Ambient Illumination Influence on *Photuris* Firefly Larval Surface Movements is not Mediated by the Stemmata

Frederick Murphy · Andrew Moiseff

Received: 12 September 2019 / Revised: 30 March 2020 / Accepted: 27 April 2020 / Published online: 18 May 2020
© The Author(s) 2020

Abstract Movements of fireflies visible on the surface of soil were measured under controlled laboratory conditions consisting of high and low ambient illumination. High illumination approximating the intensity of light prior to sunset constituted our *light* condition. Low illumination was consistent with ambient light levels after civil sunset, which we referred to as our *dark* condition. Surface movements were significantly more frequent during *dark* conditions compared to *light* conditions. Stemmata are the larval form of the insect eye and were the only identified visual organs present in *Photuris* larvae. We investigated whether stemmata provided larvae with the sensory information facilitating the light dependency of surface movements. We disrupted transmission of visual information from the larval eyes to the brain by severing the optic nerves. The amount of surface movement was compared between larvae with intact and severed optic nerves, under *light* and *dark* conditions. Light dependency of surface movements was preserved in larvae with cut optic nerves. The presence of the light dependency after cutting the optic nerves indicated that an alternative, extrastemmatal sensory pathway must be providing light intensity information to the animal. Light dependency was abolished upon removal of the head. Thus, these results suggested that the extraocular system providing light intensity

information for regulating the frequency of surface movement was located in the head. The precise location of the suggested extraocular receptor and the nature of the associated sensory system remains unknown.

Keywords *Photuris* firefly larva · stemmata · extraocular receptor · light avoidance · nocturnal behavior · larval movement

Introduction

In insects, light is detected by multiple sensory organs with differing downstream pathways that facilitate a suite of light-dependent behaviors. Insect eyes and their visual system are a primary pathway that informs behaviors mediated by light. Insects have three principal forms of photosensitive organs: compound eyes, stemmata, and ocelli. Compound eyes are featured in all adults and hemimetabolous larvae, and single lensed eyes known as stemmata are found in all holometabolous larvae (Gilbert 1994; Buschbeck and Friedrich 2008). Compound eyes are necessary for many light-dependent behaviors. For example, photoreceptors within compound eye ommatidia facilitate wavelength specific phototaxis in the green rice leafhopper (Wakakuwa et al. 2014). Stemmata are also capable of mediating phototaxis. This is seen in *Drosophila melanogaster* larvae where Bolwig's organ mediates robust light avoidance behaviors during early developmental stages (Rieger et al. 2007; Keene and Sprecher 2012). Ocelli which are single lensed, 'simple eyes,'

F. Murphy (✉) · A. Moiseff
Department of Physiology and Neurobiology, University of
Connecticut, 75 N. Eagleville Road, Unit 3156, Storrs, CT 06269,
USA
e-mail: Fred.murphy@uconn.edu

detect changes in ambient light intensity but are not capable of image formation (Mizunami 1995). Dorsal ocelli have been shown to have a role in light-dependent behaviors. For example, in some dragonflies, occlusion of the dorsal ocelli resulted in atypical flight patterns demonstrating that ocelli are critical for stabilization during flight (Stange and Howard 1979).

In addition to the compound eyes, stemmata, and ocelli, some insects have photosensitive extraocular receptors located within neural and dermal tissues (Arikawa et al. 1980; Felisberti et al. 1997; Wertman et al. 2018). Extraocular receptors have been shown to preserve flight activity patterns in moths, *Manduca sexta*. Removal of both compound eyes and ocelli had no effect on the light-dependent flight activity patterns of moths. This led to the identification of an extraocular receptor that entrains the circadian rhythm of the moth flight clock (Lundquist et al. 1996). Another example of extraocular photoreception was described in the mountain pine beetle (*Dendroctonus ponderosae*). This beetle does not possess larval eyes (stemmata) or ocelli yet are negatively phototactic, indicating photosensitivity (Wertman et al. 2018).

