**Title & Abstract**

**Introduction**

Undergraduate neuroscience programs are mushrooming and produce a substantial number of life sciences graduates in the US (Ramos 2016). Although neuroscience is growingly an attractive major choice for students and institutions alike, course instructors and students both still face the challenge of incorporating meaningful and effective hands-on learning experiences due to lack of budget and engaging teaching tools.

Aiming to build enthusiasm for the principles of neurophysiology in the undergraduate classroom, here we provide a tractable electrophysiology procedure using open-source and low-cost electrophysiology equipment from Backyard Brains (BYB, backyardbrains.com) to extracellularly record and analyze the descending contralateral movement detector (DCMD) neuron that underlies grasshoppers’ motor sensitivity to looming monocular visual cues.

The lab activities presented in this paper can guide students from an observational question of “why are grasshoppers hard to catch?” to answers regarding their escape abilities using eyesight to detect dangerous objects and their powerful hind legs to jump away. Then as the final destination to the original observation, in the neuroscience context, students can explore the very neurons responsible for this see-and-jump escape mechanism and those neurons’ electrical transmission of visual information.

We choose the grasshopper for ease of obtaining and handling. In some locations in the world, grasshoppers are available for sale for cheap. They can also be collected in the field, and this activity may allow for concrete observations of the insects’ escape mechanism. Moreover, there has been substantial publications on the motion detector neurons in locusts, a kind of grasshoppers that forms swarms, showing the reproducibility of the DCMD neuronal signals when the locusts were presented with simulated looming objects.

As known, the ability to avoid collision with surrounding objects or escape a predator is crucial to the survival of an animal and requires the visual detection of approaching stimuli and a motor reaction. In the grasshopper visual system (Fig. 1), two pairs of well-studied monocular motion-detecting neurons, lobula giant movement detectors (LGMDs) and the descending contralateral movement detectors (DCMDs), underlie the animal’s ability to jump away to escape a potential predator or avoid collision with a looming object. The LGMDs reside in the optic lobe and are excited by approaching visual stimuli. In turn, they send the neural signals to their postsynaptic targets, the DCMDs, which respond to object movements detected by the contralateral eye and activate motoneurons in the thoracic ganglia appropriate to produce the jumping reaction. The LGMDs and DCMDs together make up an early warning system to generate the escape behavior in the face of possible collision with approaching objects.

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| **Fig 1. Neuroanatomy of the grasshopper’s motion detector neurons.** The grasshopper optic lobes (lamina, medulla and lobula) lie in the central brain beneath each of the compound eyes and processes visual information. The lobula giant motion detectors (LGMDs) receive visual signals from the lobula and pass these inputs to the descending contralateral motion detectors (DCMDs). The LGMDs respond to motion of an object seen moving across the insect eye as well as to the looming effect, where the object increases in size as it approaches the eye. The DCMD activates motor neurons in the thoracic ganglia. These primary motion detector neurons underpin the animal’s detector and motor response to a looming object, forming the see-and-jump escape mechanism. |

We performed extracellular recordings of the DCMD response to approaching stimuli in grasshoppers using the BYB SpikerBox. The SpikerBox is a bioamplifier that connects to the SpikeRecorder, a smartphone/tablet/computer oscilloscope application that provides the computer-generated visual stimuli as well as serves as the recorder and analyzer of neuronal activity. With this simple and affordable apparatus, students can observe neural responses to visual stimuli in a model invertebrate, and instructors can, in addition to traditional lectures, enhancing the undergraduate understanding of the fundamental properties of the brain.

Here, we present three possible experiments: 1) Classic recording of DCMD signals when the animal is exposed to approaching stimuli, 2) Testing the ideal inter-trial (a break from stimulus presentations to prevent habituation and therefore lack of responses), and 3) Testing the effect of a less visible stimulus on DCMD response by adjusting screen brightness. Instructors are welcome to modify the methodology for their specific teaching settings or other optimizations.

