New, sensitive behavioral assay shows scorpions are attracted to multiple wavelengths of light

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Abstract. Scorpions fluoresce a bright green when under ultraviolet light, but the functional significance of the fluorescence is still unknown. A major challenge in studying scorpion fluorescence is the lack of efficient methods for testing the behavioral photosensitivity of scorpions. We have modified previous assays to produce a more sensitive testing device. The apparatus consists of a circular track made of a small Petri dish nested inside a larger one, with an LED shining from the inner chamber across a small sector of the track. We monitored the scorpions' movements in the arenas under three light wavelengths: ultraviolet (399 nm), yellow-green (566 nm), and red (630 nm); all wavelengths were matched to a nighttime light intensity (0.01 irradians). We also tracked each animal's movements in the absence of light as a control. The animals were attracted to 399 and 566 nm light and also showed some attraction to 630 nm. Furthermore, earlier studies suggest that scorpion photoreceptors form a homogeneous population that has been physiologically shown to be maximally sensitive to green wavelengths. We hypothesize that the photoreceptor population might be somewhat responsive to red light too, suggesting that the photoreceptors may respond to a broad spectrum of light or that the photoreceptor population may not be as homogeneous as previously thought. The strong response to UV light, as has been seen in other behavioral assays, remains enigmatic. Overall, this new assay is more sensitive than previous assays for detecting scorpion photoresponse and will be useful for future studies.

Keywords: Photoreception, orientation, behavior, fluorescence

There is limited information on scorpion vision and light response. Scorpions typically have eight eyes: paired medial eyes that have lenses and may form images, and three pairs of lateral eyes that are sensitive to very low light levels (Schliwa & Fleissner 1980; Hjelle 1990). Physiological studies have shown a primary neural response of the medial eyes to green and a secondary response to UV light, but not red or infrared light (Fleissner & Fleissner 2001). Photosensitivity to green light has also been found in parts of the scorpion tail (Zwicky 1968, 1970a,b; Rao & Rao 1973).

Some behavioral studies have explored scorpions' responses to different wavelengths. Blass & Gaffin (2008) found that scorpions moved more sporadically under UV and green light compared to other wavelengths. Gaffin et al. (2012) found that the scorpions had a strong locomotor response to both 505 nm (green) and 395 nm (UV) with their eyes uncovered. When the eyes were covered with foil, the scorpions had a larger response to UV than to the green, and animals with uncovered eyes moved more under the green light than those with covered eyes under the same condition. Furthermore, Kloock et al. (2010) found that scorpions whose fluorescence was reduced by photo-bleaching moved between UV and dark portions of a test arena more frequently than control scorpions.

Our understanding of scorpion vision and the interplay between light, fluorescence, and behavior is hindered by a lack of sensitive behavioral assays. Previous approaches have monitored scorpion responses to light projected from above (Blass & Gaffin 2008; Kloock et al. 2010; Gaffin et al. 2012; Gaffin & Barker 2014). Here we report a new behavioral assay that tests scorpion responses to low-intensity, pure wavelengths of light directed across a discrete portion of a small circular track. We tested the response of scorpions to UV, yellow-green, red, and no light. We found that scorpions were most attracted to the yellow-green and UV light and somewhat attracted to red light; they did not show any behavioral preference in the no-light control.

METHODS

Animal collection and care.—The animals used in this study were 24 female *Paruroctonus utahensis* (Williams, 1968) that were collected near Monahans, Texas. These animals were individually kept in 3.8 L glass jars containing sand (to a depth of approximately 2.5–5.0 cm) and a small piece of clay pot. Diet consisted of one cricket every two weeks, and the sand was moistened three times a week with roughly 5 mL of water to keep the animals hydrated. A small heater maintained the temperature of the room containing the jars at 23–26°C, and the room was at 50–65% RH. The light-dark phase was 23:15–11:15 (light) and 11:15–23:15 (dark).

