ARTICLE
Electrophysiology Meets Ecology: Investigating How Vision is Tuned to the Life Style of an Animal using Electroretinography

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Students learn best when projects are multidisciplinary, hands-on, and provide ample opportunity for self-driven investigation. We present a teaching unit that leads students to explore relationships between sensory function and ecology. Field studies, which are rare in neurobiology education, are combined with laboratory experiments that assess visual properties of insect eyes, using electroretinography (ERG). Comprised of nearly one million species, insects are a diverse group of animals, living in nearly all habitats and ecological niches. Each of these lifestyles puts different demands on their visual systems, and accordingly, insects display a wide array of eye organizations and specializations. Physiologically relevant differences can be measured using relatively simple extracellular electrophysiological methods that can be carried out with standard equipment, much of which is already in place in most physiology laboratories. The teaching unit takes advantage of the large pool of locally available species, some of which likely show specialized visual properties that can be measured by students. In the course of the experiments, students collect local insects or other arthropods of their choice, are guided to formulate hypotheses about how the visual system of "their" insects might be tuned to the lifestyle of the species, and use ERGs to investigate the insects' visual response dynamics, and both chromatic and temporal properties of the visual system. Students are then guided to interpret their results in both a comparative physiological and ecological context. This set of experiments closely mirrors authentic research and has proven to be a popular, informative and highly engaging teaching tool.

Key words: electroretinogram, electroretinography, ERG, behavioral ecology, visual system, electrophysiology

Among animals, vision is often a critical sensory modality, yet one that displays widely varying parameters and specific adaptations. The eyes of even closely related species can differ substantially (Land and Nilsson, 2012), being tuned by evolution to specific needs that arise from particular lifestyles (Greiner et al., 2007; Cronin et al., 2014). Visual physiology is also readily accessible to experimental investigation, and is thus very amenable for use as a teaching preparation. Therefore, vision makes a good tool to explore relationships between the ecology and sensory system function of animals. Differences in visual performance can be measured using electroretinography (ERG), a relatively simple extracellular electrophysiological method (Heisenberg, 1971; Belušič, 2011; Dolph et al., 2011) that can be readily set up with equipment that is already in place in most physiology laboratories (Krans et al., 2006; Olivo, 2012; Vilinsky and Johnson, 2012). To facilitate students' exploration of relationships between ecology and sensory function, we developed a teaching unit in which we let students use wild-caught local insects of their choice, guide them to make their own hypotheses about how the visual system of "their" insects might be tuned to accommodate the lifestyle of the species, and use ERGs to test their hypotheses.

With nearly one million species, insects are a large, diverse group of animals, inhabiting nearly all habitats and a diversity of ecological niches. For example, many insects are terrestrial, others are aquatic; some are nocturnal, others are diurnal; some are fast flyers, others are flightless; some are predators, while others are scavengers or herbivores. Each of these lifestyles puts different demands on their eyes. Accordingly, their visual systems possess an array of specializations that allow them to navigate complex environments, locate specific food sources and mates, avoid prey, or find their way back to their nests, hives, and burrows. For example, depending on the task, their visual systems might be specialized to process visual input quickly or slowly, such that the temporal response dynamics, often measured by the critical flicker fusion frequency (CFF, stimulus frequency at which the response no longer follows the stimulus), across insects species ranges from below 40 Hz to beyond 300 Hz (Agee, 1971; Buschbeck et al., 2003; Warrant and Nilsson, 2006). CFF in humans, by comparison, ranges from 40 Hz to 65 Hz, depending on wavelength and intensity (Hecht and Shlaer, 1936). Other specializations allow insects to see in bright or dim light, see the polarization of light, or see a rich spectrum of colors, with up to six distinct visual pigments and extending beyond our own visual spectrum into the ultraviolet (Briscoe and Chittka, 2001; Warrant and Nilsson, 2006; Arikawa and Stavenga, 2014). Many of these characteristics, however, require tradeoffs, as certain visual attributes are in direct conflict with others. For example, attaining high light sensitivity typically leads to a reduction in spatial and/or temporal resolution (Warrant,

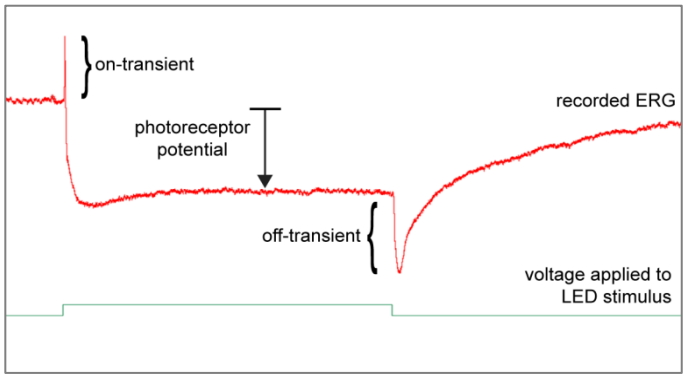

Figure 1. Typical *Drosophila* electroretinogram (ERG). *Drosophila* ERGs are composed of an on-transient, a sustained photoreceptor response and an off-transient. The on and off-transients are the pooled activity of second order neurons of the lamina, whereas the sustained photoreceptor response is the pooled activity of photoreceptors (Heisenberg, 1971). Insect photoreceptors respond by depolarization, but this extracellular method yields potentials of opposite polarity to the intracellular potentials of the cells.

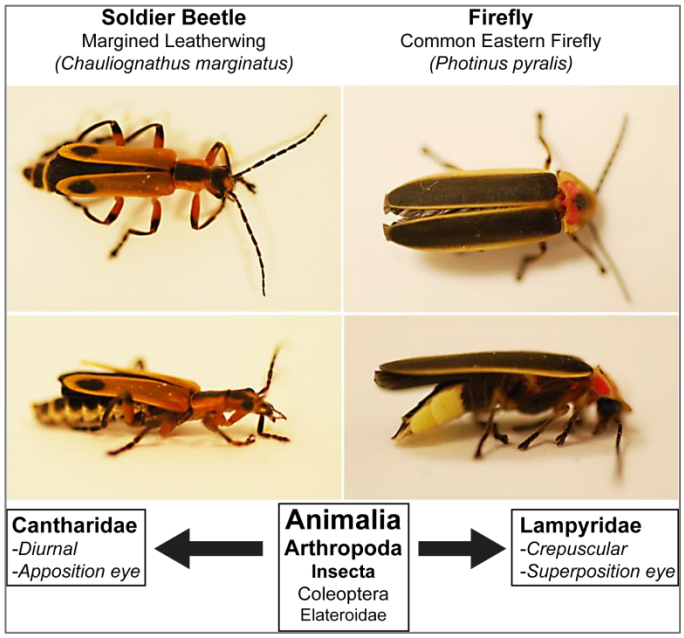

Figure 2. The soldier beetle *Chauliognathus marginatus* compared to the firefly *Photinus pyralis*. The firefly is known to have superposition eyes (Cronin et al., 2000; Land and Nilsson, 2012). While we could not find information about the eyes of our specific species of soldier beetle, a closely related species, *Chauliognathus pulchellus* has apposition eyes (Horridge et al., 1979).