**Materials & Methods**

*Animals & Electrophysiology Preparation*

Grasshoppers are inexpensive to purchase, depending on location in the world, or easily found in grass fields in summer for collection. Adult grasshoppers are best for their relative large size and are therefore easy to handle. In this study, due to dependence on local availability and catching abilities, both adult and nymph, male and female, grasshoppers (*Melanoplus femurrubrum* and *Melanoplus differentialis*) were caught in Nichols Arboretum, MI, USA, in June and July 2016. The animals were refrigerated (average temperature 3ºC) until the experiments (average 4 days).

To prepare for extracellular recording of the DCMD neuron, the grasshopper, further anesthetized in ice for 15 min after storage in the fridge since being caught, was taped ventral side up onto a corkboard on the SpikerBox, with its head and part of the thorax exposed. Its head was pulled back by thread taped to the SpikerBox to expose the neck connectives, where the contralateral nerve cord and DCMD axon could be found (Fig. 2). The neck connectives could be seen with the naked eye, but an AmScope SE400-Z binocular microscope with 20X magnification was used for more precise visualization and electrode placement. A simple small incision in the middle of the neck was made using a 0.3mm insect pin. Then, the recording hook electrode (silver wire, 0.127mm), guided by the BYB 3D printed micromanipulator, was placed into the incision and around the neck connective contralateral to the eye that would be exposed to the visual stimuli and was insulated with Vaseline, which also kept the incised part from drying out. The reference electrode (sewing needle, 0.6mm diameter) was grounded in the grasshopper’s thorax. Proper electrode position was confirmed by recording DCMD responses (listen for popcorn pops) when the grasshopper was exposed to an approaching plastic stick. For the experiment to begin, the longitudinal axis of the grasshopper’s body was oriented parallel to the iPad 10cm away such that the angle between the eye and the center of the screen was less than 1º. The setup was placed inside a cardboard box for maximal surrounding darkness during all experiments except the brightness tests.

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| **Fig 2. Ventral view of the grasshopper neck showing the neck connectives from the head to the thoracic ganglia. (Left)** Neck connectives are visible as white stripes under the neck skin. **(Right)** A simple neck cut exposes the connectives, two translucent tubes, where the part of the ventral nerve cord can be found. Placing a hook electrode around one connective and exposing the contralateral eye to looming visual stimuli allows DCMD activity to be recorded. |

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| **Fig 3. Experimental setup to measure descending contralateral movement detector (DCMD) activity during simulated stimulus presentation.** **(Top)** Schematic representation of the experimental setup, grasshopper is taped down onto the corkboard on top of the SpikerBox. The reference electrode is grounded at the thorax and the recording hook electrode, guided by the 3D printed micromanipulator for precision, is placed around the neck connective where the ventral nerve cord and DCMD neuron’s axon is found. The iPad presenting the visual stimuli is placed 10cm from the grasshopper’s eye and the angle between the grasshopper’s eye and the center of the iPad is minimized. **(Bottom left)** Photograph of the apparatus. **(Bottom right)** Visual stimuli are displayed on the iPad to simulate the looming effect of an object approaching the grasshopper. The radius S, subtending angle θ, of a filled black dot can be manipulated to simulate a fixed sized object (S) approaching at a particular constant velocity (v) on the retina of the insect. DCMD recordings can be made during visual simulations of different sized objects moving at different velocities. |

*Visual Stimuli & Spike Amplification, Recording, and Sorting*

Electrophysiological measurements were carried out using the low-cost and open-source bioamplifier, the Spikerbox v.1.3c, developed by BYB. The SpikerBox has a 4x gain instrumentation amplifier, 220x gain amplifier bandpass-filtered 340-1300Hz, and a 20x gain audio amplifier. It also contains an output port for a Radioshack mini speaker (blue port and cable) and an output port for laptops, tablets, and smartphones (green port and cable) to make neural activity audible, visual, and recordable. During all experiments, the blue and green cables connected the speaker and iPad tablet to the SpikerBox.