Arenas.—A plastic petri dish with a diameter of 55 mm was glued with chloroform to the center of a larger plastic petri dish with a diameter of 100 mm, making a circular track for the scorpions to walk. An LED was placed inside an L-shaped bracket, which was glued to the inside of the lid of the large dish so that when the lid covered the petri dishes, the LED touched the inner petri dish wall and directed its light across the track. We ran the LED's wire through a small hole in the lid and connected it to an op-amp circuit. We used a razor blade to scrape off three small ridges on the inside of the lid and used fine grit sandpaper to sand down the smaller inner petri dish to make a tight fit between the lid and dish. To ensure complete darkness in the arena, the entire lid of the large petri dish was covered with 2 layers of electrical tape. The bottom of the large petri dish also had a few layers of electrical tape on the outside of the side walls to keep out incoming light and to ensure a snug fit with the lid. We used a spectrometer (Ocean Optics USB4000 UV-VIS-E) to confirm that no light could enter the petri dishes.

We used four arenas for each set of trials, with each arena containing an LED (5 mm) emitting a different light wavelength: UV (399 nm, Newark), green (566 nm, LED-Tronics), red (630 nm Super Bright LEDs), and no light. We
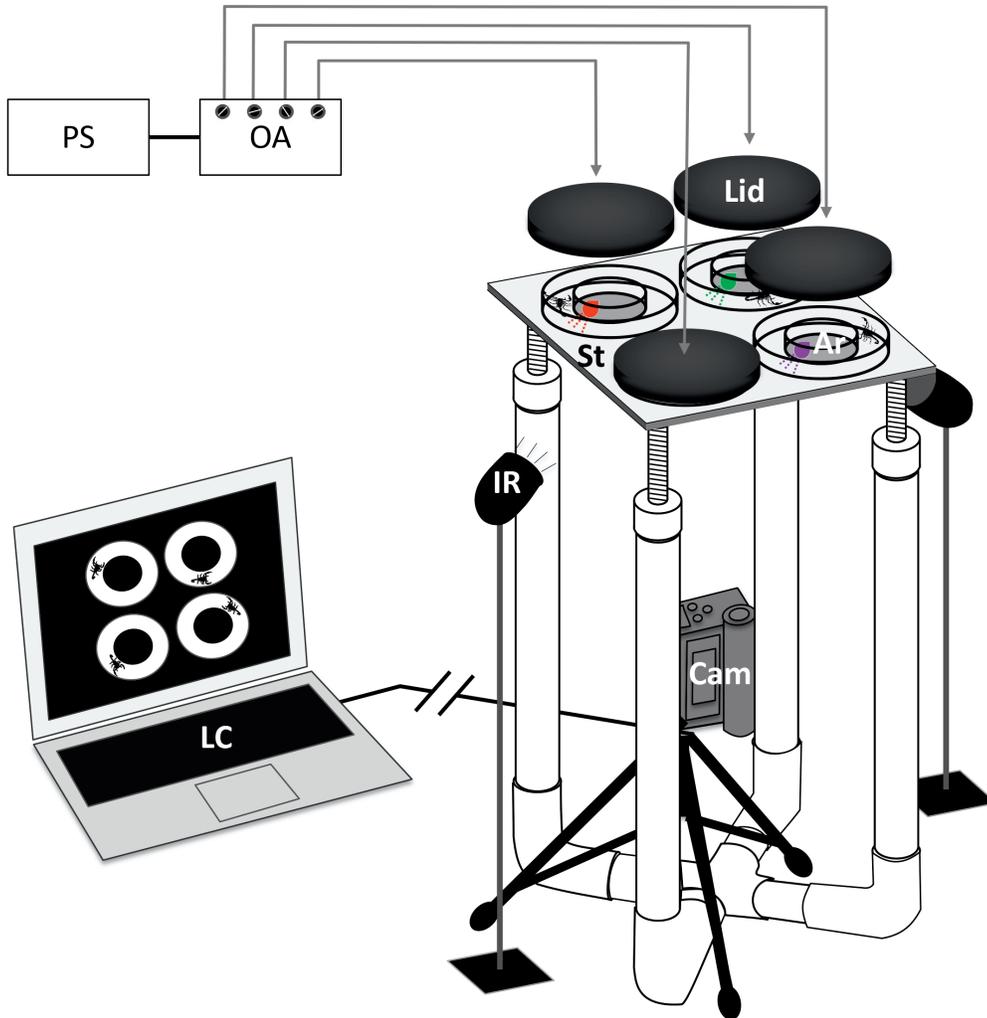
Figure 1.—Experimental apparatus. Four Petri dish arenas (Ar) sit atop a Plexiglas stage (St) supported by a frame made of PVC pipe with threaded extensions used for leveling. An infrared sensitive camera (Cam) monitors the trial from below and relays the feed to a laptop computer (LC) for storage and analysis. Supplemental infrared lamps (IR) direct light across the bottom of the arenas from the side to provide the light necessary to identify the scorpions on the camera (the camera's IR source was covered to reduce glare). LEDs are situated inside the inner Petri dish and direct light radially across the track that holds the scorpion. The LEDs, which are glued to the lids of the dishes, are pictured in their positions after the lid is shut. Wires lead from a power supply (PS) and operational amplifier circuit (OA) to control LED intensity.

