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Behavioral Changes in Crayfish after Daily Exposure to Blue LED Light.

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Ambient light settings play a crucial role in the survival of organisms within their environments. This study investigates the impact of artificial light on crayfish behavior, particularly focusing on the effects of blue light. Crayfish, specifically *Procambarus clarkii*, which possess photoreceptors sensitive to a range of light wavelengths, were exposed to various lighting conditions: 590 nm (amber), 415 nm (blue), full-spectrum blue, and white Light Emitting Diode (LED) lights. The exposure lasted for seven days under a 12 h light/dark cycle, with light intensities maintained at 8-10 lux. An aquatic plus-maze with illuminated (2-3 lux) and non-illuminated (0 lux) arms was used to assess light avoidance behavior. Compared to the 590 nm LED light, treatment with the 415 nm LED light prompted a higher percentage of crayfish to avoid light and enter the non-illuminated arms immediately upon release into the plus-maze. A similar increase in light avoidance behavior was observed with full-spectrum blue and white LED lights. The overall differences in the number of entries and total dwell time were not significantly different between the 590 nm and 415 nm light treatments. Notably, exposure to blue or white LED lights, compared to 590 nm, led to increased avoidance of illuminated areas, regardless of gender differences. These findings suggest that even low-intensity blue light significantly affects crayfish behavior, increasing their aversion to light.

Key words: behavior, blue, Light Emitting Diode (LED) lights, crayfish, photoreceptor, plus-maze.

INTRODUCTION

Understanding the impact of light pollution on animals is essential, as it can disrupt natural processes such as growth, social interaction, predator avoidance, migration, and foraging (Mayer-Pinto et al., 2022). Numerous studies have highlighted the profound effects of ambient light on emotions, moods, and behaviors (Hamid and Newport, 1989; Hong et al., 2021; Mebes, 1979; Syposz et al., 2021). For instance, exposure to blue Light Emitting

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Diode (LED) light in Drosophila increases the activation of stress-responsive genes, leading to a reduction in life expectancy for both sexes (Nash et al., 2019). Similarly, prolonged exposure of juvenile mice to subdued nocturnal lighting results in anxiety development in adulthood (Borniger et al., 2014). These effects extend significantly to aquatic life.

Ambient light settings can influence the behavior of aquatic organisms. Some fish species exhibit positive phototaxis under green and blue light but show negative phototaxis under red and yellow light (Xu et al., 2022). A brief pulse of blue or green LED light can stimulate locomotor activity in crayfish, although there is no significant difference in the velocity or acceleration of their motion (Cedeño Moreno et al., 2023). Notably, crayfish exposed to blue light tend to travel shorter distances compared to those exposed to green light (Cedeño Moreno et al., 2023). Another study on crayfish demonstrated that blue light exposure could either promote or suppress larval growth depending on the season (Toyota et al., 2022). Additionally, increased ambient white light has been associated with heightened aggression in fish (Tatemoto and Serra, 2021).

Crayfish, like many other aquatic species, possess photoreceptors sensitive to blue light wavelengths. However, the specific effects of the blue light spectrum on their behavior remain unclear. This study aims to investigate whether daily exposure to blue light affects crayfish's attraction to or aversion from light. By examining these effects, the goal is to better understand how light pollution, particularly from blue LEDs, might alter the behavior of aquatic life.

The relevance of studying the effects of prolonged daily exposure to blue light on aquatic life is also underscored by the documented harmful effects of blue light on vertebrates. The Council on Science and Public Health has issued a cautionary advisory against the use of blue LED light, as prolonged exposure has adverse effects on human health (American Medical Association, 2016). Research indicates that exposure to blue light not only interferes with hormone secretion (Figueiro and Rea, 2010) but also induces structural and physiological changes in neurons. This is demonstrated by the loss of dendritic spines in neurons of female hamsters subjected to blue light (Bedrosian et al., 2011; Bedrosian et al., 2013). Therefore, this study is valuable for assessing potential risks to a broader range of species and enhancing the understanding of how LED lighting influences animal behavior and health.

Crayfish serve as an excellent model for studying the impact of blue light on behavior due to their unique photoreceptor system. They possess blue-sensitive photoreceptors in three regions: visual photoreceptors in the retina and non-visual photoreceptors in the protocerebrum and caudal region (Larimer, 1966; Prosser, 1934; Wilkens and Larimer, 1972). While retinal photoreceptors in crayfish eyes can be stimulated maximally by either 540 nm or 440 nm wavelength of light (Kingston and Cronin, 2015), the extraretinal photoreceptors in the protocerebrum and caudal region are stimulated by blue and green light (Larimer et al., 1966; Sandeman et al., 1990). These extraretinal photoreceptors also influence the circadian system of crayfish (Arechiga et al., 1993; Prieto-Sagredo and Fanjul-Moles et al., 2004; Rodriguez-Sosa et al., 2008; Sullivan et al., 2009). Interestingly, humans also have specialized intrinsically photosensitive retinal ganglion cells (ipRGCs) that are sensitive to blue light (Berson et al., 2002; Dacey et al., 2005) and play a significant role in regulating the circadian cycle (Wahl et al., 2019; Wirz-Justice et al., 2021).

It is hypothesized that prolonged daily exposure to blue light will significantly affect crayfish exploratory behavior, altering their preference for illuminated versus dark areas. To test this hypothesis, experiments were designed in which four groups of crayfish were exposed to four distinct LED light conditions: 590 nm (amber), 415 nm (blue), full-spectrum blue, and white LED lights. The 590 nm and 415 nm wavelengths are physiologically relevant, as they can be visually detected by crayfish retinal photoreceptors (Kingston and Cronin, 2015). In all treatments, the LED light intensity was maintained at 8-10 lux, and crayfish were housed individually under a 12 h light/dark cycle for seven days. After the LED light treatments, the navigation of crayfish within an aquatic plus-maze was recorded. The plus-maze, which includes two illuminated and two non-illuminated arms, is a useful tool for assessing crayfish attraction to or aversion from illuminated areas (Fossat et al., 2015). This study represents the first attempt to assess the effects of daily blue light exposure on the exploratory behavior of crayfish within a plus-maze.

MATERIALS AND METHODS

All experiments and behavioral analyses were conducted at Central Connecticut State University. Medium-sized adult crayfish (*Procambarus clarkii*), approximately 6.5–7.6 cm in length and of mixed sex, were used in the present study (Carolina Biological, NC; Catalog No: 142512). No specific protocol or approval number was required for this study; however, the care of the crayfish adhered to the guidelines outlined in the *Carolina Arthropods Manual*. Crayfish were sexed based on the presence of the first pair of enlarged swimmerets in males and the seminal receptacle in females. They were separated by gender and housed individually in aquariums with dividers.

Each enclosure measured 23 x 15 x 16.7 cm (L x W x H), and the water temperature was maintained at 25 ± 1°C. Food pellets were provided, and the water was replaced with bubbled, fresh filtered water every two days. Prior to the start of the experiments, all crayfish were kept in the dark for three days to allow for acclimation to the laboratory environment.

Following this acclimation period, the crayfish were randomly assigned to one of four groups, each exposed to a different light treatment: 590 nm (amber), 415 nm (blue), full-spectrum blue LED, or white LED. The blue LED lights emitted wavelengths in the blue spectrum (400–500 nm), while the white LED lights emitted visible light wavelengths ranging from 400–700 nm. The lights were mounted above the terrariums and were turned on daily from 8:00

AM to 8:00 PM, with the lights off during the remaining hours. The exposure lasted for seven days. Soft or recently molted crayfish were excluded from the study.