1999; Land and Nilsson, 2012). If light is scarce, photons might be collected over larger areas in order to excite a photoreceptor, or the response of a number of photoreceptors may be pooled (Greiner, 2006; Warrant, 2008). Alternatively, the area over which light is collected may remain the same, but at low light levels photoreceptors might integrate the light over a much longer time period, resulting in slower response dynamics (Warrant, 1999). Yet another strategy to gain light sensitivity could be giving up the ability to discriminate between colors (Kelber and Lind, 2010). The teaching models presented here allow students to investigate whether selected insects could use some of these mechanisms as adaptations to their individual ecological niche.

Many excellent neurophysiology teaching modules are available and widely used (Johnson et al., 2002; Krans et al., 2006; Ramos et al., 2007; Kladt et al., 2010; Baierlein et al., 2011; Dagda et al., 2013). In addition, the introduction of authentic research for undergraduate students has been shown to be very beneficial educationally (Brownell et al., 2012; Kloser et al., 2013), and there is a need for students to be exposed to quantitative approaches (Gross, 2004). We here introduce a teaching unit that builds upon classical neurophysiology training, but encourages students to find their own study organisms and guides them and their instructors through authentic research experiences. The unit starts with fieldwork and ecological studies, which are very important in research but rare in neurobiology education. These are combined here with laboratory experimentation. Once students have defined specific hypotheses, these can be tested using ERG recordings that will allow students to explore a variety of visual attributes such as light sensitivity, ERG waveform, and both chromatic (color) and temporal properties.

When light enters the eye of an insect, it triggers a signal transduction cascade in photoreceptors that generates a depolarization in the photoreceptor, ultimately conveying visual information to the brain. The collective response of many photoreceptors, and sometimes postsynaptic neurons, can be detected as an extracellular signal from the surface of the eye, the electroretinogram (ERG). Since the ERG is relatively easy to measure, it has long been a popular tool in vision research, and is a platform for general physiological investigation (Hotta and Benzer, 1969; Stowers and Schwarz, 1999). This technique is also very conducive to teaching (Olivo, 2003; Krans et al., 2006; Olivo, 2012), and has more recently grown in popularity as a tool for integrating electrophysiology with molecular biology using the power of *Drosophila* genetics (Krans et al., 2006; Vilinsky and Johnson, 2012). Figure 1 illustrates a typical *Drosophila* ERG response to a 5 second light stimulus, and illustrates key components of that response.

In our teaching laboratory we explored the use of ERG recordings to evaluate the visual performance of a variety of wild-caught insects. The current study focuses on specimens collected between June and August, 2014 at two field sites in the Cincinnati, Ohio, USA area. Using electroretinography we measured the responses of their eyes to white and narrow-wavelength-band light. This allowed us to assess overall visual response, measure the critical flicker fusion frequency (CFF), and estimate the spectral sensitivity of their eyes. In our example, students then selected two beetle species for in-depth exploration: soldier beetles and fireflies (Figure 2). These beetles are phylogenetically closely related as they both belong to the super-family Elatroidea, but they greatly differ in regards to

their lifestyles. They both thrive in the spring and summer months, and are commonly found through the Midwest into the east coast of the USA. The soldier beetle, here tentatively identified as *Chauliognathus marginatus*, is a mostly day-active insect, whereas the firefly, tentatively identified as *Photinus pyralis*, is mostly active at dusk. In addition, *C. marginatus* is often found on various flowers, feeding on the pollen and nectar (Philips et al., 2013; Pelletier and Hebért, 2014) whereas *P. pyralis* is thought not to eat during adulthood (Milne and Milne, 2011).

Based on these lifestyle differences, students hypothesized that the eyes of these species might be specialized in different ways. First they hypothesized that the spectral sensitivity might differ between the two species, since the light spectrum differs between day and night (Johnsen et al., 2006) and the nocturnal species might be too limited by light to resolve colors. Secondly, they hypothesized that the eyes of the nocturnal species are slower, as they need to integrate over longer time to capture sufficient light. To test the first hypothesis we developed a simple stimulus method that allows assessment of spectral properties with a set of color LEDs, emitting equal light intensities. To test the second hypothesis we used series of light flashes and measured the critical flicker fusion frequency of these beetles. This unit introduces students to only a few of the most fundamental aspects of visual processing; however, the ERG approach can be powerful beyond the demonstrated exercises, and depth can be added in several different ways. For example, students could explore regional differences in response across the eye, investigate the effects of background illumination or bleaching lights to response amplitude and spectral sensitivity, evaluate temporal properties more thoroughly through power analysis, and probe the effects of temperature and light intensity on temporal response properties. A variety of specific tests within the framework of ERG recording can be readily applied to many different insects, or other arthropods, to address a large number of ecologically relevant questions.

MATERIALS AND METHODS

Animal preparation

Insects were collected in meadows, and along forest borders and pond banks, in the Cincinnati, Ohio, USA area between June and August. Identification was made using comparison of specimens to images on popular online identification guides, including insectidentification.org and bugguide.net. The soldier beetle was identified using the online key found in Pelletier and Hebért (2014).

The insects were anesthetized on ice until immobile. They were then placed into a plastic dish and immobilized with dental wax (Patterson Dental, #091-1578, St Paul, MN, USA) with the help of a micro cautery tool (Bovie AA02, Bovie Medical Corporation, Clearwater, FL, USA). We carefully immobilized the legs, wings, thorax, head, mouthparts and antennae, and the abdomen, but so that the insect still could breathe with its abdomen and one of the eyes was accessible. We made sure to leave a wax free spot to insert the reference electrode into the thorax, and to not get wax onto the eye. The animal was then placed at room temperature in a dark chamber to allow them to wake, and their eyes to dark-adapt.