used the spectrometer to measure (through the plastic petri dish wall) the LED wavelengths to the nearest nm. The no-light control was identical to the other arenas in that it still contained a bracket and an LED, but the polarity of the circuit was reversed to mimic the potential heat that may be radiating from the LED, further normalizing the experiences between the scorpions. We adjusted a variable resistor on the circuit board to set each light at 0.01 ± 0.005 $\mu W/cm^2/nm$ (as verified by the spectrometer). We chose this irradiance based on scorpion responses in a previous study (Gaffin & Barker 2014).

Apparatus.—We placed the arenas on top of a Plexiglas table supported with PVC legs (Fig. 1) and filmed the arenas from below with an IR sensitive camera (Sony Handycam CCD-TRV16). To reduce the glare on the table, we covered the IR source on the camera with electrical tape and used two additional IR spotlights (Defender) from the side. The spotlights were covered with Parafilm to further scatter the light. The irradiances of the spotlights measured from the distance of the arenas were 0.449 $\mu W/cm^2/nm$ and 0.502 $\mu W/cm^2/nm$, respectively, with a peak wavelength of 845.75 nm. The camera was connected to a computer with a video capture program (Sony Elgato). The computer was about 60 cm away from the apparatus and the screen was oriented away from the apparatus.

The location of each dish and LED color was marked on the table to standardize the placement of the arenas between scorpions. The LED inside each dish pointed towards a unique cardinal direction. The initial pattern was randomly assigned; from there, we rotated the arenas counterclockwise 90° with each trial set.

Trial protocol.—With only a single red light on in the room (oriented away from the apparatus; peak 659.5 nm; 0.0762 $\mu W/cm^2/nm$ at arenas), the four arenas were cleaned with Kimwipes and 70% ethanol. While the arenas were left to dry, the computer and the camera were turned on. Each scorpion was taken out of its jar and placed on the side of the track opposite the LED. The lid was placed on the arena, making sure that the Petri dishes
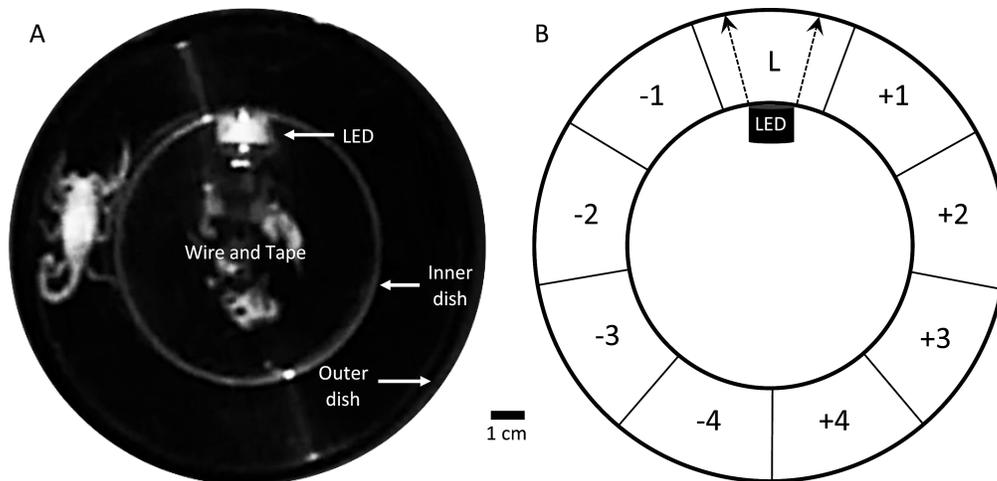
Figure 2.—Close-up and division of arena. A. Scorpions were initially placed in the side of the arena opposite the LED. B. The X and Y coordinates of scorpion location (as determined by MATLAB) were converted into degrees, and each location corresponded to one of the nine sectors of the track; the sector labels correspond to the plots shown in Figure 3. The lines at about 5 and 11 o'clock in the image to the left are glare from the IR beams directed across the bottom of the dishes.

