying mantis (Yager and Fenton, 1999). A tympanum ear is what humans and most vertebrates use to encode sound. It consists of an

external ear that transduces sound waves in the environment into electrical signals that are encoded by the nervous system. Alternatively cockroaches have been found to respond to sound using their tracheal system that discriminates small vibrations of sound waves invoking leg movements (Shaw, 1994). This type of hearing starts to diverge from the tympanic membrane into the second group of hearing systems, mechanosensory hairs. In more recent years *Drosophila* has been found to respond to auditory stimuli through a chordotonal organ at the base of the antennae called the Johnston's organ. These types of organs are non-external stretch receptors that have modified cilium or outer segments at the distal tip that take vibrating air particles of sound and open channels to invoke neuronal responses (Kernan, 2007). Other insects like mosquitoes, hawk moths, and honeybees have also been found to have similar organs in their antennae. These receptor organs are essential to finding potential mates in *Drosophila* courting, honeybee dancing, and the wing beats of mosquitoes (Kernan, 2007). Only a small fraction of insects have been found to hear and they implement both tympanic and receptor hair hearing systems. As more discoveries about insect audition emerge, the historical picture that insects cannot hear is beginning to disappear.

C: Mechanoreception and the Physics of Sound

A mechanoreceptor is a sensory receptor that responds to physical pressure or distortion like the various corpuscles in the human skin. As seen with insects like *Drosophila* certain mechanoreceptors can do more than just respond to a physical pressure, they can also respond to sound waves that are essentially vibrating air particles. A sound wave travelling through a medium has two distinct areas, the near field and the far field of the sound wave. The near field is where a sound wave is circulating and propagating in the medium whereas the far field is

where the sound wave is just propagating in one direction. Where the sound wave transitions from near field to far field is approximately one wavelength from the sound source of a particular frequency. Comparatively the loudness of a sound decreases throughout the far field and is constant in the near field (Hansen, 2001). Ultimately the pressure and loudness of a sound wave in the near can invoke a “feeling” of sound that vibrates sensory receptors on the body where it does not in the far field. This is an important aspect of auditory sensation because sensory receptors like Johnston’s organ have been found to detect air-driven vibration of its distal segments to near field sound sources (Kernan, 2007). This type of sensation to sound is effective because during *Drosophila* courting the male stays less than 5 mm away from the female, which is less than one wavelength of the sound produced by the female (Bennett-Clark, 1971 as found in Caldwell and Eberl, 2002). So the Johnston organ of the male is able to pick up near field sound for mating and not far field sound. The same Johnston organ in *Drosophila* has been found to also respond to small wind gusts, which are not sound waves but a rather a different type of mechanoreception (Yorozu, Wong, Fischer, Dankert, Kernan, Kamikouchi, Ito, and Anderson, 2009). Because many auditory sensations in insects are transduced through mechanoreceptors like the Johnston organ the discovery of audition in novel insects needs to take into account the physics and applications of sound.

D: The Possibility of Dragonfly Audition

Although most of the research in dragonfly physiology has centered around visual and aerial mechanics the possibility that dragonflies can hear is still on the horizon. In a study on the abdominal ganglia of the dragonfly it was found that fibers in the ventral nerve cord of late instar dragonfly nymphs of *Anax imperator* ranged up to 16 μm in diameter and were comparable to

the giant fibers of a cockroach or locust (Fielden, 1960). This is interesting because the cockroach and locust are two insects that have also been found to have an auditory sense. That same study concluded that the escape response of the dragonfly nymph depended on neuronal pathways and connections very similar to a cockroach. The dragonfly nymph contains three thoracic and seven abdominal ganglia and if the escape response runs through these ganglia similar to a cockroach then it could be possible that encoding sound works through the same pathways.

It has been well documented that dragonflies have mechanoreceptor hairs all over their body as well as specialized antennae. A comparative study in damselflies found that certain sensilla on these creatures might play roles in olfaction, ability to perceive temperature, humidity, or air speed (Barsagade, Thakr, Gathalkar, and Kirsan, 2017). Furthermore it was found that the dragonflies have wind sensitive hairs found in the neck region that responded to wind puffs (Olberg, 1980). It has already been proven that Johnston's organ in *Drosophila* encoded for both wind vibrations and auditory vibrations, so the same could be possible for dragonflies. In another study on insect auditory systems it was stated that some insects like *holometabolous Diptera* have no auditory precursors in their larval stages but have a rearrangement and develop an auditory sensation after metamorphosis (Lakes-Harlan and Strauß, 2006). When audition was discovered in the preying mantis it was also discovered that only males had this newly discovered sensory modality and that females hearing was significantly reduced (Yager and Fenton, 1990). With all of this information it is evident that if dragonflies can hear there may be some confounding elements that play a role in this modality.

Last summer at Union College, student Andrew Hamlin and Professor Robert Olberg found neuronal responses to auditory frequencies in the ventral nerve cord of a dragonfly (Fig.1).

The recording used a silver wire hook electrode that was wrapped around one of the ventral nerve cord connectives of the dragonfly while computer generated frequencies were played in the direction of the mounted animal. The frequencies were from 100-2000 Hz and there was clear spiking activity during the onset on the sound. This recording was done twice on two different animals in consecutive days and the data was similar for both, there was neuronal activity in response to sound stimuli.

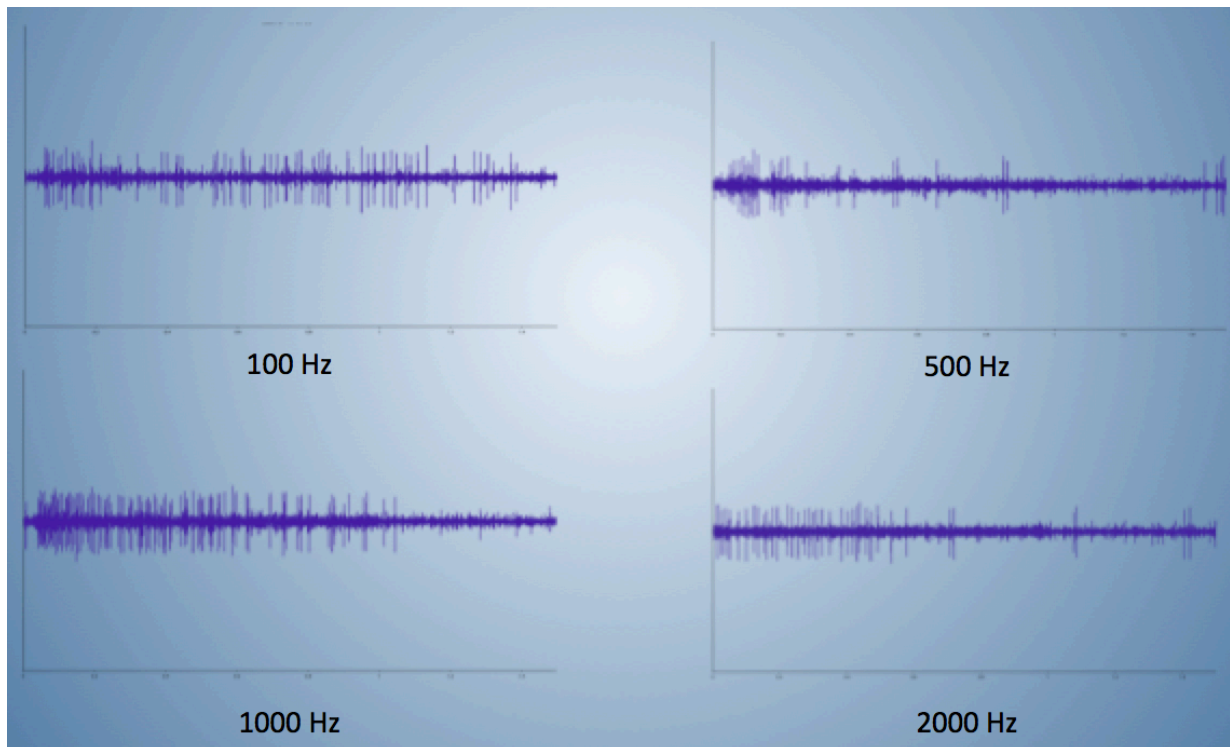


Figure 1: Spike trace data to auditory stimuli from a silver wire electrode recording of a connective in the ventral nerve cord of a dragonfly, *Anax junius*. Computer generated frequencies were presented to the animal from 100-2000 Hz and the responses were recorded by LabChart7. All of the spike traces show clear neuronal responses to sound stimuli.

This was the first recorded data of a dragonfly nervous system responding to auditory stimuli. Hamlin and Olberg did no further work during the summer of 2017 since it was not their initial research project but these findings sparked a new direction for research in dragonfly physiology. The current study is an exploration of the sense of audition in dragonflies and uses

electrophysiological and behavioral techniques similar to prior physiological studies of other insects.

III. Materials and Methods

Specimens

Dragonfly specimens were caught in the field as fully emerged adults or caught as larvae and reared in the laboratory until emergence. Each adult specimen that was caught would spend up to a week in a refrigerator at 4° Celsius. Larvae caught in the field and reared in the lab were *Anax junius* and various *Aeshna* species. These larvae were kept in water filled holding tanks until deemed close to emergence. The specimens were then put into a larger tank with screened walls inside, and a fly netting covering the top so they could not escape once emerged. Once emergence occurred the specimens were put into a window chamber for 24 hours and then placed in a refrigerator (4° C) similar to the captured adults. During Fall of 2017 I performed one *Aeshnia constricta* dissection and 8 *Anax junius* dissections. During the winter and spring of 2018 I performed 2 *Aeshnia Constricta* dissections, 7 *Anax junius* dissections, and performed 6 behavioral experiments combined with both species.

Dissection

The basic dissection aimed to expose thoracic ganglion 1 and 2, the pro- and mesothoracic ganglia. The adult dragonfly was first placed in a tub of ice for 15-20 minutes while a bee's wax/rosin mixture (insect wax) was melted on a hotplate at approximately 110° Celsius. The hot wax was then placed on the dragonfly's ventral thorax behind the posterior set of legs and stuck to a metal holder. Additional hot wax was placed around the holder to lock the specimen in place as well as around the head to immobilize it during recording. The holder was

placed in a magnetic stand and lined up under a swing-arm dissecting microscope. Under the microscope the legs and lower mouthparts were cut off using a pair Vannas spring scissors from Fine Science Tools. Next using a smaller pair of Vannas spring scissors the prosternum was cut open horizontally. Following this the prosternum was cut vertically on both sides of the original horizontal cut so the prosternum could be removed using Dumount forceps. Two apodemes that are attachment points for flight muscles connect this area of the exoskeleton to the body were carefully cut as well to remove connective tissues and muscles that covered the nerve cord. This made the prothoracic and mesothoracic ganglia visible under the microscope. Using a flame-polished glass probe the nerve cord connectives that run between T1 and T2 were gently moved around to ensure they were free for recording with electrodes. The nerve cord was kept moist with Miller's dragonfly saline. The specimen was then ready for recording.

For alternative dissections that aimed at exposing the third thoracic ganglia, the same process was followed, except that two additional cuts along the third pair of leg sockets were done to remove more posterior exoskeleton on the specimen and expose the third thoracic ganglion.

Recording Setup: Silver Wire and Suction Electrode Recordings

The dragonfly was placed on a different magnetic holder under an Olympus SZ40 dissecting microscope. On the left was a Leitz micromanipulator that held a silver wire hook electrode that was shaped with forceps under a microscope and soldered to a BNC connection cord. On the right side of the setup was a Narishige micromanipulator that held a silver ground wire. The signal was amplified with a custom electrophysiology amplifier and digitized (PowerLab, AD Instruments) and displayed and stored on a computer using LabChart7 software.