The BYB SpikeRecorder iPad application provides both the monocular visual stimuli and recording of DCMD activity. Visual stimuli consisted of expanding dark circles (of various radii, S, and velocities, v) on a white background to simulate an approaching and colliding object. Adjustable parameters in the application include: number of trials, distance between subject and stimulus/screen (cm), object size (cm) and velocity (m/s), inter-trial interval or ITI (s). The ITI is the amount of time between the end of the previous visual display and the commencement of the next looming trial. The iPad displays a blank white screen during this interval. Stimuli of particular parameters S and v are pseudo-randomly presented by the application such that each parameter is repeated equal amount of times. Recordings were done in the dark for maximal contrast intensity.

Spikes were sorted from noise after recording in the iPad application (Fig. 4). Noise is identified as false spikes with constant and consistent amplitudes (mV) over time. These spikes have noticeably lower amplitude than the real DCMD spikes. The app allows us to set minimum and maximum threshold values (mV), and spikes within the range are counted as spikes for further analysis. When we sorted the spikes from one recording for one trial (one S, one v), the app automatically performed global sorting for the rest of the trials in one experiment.

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| **Fig 4. Spike sorting in the SpikeRecorder iPad app.** SpikeRecorder provides both the looming visual stimuli and recording and analysis of DCMD activity. DCMD spikes can be sorted from noise after recording by providing minimum and maximum spike threshold values. These values can be applied to all recordings made within a single grasshopper’s experimental session. The image above shows the app’s output of the DCMD recordings during a looming experiment (object size S = 6cm, velocity v = -2m/s) with a minimum spike amplitude of approximately 0.30mV, maximum of 0.63mV. Red dots are spikes recorded within the thresholds. |

*Experiment 1: Classic DCMD Experiments*

**Objective:** This particular experiment, coupled with instructors’ lectures, can demonstrate to students the basic electrical properties of the brain and how sensory input stimulates a particular neuronal system to drive a certain behavior, using a simplified version of electrophysiology and model invertebrate. Students can follow the procedure below, with modifications appropriate for a classroom lab or other learning settings, after developing their own hypotheses, such as when the DCMD neuron would peak in firing frequency when the object approaches the grasshopper’s eye or how different object sizes and velocities may affect DCMD response.

**Procedure:** In this replication of original DCMD studies by Rind & Simmons (1992) and Hatsopoulos et al. (1995), 19 grasshoppers were tested and 11 were selected for analysis. The grasshopper was exposed to visual stimuli on the iPad while the SpikeRecorder application recorded DCMD activity amplified by the SpikerBox. Stimuli approach the grasshopper’s eye on a direct collision course. Parameters kept constant across all DCMD experiments were: 10cm distance between the grasshopper’s eye and the iPad; 16 trials per pair of S and v; and intertrial intervals, ITI, of 45s to reduce habituation of the DCMD response to visual stimuli. Each stimulus is a pair of S (6 and 8cm) and v (-2, -4, -6, -8m/s). Initially, two separate experiments of stimuli with constant S and varying v were performed on the same grasshopper. However, to prevent the effect of habituation of the DCMD to the stimuli presented later in a second experiment, the SpikeRecorder application was modified to pseudo-randomly present stimuli of varying S and v throughout a continuous experiment of approximately 2 hours, 128 trials.

*Experiment 2: Intertrial Interval Experiments*

**Objective:** In this experiment, students will exposed to the concept of inter-trial intervals (ITIs), often used in behavior studies, such as the above DCMD experiment. ITIs, the time between trials, ensure that each trial’s stimulus is distinct and thus the animal would not develop habituation to the stimulus and stop responding. Instructors can discuss the use of ITIs at least 40s long used in literature papers (e.g. Ring & Simmons 1992) and implement this experiment to justify the use of an ITI of 45s as a sufficiently long interval to avoid habituation to the stimuli. Students can hypothesize the effect of different ITIs on DCMD activity.