were flush with the lid. The arena was placed in its corresponding spot on the table, facing the appropriate cardinal direction, and the red room light was turned off. The video capture program was set to record for 60 minutes. Once the IR spotlights and LEDs were turned on and the computer screen was turned off, the recording was immediately started. After the 60-minute recording, the red room light was turned on, the video was saved to the computer, and the spotlights and LEDs were turned off. The scorpions were returned to their jars and the arenas were cleaned once again with 70% ethanol. Then, the next set of scorpions was placed in the arenas and the procedure repeated.

The experiment was conducted for three days (Monday, Tuesday, and Thursday) in each of four weeks, with two consecutive trial sets recorded each day (total of 24 scorpions). Each trial set consisted of four scorpions, one for each light variable. Every scorpion experienced each light variable only once, and each animal had a week-long break between trials. After two sets of trials (or one experimental day), the arenas were rotated clockwise approximately 90 degrees to minimize any room effects, such as the Earth's geomagnetic field. The order the scorpions experienced the lights was also randomized.

Two minor departures from the specified schedule and temperature conditions occurred. The first two sets of animals repeated their first light variable twice due to a software glitch that prevented saving the video file of the animals. These animals were still given a week off to reduce acclimation to the test environment. Another deviation from the stated conditions happened unexpectedly in the middle of the second week of trials when the temperature increased from 26°C to 28°C. The temperature returned to 26°C at the beginning of the fourth and final week of trials.

Location mapping / analysis.—Only the scorpions that entered the light sector at least once for each of the four light variables were considered legitimate and analyzed for this assay. Once those scorpions were identified, the footage from each hour-long recording was time-lapsed to 1 frame / 2 seconds (using *iMovie*, Apple Inc.) and condensed further with a MATLAB script to one frame every 3.40 seconds. Another

MATLAB script was used to determine the X and Y coordinates of the location of each scorpion based on the centroid of blob generated by the frame-by-frame subtraction. The size of the centroid was not artificially enlarged near the LED light sources for two reasons: (1) each LED produced a narrow range of wavelengths, which the IR-sensitive camera did not pick up; and (2) the arenas were illuminated and recorded from below, so the IR illumination was distributed evenly across the entire lower surface of each arena. Only frames that differed from the previous frame were retained for analysis (i.e. non-movement frames were removed). Several MATLAB functions were used to convert the linear coordinates for legitimate trials into degrees of a circle. We then computed the frequencies of the scorpions' occupancy in nine equal sectors as shown in Fig. 2 (with the light orientation determining the midpoint of the light-indexed sector). For our final analysis, we averaged the + and − sectors (essentially ignoring differences between left and right of the light and focusing on proximity to the light). Using the average corrected for the fact that there is only one L sector, but two sectors each for +/- 1, +/- 2 etc. This reduced the number of categories being tested in the sector comparison to five.

Thirteen of the 24 scorpions entered the light sector at least once for all four light conditions. We used a repeated measures ANOVA and a Tukey-Kramer multiple comparisons test to assess the average time that these scorpions spent in the light sector compared to the other four sectors and the average time spent in the light sector among conditions (with significance set at $P < 0.05$). We used Prism 6 statistical software (Graph Pad Software, Inc., San Diego, CA, U.S.A.) for our statistical analyses.