The amplified signal was also played through an audio monitor. In earlier work we amplified sine wave stimuli from a function generator to produce the stimulus tone pulses. In later work we used the built-in sine wave generator in LabChart (AD Instruments) as our sound source.

For silver wire electrode recordings once the dragonfly was mounted a light beam was aimed on the area for visibility. Then the silver hook electrode was placed under one of the nerve cord connectives and the ground wire was placed in the thorax or one of the leg sockets. The silver wire electrode was manipulated to pull the left connective up and away from the right connective to eliminate neuronal crosstalk. Once the silver wire electrode was in place the area was lightly dried using Kimwipes and Vaseline was placed in and around the nerve cord and wire to isolate the connection between the electrode and left connective.

For suction electrode recordings the silver wire that was mounted on the Leitz manipulator was replaced with a glass suction electrode. The exposed area was filled with saline via a syringe. The suction electrode was placed over a thoracic ganglion. Then a small amount of the saline was sucked up into the electrode and the electrode was placed in contact with the ganglion. Then the surface of the ganglion was sucked up slightly into the electrode for a vacuum connection. The area did not have to be dried for suction electrode recordings.

A 10 second tone pulse was played from the amplifier and adjusted so the amplitude of the tone was between 70 and 95dB at the head of the dragonfly. This was measured using a portable sound level meter. Sine wave sound stimuli (100Hz – 16KHz) tests were done with the speaker at different distances and angles from the dragonfly's head.

Recording Setup and Analysis: Behavioral Testing

For behavioral tests a newly emerged dragonfly that had not yet been in the refrigerator and only in a window cage was tethered to a wooden pole on a table using insect wax and clear fishing line. A Sony RX-10 camera was mounted on a tripod facing the animal and a grey background was placed behind the animal so there was no clutter in the recording. A Grass instruments speaker/amplifier unit was positioned at different distances away from the animal out of the field of the camera and three-second tone pulses were played at varying frequencies (100 – 2000Hz). The analysis of the behavioral data was done by playback of each video at 30 frames per second. Since the tone pulse was three seconds long a comparison between the three seconds preceding the tone pulse and the three seconds of the tone pulse was done to see movement differences. Operational definitions of animal movements were completed prior to the experiments and can be found in the Appendix.

Electrophysiological Data Analysis

Spike traces of raw data were sorted by amplitude and half-width in LabChart and displayed as peristimulus time histograms (PST's). PST's were used to bin the number of neuronal responses before and during and after the tone pulse.

Raw data from LabChart7 was saved as MatLab data. This data was then uploaded to MATLAB_R2016a for processing. A script for signal averaging was written in Matlab to analyze all data points collected in each recording and to filter out background noise to examine the differences in response during the tone pulse.

Scripts for Fast Fourier Transform were written in MatLab to quantify the signal-averaged data and discriminate between played frequencies that the silver wire and suction

electrode could have picked up and actual neuronal signals. Calculations for integration were done to find the area under each graph to further quantify the differences between neuronal response before the tone pulse and during the tone pulse. All MatLab scripts used for analysis are included in the Appendix.

In later work, originally written MatLab scripts for “sound clipping” that extracted the recorded signal in a narrow time window surrounding the stimulus presentation were used to analyze each sound stimulus and response on its own from electrophysiology recordings. For this data LabChart was used to zoom in and analyze the time delay of responses using the marker feature.

Microsoft Excel was used to graph operationally defined movements for behavioral tests as well as perform statistical testing to try and expand the breadth of auditory response data that may be occurring in dragonflies.

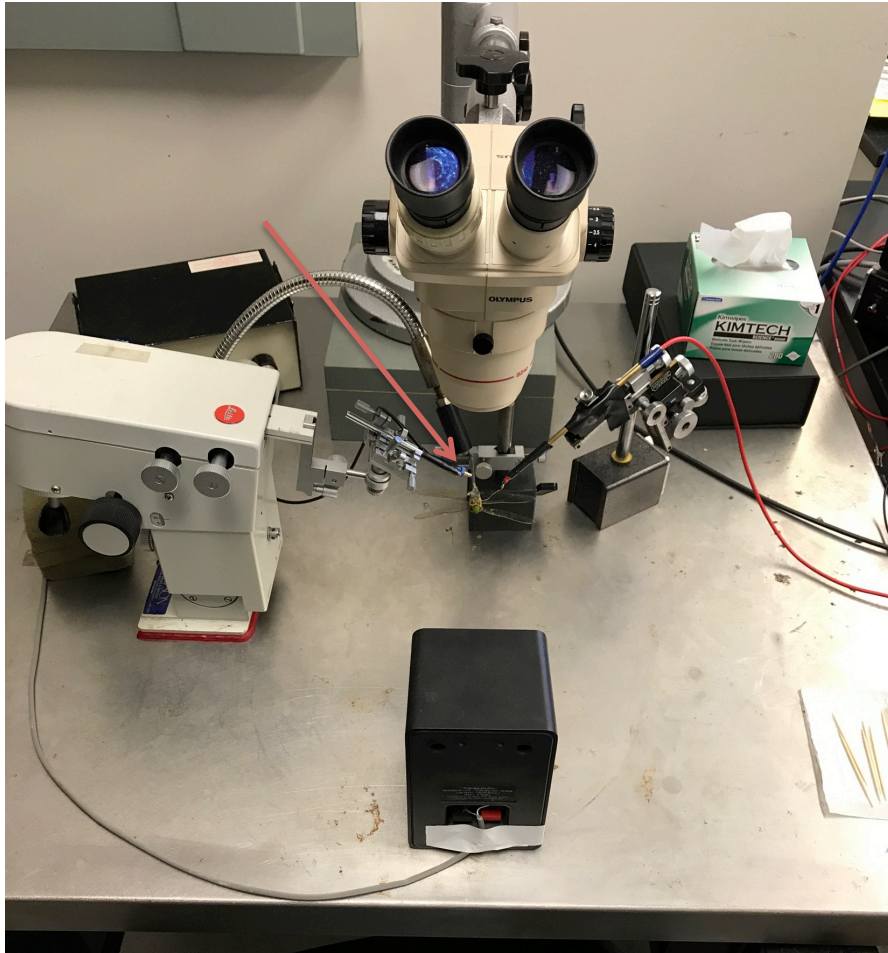


Figure 2: Example of a basic electrophysiology recording set up. The dragonfly was immobilized on a stand under the microscope with the silver hook/ suction electrode and ground wire placed inside the dissection area. A smaller Realistic SA-10 solid-state stereo speaker was moved around the animal in close range while a Grass Instruments AM7 Audio Monitor speaker was placed on a moving cart that could be placed farther away from the animal. The arrow points to the electrode placed inside the dragonfly either around or on one of the connectives.

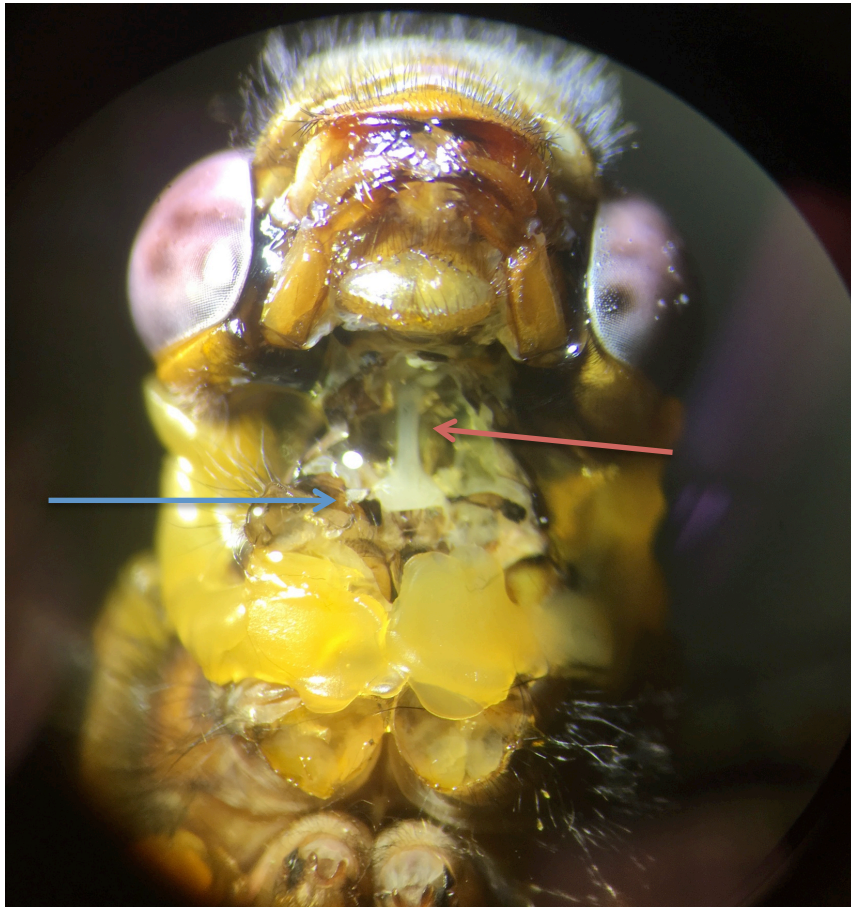


Figure 3: Picture from under the microscope prior to placing the electrode and ground wire in the dissection area on an *Anax junius* dragonfly for an electrophysiology recording. The red arrow points to the two connectives of the ventral nerve cord that were the primary recording areas. The blue arrow points to the prothoracic ganglion.

IV. Data and Results

A. Neuronal Responses to Early Sound Stimuli

The dragonflies used in this study had consistent and characteristic neuronal responses to auditory stimuli in the far field, eliminating near-field mechanosensory effects. However this conclusion was not revealed immediately. In original electrophysiology recordings, specific frequency auditory stimuli were presented for 75-150 repeats and the responses picked up by the electrode were signal averaged over time.

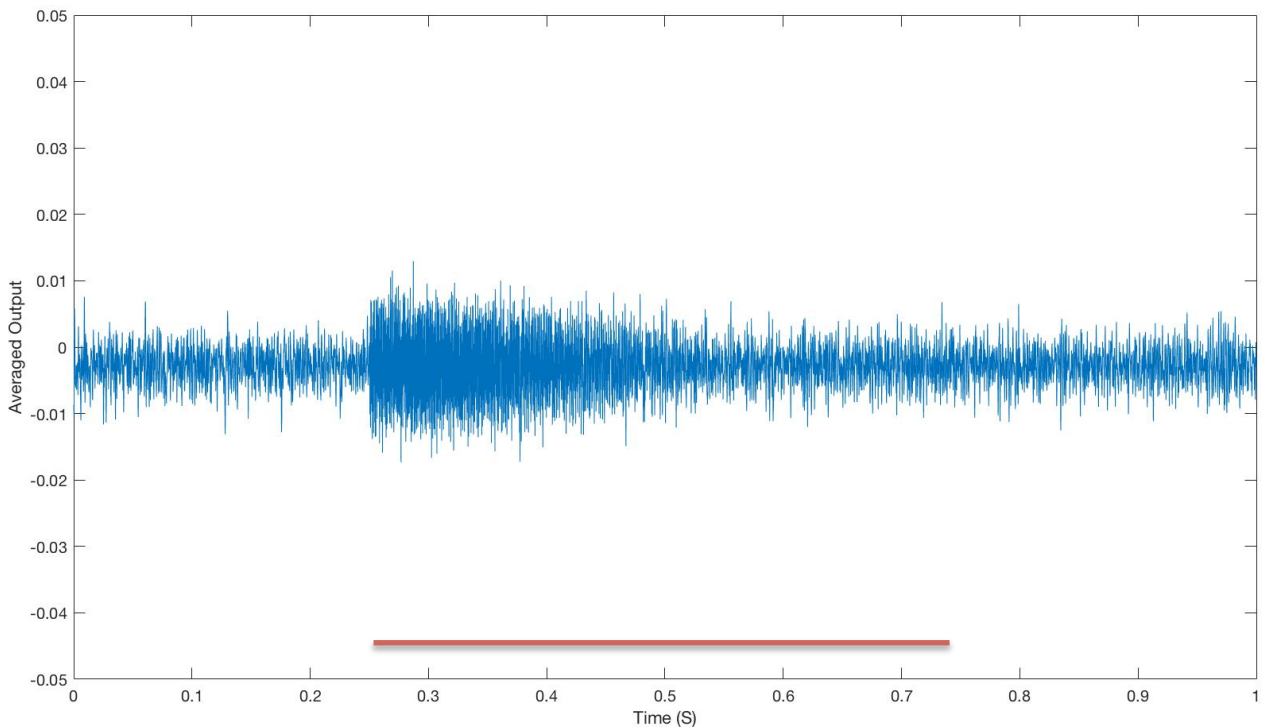


Figure 4: Signal averaged data from a silver wire hook electrode recording from the left connective of an *Anax junius* dragonfly at 2000Hz on 9/26/2017. The speaker was placed 19cm away from the head of the dragonfly at 180 degrees. An amplifier, frequency modulator, and LacChart7 produced a 500ms sound signal for 150 repeats with a 4 second delay between each stimulus. The 500ms sound signal along with the 250ms before and after the stimulus were recorded by LabChart7 for comparison. This graph was generated by originally written MatLab scripts for signal averaging. As seen by the graph the output from the electrode increases at the 250ms mark when the sound stimulus starts and decreases as the sound plays out. This is characteristic of a neuronal response.