After the seven-day exposure period, behavioral tests were conducted in complete darkness using a custom-built aquatic plus-shaped maze. The maze consisted of two illuminated arms and two non-illuminated arms to assess the crayfish's light preference and avoidance behavior.

The plus shaped maze

The behavioral tests for crayfish were set up following the protocol outlined by Fossat et al. (2015). Each arm of the plus-maze measured 25 x 10 x 20 cm (L x W x H) (Figure S1). The central platform area (neutral space) where all four arms converged had dimensions of 10 x 10 x 20 cm (L x W x H). The outer walls of the two illuminated arms were covered with white paper, while the outer walls of the two non-illuminated arms were covered with opaque black paper. The maze was placed inside a custom-made circular aluminum drum, with the inner wall covered in black cloth to prevent the crayfish from seeing their surroundings. The drum was 60 cm in height, further ensuring the crayfish were unaware of their external environment.

Different LED light sources were tested to illuminate the maze arms, and the optimal source was determined to be an AC-powered, dimmable white LED linear light bar (4000 Kelvin) placed along the bottom sides of each illuminated arm outside the maze (Figure S1). The light bars were directed upward toward the ceiling to maintain a low light intensity within the illuminated arms. Both the light bars and the illuminated arms were 25 cm in length. The light intensity inside the illuminated arms was adjusted to 2–3 lux, as measured from within the arms themselves. The light intensity in the neutral space was approximately 1 lux, while the non-illuminated arms were kept at zero lux. All recordings were conducted in a laboratory setting devoid of any ambient light.

LED lights used for behavioral tests

The following LED lights were used in the study: 590 nm LED light (Amazon.com, Seattle, WA; Cat No. B097CJ6674), 415 nm LED light (Amazon.com; Cat No. B097CGJ33Z), blue LED light, and white LED light strip (Homedepot.com, Atlanta, GA; Cat No. 1003620390), as well as white LED light bars for the illuminated arms (Amazon.com; Cat No. B01M22BSBE). All white LED lights were bright white (4000 Kelvin). Light intensity was measured using a light photometer (LI-COR Biosciences, Lincoln, NE; LI-189) and adjusted to ensure that all terrariums received a uniform light intensity of 8-10 lux.

Behavioral test after treatment with LED lights

All behavior testing was conducted between 9:00 AM and 2:00 PM. At the start of each test, the maze was filled with 2.5 cm of water. A crayfish was then placed into an opaque confinement box situated at the center of the plus-maze (neutral zone) and allowed to rest for five minutes to acclimate. After this acclimation period, the crayfish was released from the confinement box and allowed to navigate the maze freely. An infrared video camera (Reolink, Hong Kong; Model No: RLC-410-5MP) was mounted 71 cm above the maze (Figure S1). The open design of the maze allowed for clear visibility of the crayfish's activity. For each crayfish, behavior inside the maze was recorded and saved to a hard drive for subsequent analysis. The videos were then replayed using VLC Media Player for Windows (VideoLan, 2006; VLC media player, Retrieved from https://www.videolan.org/vlc/index.html). An individual observer, blind to the identity of the crayfish, reviewed the videos. Behavioral data were analyzed for a 15-minute period (900 sec) following the crayfish's release from the neutral zone.

Entries from the neutral zone into either the illuminated or non-illuminated arms were scored based on the anatomical location of the crayfish's eyes, located in the cephalothorax region, as a reference point. For each entry, the crayfish's eyes had to align with the boundary marking the beginning of the arm's walls to be considered a new entry. Entry and exit times in various maze regions were recorded and later analyzed using GraphPad Prism 8.0 Software (San Diego, CA).

Statistical analysis

Data were organized and statistically analyzed using GraphPad Prism 8.0 and RStudio (Boston, MA) by an individual who was unaware of the control and experimental groups. The analyses were based on the following measurements:

1. Time to first entry into an illuminated or non-illuminated arm.
2. Total number of entries into an illuminated or non-illuminated arm by a crayfish.
3. Total dwell time in illuminated or non-illuminated arms by a crayfish.
4. Dwell time in illuminated or non-illuminated arms based on the gender of the crayfish.
5. Total neutral zone dwell time by a crayfish.

These measurements (items 1-5) were assessed for each of the light treatments: 590 nm, 415 nm, blue-LED, and white-LED. For item 1, the Mantel-Cox log-rank test was performed to evaluate the statistical difference in the percentage of crayfish that made their first entry into either the illuminated or non-illuminated arm. For items 2, 3, and 4, differences between the non-illuminated and illuminated arm measurements were calculated. Specifically, for items 2 and 3, a Kruskal-Wallis test was performed to compare the effect of the light treatments, followed by Dunn's test for post-hoc comparisons, with Holm's adjusted p-values. For item 4, a non-parametric rank transformation was performed to assess the effect of light treatments, gender, and their interaction on the differences. For item 5, a Kruskal-Wallis test was again performed to compare the effect of light treatments on the dwell time in the neutral zone, with Dunn's test for post-hoc comparisons and Holm's adjusted p-values.

The use of the Kruskal-Wallis test was due to the rejection of the normality assumption, which was assessed using the Shapiro-Wilk test. The assumption of equal variance for ANOVA was assessed using Levene's test.

The number of crayfish used in the behavior tests for items 1, 2, 3, and 5 were as follows: 16 for 590 nm, 21 for 415 nm, 37 for blue, and 36 for white light. Some blue and white light-treated crayfish were not sexed, so the analysis of gender differences in item 4 includes the following number of crayfish: 16 for 590 nm, 21 for 415 nm, 18 for blue, and 13 for white light. Data from the following scenarios were excluded from the analysis for the 900-second ALB recording:

1. Crayfish that appeared immobile and did not move after being released into the neutral zone
2. Crayfish that made only a single slow entry and remained in the same arm for the entire duration of the recording.

Only two crayfish from the white LED group and one crayfish from the 590 nm group were omitted. These crayfish were excluded because their immobility suggested they may have been unhealthy.

RESULTS

Percentage of crayfish making the first entry into an illuminated or a non-illuminated arm

Upon release from the neutral zone (middle part of the plus-maze), a crayfish chose to enter one of the four arms of the maze. Latency refers to the time elapsed between the start of an experiment and the first entry of the crayfish into a particular arm. Comparing latency values across all four light treatments could introduce statistical bias (Jahn-Eimermacher et al., 2011). This is because not all crayfish enter the same arm upon repeated measurements. Furthermore, while some crayfish enter the illuminated arm, others enter the non-illuminated arm, and vice versa. Therefore, the data were used to calculate the percentage of crayfish that made their first entry into either the illuminated or the non-illuminated arm.

A Kaplan-Meier survival curve was plotted (Figure 1). For the percentage of crayfish that made their first entry into the non-illuminated arm (solid line), those that did not enter the illuminated arm were censored at 900 sec. Similarly, for the analysis of the percentage of crayfish that made their first entry into the illuminated arm (dotted line), crayfish that first entered the non-illuminated arm were censored at 900 sec.

The control group was treated with 590 nm LED lights, which lacked the blue spectrum. Behavioral analysis showed that crayfish in the control group made random entries into either an illuminated or non-illuminated arm. Among the crayfish, 43.75% made their first entry into an illuminated arm, while 56.25% first entered the non-illuminated arm (Figure 1A).