Electrophysiological setup and ERG recording

Animals were imaged using an Olympus SZ51 stereoscope with top illumination. Electrodes were positioned using Narishige MM-3 and M-333 micromanipulators (Narishige International, East Meadow, NY, USA). The setup was stabilized on an ELpF vibration isolation platform (Kinetic Systems, Boston, MA, USA) and housed in a custom built Faraday cage using aluminum screening. Signals were recorded using an A-M Systems Neuroprobe 1600 intracellular amplifier (A-M Systems, Sequim, WA, USA). Data was acquired, digitized and stored using a PowerLab 26T and LabChart 7.2.4 software (ADInstruments) running on an iMac (Apple Inc., Cupertino, CA, USA). The recording electrode was a glass electrode rod with a cotton wick and the reference electrode was a dull glass electrode, both of which were filled with 0.9%NaCl and 20% glycerol, to reduce fluid surface tension and assist in making contact with insects' corneas. Visual signals were elicited by light pulsed from LEDs that were controlled by the positive analog output of the A/D board. To reduce noise and stimulus artifacts, the light stimulus was delivered with a custom-built LED assembly that allowed us to mount the LED outside the Faraday cage. The assembly was composed of a 1mm ID jacketed optical fiber that was inserted into a small hole drilled into the plastic LED dome and glued with optical glue (both from Edmund Optics, Barrington, NJ, USA). In order to stabilize the joint between the optical fiber and the LED, we imbedded it with hot glue into a short aluminum rod. To allow quick and convenient exchange, LEDs were inserted in a two-prong header attached to a BNC cable leading to the positive analog output of the A/D board. The optical fiber was positioned within two mm of the eye of the specimen by threading it through a homemade holder assembly, composed of a plastic pipette tip into which an aluminum rod was glued with hot glue, which was itself held in position by an adjustable dial indicator holder mounted on a magnetic base (AGPtek electronics, Brooklyn, NY, USA), see Figure 3. This setup allows consistent positioning of the fiber optic, to ensure illumination of the entire eye, thus avoiding eye region-specific differences in response amplitude. Furthermore, fixation of light source orientation and distance minimized discrepancies when changing LEDs for a given animal, and greatly reduced variation in stimulus between animals.

Testing spectral responses

In order to measure spectral sensitivity of the insects we built optical fiber assemblies with using 5mm through-hole LEDs, either cool white (7300 K color temperature), or emitting narrow color ranges, with peak wavelengths of 634, 609, 594, 523, 461, 400, 380 nm (Super Bright LEDs, superbrightleds.com). The white LED was used to elicit a maximal total ERG that presumably activated all or nearly all photoreceptor types, while the collection of color LEDs was used to estimate spectral sensitivity of each animal.

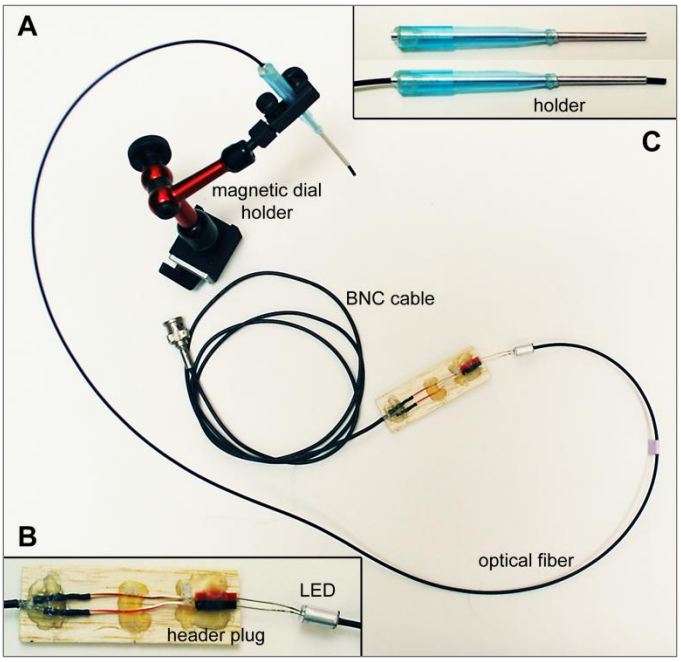

Figure 3. LED assembly. A plug for the LED was attached to a BNC cable to allow for changing out LEDs (see insert B). An optical fiber was glued to the LED with optical glue, and for stability was then glued with hot glue together with the LED into a short aluminum rod. For easy positioning of the optical fiber, a holder was assembled from a plastic pipet tip and a hollow aluminum rod though which the optical fiber was threaded (see insert C).

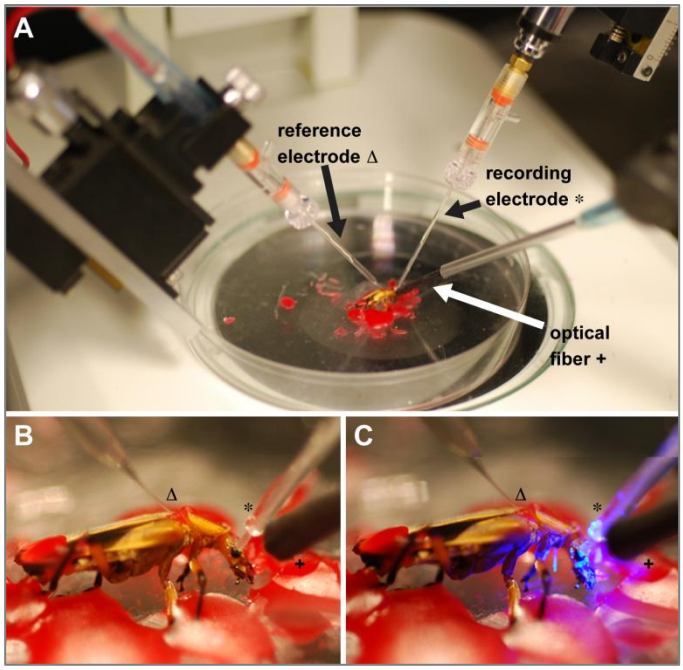

Figure 4. Experimental setup. The insects were mounted with wax onto a plastic dish. *A* Illustrates the placement of the insect, a reference and recording electrode that were mounted on a micromanipulator, and an optical fiber. *B* Shows the placement of the reference electrode (a sharp glass micropipette slighlty broken back with forceps), inserted into the thorax; and the the recording electrode (an unpulled glass cappilary with a cotton thread protruding a few mm from the tip), placed so that the wick touches the surface of the compund eye of the insect. *C* Illustrates how the light stimulus was supplied by the optic fiber during measurements.