Many repeats using the same process were conducted for frequencies of 100Hz – 16kHz at an amplitude range of 60 – 95dB. When the sound stimulus was presented the response picked up by the electrode was spot on showing responses to auditory stimuli and a lack of responses

with no sound (Fig.4). Higher amplitude sound stimuli seemed to increase the overall response during the sound. Directionality of the sound stimuli did not seem to change the response pattern for varying frequencies and amplitudes (Fig. 5).

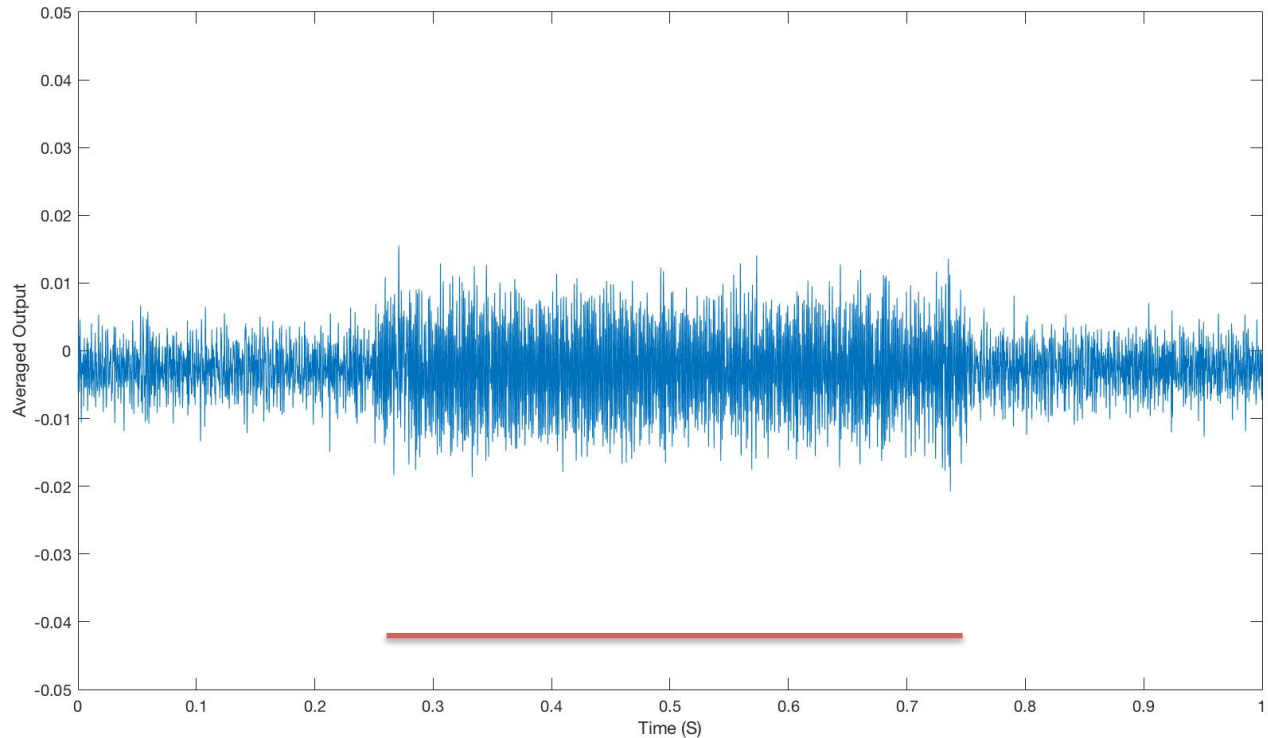


Figure 5: Signal averaged data from a silver wire hook electrode recording from the left connective of an *Anax junius* dragonfly at 1000Hz 45 degrees left of the animal on 9/26/2017. The same signal averaging process from (Fig.4) was used here. Left and right directionality did not seem to change the response pattern for varying frequencies and amplitudes. There was always a consistent response to sound stimuli of 500Hz – 8000Hz.

A glass suction electrode was used to look closer at the neuronal responses because of its higher sensitivity. The suction electrode was placed on the pro- or mesothoracic ganglion instead of being hooked around one of the connectives. The same signal averaging process was invoked for these recordings and a much greater amplitude response was found that was completely synced to the sound stimulus (Fig. 6).

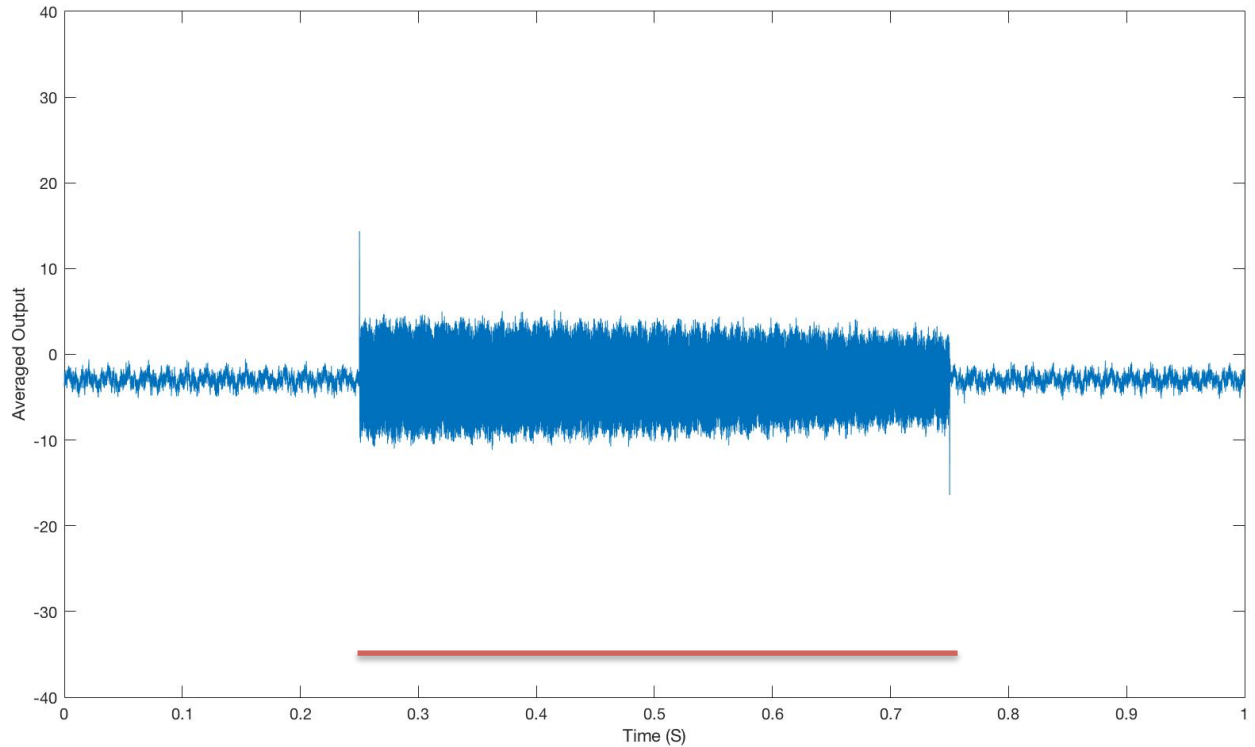


Figure 6: Signal averaged data from a suction electrode recording on T1 (thoracic ganglion) of an *Anax junius* dragonfly at 2000Hz on 10/3/2017. The speaker was placed 19cm away from the head of the dragonfly at 180 degrees. A 500ms sound stimulus was presented using an amplifier, frequency modulator, and LabChart7 with 150 repeats and 4 seconds between each stimulus. The 500ms sound stimulus and the 250ms before and after were recorded by LabChart7 and signal averaged using originally written MatLab scripts. From the graph you can see the output from the electrode increases at 250ms when the sound stimulus starts and ends exactly at 750ms when the sound stimulus ends. The y-axis limits are much greater than the ones for the silver wire electrode recordings because of the greater sensitivity of the suction electrode. This seems to display another neuronal signal to the sound stimulus.

B. Further Analysis Finds Flaws in Early Data

Further analysis on the recorded data was done to investigate this seemingly perfect sound response further using Fast Fourier Transforms.

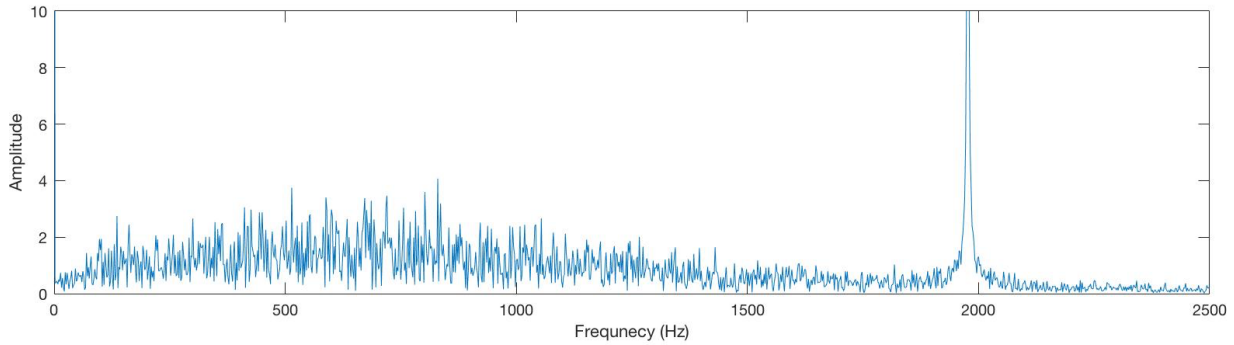
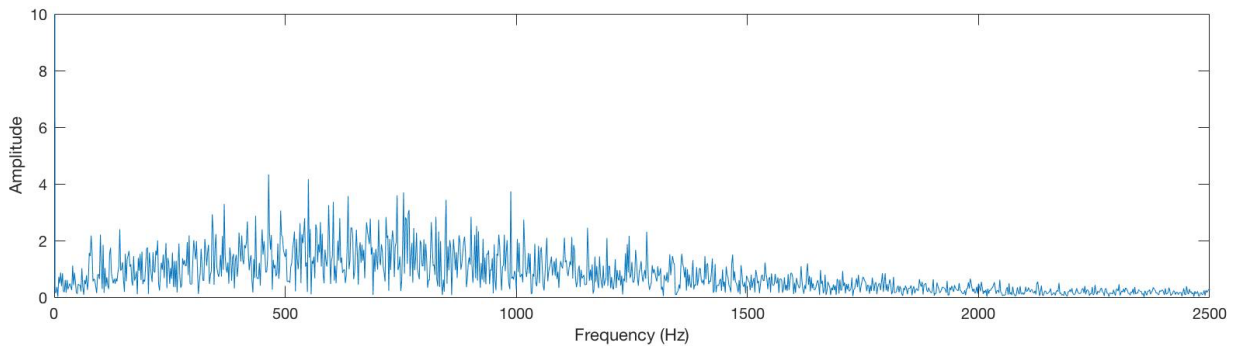
A**B**

Figure 7: Two Fast Fourier Transforms of silver wire electrode output data from the left connective of an *Anax junius* dragonfly stimulated with a 2000Hz sound stimulus on 9/26/2017. (a) Represents the FFT of the middle 500ms recording when the sound stimulus is on. A large peak at 2000Hz represents the exact frequency of the sound being played. (b) Represents the FFT of the before 250ms and after 250ms of the recording when the sound stimulus is off. These FFT's break up the input and output into their underlying frequencies. There is no obvious difference in these graphs so integrations for the areas under the curve were calculated. The integration value for (a) was 1.2245×10^3 , and the integration value for (b) was 1.0687×10^3 . Mathematically there was no significant difference meaning the output signal was not actually different when sound was on versus off.