Photuris larval surface activity was light dependent. At ambient illumination corresponding to the intensity of light prior to sunset, *Photuris* larval activity levels were reduced compared to activity levels in the light (Murphy and Moiseff, unpublished data). Given that light-dependent behaviors in different insects may rely on different sensory organs and downstream pathways, our goal was to investigate which sensory pathway facilitated light-dependent surface activity behaviors in *Photuris* larvae. The known photosensitive structures in *Photuris* larvae are the stemmata (Murphy and Moiseff 2019); firefly larvae do not have ocelli. Thus, we proposed that the stemmata of *Photuris* larvae mediated light-dependent surface activity. To test this hypothesis, we recorded larval surface movements in response to disruption of the stemmatal pathway by cutting the optic nerve.

Materials and Methods

Animal Collection and Storage

Photuris firefly larvae were collected locally near Storrs, CT, USA between September – October 2019, 2000–2200 h. Soil was collected at the collection site and

larvae were housed in containers with soil from the site and maintained at room temperature (21 °C). Containers were watered daily and lined with a water absorbent substrate (Datesand Ltd) to maintain moisture. Larvae were fed worms and slugs.

Light Dependent Movement

Arena

Larvae were placed in an arena (15 cm x 15 cm x 5 cm) (Fig. 1a). The bottom of the arena was lined with a water absorbent substrate (Datesand Ltd). This substrate was covered with ~ 1 cm of soil. A white LED light source (FEIT Electric BPAGOM800/LED) was placed ~ 25 cm above the arena and controlled by a mechanical timer (Model 50000 Indoor 24-Hour Mechanical Timer 2-C). A camera (WYZE CAM v2, Model: WYZEC2) was positioned ~ 15 cm above the arena for recording movement on the surface. Images were recorded at the rate of 1 frame every 5 s for a recording window of 12 h. Under dark conditions, the camera was set to automatically engage an infrared LED (840 nm) to enable imaging.

Tracking Larval Locomotor Behavior

Videos were analyzed using FIJI (Schindelin et al. 2012). A macro for FIJI was written to detect larval surface movement by highlighting pixel-level differences between adjacent frames. Brightness and thresholds were manually adjusted for each video and applied to all frames to enhance detection of the larval position. The enhanced video stack was binarized across all frames to extract the location of larva movements. Noise in the images was removed in all frames using a minimum filter, performing grayscale erosion on each pixel. To extract the animals position we applied the analyze particle centroid function in FIJI to all frames. The x and y pixel positional coordinates for all centroid values in the same frame were averaged to generate a single ‘x’ ‘y’ position representing the larva’s location in each frame.

Quantification of Larval Movement

Larval movement was defined as any movement made by larvae, visible on the surface of the soil. To quantify larval movement, the number of frames where movement was detected was divided by the total number of

Fig. 1 Light stimulation set-up and illumination protocol sequences. **a** Experimental set-up. Arena where larval movement was recorded in response to an overhead light source that provided a sequence of light and dark conditions. The spectrum of the LED light indicates relative power on a linear scale (y-axis) as a function of wavelength. **b** Light stimulus protocol. Individual larvae were presented with one of three different light stimulus protocols between the recording window of 1800–0600 h. Data analysis began following the first light to dark transition, indicated by the

red arrowheads. Number line indicates local time. **c** Intensity of light and dark experimental conditions compared to sunlight measurements in the field. Intensity is measured as irradiance W/m². Intensity values referring to the field are indicated by solid filled, vertical arrows. Yellow striped arrow indicates the intensity of the light condition (10.135 W/m²) and the unfilled arrow indicates the threshold sensitivity measured by our meter. The intensity of the dark condition (black filled, horizontal arrow) was below the threshold sensitivity of the meter, 8.8E⁻⁴ W/m²

frames in the recording window. The resulting value termed *percent movement*, was a measure of larval surface movement. Any larva that registered zero movement (0% movement) in both the *dark* and *light* trials was excluded from this study.