Statistical comparison revealed that this difference was not significant (Mantel-Cox test, $X^2 = 0.14$, DF = 1, P = 0.71, N = 16). In comparison to the control group, crayfish treated with 415 nm blue LED lights made significantly more first entries into the non-illuminated arm. Of the crayfish exposed to 415 nm light, 66.6% first entered a non-illuminated arm, while only 33.3% entered an illuminated arm (Figure 1B). This difference was statistically significant (Mantel-Cox test, $X^2 = 4.72$, DF = 1, P = 0.030, N = 21).

Next, groups of crayfish were subjected to similar treatments using two different LED lights. The blue LED lights emitted a slightly stronger wavelength in the blue spectrum (400-500 nm), while white LED lights emitted visible light wavelengths ranging from 400-700 nm. Kaplan-Meier plots revealed that only 21.6% of crayfish exposed to blue LED lights first entered an illuminated arm, compared to 78.4% that first entered the non-illuminated arm (Figure 1C). This difference was highly significant (Mantel-Cox test, $X^2 = 28.8$, DF = 1, P < 0.0001, N = 37).

A similar trend was observed with white LED-treated crayfish, where 69.4% made their first entry into a non-illuminated arm, and 30.6% entered the illuminated arm. The difference between the percentages of crayfish that first entered the illuminated and non-illuminated arms was also statistically significant (Figure 1D; Mantel-Cox test, $X^2 = 12.2$, DF = 1, P = 0.0005, N = 36).Upon release from the confinement box, the crayfish oriented their head toward either the illuminated arm, the non-illuminated arm, or a corner within the confinement box. It was possible that the orientation of the crayfish's head just before release could have influenced its decision to enter a particular arm. However, no significant relationship was found between the head orientation and the choice of arm (Pearson correlation coefficient, 2- tailed, 81 XY pairs, R = 0.136, P > 0.05).

Total number of entries into the illuminated and non-illuminated arms of the plus-maze

One would ask if exposure to blue wavelength light affect the frequency with which crayfish enter the non-illuminated and illuminated arms during the 900-second experimental period. To address this question, an observer, unaware of the control and experimental groups, recorded the number of times a crayfish entered the illuminated and non-illuminated arms. Crayfish entry and dwell time in a particular arm began when the eyes of a moving crayfish crossed the boundary marking the start of the arm.The number of entries into both the illuminated and non-illuminated arms after exposure to either 590 nm, 415 nm, blue, or white LED light was plotted (Figure 2A). Overall, most crayfish made more entries into the non-illuminated arms across all treatments. Since measurements of time spent in the non-illuminated and illuminated states were taken from the same crayfish, the difference in the number of entries into each arm provides a testable comparison. To statistically assess which treatments resulted in increased entries into either arm, the difference between the number of entries into the non-illuminated arm and the illuminated arm per crayfish was calculated (Figure 2B). Positive differences indicate more entries into the non-illuminated arm than into the illuminated arm, while negative differences indicate the reverse.

A Kruskal-Wallis test was performed to compare the effect of the light treatments on these differences, followed by Dunn's post-hoc test (Table 1). The results revealed a statistically significant difference in the distribution of the difference in the total number of entries between the non-illuminated and illuminated arms ($X^2(3) = 23.12$, P < 0.001).Dunn's test, with p-values adjusted using the Holm method, showed that light treatments with blue LED and 590 nm (3.76, P < 0.001), white LED and 590 nm (4.30, P = 0.0001), and white LED and 415 nm (2.73, P = 0.025) all produced statistically significant differences. The median differences were positive, indicating more entries into the non-illuminated arm for

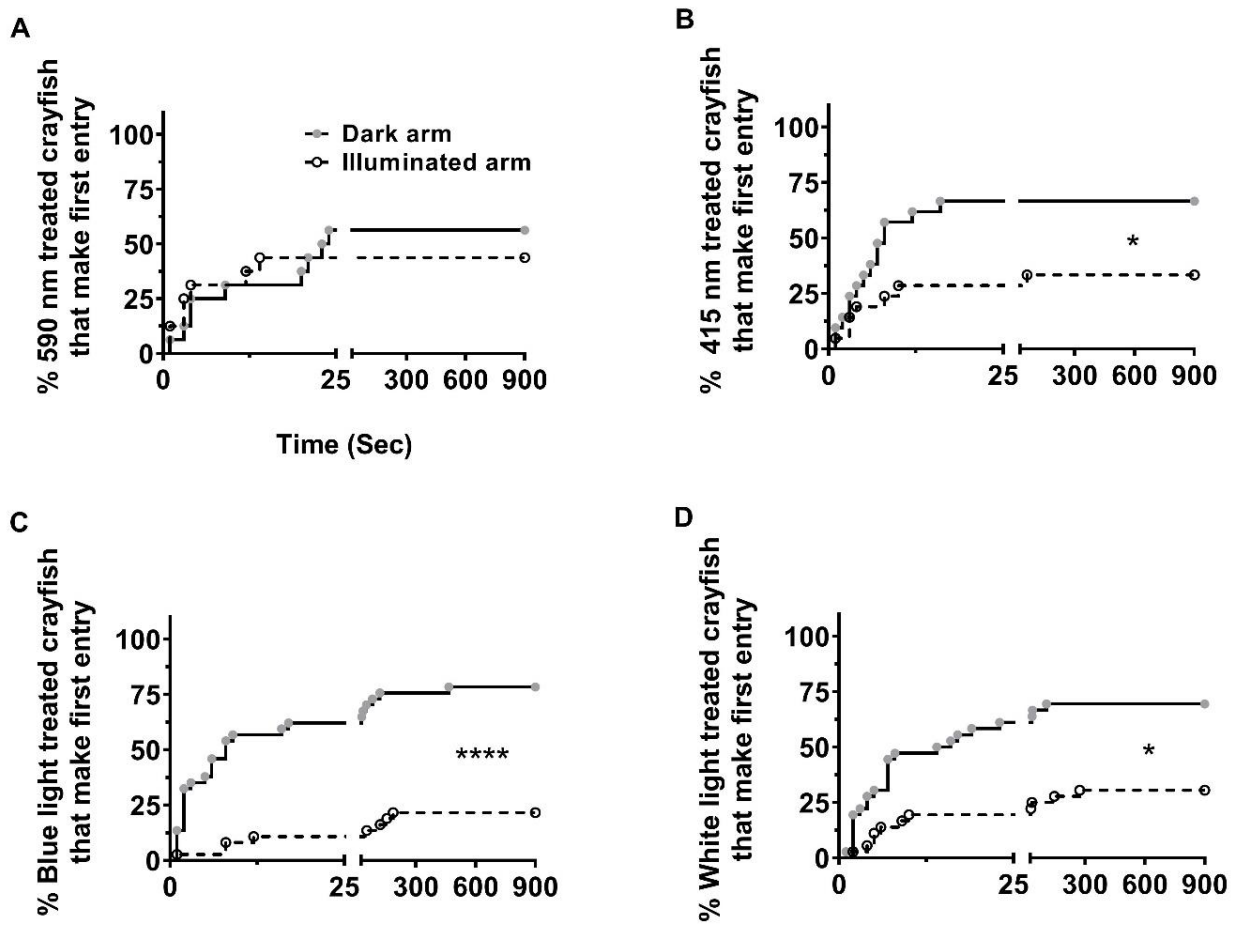

Figure 1. The impacts of different light treatments on the initial entry of crayfish into either an illuminated or non-illuminated arm. The experiment included different LED light treatments: 590 nm (control) (A), 415 nm (B), blue (C), and white light (D). The treatments were applied for 12 h per day for seven days, following a 12 h light/dark cycle. The graph illustrates the relationship between individual crayfish latency (Time; measured in sec) for their first entry into either the non-illuminated arm (represented by gray filled circles) or the illuminated arm (represented by open circles). The data is pooled to include male and female crayfish. Mantel-Cox test * and **** at $P < 0.05$ and $P < 0.0001$ respectively. Data were censored at 900 sec.

the blue and white light treatments compared to the 590 nm treatment, as well as for white light compared to 415 nm (Table 1). The remaining comparisons were not statistically significant.