The light outputs of the color LEDs were calibrated with a USB2000 spectrometer using a cosine corrector (Ocean Optics, Dunedine, FL, USA), so that they had equal quantal light output. Voltage applied to each LED was set so that the LEDs emitted 4.1×10^{14} photons/ cm^2/s.

For recording, the cotton wick of the recording electrode was gently placed onto the eye so that the contact between the cotton wick and the surface of the eye was good, but the wick only covered a small portion of the eye. The sharp end of reference electrode was inserted into the thorax (Figure 4). Animals were exposed to light pulse durations (between 1 and 2 seconds) that elicited a clear sustained photoreceptor response in all animals recorded. For measuring spectral sensitivity, the time between stimuli of the same color (each color stimulus was repeated three times) was set so that the photoreceptors recovered to baseline before the next stimulus, and responses were monitored to minimize light adaptation between pulses. In addition, the insects were given a two-minute dark adaptation period between stimuli of different colors.

Testing critical flicker fusion frequency (CFF)

Light flickers were created using the white LED fiber optic assembly, taking care to illuminate all samples with equal light intensity. The frequency of the light pulses was controlled by the analog out channel of the AD board. The brightness of the light was adjusted by adjusting the voltage setting of the stimulus of the recording software so that the response was good, but not maximal. To keep light intensity constant between individuals we kept the distance between the fiber optic and the eye constant as best as possible. The duty cycle was 50% and the number of pulses per train was chosen so that the total duration of the stimulus train was kept constant across all stimulus frequencies. We let the baseline fully recover before each new stimulus train. The stimulus frequency was increased until the response clearly failed to follow the stimulus, as determined by multiple pulses within a stimulus train failing to elicit a phase-locked response, as has been done, for example, for crustaceans (Frank, 1999).

Data analysis

Signal amplitude and time course data were derived from measurement tools in LabChart 7 software. Initial composition of figures depicting ERG traces was likewise performed in Lab Chart 7. All images were processed in Adobe Photoshop and composed using Adobe Illustrator. Microsoft Excel was used for statistical analysis.

A customized MATLAB program assisted in the data analysis of the spectral sensitivity data. This customized program extracts a variety of data points that are generally useful for the analysis of ERG recordings (see Figure 5, supplementary materials). The program computes a variety of parameters for each of the points such as the absolute response magnitude, an average within a certain time window, time delays that relate to the timing of the stimulus, and differences in response magnitudes between

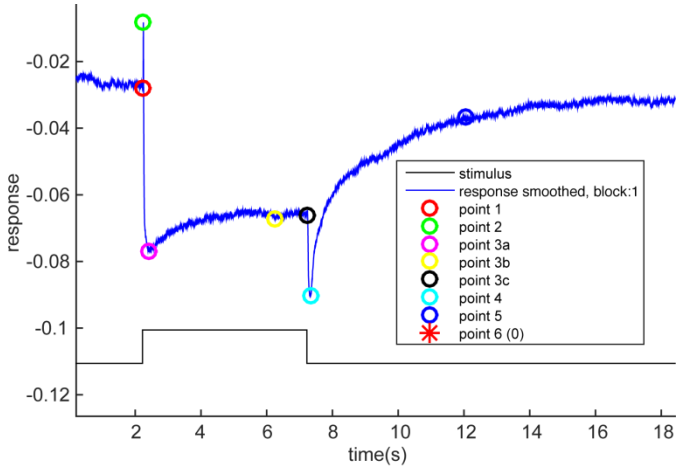

Figure 5. Example output figure of the MATLAB program (supplementary materials) demonstrating the points that are found by the program. This example is a *Drosophila* recording that includes on and off transients. Point 1 is the averaged response before the stimulus beginning. Point 2 is the maximum response between stimulus beginning and ending. Point 3a is the averaged minimum response between stimulus beginning and ending. Point 3b is a second minimum average response, between a definable number of points after stimulus beginning and ending. Point 3c is the averaged response just before the ending of the stimulus. Point 4 is the averaged minimum response within a certain time window after the ending of the stimulus. Point 5 is the average around a maximum response within a time window after the end of the stimulus. Point 6 is the first time baseline (Point 1) level is reached after stimulus ending (not present in this sample trace, as the pre-stimulus baseline is not reached in the time period displayed).

certain points (the scripts and a user guide are available as supplementary materials). This allows for analyzing ERG data in many different ways. We chose the data points that were most useful for the analysis of our recordings (Figure 5). For our recordings this was point 1 for the baseline and point 3c for the maximum sustained photoreceptor response.

The magnitude of the photoreceptor response was the difference in magnitude between points 1 and point 3c. In each sample the three stimuli per color were averaged. To account for overall differences in response magnitudes between individuals, the response magnitude was normalized against the individual's average response to white light LED. To find the critical flicker fusion frequency, we looked at the traces and found the frequency at which the responses started to fail to follow the stimulus.

Alternative materials and approaches

The exercises described here can be easily performed by any laboratory equipped for cell-level electrophysiology. Any DC-capable amplifier will work: it does not need to have high input impedance as is necessary for intracellular recordings. Vendors such as AM-Systems, WPI, Inc. and Warner Instruments have units at various price points that will work well. Likewise, the stereoscope and micromanipulators do not need to be the most precise instruments available.

If the experiments are carried out when local wild specimens are available, students are highly motivated to collect their own study animals. If this is not possible, or as a supplementary source of study subjects, a variety of vendors can supply diverse insects and arthropods. A partial list includes: Carolina Biological (http://www.carolina.com), Bugs In Cyberspace (http://shop.bugsincyberspace.com), Benzon Research (http://www.benzonresearch.com), Bugs of America (http://www.bugsofamerica.com), Niles Biological, Inc. (http://www.nilesbio.com), and Ken the Bug Guy (http://www.kenthebugguy.com). Some suppliers are larger companies with consistent stock, while others are smaller operations trading in more exotic arthropods with varying availability. Special care needs to be taken with international shipping of live specimens, as some species may be subject to licensing and controls.