Movement as a Function of Time

Our illumination protocols (Fig. 1b) contained multiple *dark/light* transitions per trial. All frames within the first hour (720 frames) following each light-dark and dark-light transition in our illumination protocols were collected and separated into 6 ten-minute intervals (120 frames per interval). All frames in which movement was detected were counted and placed within the appropriate time interval bin. This process was performed for each animal, in both conditions (*dark* and *light*) and each group tested (severed optic nerve, and headless) for all illumination transitions in each video. Since our illumination protocols (Fig. 1b) contained multiple *dark↔light* transitions per trial, each animal experienced multiple sessions in the *dark* and in the *light* over the full recording window. The number of active frames counted during each time interval were summed across all *light* and *dark* conditions. In all cases, light

movement and dark movement were summed separately, yielding a single value representing the total number of frames reflecting total larval movement under each condition for all six time intervals. The *dark* and *light* active frame values were normalized for each animal to the maximum level of movement exhibited by an individual animal during any of the *dark* or *light* conditions. This provided each individual animal with a range of movement values between 0 (no movement) and 1 (maximum movement) for each 10-minute time block.

Statistical Analyses

All movement data was compiled in Microsoft Excel and imported into the R environment for processing, visualization, and statistical analysis. Data manipulation and visualization was performed using the *Tidyverse* collection in R, specifically the *dplyr* and *ggplot2* packages. Wilcoxon rank sum tests were used to compare any two distributions and Friedman's test was applied to compare sample medians across multiple groups. Multiple pairwise comparisons were made using the Wilcoxon rank sum test followed by the Holm-Bonferroni correction methods.

Experimental Illumination Intensities

The intensities of the *light* and *dark* experimental conditions were measured (Mettrue Model Sim-2 Plus Spectral Irradiance Meter). The light meter was placed on the surface of the arena for both *light* and *dark* conditions and intensity was measured as irradiance (W/m^2). The extent of the *light* and *dark* conditions relative to natural illumination levels in the field was contextualized by sampling irradiance levels taken in the field approximately 2 h prior to sunset ($16.86 \text{ W}/\text{m}^2$) and at civil twilight ($4.35\text{E}^{-1} \text{ W}/\text{m}^2$) (Fig. 1c). The intensity of the *light* condition, $10.135 \text{ W}/\text{m}^2$, was comparable to the intensity measured prior to sunset. To put this into context, the intensity prior to sunset was less than 1/50th the peak intensity of sunlight reported in the literature, values $> 500 \text{ W}/\text{m}^2$ (Thorington 1980; Brainard et al. 1984). The intensity of the *dark* condition was below the resolution of our meter, i.e., less than $8.8\text{E}^{-4} \text{ W}/\text{m}^2$ (unfilled arrow, Fig. 1c). Our dark condition was at least 2 orders of magnitude below the intensity we measured for civil twilight (Fig. 1c). Throughout the manuscript we referred to experimental high and low level light conditions as *light* and *dark* respectively.

Severing the Optic Nerve

Firefly larvae were removed from their storage container and any excess soil in contact with the animal was removed. The head was anchored in a SYLGARD™ (Dow ®) dish with a stainless-steel pin (0.1 mm Minutien pin, Fischer Scientific). A semi-circular incision into the cuticle surrounding the stemmata was made using the tip of a 25-Gauge hypodermic needle as a microscalpel. The cut end of the cuticle was elevated to expose the stemmata and the optic nerve. The optic nerve was severed with a single transverse cut using surgical micro-scissors (Fig. 2a). The cuticle was placed back into its original position after confirming that the optic nerve was severed completely.

Removal of the Larval Head

The larval head was anchored as described for severing the optic nerve. The head was cut at the base of the cuticle surrounding the head capsule (red line, Fig. 3a). This cut severed the optic nerve while preserving the brain, which is proximal to the thorax and ventral to the pronotum.

Results

Are Stemmata Responsible for Light-dependent Movement?

We posited that stemmata would be the sensory organ responsible for facilitating light-dependent surface movement in *Photuris* larvae. Larvae were collected in the field and maintained and stored in the laboratory with intact stemmata and visual systems served as controls. To disrupt the stemmata-visual pathway, we severed the optic nerves of control larvae and recorded larval surface movement in response to *light* and *dark* conditions (Fig. 1).

Severing the optic nerve interrupted all visual input to the brain from photoreceptors in the stemmata (Fig. 2a). We predicted that larvae whose visual input was removed would behave as if they were always in the dark, even if the light was on. This would manifest itself as equal larval movement levels in *dark* and *light* states with no sensitivity to the *light* ↔ *dark* transitions (shown schematically in Fig. 1b).