Fast Fourier Transforms of the sound response data during the sound stimulus and without the sound stimulus did not show a significant difference (Fig.7). The integration values between the two FFT's were 1.2245×10^3 and 1.0687×10^3 respectively. The next step was to dive deeper and analyze the raw data as best as possible to see if there is any difference in the responses being picked up.

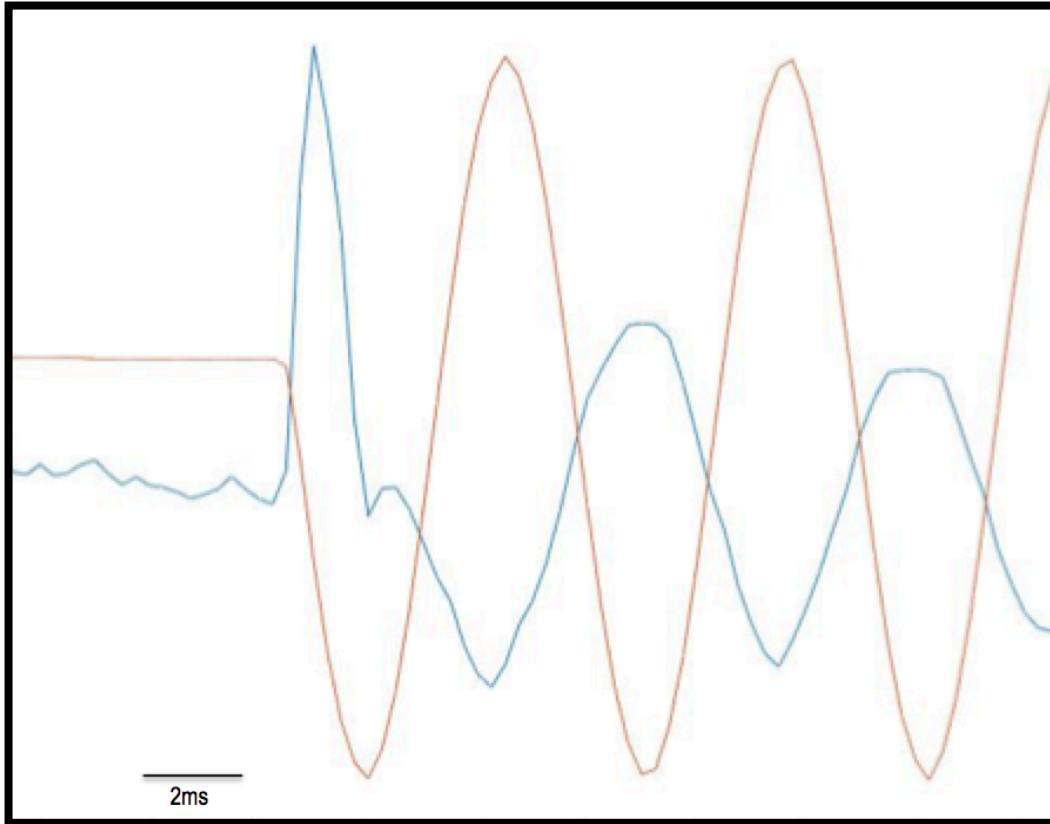


Figure 8: Signal averaged data from a silver wire recording of the left connective of a dead *Anax junius* dragonfly to a 2000Hz sound stimulus with the stimulus sine wave superimposed on the graph to a near-field sound on 10/10/2017. The tan line represents the sine wave and the blue line represents the electrode output. This graph shows that when the sound signal is on the electrode output is essentially phase locked with the sound meaning that the electrode is acting as an antenna and picking up signal directly from the speaker as well as the neural activity in the dragonfly connective.

Upon further analysis with the sound stimulus sine wave superimposed on the electrode output graph it was seen that the sine wave and response were phase locked indicating that there was direct transmission between the speaker and the electrode. So the next step was to use far-field sound stimuli.

C. Far Field Sound Data

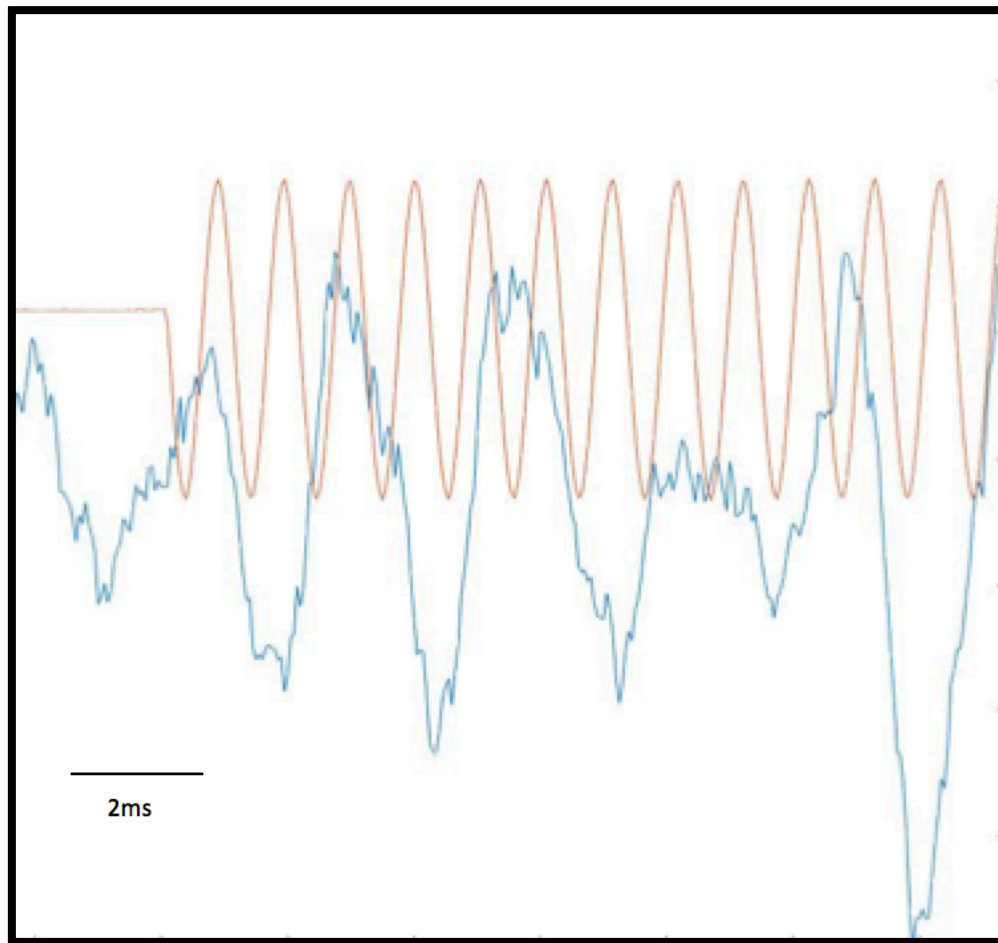


Figure 9: Sine wave from a 2000Hz sound stimulus superimposed on electrode output data to far-field sound. This figure shows that the sine wave is no longer phase locked with the electrode meaning the direct transmission between the speaker and electrode has been overcome.

Far-field sounds could be produced outside of the range direct speaker-to-electrode transmission by placing the speaker on a portable cart and using higher frequency sounds. By doing so the sine wave generated from the sound stimuli no longer directly transmitted to the electrode (Fig. 9).

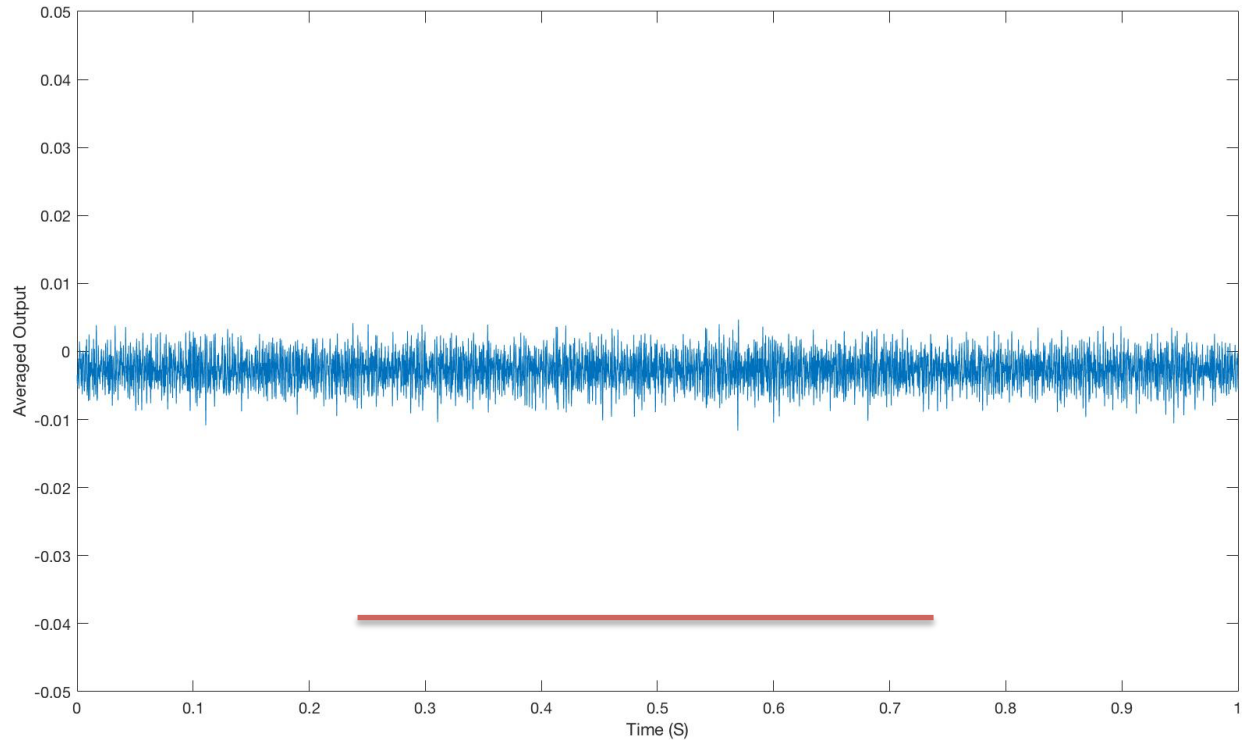


Figure 10: Signal averaged data from a silver hook electrode recording on of an *Anax junius* dragonfly at 2000Hz on 10/10/2017. The speaker was placed 2 meters away from the head of the dragonfly at 180 degrees. A 500ms sound stimulus was presented using an amplifier, frequency modulator, and LabChart7 with 150 repeats and 4 seconds between each stimulus. The 500ms sound stimulus and the 250ms before and after were recorded by LabChart7 and signal averaged using originally written MatLab scripts. From the graph you can see that there is no difference from when the sound is on (indicated by the red line) and when the sound is off. This was measured over and over from different angles using different frequencies and shows no indication of a sound response in dragonflies.