All 5mm LEDs used here are very inexpensive and are readily available from a variety of vendors. The magnetic dial holder positioner is a relatively inexpensive item with many similar units available from online retailers. It is even possible to omit the fiber optic and place the LED directly in the rig, at the cost of some noise at the very beginning of the light pulse. While calibration of light output from different LEDs requires a spectrometer, this needs to be done only once per set of LEDs. If temporary use of a spectrometer is not possible, a more qualitative estimation of spectral sensitivity would still be informative and educational.

RESULTS

Students successfully recorded ERGs from a wide range of wild-caught insects. Between species, recordings varied greatly in their characteristics (Figure 6). In contrast to a typical ERG recording of *Drosophila* (Figure 1), most did not have an on-transient, as for example the pearl crescent butterfly (Fig. 6*A*), which generally showed a relatively low amplitude receptor potential, but consistently showed a post-stimulus “overshoot”, or positive shift in baseline potential. Such a baseline shift could be the result of receptors rapidly adapting to the light exposure. Some insects, such as the clouded sulfur butterfly (Fig. 6*B*) and the blue-fronted dancer damselfly (Fig. 6*C*) showed a small off-transient even though no on-transient was visible. The augochlora sweat bee (Fig. 6*D*) revealed very fast and striking photoreceptor adaptation resulting in a large initial negative photoreceptor spike, which was rapidly followed by an unusually positive sustained receptor potential. After stimulus end, a strongly positive baseline shift persisted for several seconds. Other insects, such as the barberry geometer moth, the multi-colored asian beetle, and the polyphemus moth (Fig. 6*E-F*) showed the opposite after stimulus end: their response took a long time to repolarize to the levels of the original baseline, possibly because of excessive rhodopsin to metarhodopsin conversion as is known for the Prolonged Depolarizing Afterpotential (PDA) response in *Drosophila* (Pak and Leung, 2003). While the traces for these three species are also similar in that they do not exhibit on transients, they exhibit notable

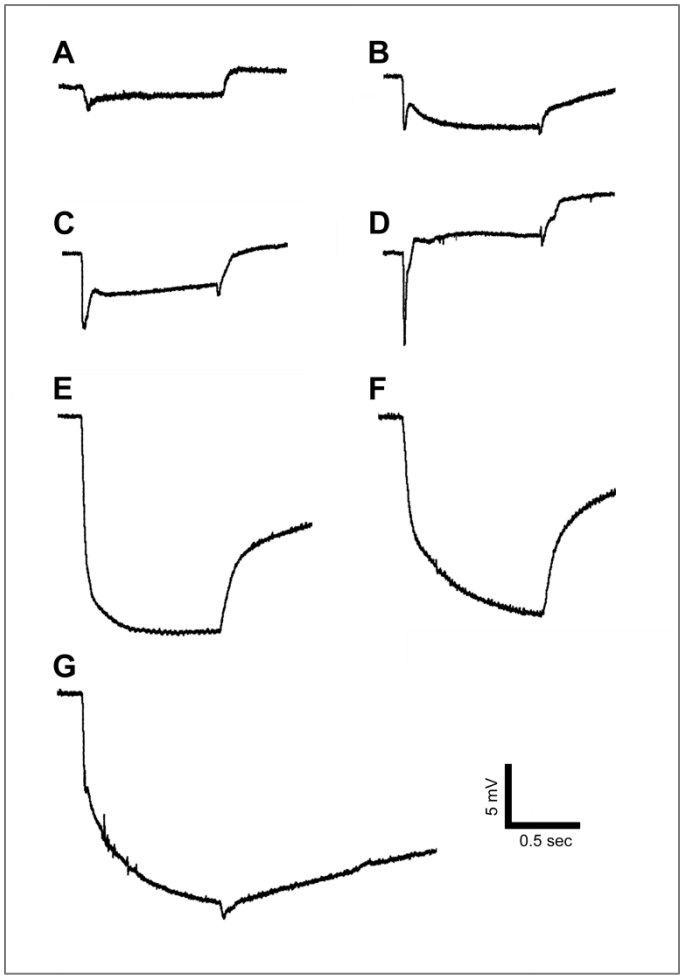

Figure 6. Representative electroretinograms from seven insects, demonstrating variation in different functional aspects of the ERG trace. All recordings were performed on dark-adapted animals and elicited by a 1 second pulse of light from a white LED. The insects were tentatively identified as follows: *A* pearl crescent butterfly (*Phyciodes tharos* species group); *B* clouded sulfur butterfly (*Colias philodice*); *C* blue-fronted dancer damselfly (*Argia apicalis*); *D* augochlora sweat bee (*Augochlora leptoloba*); *E* multi-colored asian beetle (*Harmonia axyridis*); *F* geometer moth (*Scopula* sp.); *G* polyphemus moth (*Antheraea polyphemus*).

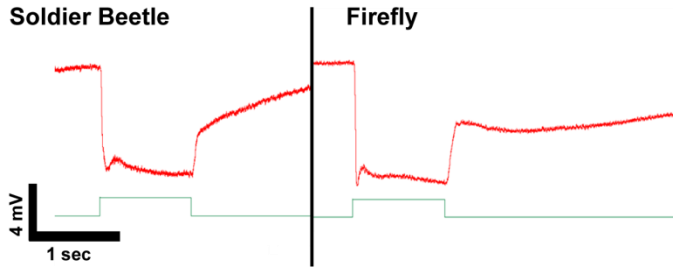

Figure 7. Typical ERG trace of a soldier beetle and a firefly. The photoreceptor response is in red (see scale bar for response magnitude), and the timing of the white LED stimulus is indicated in green. Unlike ERGs of *Drosophila*, these ERGs generally did not show on and off-transients. After stimulation it could take several seconds until full recovery to baseline. During our measurements we made sure that original base line level was recovered before presenting the next stimulus.

differences in how quickly they reach the response maximum.

ERG of soldier beetle and firefly

In contrast to ERGs of *Drosophila*, but similar to many recordings of other wild-caught insects, the ERGs of the soldier beetle and the firefly do not show on- and off-transients. Representative example recording of responses to a white light stimulus are shown in Figure 7. There was no significant difference in the response magnitude to white light stimulation between the two beetles.