RESULTS

Most animals moved readily in the behavioral arenas. Though only 13 of the 24 animals ventured into the light-containing sector for all four light conditions, animals moved away from their starting position in 84 of the 96 trials (87.5%). The animals' typical walking pattern consisted of a few
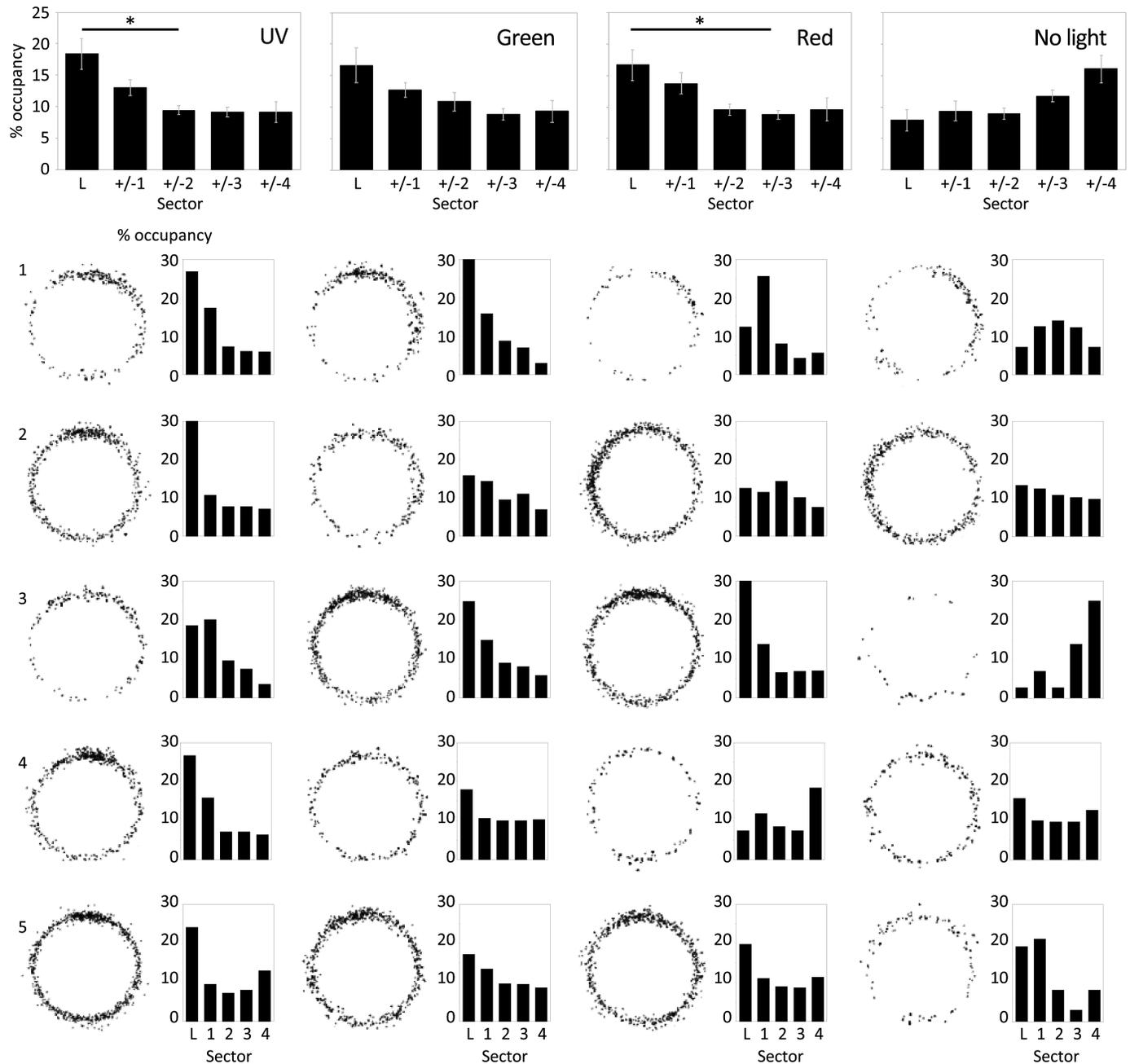
Figure 3.—Analysis of scorpion positions relative to light. Rows 1-5 show plots of activity in Petri dish arenas by 5 of the 13 animals that completed at least one full lap of the arena under all four light conditions during the 1 h test periods, arranged by animal and light condition. Dots represent animal location every 3.40 s. All arenas have been oriented so that the light direction is toward the top of the figure. Histograms next to plots show occupancy by arena sector; L is the light sector and +/- 1 being the average occupancy of the sectors immediately adjacent to the light sector, +/- 2 the average occupancy of sectors two away from the light sector, etc. The histograms at the top of the figure show mean occupancy (+SE) of these five sectors by all 13 animals under each light condition. Significant sector differences ($P<0.05$) compared to the light sector based on Tukey-Kramer multiple comparisons test are indicated by "*".

forward steps followed by a pause lasting a few seconds. While walking, their anterior prosoma was typically angled toward the periphery of the outer Petri dish, with their pedipalps making intermittent contact with the outer wall. Also, we noticed numerous U-turns and rapid walking motions that indicated that animals were free to move in any desired direction if they chose to do so.