In order to reevaluate the findings up to date, behavioral experiments were run using a tethered dragonfly recorded by a video camera to watch for operationally defined movements in response to sound stimuli (See Appendix).

D. Reevaluating With Behavioral Testing

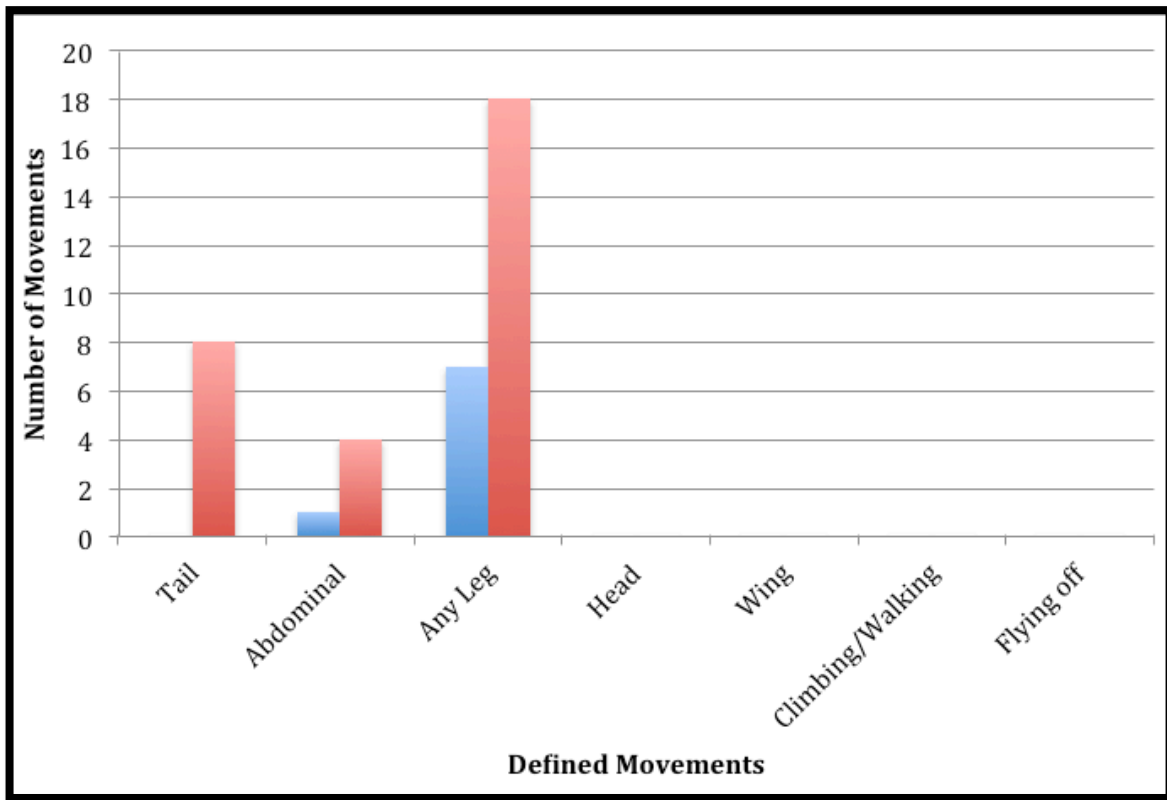


Figure 11: Behavioral movements to near-field sound stimuli in an *Anax junius* dragonfly to 200Hz and 100Hz sound on 1/5/2018. The dragonfly was tethered to an upright pole on a table with the speaker approximately 1 meter away. The whole procedure was recorded on a video camera and analysis of movements to the 3 second sound stimuli compared to the 3 seconds prior to the sound stimuli were done frame by frame using the video recording. The sound was repeated 10 times for each condition with 20 seconds in between repeats. The red bars indicate movements during the sound stimulus and the blue bars represent movements without the sound stimulus. Movements were operationally defined prior to the experiment. This data set shows that movements during the sound were much more common than without the sound. This difference was significant ($p < 0.05$)

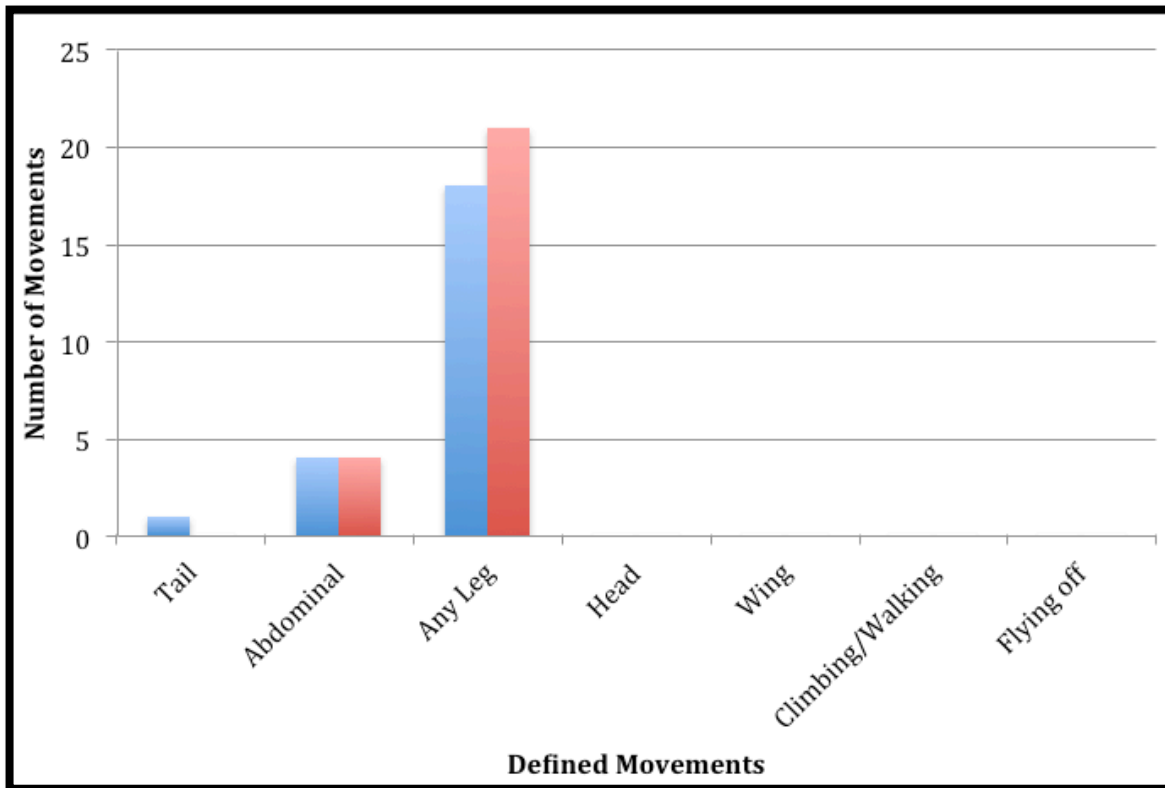


Figure 12: Behavioral movements to far-field sound stimuli in the same *Anax junius* dragonfly to 200Hz sound for comparison of movements on 1/5/2018. The same set up was used as before except the speaker was placed 2 meters away from the animal to get out of the near-field for the 200Hz sound wave (100Hz could not be used because its wavelength is too long). This data set shows a much more even distribution of movements of sound on versus sound off in the far-field. This difference was not significant ($p > 0.05$). This further indicated that dragonfly hearing may be mechanosensory and regulated by the physics of sound. A chi-squared test was also run for this data set compared to the near-field data and the value was 0.00 for near-field, and 0.736 for far-field. This suggest that for this one animal it is definitely significant that it moves to near-field sound more than far-field sound.

A single dragonfly was used for each near-field vs. far-field test and a total of three dragonflies were used for this analysis. Movement during near-field sounds compared to no sound was significant (Fig. 11, $p = 0.000513$). Movement during far-field sound compared to no sound was not significant (Fig.12, $p = 0.19229$). A chi squared run to compare the two tests was highly significant for near-field movements to sound (0.00) and not significant for movement to far field sounds (0.736). These statistical tests were only run for three animals so they only show a possible trend and not a conclusion.

E. Finding a True Far Field Sound Response

Next the set up for electrophysiology was remade using the same techniques except for the use of the stimulator panel within LabChart7 to manually control when sound stimuli were played instead of being run on a timed circuit.

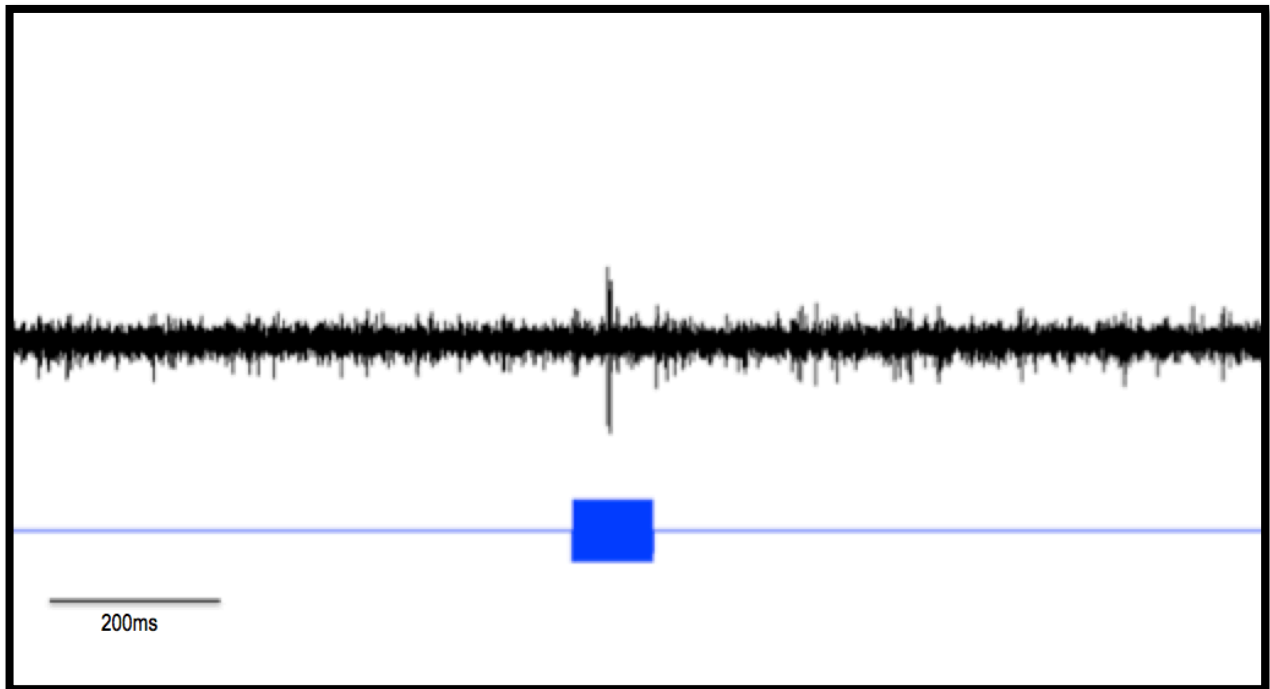


Figure 13: Silver wire hook electrode recording of the left connective in an *Anax junius* dragonfly with 1000Hz sound in the far-field on 3/15/2018. The same set up was employed except we moved to manual stimulation using the stimulator panel in LabChart7 so we could control when the sound was played so the animal was not moving when the stimulus was played. The small speaker was placed 40cm behind the animal at amplitude of 0.15V. The sound level at the head of the animal was approximately 75dB. This is much less than some of the previous experiments so the sound response does not have a very high threshold. This raw data shows a very characteristic neural response to a 100ms sound stimulation. Upon further zooming and using the marker the response was found to have a 40ms latency, which is characteristic of dragonfly neural activity.