Spectral response of the soldier beetle and firefly

Both insects showed a clear difference in their response magnitudes between stimuli of different wavelength as illustrated in Figure 8. After taking the averages, we could observe differences in the spectral responses between fireflies and soldier beetles (Figure 9). Comparing our data to the literature, we found a good correspondence to the two spectral maxima (400nm and ~570nm; Lall et al., 1980a; Lall et al., 1980b) and their relative response strength for the firefly. To the best of our knowledge there is no data available for the soldier beetle, so our student's data on this species is novel. Specifically, we found that the soldier beetle has a relatively stronger response to UV than the firefly, while the firefly has a relatively stronger response to orange and red than the soldier beetle.

Responses to flicker

An example series of recordings is shown in Figure 10*A* and Figure 10*B* illustrates the average critical flicker fusion frequency for both beetles. The flicker fusion frequency of the soldier beetle is 45 Hz (± 12 std. dev.) and for the firefly it is 40 Hz (± 10 std. dev.). The latter corresponds well to a previous report on *Photuris versicolor* (Lloyd, 1978). There was no significant difference between the two beetles (Student's t-test, p = 0.33).

DISCUSSION

Electroretinography is an accessible, flexible and adaptable electrophysiology preparation that is of great value in research, and is becoming increasingly popular in undergraduate laboratories. As is the case for many research programs (Hotta and Benzer, 1969; Heisenberg, 1971; Pak and Leung, 2003) one focus of teaching laboratories has been to use ERG recordings on *Drosophila*, which allows for the analysis of mutants (Vilinsky and Johnson, 2012). While this is a valuable teaching tool, ERG recordings also can be used as a powerful tool to investigate the visual systems of a great variety of species, and to apply neurophysiology to ecological questions, which is relatively novel. Modern neuroscience education does not usually incorporate natural history, zoology or fieldwork, but instead heavily focuses on well-established model organisms that demonstrate specific concepts. However, investigating a variety of largely unknown organisms has been critical to the advance of neurobiological research in the past, and

the incorporation of authentic research into undergraduate education has been shown to be highly beneficial (Brownell et al., 2012; Kloser et al., 2013). Our report is in this spirit, and extends physiological measurements into ecology in the context of the undergraduate laboratory.

The large diversity of eye organizations and functions in insects makes it intriguing to investigate them from an ecological perspective, which can be done in different ways. Our report primarily focuses on the spectral response and temporal dynamics of two beetle species, but we also investigated the feasibility of our ERG recording methods in a variety of other species (Figure 6). Differences in how quickly receptors respond, to what extent they adapt, and how potentials recover post stimulation are notable. These parameters provide a great framework for students to investigate specific attributes of visual systems, compare them, and interpret findings in context of the ecology of the species. In addition to typical parameters of photoreceptors (such as the sustained response and recovery), other parameters of the wave-shape (such as transient signals) give insights into higher order processing (Heisenberg, 1971; Belušič, 2011).

In our example, we tested light sensitivity, critical flicker fusion frequency, and spectral responses of a crepuscular firefly (*Photinus pyralis*) and a diurnal soldier beetle (*Chauliognathus marginatus*). We could see significant differences in spectral responses, but not in the light sensitivity and flicker fusion. Efficient and specific color vision has been implicated in regards to mate choice and is often crucial for navigation through habitats, including finding appropriate flowers (Briscoe and Chittka, 2001; Kelber, 2006). Color vision also often is influenced by how much light there is available, and nocturnal species tend to be relatively poor at seeing colors when compared to diurnal species, though there are exceptions (Kelber and Lind, 2010).

No matter what students' results are, they easily lead to fruitful discussions, the development of new hypotheses, and design of future experiments that could test these, just like in "real" research. For instance, in our example, the soldier beetle and the firefly showed responses in UV and in a longer wavelength (LW). However, the UV response (relative to the LW response) was significantly lower for the nocturnal species than for the diurnal species and the LW response of the firefly was red-shifted compared to the response of the soldier beetle. Based on these findings students might raise the following question: Could this be due to soldier beetles relying on UV light to find flowers, and fireflies "only" needing LW light to find signaling mates? While necessary experiments to address these questions are beyond the scope of the physiology laboratory, a good discussion could focus on conceptual ideas for behavioral experiments (Kelber et al., 2003) that could provide answers. On a more proximal level, the shape of the spectral sensitivity curves raises the possibility that these species have only a LW visual pigment (opsin), since LW opsins have an intrinsic, typically less sensitive secondary UV sensitivity (Briscoe and Chittka, 2001). Alternatively either species could have an independent UV sensitivity, a property that could be investigated further by, for example, repeating measurements after bleaching the eye with monochromatic light. In this case, if only one receptor type is present, one expects that a green or UV monochromatic bleach light would lead to equal reduction of both these peaks. However, if the response ratio between these two peaks shifts, this is an indication for the existence of multiple receptor classes, as indeed has been demonstrated to be the case in the firefly *Photinus pyralis* (Lall et al., 1980a). Discussion also could be geared towards how one could find out how many distinct photopigments these species actually have, and consider