Plots of the data for 5 of the 13 animals that completed at least one full lap of the arena under all four light conditions are shown in Fig. 3. We have rotated the plots so that the LED positions are to the top of the figure (even though the LEDs faced various cardinal directions during the trials). The histogram next to each plot shows percent occupancy of the five designated sectors. Over all 13 animals that completed at
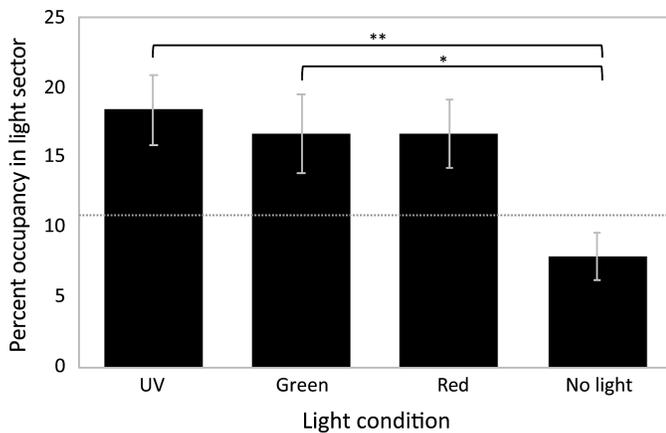
Figure 4.—Summary of behavioral responses to light. Mean (+ SE) percent occupancy in light sector by condition; the dotted line indicates level of random choice (100/9 = 11.1%). Asterisks indicate conditions that are statistically different (** = $P<0.01$, * = $P<0.05$).

least one full lap of the arena under all four light conditions, the sector with the greatest occupancy coincided with the light sector in eight of the UV trials, nine of the green trials, seven of the red trials, and two of the no-light trials. Mean percent quadrant occupancy by light condition for all 13 animals is shown at the top of Fig. 3. Significant differences in the distribution of sector occupancy were seen for the UV and red light conditions (ANOVA, repeated measures, df = 12, UV: F = 5.855, $P = 0.0125$; green: F = 2.659, $P = 0.0899$; red: F = 3.663, $P = 0.0317$; no light: F = 3.649, $P = 0.0630$). Occupancy of the light sector was significantly higher than sector 2 for the UV trials and sector 3 for the red trials (Tukey-Kramer multiple comparisons test). The plot of sector occupancy for the control trials appears skewed toward the sectors farthest away from the LED-containing sector. This is likely because the animals were always started opposite the LED and were not drawn repeatedly to the LED-containing sector as were the light-stimulated animals. When the sector occupancy of the 13 control trials was calculated based on a consistent geocentric orientation, no significant differences were detected among the sectors, indicating that no extraneous cues affected orientation in the test arenas.

Fig. 4 shows the mean percent occupancy in the light sector for the three light conditions and the control (the expectation for random occupancy is indicated by the horizontal dotted line at 11.1% (100% divided by 9 sectors). The repeated measures ANOVA P value was 0.0167 (F = 4.383, df = 12) and considered significant. Significant behavioral differences also existed between UV and no light ($P<0.01$) and green and no light ($P<0.05$) conditions.

DISCUSSION

In this assay, we found significant behavioral responses to yellow-green and UV light and also a response to red. In all of the arenas but the control, the scorpions lingered in the light-containing sector and oriented toward the light, which is different from their typical light-avoiding behavior.

These results differ from what we expected, based on previous physiological and anatomical studies. Gaffin et al.

(2012) found no response to the 565-nm wavelength in their study and they used a higher irradiance (0.15 $\mu W/cm^2/nm$). The physiological response of the median and lateral eyes of *Androctonus australis* (Ewing, 1928) peaks in the blue-green range (500 nm) and falls to half this peak response by 565 nm and essentially no response by 630 nm (Fleissner & Fleissner 2001). Furthermore, a secondary "shoulder" of responsiveness (about 60% of maximum) exists in the 350–400 nm range. Curiously, when illuminated with UV light in the 350–400 nm range, the peak fluorescence emission also falls in the 500-nm range (Fasel et al. 1997). In addition, Fleissner & Fleissner (2001) suggested that scorpions have a homogeneous population of photoreceptors, indicating that scorpions are color blind. However, the results of this assay suggest that scorpions may detect a broader spectrum of light than initially thought. It is possible that the photoreceptors are homogeneous, but respond to a broad spectrum of wavelengths, or that the photoreceptors are heterogeneous, and the scorpions have more than one response maximum.