**Procedure:** ITI experiments were carried out with 2 grasshoppers. The setup was the same as Experiment 1. Constant parameters included 10cm distance between eye and iPad, 30 trials per ITI, and one pair of S (6cm) and V (-2m/s). Three ITIs were chosen: 45, 22.5, and 1 second and the total experiment consisted of 90 trials.

*Experiment 3: Screen Brightness Experiments*

**Objective:** This experiment aims to engage students in further experimental inquiry after they have performed the previous two activities. Here, they can hypothesize and test the effect of screen brightness, which affects the contrast intensity and thus visibility of the black stimuli against the white background, on DCMD response.

**Procedure:** Brightness of the screen was adjusted in the settings of the iPad, whose brightness is 426 cd/m2 at maximum and 6 cd/m2 at minimum. Maximum and minimum (not complete darkness) brightness levels were set for two separate experiments. Constant parameters included 10cm distance between eye and iPad, 45s ITI, 20 trials per brightness level for 40 trials total, and one pair of S (6cm) and v (-2m/s).

*Data Analysis*

The SpikeRecorder app includes data analysis and visualization functionality. DCMD activity in conjunction with change in angular size of the visual stimulus over time can be visualized in perievent histograms and raster plots, with time to collision defined as 0 seconds. Students may save the data graphs from the app and engage with the biostatistics as a post-lab assignment.

For more in-depth analysis, data, recorded and contained by the SpikeRecorder application in JSON files, were imported into MatLab (MathWorks, Inc.) using the open-source JSONlab toolbox. A database of the experimental data (recording and spike timestamps, stimulus angles, time of collision) was created. Perievent time histograms and raster plots were plotted to visualize firing rate and timing of spikes in relation to stimulus.

**Results**

*Technical considerations: The synchronization between simulated and recorded time of collision*

To confirm that the SpikeRecorder accurately records the simulated time of collision, we performed tests to check for the synchronization between the recorded and stimulated time of collision (0s on the perievent and raster plots).

First, the refresh rate of the iPad screen was measured. A 5mm phototransistor (EverLight, PT334-6C model) was placed 10cm from the screen, to ensure that it would get light from the whole screen, and a single trial of the approaching stimulus was run. The recording output of the phototransistor was observed on an oscilloscope (Tektronix, TDS2000 model). The duration of one frame was 16.8ms, corresponding to 60Hz refresh rate (60 frames per sec) of the iPad screen. We then calculated the delay between the recorded time of collision on the software and the actual time when the stimulus filled up the whole iPad screen for each S and v parameter (see above). A delay of up to 0.06s for the fastest stimuli (e.g. velocities v of -6 and -8m/s) was recorded.

*Experiment 1: DCMD response to simulated approaching stimuli*

The visual stimuli in form of enlarging circles simulate approach toward the grasshopper. The circles started at a distance from the eye where their images subtend a minimal angle. Example extracellular recordings of DCMD response for circles 6cm in radius approaching at four velocities (-2, -4, -6, -8m/s) are shown in Fig. 5. DCMD firing frequency increased approximately 1.8s after the onset of the stimulus and peaked around the time of collision (0s) between the object and the eye, when the object's image on the grasshopper's retina reached a certain angular size (80º) (Fig. 5A). The faster the velocity of the stimulus, the later the DCMD peak firing occurred. As shown in Fig. 5B and 5C, with the circles at the slowest velocity (-2m/s), peak firing occurred approximately at the time to collision between the eye and the stimuli. With increasingly faster velocities (-4, -6, -8m/s), DCMD firing peaked up to approximately 0.05s after collision, with possible delay above-discussed.