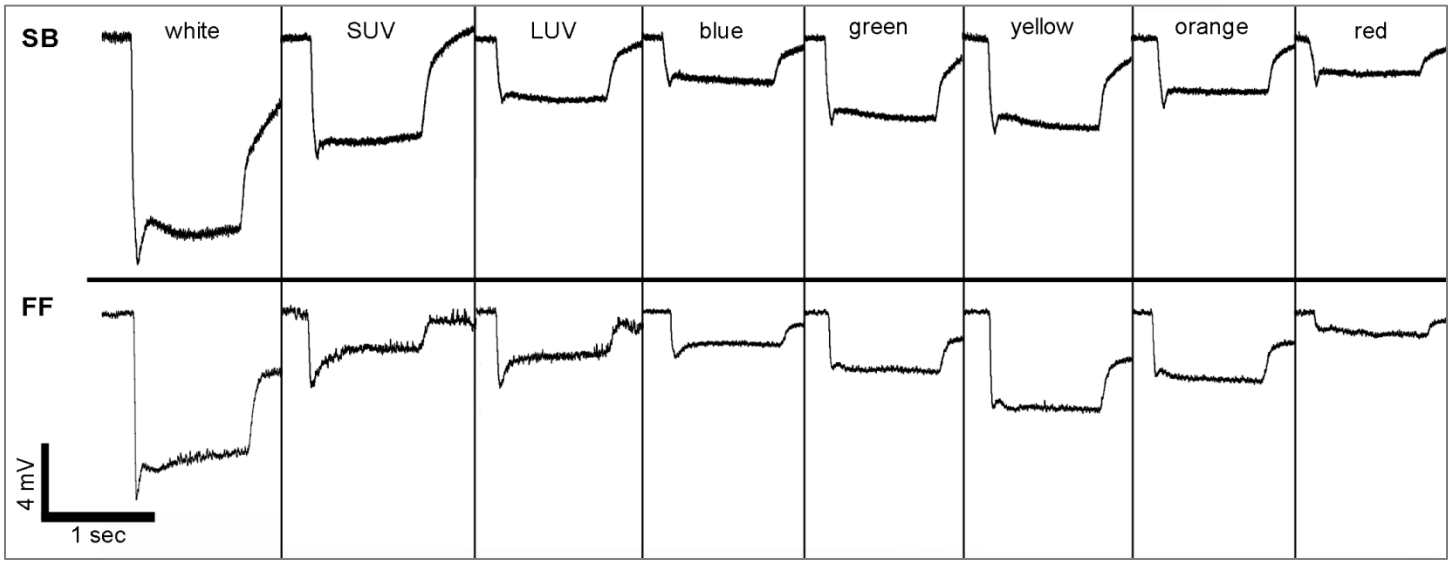

Figure 8: Example sequences of the responses of an individual soldier beetle (SB) and a firefly (FF) to stimulation with color LEDs. The peak wavelengths of the LEDs were 380 nm (SUV), 400 nm (LUV), 461 nm (blue), 523 nm (green), 594 nm (yellow), 609 nm (orange) and 634 nm (red). The stimulus duration was 1 second.

the possibility of regional differences in spectral sensitivity across the eye as has been demonstrated for the dragonfly *Sympetrum* (Labhart and Nilsson, 1995) This then allows the instructor to guide students towards incorporating genetic and molecular methods that could provide answers, and to discuss limitations of the ERG approach.

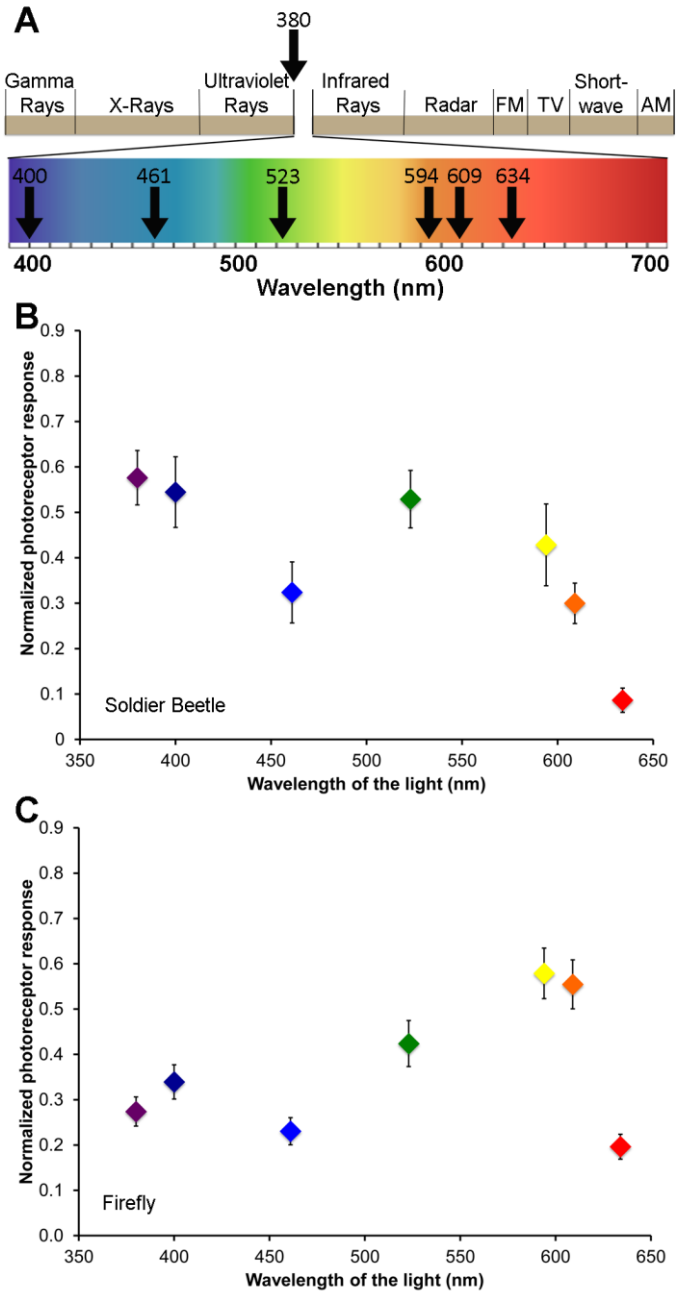

Figure 9. Comparing spectral sensitivity. *A* Illustrates the spectral range of light. The arrows indicated the peak wavelengths of our stimulus (380, 400, 461, 523, 594, 609 and 634 nm). *B* The average photoreceptor response of the soldier beetle with standard error (N = 7). *C* The average photoreceptor response of the firefly with standard error (N = 15). The spectral response of the firefly seems to be red-shifted and is relatively low in the UV-range compared to the spectral response of the soldier beetle. Spectral-specific responses in B and C were normalized to the average white light response amplitude.

A similarly fruitful in-depth discussion arises from our flicker fusion results, which led us to reject our initial hypothesis. The critical flicker fusion frequency (CFF) is another important eco-physiological parameter of eyes, because it refers to how fast eyes can respond to changes (temporal resolution). One way to assess temporal resolution is to determine at what frequency photo-receptors become incapable of following individual light pulses (Frank, 1999; Miall, 1978). We presented each of the two beetle species with series of consecutive light pulses and evaluated responses accordingly. Surprisingly, there was no detectable difference between the two species, which was in contrast to the initial hypothesis, that the nocturnal species might be slower because the lower light level might require longer integration times. This is a very interesting finding, and a good basis for a discussion on the trade-offs that are observed in eyes in regards to spatial resolution, temporal resolution and light sensitivity (Land and Nilsson, 2012). Could it be that there is spatial instead of temporal summation in the firefly in order to maintain high flicker fusion frequency for flying? How might they benefit from this, and how could one test this hypothesis? While assessment of temporal properties through the critical flicker fusion frequency (CFF) is fast, there is some level of subjectivity. A more analytically thorough, but more time-intensive, approach uses a more objective method that at the same time introduces students to the concept of biological filters, by plotting the normalized response magnitude across a wide range of frequencies. From this data students then could establish the corner frequency, often defined as a 3 dB attenuation in response amplitude. For this method to work, however, students need to collect data to stimuli frequencies ranging incrementally from very low frequencies (at which the response magnitude to each pulse is undistinguishable from a response to a single stimulus), to a stimulus frequency that fails to elicit a response above noise levels. This method is particularly suitable for comparisons of CFF between different temperatures or light levels.