Although behavioral patterns were not further quantified, we did notice that responsive animals often turned toward the LED, and their pedipalps and forward pairs of legs appeared to make deliberate contact with the inner wall (Fig. 5). Such animals often reversed their course, repeating this movement towards the LED several times; some animals even contorted their bodies in front of the LED, appearing to survey the floor and ceiling of the chamber. The motivations for this behavior suggest an opportunity for follow-up studies.

The configuration of previous behavioral studies used light that flooded the entire arena from above to detect locomotor changes to different wavelengths (Gaffin et al. 2012). In the current study the animals only experienced light when they passed through the light-containing sector of the arena and they could adjust their exposure by their behavior. Scorpions have a protective pigment that shifts on and off the eyes across a day and changes visual sensitivity up to 4 log units (Fleissner & Fleissner 2001). The animals of this and previous studies were dark adapted and the sunset irradiance of 0.01 $\mu W/cm^2/nm$ that we used here was also used in the previous configuration. It is possible that the constant light of previous studies light-blinded the animals and rendered them less sensitive; the limited exposure of the current configuration may have allowed for responses to the longer wavelengths.

There were a few challenges with some components of this study. One concerns the starting position of the scorpions. While each scorpion was initially placed on the side of the arena opposite the LED, some scorpions scurried along the track and immediately experienced the light, whereas the animals that remained motionless started in the dark. To normalize the experience among scorpions, the arenas could be tilted on their sides to shift the scorpions away from the LED until the recording is started. Another issue is the lack of movement from some scorpions, which could be mitigated somewhat by elevating the temperature of the room or the test chambers.

Given these results, future assays can use the 0.01 irradians to explore behavior and perception. It will be interesting to test many additional wavelengths, including below 399 nm and at regular intervals from 400 to 630 nm and beyond. In addition, sensory ablation studies and the use of photo-
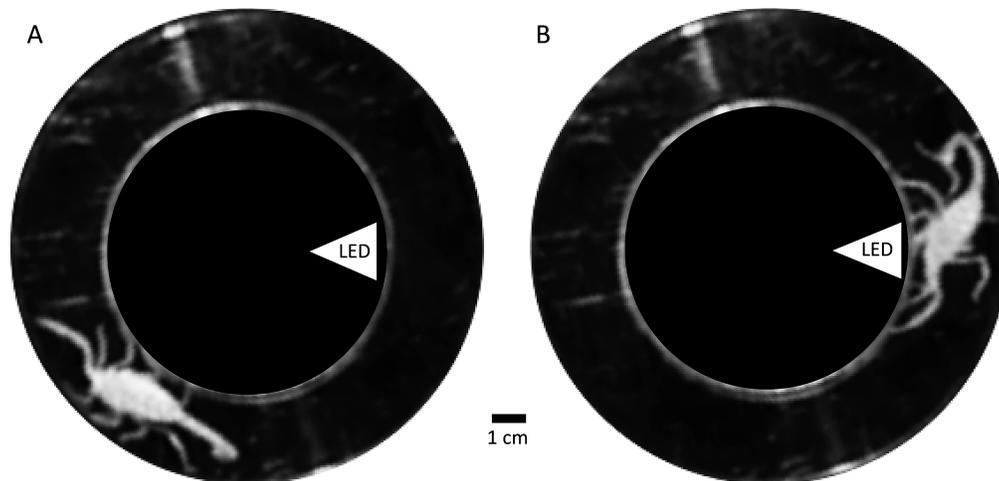
Figure 5.—Typical animal body position in front of and away from LED. A. Animal away from LED with pedipalps surveying outer wall. B. Animal in front of LED (UV in this case) with pedipalps, front legs, and anterior prosoma against inner wall.

bleached scorpions (Kloock 2009) can be used to investigate the contributions of ocular and extra-ocular (Zwicky 1970a,b) photodetection in mediating these responses. Most importantly, the connection to the scorpion's fluorescence, behavior in its natural habitat, and other aspects of its biology need to be better understood and should be investigated.

ACKNOWLEDGMENTS

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