Raw data abstracted from the most recent recordings showed a consistent and characteristic neuronal response to far-field sound stimuli. The latencies for these large spikes were between 40-50ms behind the onset of the sound (Fig. 13). An originally written Sound Clipper MatLab script extracted the recorded signal in a narrow time window surrounding the stimulus presentation from this raw data and graphed it to enhance the spiking activity within the electrode output (Fig. 14).

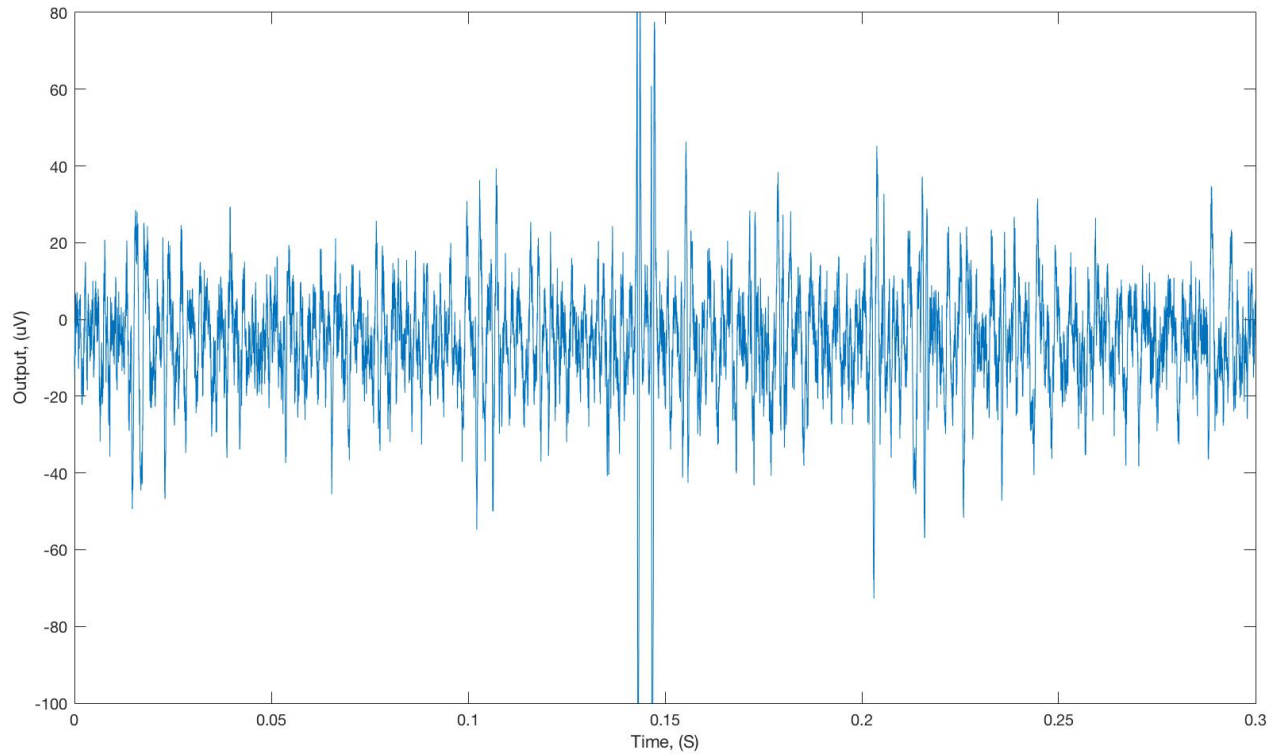


Figure 14: Sound clipped data from a silver wire hook electrode recording of the left connective in an *Anax junius* dragonfly to 1000Hz sound on 3/15/2018. This graph corresponds to the raw data in figure 9. (a) Graph of raw data obtained using an originally written MatLab script. The sound stimulus was 100ms and started at 0.1S on the graph and went until 0.2S. This data shows a clear sound stimulus just like the raw data but with an arbitrary threshold selected showing that this response is clearly different from anything else in the plot.

The most recent data was presented for frequencies between 500-1000Hz in the far field and consistently showed characteristic neuronal spiking activity to sound stimuli.

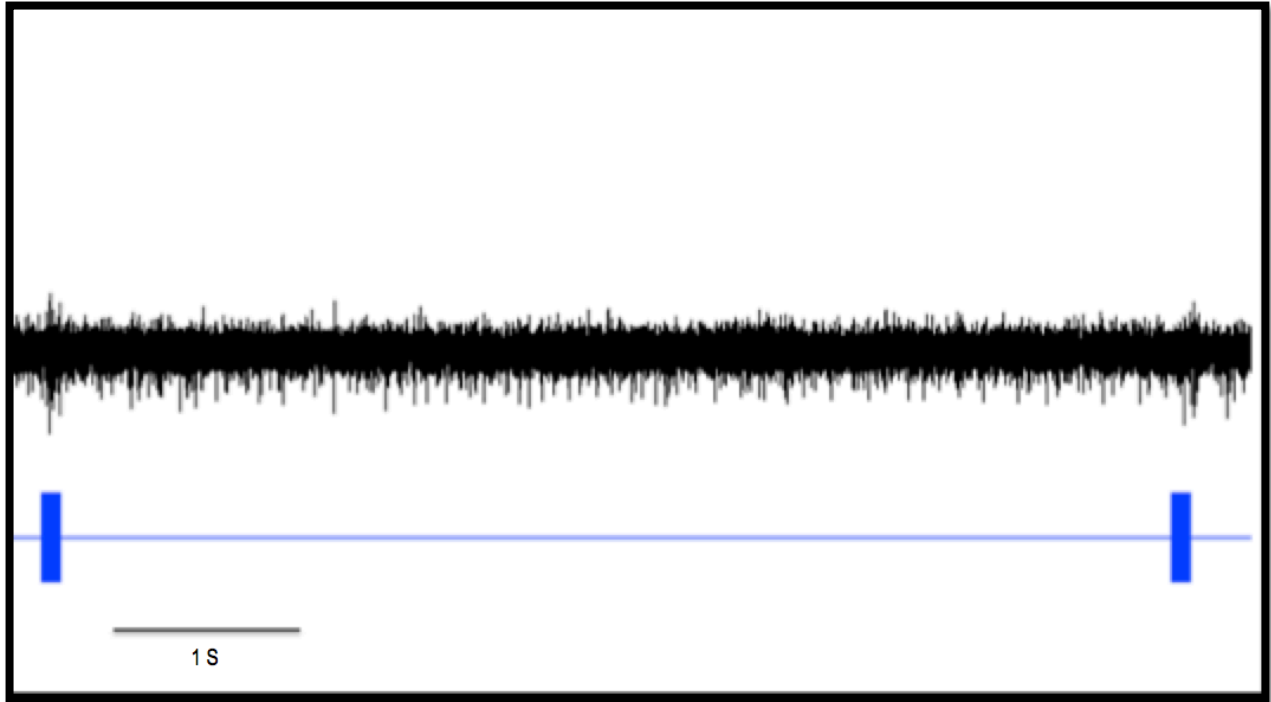


Figure 15: Silver wire electrode recording of the left connective in an *Anax junius* dragonfly with 1000Hz sound in the far field on 3/15/2018. The speaker was placed 40cm behind the animal at an amplitude of 0.15V. The sound level at the head of the animal was approximately 75dB. This zoomed in data shows two manual stimulations in succession and the clear increase in the baseline of neural activity when those stimulations happen. This raw data just further shows how the neural signal is occurring due to sound.

The final goal of this study was to try and locate an external ear or the source of dragonfly audition if there was one. Early attempts at locating such a source on dragonflies was done by using Vaseline and covering sections of the body to see if there was a decrease in neuronal sound response. Figure 16 shows one of these trials with Vaseline applied to all sides of the thorax and the wings, legs, head, and lower abdomen cut off.

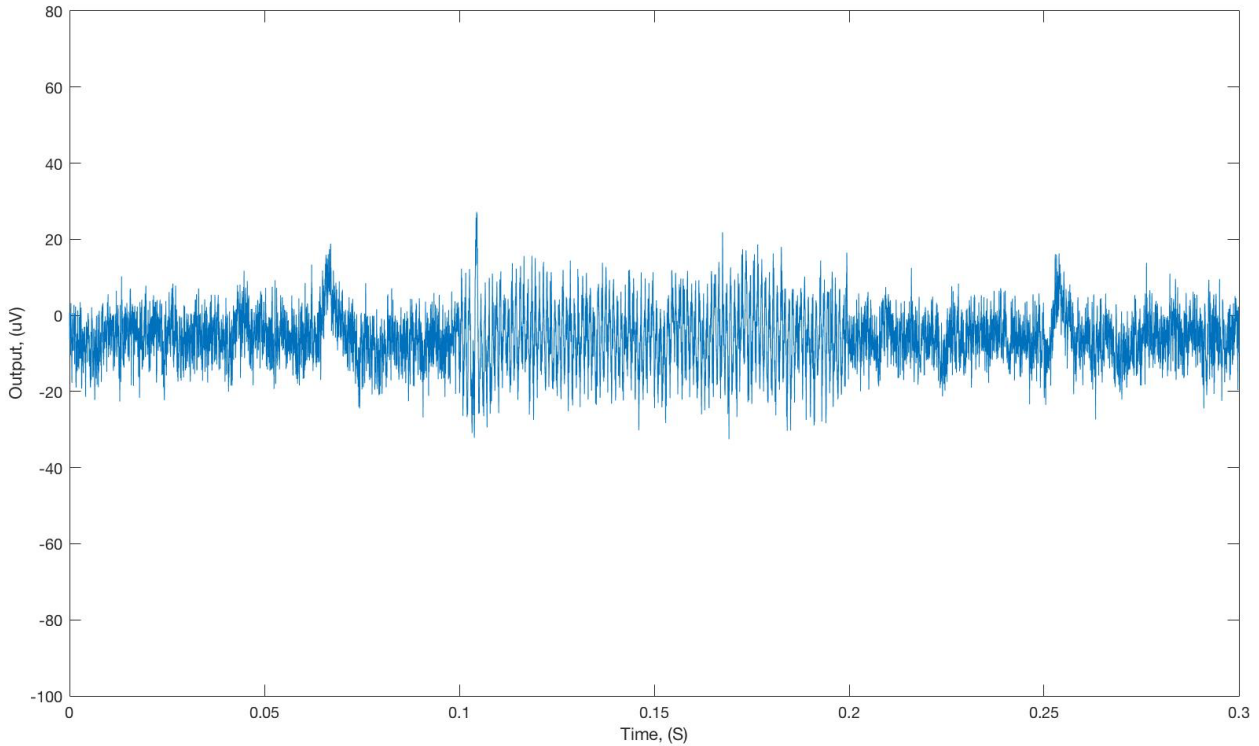


Figure 16: Sound clipped data from a silver wire electrode recording of the left connective of an *Anax junius* dragonfly with cut off lower abdomen, wings, head, and Vaseline applied to sides of the thorax on 3/15/2018 with 1000Hz sound. We still found a response with the wings and lower abdomen cut off, but with both of those manipulations and Vaseline applied to all sides of the thorax this is the response seen. It is a much lower amplitude and is very similar in size and shape to the sine wave seen when the animal is dead. This suggests that the ear for the dragonfly is somewhere on the thorax.