Total dwell time inside the illuminated and non-illuminated arms

The effect of blue light treatment on the duration of crayfish dwelling in either the illuminated or non-illuminated arms during the 900-second navigation activity was examined. To determine this, all time intervals during which a crayfish entered and explored the illuminated arm were summed. This summed value represents the total dwell time in the illuminated arm for each crayfish. The total dwell time in the non-illuminated arm was calculated in a similar manner.

No significant difference in the total dwell time in either the illuminated or non-illuminated arms was observed among control crayfish treated with 590 nm LED light (Figure 3A). However, a notable contrast emerged between the effects of the 415 nm, blue, and white LED light treatments, with most crayfish spending a longer duration in the non-illuminated arm compared to the illuminated arm. To statistically assess whether crayfish dwell longer in the non-illuminated or illuminated arms, the difference in total dwell time between the non-illuminated and illuminated arms was calculated for each crayfish (Figure 3B). Positive differences indicate longer dwell time in the non-illuminated arm, while negative differences indicate longer dwell time in the illuminated arm.

A Kruskal-Wallis test was performed to compare the effect of light treatments on these differences, followed by Dunn's test for post-hoc comparisons. A significant

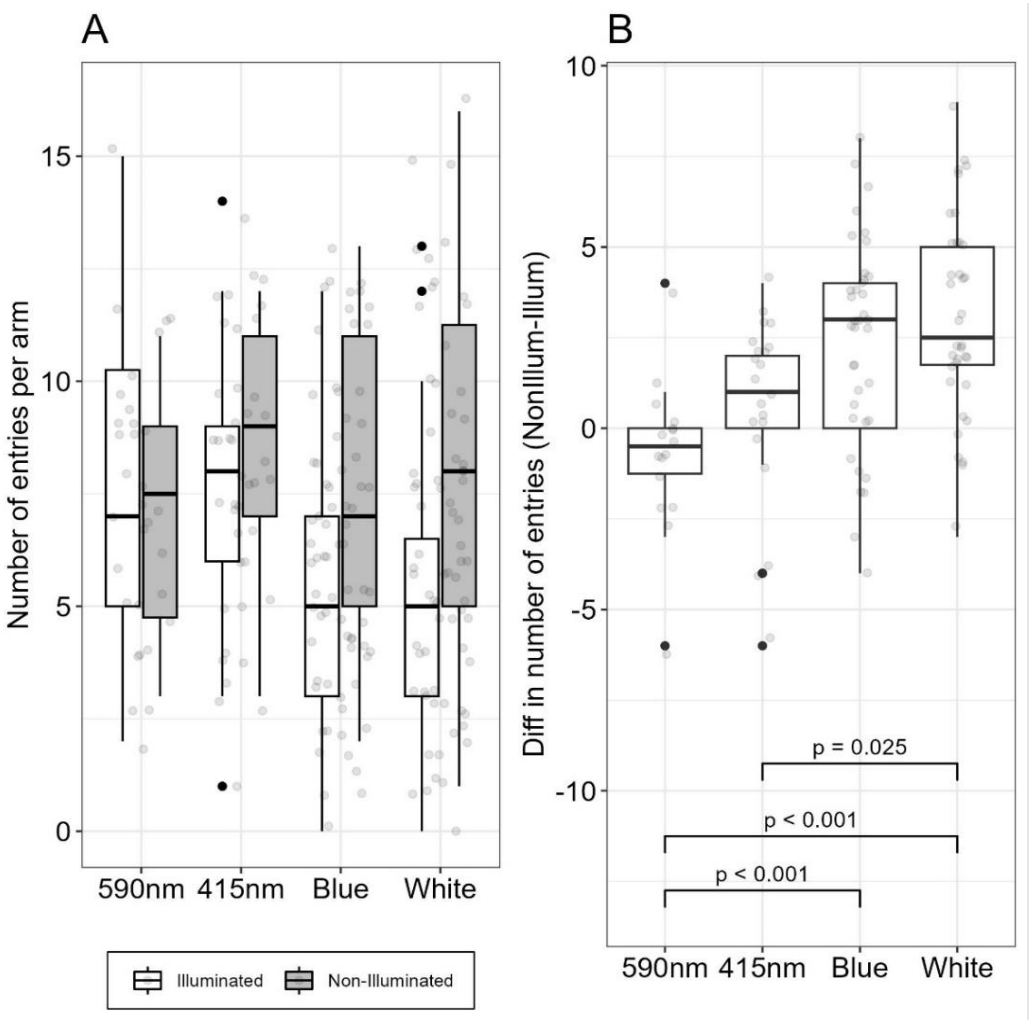

Figure 2. A box-plot diagram showing the influence of light treatments on the total number of crayfish entries into an illuminated or non-illuminated arm. A: Actual number of overall entries into the illuminated (white boxes) or non-illuminated arms (gray boxes) after different light treatments. B: Difference between the number of entries into non-illuminated and illuminated arm. Positive values represent more entries into the non-illuminated arms. Each box visually represents the interquartile range (IQR), encompassing 50% of the dataset. A median value is denoted by a central line within each box. The upper and lower quartiles are symbolized by the top and bottom whiskers, respectively. Every gray circle on the plot represents the value for an individual crayfish, while black circles signify outlier data points. At the bottom of the boxplots are p-values and lines indicating statistically significant differences in the median difference in the number of entries based on comparing light conditions using Dunn's post-hoc test.

Table 1. A statistical analysis comparing the differences in the total number of entries into either the illuminated or non-illuminated arms following treatment with different lighting conditions. Differences are non-illuminated minus illuminated total number of entries.

Light treatment	Difference in total number of entries (Median ± IQR)	Dunn's post-hoc comparisons (Test stat, Holm's adjusted p-value)
590 nm	-0.5 ± 1.25	White and 415nm (2.73, P =0.025) ; Blue and 590nm (3.76, P< 0.001) ; White and 590nm (4.30, P < 0.001).
415 nm	1.0 ± 2.00	
Blue LED	3.0 ± 4.00	
White LED	2.5 ± 3.25	
Kruskal-Wallis	$X^2(3) = 23.12$; P = 0.000038	