The range of possible projects using these tools is vast, yet at the same time centered on the local ecology. Experiments can be based on different comparison groups, with hypotheses and predictions developed by the students based on what is known of the behavioral ecology of the animals selected. In addition to the day vs. night- flying example described here, other comparisons can be made using a variety of behavioral/ecological parameters, including:

1. Flying vs. walking, or fast vs. slow flying
2. Bright, polychromatic environments such as meadows vs. dim environments with muted colors, such as forests
3. Surface vs. leaf litter or burrowing
4. Nectar or pollen feeding vs. predatory vs. scavenging vs. non-feeding as adults
5. Different castes of the same species of a social insect

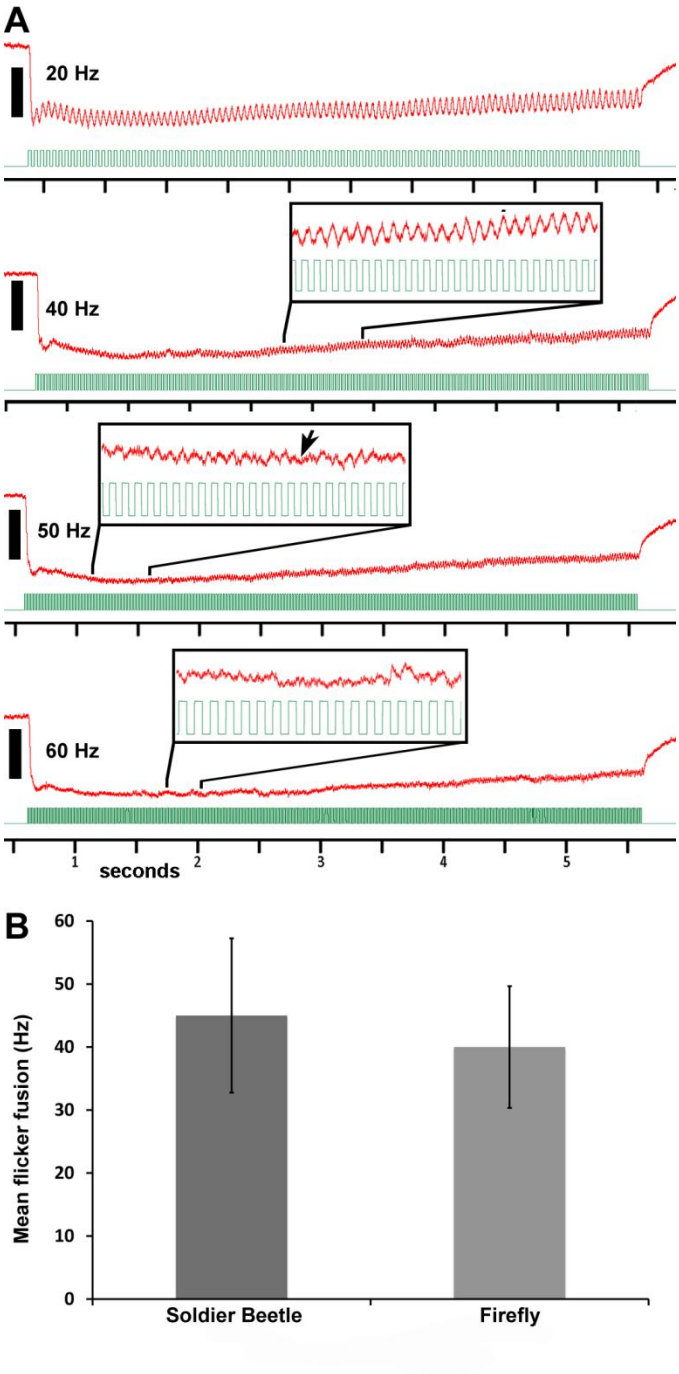

Figure 10. Critical flicker fusion measurements. *A* Sample recordings with photoreceptor response (red) and white LED flash stimulus (green) with a duty cycle of 50% and frequencies of 20, 40, 50, and 60 Hz. Inserts are magnified traces matching photoreceptor response to stimulus. Here, the photoreceptor responses followed the stimulus reliably up to 40 Hz, started to skip at 50Hz (see arrows), and failed to follow at 60 Hz, yielding a CFF estimate of 50 Hz. *B* Average CFF of the soldier beetle (SB) and the firefly (FF) with standard deviation (SB N = 6; FF N = 16). There was no significant difference between the flicker fusion frequency of the soldier beetle and the firefly (p = 0.33). Scale bar = 2 mV.

Alternatively, students could focus on collecting and cataloging a wide range of insects or arthropods and their visual responses. Capturing and maintaining wild-caught specimens gives students insight into the full range of experimentation techniques, from field to electrophysiology rig, and the process of identifying animals is in itself a rich and highly engaging educational experience.

The soldier beetle vs. firefly example illustrates how students can apply physiology to ecological questions, and interpret and test their results and resulting hypothesis across different levels of organization. In more general terms, as a starting point students learn about the overall organization of insect eyes, and about trade-offs in the physiological construction of visual systems. At the same time students are encouraged to survey and collect local species of insects, identify them, and learn about their known life history. Students then use their new knowledge to develop a hypothesis about how specific vision systems might differ based on the specific visual needs of their collected species. Students perform ERG recordings and analyze their data, for example with the MATLAB code that we provide in the supplementary materials and on our website (http://www.artsci.uc.edu/departments/biology/byDeptMembers/faculty.html?eid=buschbek&thecomp=uceprof). This way they can test their specific hypothesis, discuss their results, and generate follow-up question and hypotheses, as would be done in original research studies.

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