Neuronal responses seemed to still exist with many manipulations to the animal. So an animal's nervous system was killed using a formalin and methanol mixture and sound stimuli were played once again to see if anything was picked up.

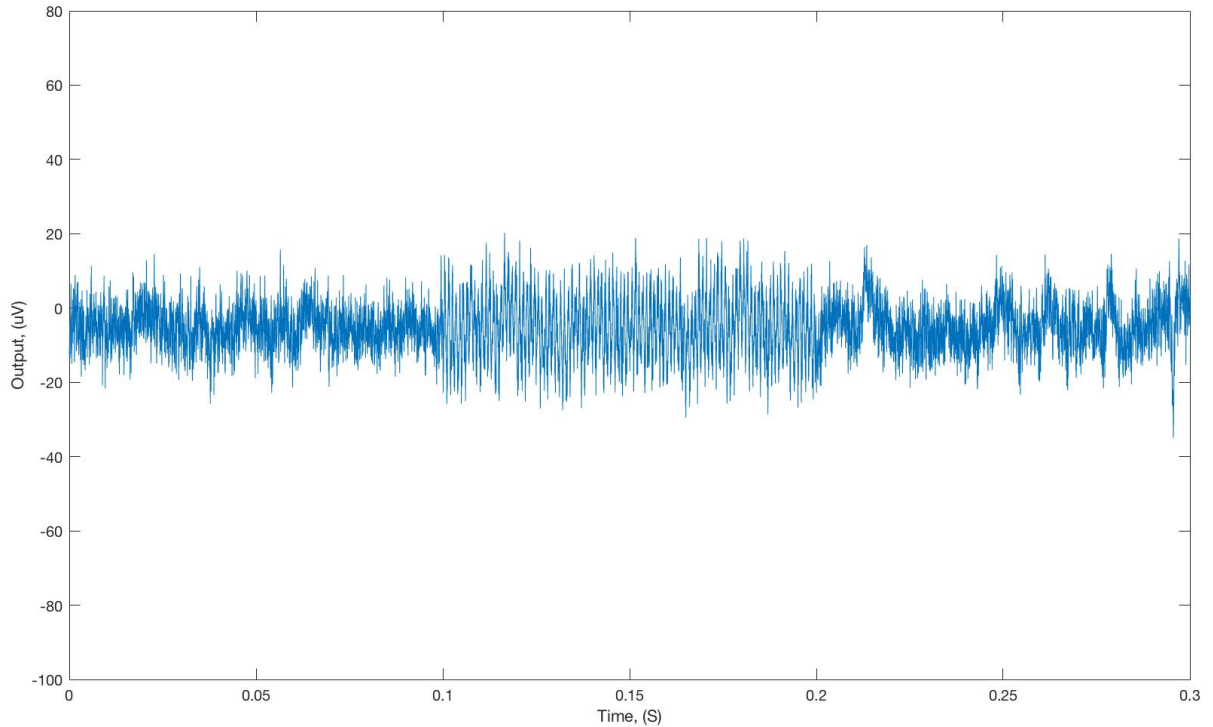


Figure 17: Sound clipped data from a silver wire electrode recording of the left connective of a dead *Anax junius* dragonfly to 1000Hz sound on 3/15/2018. The animal's nervous system was killed using formalin and methanol and then the same sound stimulation was manually played to see what the electrode picked up. This graph shows that there is slight direct electrical communication between the speaker and the electrode but much less than what we had in earlier experiments. This further suggests that our data is from only neural responses to the sound stimuli and not from outside sources.

Figure 17 shows that even with a dying nervous system there was still slight imbalance on the baseline that could be some minor electrical interference from the speaker and the falling off sound wave over distance.

V. Discussion

Over the past few decades with new technologies and heightened research in the field, many new insect species have been found to have a nervous system equipped to process auditory stimuli where as, traditionally, many insects have been thought to not hear at all. Last year student Andrew Hamlin and Professor Robert Olberg recorded neuronal responses to auditory frequencies in the ventral nerve cord of a dragonfly (Fig.1) but their results were not published. The current study followed up on these brief findings and investigated a sensory modality that was previously unknown in dragonflies, the sense of hearing. Entering this study the hypothesis was that dragonflies do have an auditory sense using an external/tympanic hearing system.

A. The Process of Finding a True Auditory Sense

To investigate an auditory sense in dragonflies we used electrophysiological and behavioral techniques that had been used in prior insect audition research (Yager and Fenton, 1990, Roeder, 1967, and Hoy, Nolen, and Brodfuehrer 1989). At the start, electrophysiological studies were employed using a silver wire hook electrode and computer generated sine wave sound stimuli recording from one of the connectives in the nerve cord. Recording here is a great starting point because if dragonflies do have an external/tympanic ear then the transmission through the nervous system will mostly likely go upstream from the nerve cord to the brain or downstream from the brain through the nerve cord to the body. Either way it was the best chance to record neuronal transmission if there was an auditory sense in these animals. This original data was signal averaged over 75-150 repeats to eliminate noise and variable responses that the electrode picked up so only consistent signals into the electrode were emphasized. If dragonflies responded to sound stimuli then these responses would be consistent over many trials and be obvious on the signal averaged data. At first this is what seemed to be occurring with large

activity at onset of the sound stimulus that decreased over time (Fig.4). Many trials with sine wave stimuli between 500 – 8000Hz were completed and regularly showed the same activity pattern with a lot of activity at the onset of the sound that either decreased over time or continued until the stimulus stopped, both are possible neuronal responses. There was no directional difference for sound responses (Fig.5) from the left, right, back, or front of the animal meaning that if this was a true sound response than the source of this auditory reception may be all over the animal or centralized pretty well on the body. There was a range of sound amplitudes to elicit a response. Sound stimuli from 60 – 95dB consistently elicited activity the best. Sound stimuli under 60dB were often too quiet and sound stimuli greater than 95dB were deafening and could create a sensory overload for the animal that could interfere with the response we were looking for.

Next we replaced the silver wire hook electrode for a glass suction electrode and placed it on one of the thoracic ganglion. The suction electrode has a much greater sensitivity so the Y-axes are much larger for the graphs. The suction electrode data was also signal averaged over 75-150 repeats and showed a near perfect onset and stop to sound stimuli for the whole 500ms duration (Fig.6). This data seemed almost too perfect and it occurred to us that there was no obvious neuronal latency for this response. We decided to examine this data further using Fast Fourier Transforms of the signal-averaged data. The FFT's basically took apart the response the electrode was picking up and broke it up into the underlying frequencies. By comparing FFT's when the sound stimulus was on and when the sound stimulus was off it was obvious that the only difference was the large peak of the exact sound stimulus frequency (Fig.7). We took the integration values to see if there was any mathematical difference not visible to the naked eye and we found that there was no significant difference. These analyzed results created some worry

because the activity and responses seen from the original signal averaged data may not be auditory responses at all. We went back to the raw electrode output data and superimposed the sine wave sound stimulus on the responses the electrode was picking up and found that the two were phase locked. As soon as the sine wave sound stimulus started the output from the electrode started and its peaks were in sync (Fig.8). We figured out that the problem was physics. In all of the original electrophysiology recordings the speaker was approximately 19cm away from the animal containing the electrode. With this comes two problems, one is that the speaker itself produces an electrical field because of the magnet and coil within the speaker. It is hard to tell how far this electrical field extends but with some brief research we concluded that it may be anywhere from 10 – 30cm. The second problem is that a lot of the sine wave sound stimuli being produced were frequencies from 500 – 4000Hz. The wavelengths for these sound stimuli were from 68 – 34.3cm (wavelength = the speed of sound (343m/s / frequency)). This means that many of the sine wave sound stimuli played created near field sound effects. A sound wave travelling through a medium has two areas, the near field and the far field. The near field is where a sound wave is circulating and propagating in the medium whereas the far field is where the sound wave is just propagating in one direction. Where the sound wave transitions from near field to far field is approximately one wavelength from the sound source of a particular frequency. Comparatively the loudness of a sound decreases throughout the far field and is constant in the near field (Hansen, 2001). The near field sound can act directly on the animal as a “feeling” like a gust of wind especially with all of the mechanosensory hairs that dragonflies have on their bodies. If this was occurring then even if there was an auditory response it may be caused from mechanoreception to the sound wave and not from actual external/tympanic ear hearing.

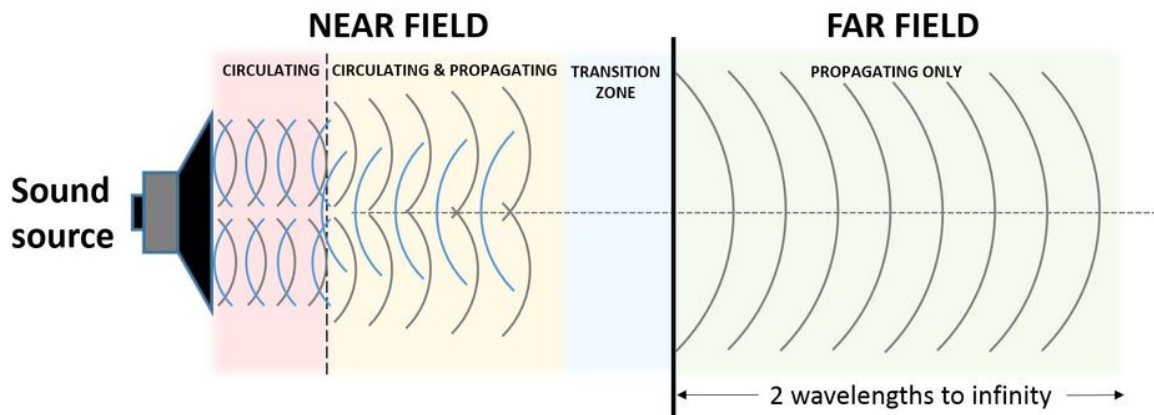


Figure.18: Example of the two distinct areas of a sound wave travelling through a medium. If near field sound waves were creating the activity response that was seen in the original recordings then the auditory response could be purely mechnosensory instead of a true hearing response.

<https://community.plm.automation.siemens.com/t5/Testing-Knowledge-Base/Sound-Fields-Free-versus-Diffuse-Field-Near-versus-Far-Field/ta-p/387463>

To solve these physics problems, the speaker was moved well out of the range of near field sound and the electrical field of the speaker. The same techniques were employed using a silver wire recording and signal averaged data, and this new method did negate the interference of the speaker and near field sound waves (Fig.9), but it was not producing any kind of visible response to the auditory stimuli in dragonflies (Fig.10). Repeated trials with no obvious response or activity to auditory stimuli made us take a step back. We decided to employ behavioral methods using a tethered dragonfly that was free-flying and far-field/near-field sound to see if there was any kind of behavioral/movement differences. Behavioral testing would show that if there was consistent activity/responses to auditory stimuli then something had to be happening in the nervous system to process those stimuli. The same animal was used for near-field and far-field sound stimuli to accurately compare them. This was done with 3 separate animals recording their behaviors to a 3 second sound stimulus compared to the 3 seconds prior to the sound stimulus. These tests were recorded with a Sony RX-10 camera and then operationally defined movements were sought out frame by frame from the recordings. Overall it was found that near

field sound stimuli invoked movements significantly more than far field stimuli (Fig.11 and Fig.12). These tests were only done for a few animals and cannot be generalized as a conclusion but only as a possible indication. Even though it was a small sample size it brought up the possibility that auditory stimuli may only activate mechanosensory hairs and not a true hearing sense.