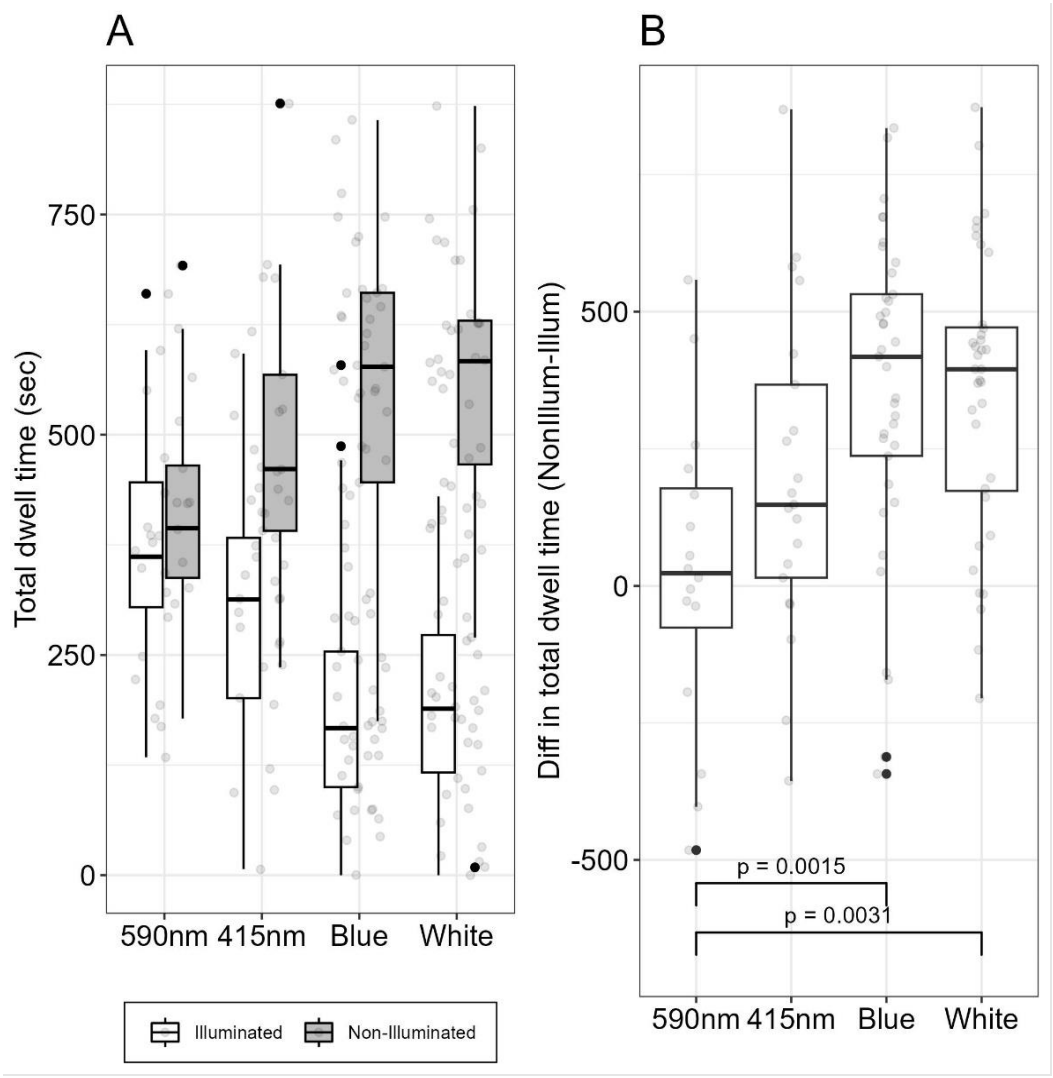

Figure 3. Graphs illustrating how different light treatments impact the cumulative dwell time of crayfish within illuminated or non-illuminated arms. A: The dwell time is represented by white boxes (illuminated arms) and gray boxes (non-illuminated arms). B: Difference in the dwell time inside the non-illuminated and illuminated arm. Positive values indicate greater dwell time inside the non-illuminated arms. A median value is denoted by a central line within each box. Every gray circle on the plot represents the value for an individual crayfish, while black circles signify outlier data points. At the bottom of the boxplots are p-values and lines indicating statistically significant differences in the median difference in the total dwell time based on comparing light conditions using Dunn's post-hoc test.

statistical difference ($X^2(3) = 17.65$; $P < 0.001$) was found in the distribution of the total dwell time differences among the treated crayfish (Table 2). Dunn's post-hoc comparisons revealed that the distribution of dwell time differences between the illuminated and non-illuminated arms was significantly different for the blue LED and 590 nm treatments (3.65, P = 0.0015), as well as for the white LED and 590 nm treatments (3.93, P = 0.0031). The median differences were positive, indicating more time spent in the non-illuminated arm for the blue and white light treatments compared to the 590 nm treatment (Table 2). The remaining comparisons were not statistically significant.

The effects of gender on total dwell time in either illuminated or non-illuminated arms

The potential gender-specific effects of 590 nm, 415 nm, blue, and white LED light treatments were also investigated (Figure 4A). Consistent with the findings in section 3.3 regarding total dwell time, most crayfish in all treatments spent more time dwelling in the non-

Table 2. A statistical analysis comparing the differences in the total dwell time into either the illuminated or non-illuminated arms following treatment with different lighting conditions. The differences are non-illuminated minus illuminated total dwell time.

Light treatment	Difference in total dwell time (Median ± IQR)	Dunn's post-hoc comparisons (Test stat, Holm's adjusted p-value)
590 nm	23 ± 254.25 sec	
415 nm	148 ± 352.00 sec	Blue and 590nm (3.65, P = 0.0015);
Blue LED	418 ± 295.00 sec	White and 590nm (3.93, P = 0.0031).
White LED	395 ± 298.25 sec	
Kruskal-Wallis	$X^2(3) = 17.65$; $P = 0.00052$	

illuminated arm, regardless of gender. The impact of gender on the difference in total dwell time between the non-illuminated and illuminated arms was assessed using statistical analysis (Figure 4B). Positive differences indicate longer dwell time in the non-illuminated arm, while negative differences indicate longer dwell time in the illuminated arm.

A nonparametric rank transformation (Conover and Iman, 1981) of the mean rank differences was applied to analyze the interaction between gender and light treatment on the difference in average total dwell time (non-illuminated – illuminated; Table 3). The analysis revealed no statistically significant interaction between gender and light treatments ($F(3, 60) = 0.19$, $P = 0.900$). Simple main effects analysis showed that light treatment had a statistically significant effect on the difference in dwell time ($F(3, 60) = 3.358$, $P = 0.025$), whereas gender did not ($F(1, 60) = 0.068$, $P = 0.795$).

Total dwell time inside the neutral zone

At the beginning of the experiment, each crayfish started navigation in the center of the maze, known as the neutral zone. It is at this location that a crayfish first decides whether to enter an illuminated or non-illuminated arm. The neutral zone is not completely dark and has minimal illumination (~1 lux). Dwell time in the neutral zone began when a crayfish entered the neutral space from an arm. An entry into, and exit from, the neutral zone was defined when the anatomical location of the moving crayfish's eyes aligned with the edge of an arm wall.

Behavioral analysis assessed the amount of time crayfish spent in the neutral zone during their 900-second plus-maze navigation (Figure 5). A Kruskal-Wallis test was conducted to compare the effect of the light treatments on time spent in the neutral zone (Table 4). The test revealed no statistically significant difference in the distribution of time spent in the neutral zone for crayfish based on light treatments ($X^2(3) = 4.85$, $P = 0.1831$).

DISCUSSION

The use of artificial light and light pollution can create stressful conditions for aquatic animals, including crayfish, which possess retinal, central, and caudal photoreceptors sensitive to both short and long light wavelengths. The behavioral impact of prolonged exposure to blue light on crayfish is not well understood. This study tested whether daily exposure to blue light makes crayfish more or less averse to light. Using *Procambarus clarkii* as a model organism, navigation in a plus-maze with both illuminated and non-illuminated arms was recorded to assess exploratory behavior in lit and dark regions. Four groups of crayfish were subjected to four different light treatments: 590 nm, 415 nm, blue, and white LED lights. Both 590 nm and 415 nm LED lights fall within the range of wavelengths visually detectable by crayfish. All LED lights were administered under similar treatment conditions. While blue LED lights primarily emit a greater concentration of wavelengths within the blue spectrum (400–500 nm), white LED lights emit a visible wavelength spectrum spanning 400–700 nm.