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| **Fig 5. DCMD response to approaching objects.** d: 10cm; S: 6cm; v: -2, -4, -6, -8m/s; trials per pair of S and v: 30; ITI: 45s. Collision at 0s. **(A)** DCMD activity over time and change in angular size. **(B)** Perievent histogram (PETH; bin size: 0.03s) of DCMD firing frequency 2s before and after simulated collision between eye and object. **(C)** Raster plot of DCMD spiking pattern across each pair of S and v over time. DCMD firing peaks around collision for objects approaching at -2m/s, and after collision for objects approaching faster. |

*Experiment 2: DCMD activity during various intertrial trials*

It is known that repeated stimuli could induce habituation of a response. We investigated the effect of various ITIs on DCMD activity, shown in Fig. 6. As expected, an ITI of 1s yields low firing frequency (approximately 20Hz), compared to the frequencies with ITIs of 22.5s (45Hz) and 45s (75Hz). The 45s ITI showed the most consistent and frequent firing, and was the ITI of choice for the rest of the experiments.

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| **Fig 6. DCMD response to varying intertrial intervals.** Peak firing frequency is around 20, 45, and 75Hz for 1, 22.5, 45s ITI, respectively. The 45s ITI shows the most consistent and frequent firing of the DCMD, and is the ITI of choice for other experiments. |

*Experiment 3: Effect of screen brightness on DCMD response to approaching stimuli*

We also investigated whether screen brightness, which affected the contrast between the stimulus and the screen background, affected the peak response of the DCMD. As shown in Fig. 7, the DCMD activity profiles at maximum and minimum iPad screen brightness are not significantly different, with 90Hz and 95Hz peak frequency, respectively.

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| **Fig 7. DCMD response to varying iPad screen brightness.** **(Top)** At maximum brightness, DCMD firing rate is consistent and peaks at 95Hz. **(Bottom)** At minimum brightness, firing rate peaks at 90Hz and is not significantly different from activity pattern at full screen brightness. |

**Discussion**

Our results demonstrate the efficacy of the tools, as the SpikerBox and SpikeRecorder app successfully reproduce grasshopper DCMD responses seen in literature. Thus, the affordable and simple experiments discussed in this paper could be implemented in undergraduate laboratories to provide students with hands-on activities to better understand the neurophysiology concepts of neuroanatomy, sensory responses, and the electrical properties of the brain.

**Procedure challenges**

Our results also serve as points of reference for students to compare their results. Possible procedural challenges may arise, especially given the limited time in a classroom lab, and here we discuss several potential problems and solutions. First, noise signals are likely to be present during neural recordings and may drown out the desired signals. Since a noise-free environment is not realistic in a classroom, Faraday cages may be used. This provides the instructor with an opportunity to briefly explain how a closed conductive container, like a wire mesh box, can shield the inside materials from outside electromagnetic interference. If desired, this can also be an additional experiment, in which students can observe the differences in noise and neural signals during various levels of interference. Another challenge students may encounter is the animal prep and the time it may take. If the grasshopper is waking up from the anesthesia and becomes active during the surgery part, it should be placed in ice until it is inactive. Electrode placement, especially the recording electrode around the neck connective, is relatively simple but does demand using a microscope and micromanipulator for precision. Adult grasshoppers should be used if possible, as their neck connectives are more visible and larger. If the electrode placement around a neck connective proves too difficult, students may modify the hook electrode into a simple straight wire and place it between the two neck connectives. It is less precise, but the amplification would be sufficient for DCMD spikes to be recorded.

**Effectiveness of the experiments**

**Further experiments**

We encourage modifications and experiments that may arise from our procedures. Example research ideas using the SpikerBox amplifier include: somatotopy with grasshopper or cricket legs, the effect of temperature on neuronal responses or spikes, and the effect of drugs (e.g. nicotine) on the nervous system.

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* Example FUN papers:
  + Nesbit 2015 - Extracellular Recording of Light Responses from Optic Nerve Fibers and the Caudal Photoreceptor in the Crayfish
  + Dagda 2013 - Using Crickets to Introduce Neurophysiology to Early Undergrad Students
* More locust DCMD papers:
  + Rind 1999 - Seeing What is Coming...
  + Gray 2001 - Activity of DCMD Neurons...
  + Card 2012 - Escape behaviors in insects
* Other papers:

**Audience/Journals**

**JUNE**

* Deadline for spring issue: Jan 15
* Cover letter
* Formatting guideline: <http://www.funjournal.org/for-authors/>