It was beginning to look like the dragonfly auditory sense we were after was being mediated by mechanoreception. Mechanoreception in dragonflies has been well documented due to the many sensory hairs throughout their body and if the near field sound waves were just activating some of these sensory hairs then the air molecules displacing these hairs were acting no differently than wind puffs and other mechanosensory stimuli (Barsagade, Thakr, Gathalkar, and Kirsan, 2017 and Olberg, 1980). In a last effort to see if this was true the electrophysiology set up was remade and we used the stimulator panel on LabChart7 to manually produce the computer generated sound stimuli instead of them being run on closed time circuit. This allowed us to control when the sound was being played so we could make sure the animal was not restless or moving during the sound stimulus as sometimes occurs because the dragonfly is immobilized on a stand and awake. The new experiments were also conducted in two different rooms on two different set ups to control environmental effects on the recordings. From these new recordings we first looked at the raw data of electrode output with the matched up sound stimulus and found what we were initially looking for. There was a very consistent and characteristic spiking activity to far field sound stimuli with a response latency of 40-50ms that is highly characteristic of dragonfly neuronal responses (Fig.13). This was done many times with the same results. Next we took this raw data and used a Sound Clipper MatLab script that took an arbitrary threshold and extracted the recorded signal in a narrow time window surrounding the

stimulus presentation. This amplified the response coming into the electrode and presented the spiking activity in a clear way over time (Fig.14).

Overall what we found at the end of this study was consistent and characteristic neuronal responses to far field sound stimuli in dragonflies. From our data this means that dragonflies may have an external/tympanic hearing system similar to humans or insects like the preying mantis. But why did this take so long to find?

B. Limitations of This Study and Future Work

There has not been a thorough examination of dragonfly audition in the scientific literature to date. Because many insects had traditionally been found to not have a sense of audition many researchers have ignored this sensory modality in their research. The techniques for this study came from classical electrophysiological tests that have been used to study many different sensory modalities in insects and from some pioneering research in insect audition (Yager and Fenton, 1990, Roeder, 1967, and Hoy, Nolen, and Brodfuehrer 1989). Furthermore this study had to overcome some problems in data collection and analysis. In original recordings we were using near-field sound and were within the electrical field of the speaker as was previously discussed. However another problem at the start was signal averaging. What we have found throughout this study is that this auditory sense is somewhat subtle and overpowered by more of the major sensory modalities of the dragonfly. Signal averaging the original data even when we employed far-field sound and found nothing (Fig.10) eliminated small varying activity picked up by the electrode. The animal sometimes moved on and around the sound stimulus so a small auditory sense that is overpowered would easily be lost in the signal averaged data done over many repeats.

One major problem that affected how this study proceeded was the Vaseline job needed for each electrophysiology recording. Once the electrode and ground were placed within the animal the entire area had to be filled with Vaseline so the connectives did not touch and that no saline or bodily fluid touched the electrode. This was extremely hard because it had to be done by hand in a very small and delicate area. If there was not a perfect Vaseline job then the output to the electrode was not clear and resulted in a possible loss of this subtle response to be seen anywhere in the data.

Another possible limitation of this study came from using the manual stimulator panel with the new data instead of a timed circuit. This could create experimenter bias because we controlled when each sound stimulus was played but it was also necessary to eliminate movement problems that resulted in the overpowering of this subtle auditory response. One thing that we have still not figured out is why there was a fall off in the response over time in some of the original recordings that had the direct electrical transmission of the speaker to electrode.

Future work for this study would be to locate the external ear source used for this auditory sense in dragonflies. That is the ultimate goal because if done then there is no doubt that dragonflies have an external/tympanic hearing system that has been found in other insects like the preying mantis. This study started to explore this problem but was limited with time. In preliminary experiments we covered parts of the body with Vaseline and cut other parts off to see if there was a decrease in the sound response. What we found is that with the wings, lower abdomen, and head cut off along with the thorax covered in Vaseline that there was a decrease in the sound response (Fig.16). There was still an increase on the baseline however so we killed the animal's nervous system with a formalin/methanol mixture and found this same disturbance in the baseline. This means that in these isolation experiments there was a small interference

between the speaker and the electrode (Fig.17) but nothing that would affect the large spiking activity seen from the auditory responses. Rather what was seen is that the (Fig.16) isolation experiment and the dead nervous system baselines (Fig.17) were very similar. This could possibly mean that the external ear source may be on the thorax of the dragonfly because it was the last manipulation between a sound response and a dead nervous system to the same auditory stimuli.

In summary this study suggests that dragonflies do have a nervous system equipped to process auditory stimuli using an external/tympanic hearing system (Fig.13, Fig.14, and Fig.15). This is a subtle sense that took a lot of trial and error to confirm. This study adds to the existing literature on insect audition and opens up a new chapter for audition in dragonflies that should be continued to explore this new sensory modality in these creatures.

VI. Acknowledgments

First I would like to thank Professor Robert Olberg for allowing me to conduct research under him in his laboratory and for all of his assistance and guidance that allowed me to conduct this study. I would also like to thank Union College for a grant to buy necessary equipment for this study as well as the National Science Foundation. We would not have been able to conduct this research without it. I would also like to thank Caroline Roberto and Nicholas Garcia for laboratory help and communication throughout the year. Finally I would like to thank Professor Stephen Romero for his assistance and guidance in statistical tests.

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VIII. Appendix

MatLab Data Analyses Scripts

1.) Signal Averaging

```
nb = 76 % Enter the number of blocks to average;

frequency = '1000Hz';

blockarray = zeros([40000,nb]);

for blocknum = 1:nb;

    blockname = ['C1B' num2str(blocknum)] ;

    data = eval(blockname);

    blockarray(:,blocknum) = data;

end;

averagetrace=sum(blockarray)/nb;

times = [1:40000]/40000;

plot(times,averagetrace)

ylim([-0.1 0.1]);
title('9/26/2017 1000hz', 'Color', 'm')
xlabel('signal time')
ylabel('output')
```

2.) Fast Fourier Transforms

```
nb = 150; % Enter the number of blocks to average;

frequency = '2000Hz';

lowerlim = 10001; %limits for 500msec of data, change accordingly
upperlim = 30000;
usedata = 20000;

blockarray = zeros([usedata,nb]);

for blocknum = 1:nb;

    blockname = ['C1B' num2str(blocknum)] ;
```

```

    data = eval(blockname);
    datatrim = data(lowerlim:upperlim);  %"datatrim" gives middle 500msec

blockarray(:,blocknum) = datatrim;

end;

averagetrace=sum(blockarray')/nb;

times = [1:40000]/40000;

signal = averagetrace;

Fs = 40000;
stim = abs(fft(signal));
    % assume x is even length
    stim = stim(1:length(signal)/2+1);
    freq = 0:Fs/length(signal):Fs/2;
    figure
    subplot(2,1,1);
    plot(freq,stim);  %plot middle 500msec
    ylim([0 10])
    xlim ([0 2500])
    xlabel('Frequency (Hz)');
    ylabel('Amplitude');
    title('FFT of 500ms Sound Signal')

blockarray = zeros([40000,nb]);

for blocknum = 1:nb;

    blockname = ['C1B' num2str(blocknum)] ;

    data = eval(blockname);

blockarray(:,blocknum) = data;

end;

averagetrace=sum(blockarray')/nb;

times = [1:40000]/40000;

signalb = averagetrace([1:10000 30001:40000]);

Fs = 40000;
nostim = abs(fft(signalb));
    % assume x is even length
    nostim = nostim(1:length(signalb)/2+1);
    freq = 0:Fs/length(signalb):Fs/2;
    subplot(2,1,2);

```

```

    plot(freq,nostim);    %plot first 250msec and last 250msec
    ylim([0 10]) ;
    xlim ([0 2500])
    xlabel('Frequency (Hz)');
    ylabel('Amplitude');
    title('FFT of 250ms Before, 250ms After Sound Signal')

area_stim = sum(stim (1:1500));    %1.0912e3

area_nostim = sum(nostim (1:1500)); %area under the curve up to 1500,
excluding sound peak
                                %1.2670e3

stim_hist = zeros(1,50);

nostim_hist = zeros(1,50);

for n = (1:50)

    stim_hist(n) = sum(stim((30*(n-1)+1):n*30));
end

for n = (1:50)

    nostim_hist(n) = sum(nostim((30*(n-1)+1):n*30));
end

figure
plot(stim_hist) %loop: 1-50, 51-100,etc of areas under curve finding
difference
ylim([-1 10])

figure
plot(nostim_hist)
ylim([-1 10])

diff_plot = (stim_hist - nostim_hist);
figure
plot(diff_plot)
ylim([-1 10])

3.) Sound Clipper
% Find sound starts.
close all

isi = 1;
samplerate = 40000;

```

```

threshold = -0.1;
SoundChannel = '2';
TraceChannel = '1'
Block = '3';
cliplengthsec = .3
cliplength = cliplengthsec*samplerate
TimeBefore = .1; %in seconds
TimeAfter = .1; %in seconds
StimLength = .1; %in seconds
BlockName = ['C' SoundChannel 'B' Block];
TraceBlock = ['C' TraceChannel 'B' Block];
sounds = eval(BlockName);

traces = eval(TraceBlock);
negind = find(sounds<threshold); %finding below threshold before sound (neg
index)
negminus = zeros(length(negind),1);
negminus(2:length(negind)) = negind(1:length(negind)-1);
negminus(1) = 1;
diffs = negind-negminus;
startsind = find(diffs>1000); %start of sound is where threshold breaks
starts = negind(startsind);
starttimes = starts/samplerate;
cliplength = (TimeBefore+TimeAfter+StimLength)*samplerate;
cliplength=12000;
clips = zeros(cliplength,length(starts));

for i = 1:length(starttimes);
    startpt = starts(i)-(samplerate*TimeBefore);
    clips(:,i) = traces(startpt:startpt+cliplength-1);
end

stim = eval(BlockName);
sine = stim(starts(1): starts(1) + StimLength*samplerate-1);
sinescaled = sine*133*-1;
x = (1:cliplength)/samplerate;
before = zeros(TimeBefore*samplerate,1);
after = zeros(TimeAfter*samplerate,1);
takeaway = [before' sinescaled' after]';

for j = 1:length(starts);
    h = figure
    plot(x, clips(:,j));
    ylim([-100,80])
    ylabel('Output, (uV)');
    xlabel('Time, (S)');
    t = title(['Electrode output as a Function of Time ', num2str(j)],
'FontSize', 12);
end;

corrclips = zeros(cliplengthsec*samplerate,length(starts));

```

```

for i= 1:length(starts);
corrclips(:,i) = clips(:,i)-takeaway;
end

for j = 1:length(starts);
h = figure
plot(x, corrclips(:,j));
ylim([-100,80])
ylabel('Output, (mV)');
xlabel('Time, (ms)');
title(['Corrected Electrode output as a Function of Time ' num2str(j)],
'FontSize', 14);
end;

```

Operationally Defined Movements for Behavioral Tests

- Tail Movement = any movement where the tail moves independent of the rest of the body, this includes a flexion or extension up from the bodyline.
- Abdominal/body segment movement = any movement where the middle abdominal body segments move up, down, or to the side. These movements can cause other parts of the body to move as well but an abdominal segment movement must be the root.
- Leg movement = when any of the legs moves more than a twitch. Must be a full leg movement of a bend at the joint or a swing of the leg. This movement also includes swiping at the head that can make the head move as well, but the movement will only be counted as a leg movement.
- Head movement = head moving in any direction or twisting independent of the legs. A leg movement that swipes at the head (usually to clean the eyes) is counted as a leg movement.
- Wing movements = movements where the wings flap independent from another body movement. So an abdominal movement that moves the wings up is only an abdominal movement.

- Walking/climbing pole movements = any movement where the dragonfly moves itself on the pole in a direct walking or climbing fashion to change positions.
- Flying away = a movement where the dragonfly releases from the pole and attempts to fly off in normal flying behavior.