Compared to the control group treated with amber-colored 590 nm LED light, a higher percentage of crayfish exposed to 415 nm blue LED light made their first entry into the non-illuminated arm (Figure 1).

This effect was more pronounced for the treatments with either blue or white LED lights, which contain a broader spectrum of blue wavelengths. While 415 nm LED light showed no significant effect on the number of entries into the illuminated or non-illuminated arms, both blue and white LED light treatments prompted a significant increase in crayfish entries into the non-illuminated arms (Figure 2). Both blue and white LED treatments also led to significantly longer periods of time spent dwelling within the non-illuminated arms, with the difference in dwell time between the non-illuminated and illuminated arms being significantly greater compared to the 590 nm treatment (Figure 3).

This behavioral response was not influenced by gender (Figure 4). No variations were observed in the median dwell time per entry into either arm or in the time spent

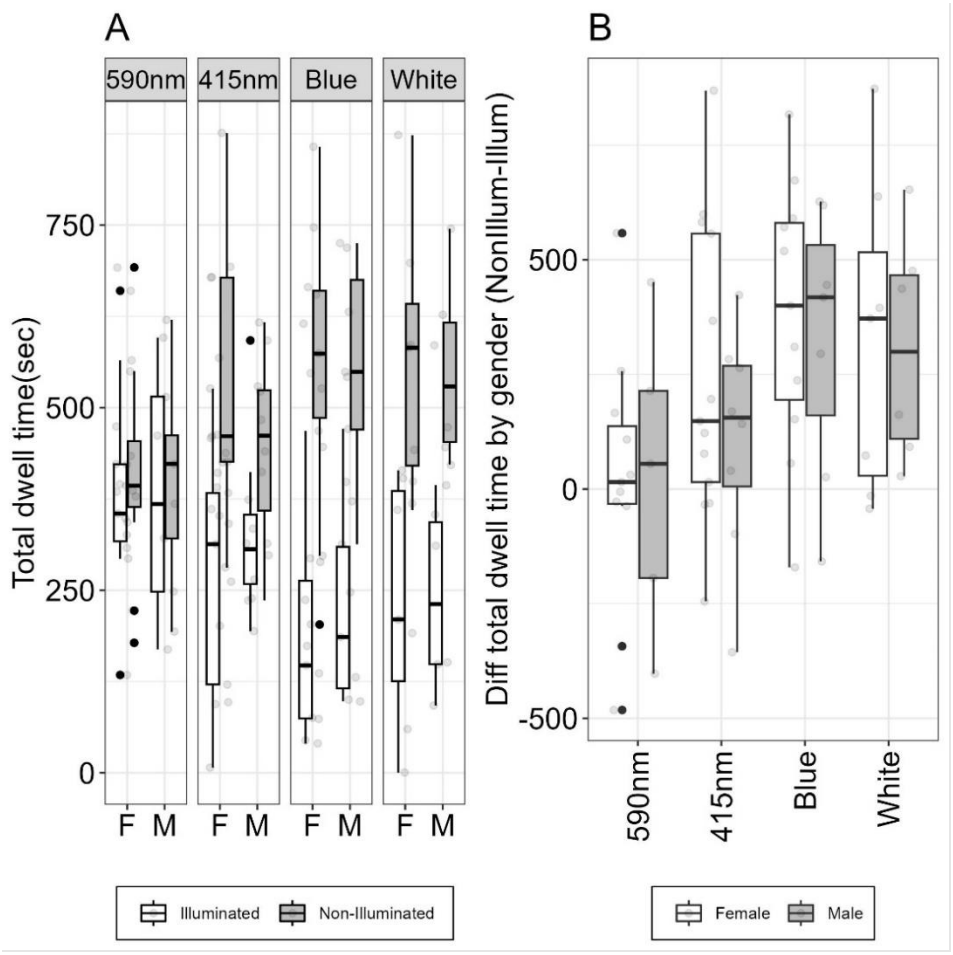

Figure 4. Box-plots graphs depicting how different light treatments affect the cumulative dwell times within illuminated and non-illuminated arms, with data segmented by gender. A: Dwell times of male crayfish female crayfish treated with either 590 nm, 415 nm, blue or white LED lights. White and gray boxes represent illuminated and non-illuminated arms, respectively. B: Difference in the dwell time inside the non-illuminated and illuminated arm between genders. Positive values indicate greater dwell time inside the non-illuminated arms. A median value is denoted by a central line within each box. Every gray circle on the plot represents the value for an individual crayfish, while black circles signify outlier data points.

within the neutral zone after the 590 nm, 415 nm, blue, and white LED light treatments (Figure 5). Overall, these results reveal a notable pattern: treatment with broad-spectrum blue light reduces the crayfish's drive to explore the illuminated section of the plus maze.

It is important to note that the LED light intensity in these experiments was set to 8–10 lux for daily exposure. This intensity is equivalent to a twilight setting during a full moon and cannot be considered a noxious stimulus. For reference, the lighting in a standard office is typically around 500 lux, often lower (Spitschan et al., 2016). Exposure to bright, noxious light could result in significant alterations in neuroendocrine hormones and neurotransmitters. In fact, bright white polychromatic light with an intensity of 2,500 lux has an acute effect on the suppression of melatonin in crayfish (Holzman, 2010). Prolonged exposure to high-intensity light can alter metabolic and behavioral activities, such as oxygen uptake, lactate concentrations, and motor activities, in *Procambarus clarkii* (Fanjul-Moles et al., 1998). In the present experiments, the blue light treatment may have increased crayfish sensitivity to the white light inside the illuminated arms. However, this possibility is ruled out by the results, which show that treatment with 590 nm LED light produced no significant difference in the percentage of crayfish entering either illuminated or non-illuminated arms upon release in the plus-maze (Figure 1A).

Many species exhibit gender-based variations in behavior and physiology when responding to different light stimuli. In this study, treatments with broad-wavelength blue LED light resulted in significantly higher dwell times in the arms devoid of light, and this behavior was not gender-specific (Figure 4). These findings align with other studies indicating that light stimuli similarly

Table 3. A statistical analysis assessing the variations in total dwell time within the illuminated and non-illuminated arms among males and females under different lighting conditions after treatment. Differences are non-illuminated minus illuminated total dwell time by gender.