Larvae with severed optic nerves were significantly more active in the *dark* condition (median movement 10.2%) than in the *light* condition (median movement 0.6%) (Fig. 2b). Median percent movement declined in larvae within the cut optic nerve group. This decrease was not significantly different from the behavioral results observed in control larvae (Fig. 2d). The percent movement in the *light* condition for both the control and cut optic nerve groups were also not significantly different (Fig. 2e). This suggested that overall movement during the *light* and *dark* conditions was not affected by removing all sensory input from the stemmata.

Larval movement during the *dark* condition increased over time relative to the *light-dark* transition, this result was consistent with control larvae. Median normalized movement was 0 during the first 10 min and increased significantly during the duration of the *dark* condition, $p < 0.05$, Friedman's test (Fig. 2c). However, precise groups that differed significantly from the 10 min time interval during the dark condition could not be detected, $p > 0.05$, pairwise post hoc Wilcoxon rank sum with Holm-Bonferroni correction. The maximum movement for 80% of the larvae in this group ($n = 5$) occurred during the *dark*. Only 1 individual larva registered maximum movement during the *light* condition, indicated by the outlier in the 10-minute interval (Fig. 2c). This animal's movement declined in each

Fig. 2 Effect of cutting optic nerves on movement. **a** Optic nerve surgery. Scissors approximate the location where the optic nerve was severed. Purple ovals = stemmata. mp = mouth parts. ptm = pronotum. bn = brain. **b** Percent of video frames where movement was detected during dark and light; $n = 05$. Asterisk indicates a significant difference between dark and light conditions ($p < 0.05$; Wilcoxon rank sum test). **c** Normalized movement as a function of time. X-axis: labels indicate 10 min epochs, ending at the time shown. Asterisks indicate significant

difference to the corresponding condition during the first 10 min time interval ($p < 0.05$, Wilcoxon rank sum with Holm-Bonferroni correction), $n = 05$. **d** and **e** Percent movement comparing control and severed optic nerve groups in dark (**d**) and light (**e**) conditions respectively. All data presented as box plots indicating interquartile range (IQR) with the median displayed as a horizontal line. Whiskers (+/-) indicate minimum and maximum values within $1.5 \times$ IQR. Outliers, plotted as dots, are values outside the $1.5 \times$ IQR range

successive time interval until burrowing in the soil during the 44th minute (outlier in the 50-minute time interval, Fig. 2c). During the *light* environment, 3 larvae were moving on the surface during the first 10-minute interval, which was the only interval where mean movement was $> 0\%$. While two animals were moving on the surface beyond the 10-minute interval there were no statistical differences between the normalized movement levels during the *light* phase (Friedman test, $p > 0.05$).

The disruption of the stemmata-visual pathway to the brain did not eliminate the relationship of larval movement levels between *dark* and *light* conditions. Larval surface movement remained greater during the *dark* state and displayed minimal movement during the *light* condition. Our finding that light-dependent movement remained when the optic nerves were cut suggested that an extraocular mechanism mediated the observed behaviors.

To identify potential sources of the extraocular input, we successively removed different areas of the body. To determine if the proposed extraocular receptor was located in the head, we removed the heads of larvae whose

optic nerve had been previously severed (Fig. 3). If the mechanism was located in the head, removal of the head would be expected to eliminate light-dependent surface movement preferences.

Headless larvae did not exhibit light-dependent surface movement behavior (Fig. 3). Differences in larval movement between the *dark* and *light* states were not significant (Fig. 3b). Larval movement in the *dark* was not significantly different between headless (11.2%) and the cut optic nerve group with intact heads (10.2%) (Fig. 3d). However, movement in the *light* condition increased significantly between headless larvae (9.9%) and larvae with intact heads with severed optic nerves (0.6%) (Fig. 2e).