Light treatment	Gender	Difference in total dwell time (Non-Illuminated-Light) (Mean ± SD)
590 nm	Female	21.73 ± 276.55 sec
590 nm	Male	24.60 ± 335.13 sec
415 nm	Female	247.85 ± 321.79 sec
415 nm	Male	108.38 ± 245.36 sec
Blue	Female	377.64 ± 293.76 sec
	Male	324.57 ± 295.60 sec
White	Female	327.57 ± 346.38 sec
	Male	308.00 ± 249.10 sec
Rank transformation of the differences		Light ($F_{(3,60)} = 3.356$, $P = 0.025$)* Gender ($F_{(1,60)} = 0.068$, $P = 0.795$) Interaction ($F_{(3,60)} = 0.195$, $P = 0.900$)

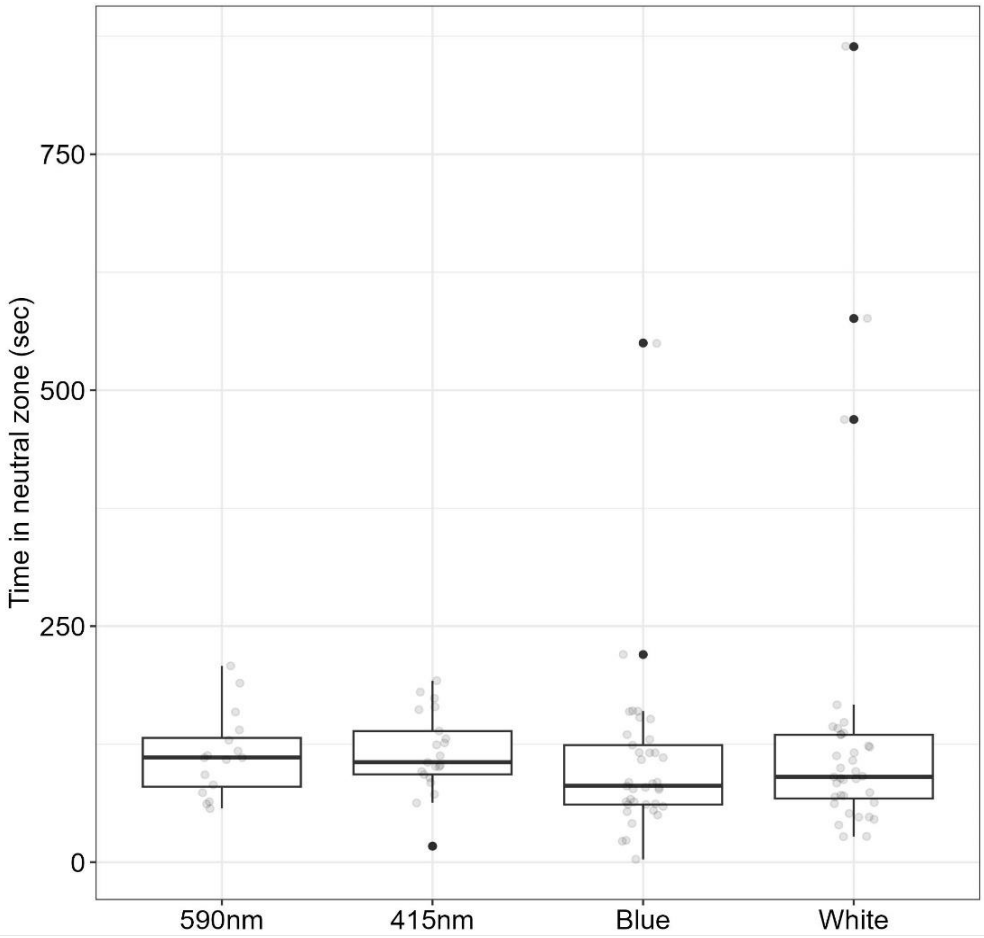

Figure 5. A box-plot graph depicting the duration of crayfish dwell time within the neutral zone of a plus-maze after exposure to various LED light treatments. The neutral zone is the middle part of the plus-maze where crayfish were released at the beginning of every experiment. A median value is denoted by a central line within each box. Every gray circle on the plot represents the value for an individual crayfish, while black circles signify outlier data points.

Table 4. A statistical analysis comparing the neutral zone following treatment with different lighting conditions.

Light treatment	Dwell time in Neutral (Median ± IQR)	Dunn's post-hoc comparisons (Test stat, Holm's adjusted p-value)
590 nm	111.0 ± 51.75 sec	
415 nm	106.0 ± 46.00 sec	
Blue LED	81.0 ± 63.00 sec	
White LED	90.5 ± 67.50 sec	
Kruskal-Wallis	$X^2(3) = 4.85$; $P = 0.1831$	

affect the behavior of both males and females. For example, blue LED light increases the expression of stress-responsive genes, shortening lifespan in both males and females of Drosophila (Nash et al., 2019). Similarly, different species of crayfish have unique preferences for colors that do not vary by sex (Suryanto et al., 2023).

In contrast, some studies show that blue light can affect crayfish in a sex- and season-dependent manner. For example, female crayfish grow faster than males during the spawning months in spring and summer (Toyota et al., 2022). However, in winter, female crayfish remain more sensitive to blue light than their male counterparts, although their growth declines (Toyota et al., 2022). It remains unclear whether these differences result from seasonal or temperature-dependent genetic expression changes in photoreceptor visual pigments.

Additionally, prolonged exposure to very dim blue light may lead to increased endogenous levels of stress-related hormones equally in both male and female crayfish.

During stressful conditions, an increase in serotonin can augment the release of stress hormones such as crustacean hyperglycemic hormone (CHH), which elevates hemolymph glucose levels (Escamilla-Chimal et al., 2002; Lorenzon et al., 2005). Higher levels of endogenous serotonin and stress hormones can increase anxiety by desensitizing or downregulating serotonin receptors (Abbas et al., 2007; Albert et al., 2014; Nishi and Azmitia, 1996).

An aquatic plus-shaped maze was used to gather behavioral data, serving as a reliable tool for assessing crayfish light aversion responses. The maze's design, featuring both illuminated and non-illuminated arms, capitalizes on crayfish's natural tendency to seek darkness or avoid light in new environments (Abeel et al.; Fossat et al., 2015). The term "anxiety-like behavior" is used to describe the behavioral response in which stressed crayfish seek refuge in dark places (Fossat et al., 2014). Studies have shown that stress induction by electric shocks or serotonin injections causes crayfish to avoid illuminated arms (Fossat et al., 2014). It is important to note that the plus-maze in the study by Fossat et al. (2014) consisted of two dark arms at 10 lux and two illuminated arms at 50 lux. The light intensities were different in the present study's illuminated and non-illuminated arms. Crayfish are attracted to light intensities between 0.17 and 1.4 lux and withdraw from light above 5.6 lux (Fernández-De-Miguel and Aréchiga, 1992). Given this threshold, the illuminated arms were set to 2-3 lux, and the non-illuminated arms were kept at 0 lux. In unpublished data, regardless of the LED light type, crayfish showed a higher preference for non-illuminated arms when the brightness in the illuminated arms exceeded 10 lux. Reducing the light intensity of the illuminated arms to 2-3 lux helped balance the crayfish's natural aversion to light and their tendency to explore a new environment after different LED light treatments.

A few limitations of this study should be noted. First, there was a lack of native habitat for the crayfish. As nocturnal creatures that typically live in murky water, crayfish were exposed to light only during the day (8 am–8 pm), and all behavioral tests were performed during this time. This controlled environment did not affect measurements of multiple parameters, as supported by the data showing that crayfish treated with 590 nm light entered either the illuminated or non-illuminated arms randomly (Figure 1A). While 590 nm-treated crayfish served as a control, those exposed to blue wavelength light exhibited a significant avoidance of illuminated arms. Second, crayfish were kept in isolation, preventing social interactions with other members of their species. Studies show that prior social experience, such as isolation or communal living, can influence crayfish neurobehavioral responses to pharmacological treatments (Swierzbinski et al., 2017; Venuti et al., 2021). It is possible that the lack of social interaction could have affected their aversion to light, but this was not tested in the present study. Third, a study revealed that after 5 minutes of light stimulation, locomotor activity varied between crayfish subjected to blue light and those exposed to green light (Cedeño Moreno et al., 2023). Although it is possible that blue or white LED light could affect the locomotor activity of crayfish, changes in locomotor activity were not measured in the present experiments.