The headless group were the only larvae to remain active in the *light* condition throughout the entire hour, median normalized movement for all light intervals was > 0.25 (Fig. 3c). Unlike the control and cut optic nerve groups, there was no significance in movement levels, during the *dark*, compared to the 10-minute time interval. Larvae were equally active during the first 10 min of the *light* to *dark* transition as the final 10 min (Fig. 3c), a

Fig. 3 Removal of the head abolishes light dependent movement behavior. **a** The vertical red line indicates the location where the head is removed, eliminating the nerve bundle that contains axons from the stemmata (purple ovals), antennae (an) and mouth parts (mp). The brain (bn) is left intact, located ~2 mm caudal to the stemmata, beneath the first cuticular segment of the body, the pronotum (ptm). The red 'X's indicate that the optic nerves were severed during a previous surgery (see Fig. 2a). **b** Percent of frames where movement was detected in headless larvae during exposure to light and dark; $n = 4$. **c** Normalized movement of headless larvae as a function of time. X-axis: labels indicate 10

minute epochs, ending at the time shown. Asterisks indicate significant difference to the corresponding condition during the first 10 minute time interval ($p < 0.05$, Wilcoxon rank sum with Holm-Bonferroni correction), $n = 4$. **d** and **e** Percent movement of larvae with head intact and optic nerve severed (cut optic nerve) compared to headless larvae (headless) in dark (**d**) and light (**e**). Asterisk indicates $p < 0.05$; Wilcoxon rank sum. All data presented as box plots indicating interquartile range (IQR) with the median displayed as a horizontal line. Whiskers (+/-) indicate minimum and maximum values within $1.5 \times$ IQR. Outliers, plotted as dots, are values outside the $1.5 \times$ IQR range

finding that was not observed in the control and severed nerve groups. These results suggest that the headless animals did not sense the illumination changes.

Discussion

Photosensitive Behaviors in Larvae With Severed Optic Nerves

Photuris larval surface movement is light-dependent. In *Photuris* larvae, the only identified pathway conferring light intensity information was the stemmata. Thus, we expected that stemmata would be necessary for the mediation of light-dependent movement behaviors. While our results indicated that the stemmata were not necessary for light-dependent surface movement, these findings do not preclude the stemmata from having some supplemental role in light-dependent behaviors. Larvae retained their sensitivity to ambient light upon removal of sensory input from the stemmata (Fig. 2).

Furthermore, the measured decrease in larval movement during both the *dark* and *light* conditions were not significantly different from the movement measured in the *dark* and *light* conditions of the control animals (Fig. 2d,e). This suggested that the effects of cutting the optic nerve did not eliminate the sensory signals that mediated the larva's light-dependent responses.

Loss of Light-dependent Movement Behavior in Headless Larvae

Because light dependent surface movement persisted after cutting the optic nerve, this indicated the presence of an extraocular, photoreceptor mechanism. Such extraocular systems have been identified in a variety of insects (For a review see Cronin and Johnsen 2016). To identify a possible location of an extraocular receptor, the heads of animals whose optic nerves were previously severed were removed and we tested their movement responses to both *light* and *dark* conditions. We

investigated the possibility that the extraocular receptor was located on the head. The head in firefly larvae is a retractable *appendage* responsible for critical sensory and motor functions including vision, olfaction and feeding. Furthermore, the head can be removed separately from the brain, which is not located in the head (schematic, Fig. 3a).

If the putative extraocular mechanism was located in the head, headless larvae would be expected to lose the ability to detect differences in light intensity. In such a case, larvae should not perceive changes in light intensity and thus their movement should not be light dependent. We would expect this to result in no change between the movement levels between the *light* and *dark* conditions. However, if movement correlated with changes in light level persisted, this would be evidence that the extraocular photoreceptor location is not located in the head.

Larvae with their head removed did not exhibit light dependent changes in movement (Fig. 3). This was supported by the fact that light movement maintained a steady median value across all time intervals (min = 0.36, max = 0.50) (Fig. 3c). In the *light* condition, surface movement exhibited by the headless larvae were increased significantly compared to the surface movement of larvae with severed optic nerves (Fig. 3e). No significant relationship between the time intervals during the *dark* environment was detected (Fig. 3c). Larvae were active during the first 10-minute interval following the *light* to *dark* transition, a time interval where movement was suppressed in both control and cut optic nerve groups (Fig. 3c). There was no significant difference between larval movement in the *dark* environments between the severed optic nerve and headless groups (Fig. 3d).