Effects of blue light on human and crayfish photoreceptors

The human brain is stimulated by blue wavelength light through specialized photoreceptors in the eye, including

rods, cones, and intrinsically photosensitive retinal ganglion cells (ipRGCs). Rods and cones are responsible for image-forming vision, while ipRGCs are uniquely photosensitive and play a critical role in entraining circadian responses to light (Wahl et al., 2019; Wirz-Justice et al., 2021). ipRGCs contain melanopsin, a pigment that absorbs light and is sensitive to short-wavelength blue light, with peak sensitivity around 482 nm (Berson et al., 2002; Dacey et al., 2005). Upon stimulation, light is converted into electrical signals that are processed by the nervous system, ultimately influencing higher brain functions. ipRGCs transmit these signals to the brain's master clock, the Suprachiasmatic Nucleus (SCN), which regulates the body's circadian rhythm. The SCN neurons synchronize their activity in response to variations in light intensity and spectral characteristics, and this information is relayed to the rest of the body through both humoral and autonomic nervous system signals (Mohawk et al., 2012; Schibler et al., 2015).

The presence of both a circadian rhythm and blue-light sensitive photoreceptors makes crayfish an excellent model for studying the impacts of blue wavelength light on behavior. Unlike humans, crayfish possess compound eyes with individual units called ommatidia. In *Procambarus clarkii* crayfish, each ommatidium contains eight photoreceptor cells (R1–R8). The R1–R7 cells contain visual pigments with peak sensitivity at around 530 nm (long-wavelength), while the R8 cell is sensitive to light with a wavelength of approximately 440 nm (short-wavelength) (Eguchi et al., 1973; Kingston and Cronin, 2015). While humans rely on a single circadian pacemaker, the SCN, crayfish have two mutually coupled pacemakers: one in the eye stalk and another in the protocerebrum of the brain (Arechiga et al., 1993; Barrera-Mera and Block, 1990; Nelson-Mora et al., 2013). These crayfish pacemakers can be entrained by light through extraretinal photoreceptors.

In addition to the visual photoreceptors in the compound eyes, crayfish possess non-visual, extraretinal photoreceptors located in two distinct regions. One set is located in the supraesophageal ganglion of the protocerebrum (Sandeman et al., 1990; Sullivan et al., 2009), and another pair resides in the terminal abdominal ganglion at the caudal region of the body (Petrowski et al., 2019; Rodriguez-Sosa et al., 2008; Wilkens and Douglass, 1994). These extraretinal photoreceptors are sensitive to short-wavelength blue light. Particularly important are the blue-sensitive extraretinal photoreceptors in the protocerebrum, which can independently sustain biological rhythms in crayfish. Studies have shown that even in the absence of compound eyes and caudal photoreceptors, crayfish can maintain circadian rhythms and synchronize locomotor activity patterns based solely on light signals (Hammond and Fingerman, 1975; Sullivan et al., 2009). The protocerebrum's photoreceptors contain a blue-light-absorbing pigment called cryptochrome (Fanjul-Moles et al., 2004; Sullivan et al., 2009). In addition to responding to blue light, these photoreceptors also respond to green light (Sandeman et al., 1990).

Crayfish also possess caudal photoreceptors, which are sensory neurons located in the sixth abdominal ganglion (Larimer, 1966; Prosser, 1934; Wilkens and Larimer, 1972). When these photoreceptors are stimulated by blue or green light, they signal motor neurons to initiate backward walking in crayfish (Larimer et al., 1966). These caudal photoreceptors are essential for the crayfish's circadian system, generating locomotor activity in response to daily light/dark cycles (Arechiga et al., 1993; Prieto-Sagredo and Fanjul-Moles, 2004; Rodriguez-Sosa et al., 2008; Sullivan et al., 2009).

As human populations increasingly encroach on natural aquatic habitats and light pollution grows, the impact of artificial lighting on biodiversity has gained attention. Studies have explored how various light spectra influence crayfish behavior and color preferences. For instance, marbled crayfish exhibit negative phototactic behavior in response to white light but display positive phototaxis toward blue light (Okada et al., 2021). Color preference in crayfish may be species-specific, as *Procambarus clarkii* shows a strong preference for red light, followed by green, blue, and yellow light (Suryanto et al., 2023). Despite efforts to use LED lights to attract crayfish for fisheries, these attempts have generally been unsuccessful (Ruokonen et al., 2021).

Interestingly, artificial light can affect crayfish even when they lack visual photoreceptors and rely solely on non-visual, extraretinal caudal photoreceptors. For example, exposure to white light significantly reduced the duration of social interactions among blind cave fish compared to infrared light (Li and Cooper, 2002). These findings underscore the complex and often detrimental effects of artificial lighting on aquatic species.

The research evaluated the light avoidance response of crayfish after daily exposure to blue light. The results suggest that this exposure increases crayfish aversion to light, which could impact their ability to find food, avoid predators, and seek shelter. Specifically, treatment with 590 nm light had a limited impact on crayfish behavior, likely due to insufficient stimulation of extraretinal photoreceptors. Crayfish exposed to 590 nm LED light did not show a preference for dark versus illuminated areas in a plus-maze, unlike those exposed to blue or white LED lights.

White light, which stimulates both blue and green extraretinal photoreceptors, led to a stronger avoidance of illuminated areas compared to blue light.

This altered behavior could negatively affect crayfish's ability to capture prey or evade predators in their natural aquatic environments.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Materials

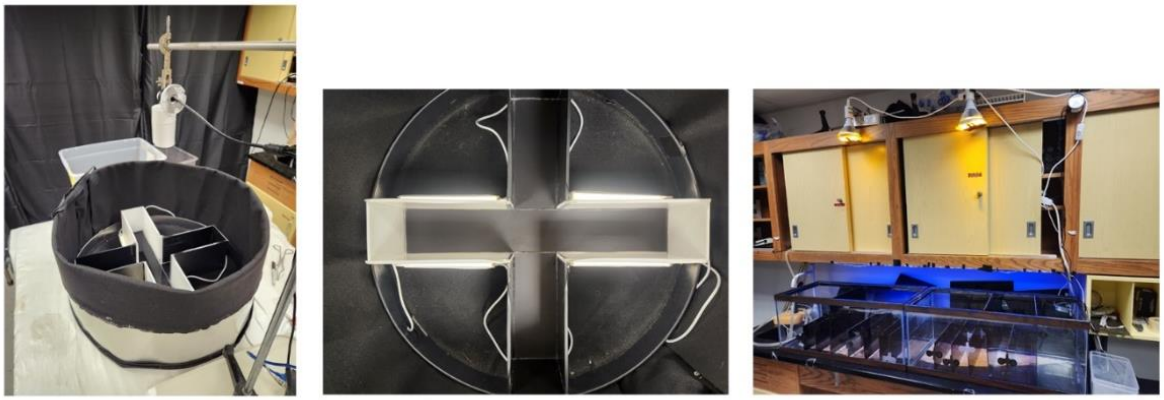

Fig S1. Plus-maze and LED light schematics. **Left:** Photograph showing the behavioral recording apparatus with camera mounted above the plus-maze. **Middle:** plus-maze with two non-illuminated arms. The illuminated arms have led track lights outside. **Right:** Space where crayfish were housed. LED lights were mounted above.