Extraocular photoreceptors have not previously been reported in firefly larvae. However, our behavioral results indicated photosensitivity after removal of the stemmata suggesting that *Photuris* larvae have an extraocular receptor located somewhere on the head. Adult fireflies have been shown to possess extraocular photoreceptors in the form of intracerebral ocelli (Hariyama 2000). No such organ has been identified in firefly larvae. Although our results support the existence of an extraocular receptor in larvae, the nature and location of such a receptor are unknown. In sawfly larvae it has been proposed that transparent hairs on their head may direct light onto putative extraocular photo-sensing organs (Meyer-Rochow 1974). An area of the larval firefly head, where the cuticle is devoid of

Fig. 4 Proposed site on the *Photuris* larval head for investigating an extraocular receptor. Arrows indicate the stemmata. Circle indicates an area devoid of pigmentation on the dorsal surface of the larval head. mp = mouth parts, an = antenna, r = rostral, c = caudal. Scale = 150 μ m. Figure modified from Murphy and Moiseff 2019

pigment, may serve a similar purpose (Fig. 4). Thus, we recommend that this relatively transparent region be further investigated to see if this area is the site of an extraocular receptor.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Arikawa K, Eguchi E, Yoshida A, Aoki K (1980) Multiple extraocular photoreceptive areas on genitalia of butterfly *Papilio xuthus*. *Nat* 288:700–702
- Brainard GC, Richardson BA, King TS, Reiter RJ (1984) The influence of different light spectra on the suppression of pineal melatonin content in the Syrian hamster. *Brain Res* 294:333–339
- Buschbeck EK, Friedrich M (2008) Evolution of insect eyes: tales of ancient heritage, deconstruction, reconstruction, remodeling, and recycling. *Evol: Educ Outreach* 1:448–462
- Cronin TW, Johnsen S (2016) Extraocular, non-visual, and simple photoreceptors: an introduction to the symposium. *Integr Comp Biol* 56:758–763

- Felisberti F, Ventura DF, Hertel H (1997) Cerebral extraocular photoreceptors in beetles. *Comp Biochem Physiol Part A: Physiol* 118:1353–1357
- Gilbert C (1994) Form and function of stemmata in larvae of holometabolous insects. *Annu Rev of Entomol* 39:323–349
- Hariyama T (2000) The brain as a photoreceptor: intracerebral ocelli in the firefly. *Naturwissenschaften* 87:327–330
- Keene AC, Sprecher SG (2012) Seeing the light: photobehavior in fruit fly larvae. *Trends Neurosci* 35:104–110
- Lundquist C, Baines R, Bacon J (1996) Evidence that histamine is a neurotransmitter in an insect extraocular photoreceptor pathway. *J Exp Biol* 199:1973–1982
- Meyer-Rochow VB (1974) Structure and function of the larval eye of the sawfly, *Perga*. *J Insect Physiol* 20:1565–1591
- Mizunami M (1995) Functional diversity of neural organization in insect ocellar systems. *Vis Res* 35:443–452
- Murphy F, Moiseff A (2019) Anatomy of the stemmata in the *Photuris* firefly larva. *J Comp Physiol A* 205:151–161
- Rieger D, Fraunholz C, Popp J et al (2007) The fruit fly *Drosophila melanogaster* favors dim light and times its activity peaks to early dawn and late dusk. *J Biol Rhythms* 22:387–399
- Schindelin J, Arganda-Carreras I, Frise E et al (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676–682
- Stange G, Howard J (1979) An ocellar dorsal light response in a dragonfly. *J Exp Biol* 83:351–355
- Thorington L (1980) Actinic effects of light and biological implications. *Photochem Photobiol* 32:117–129
- Wakakuwa M, Stewart F, Matsumoto Y et al (2014) Physiological basis of phototaxis to near-infrared light in *Nephotettix cincticeps*. *J Comp Physiol A* 200:527–536
- Wertman DL, Bleiker KP, Perlman SJ (2018) The light at the end of the tunnel: photosensitivity in larvae of the mountain pine beetle (Coleoptera: Curculionidae: Scolytinae). *Can Entomol* 150:622–631

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.