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Crayfish-tracking motion vision to analyze the locomotor activity induced by changes in environmental lighting

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Abstract: Crayfish show locomotor activity modulated by environmental lighting. Yet, little is known about different light spectra stimulation effects on the locomotor activity of crayfish, and laboratory instruments are generally limited to the study of a single animal. As such, this research describes the development and implementation of the Crayfish-Tracking Motion Vision (CTMV) system, as a tool for experimentation. Based on artificial vision, this system allowed for the observation and recording of locomotor activity in one to four *Procambarus clarkii* crayfish that were placed in individual compartments. The effect of blue or green monochromatic light stimulation was explored in two groups of dark-adapted crayfish (with a total of 15 adult crayfish). Light-emitting-diodes of blue or green spectra were applied for 5 min while locomotor activity in crayfish was monitored in a large container. Both light pulses caused similar reaction times of locomotor activity (2.9 ± 3.5 s). Meanwhile, the displacement score of "blue light" animals (2.1 ± 0.9 m) was lower than that of "green light" animals (2.8 ± 0.6 m). In contrast, speed (1.1 ± 1 cm/s) and acceleration data (0 ± 0.8 cm/s2) of the two groups were similar. Finally, the locomotor activity decreased significantly when container size was decreased. These results showed that the CTMV system is appropriate for measuring the locomotor activities of multiple crayfish and suggested that both monochromatic lights induced different locomotor behavior in the crayfish. It has been postulated that this light-induced reflex activity, is caused by an interplay of the photoreceptor system output with a circadian system modulating light sensitivity and locomotor behavior in the crayfish. It can be inferred that the CTMV system will benefit subsequent studies focused on light-induced reflex activity in crustaceans.

Keywords: Computer vision, 2D image analysis, *Procambarus clarkii*, monochrome light, locomotor activity

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1. Introduction

In decapod crustaceans, environmental lighting modulates diverse functions and locomotor activity (Aréchiga & Rodríguez-Sosa, 1997). Freshwater crayfish is a suitable model organism to study the functional role of three photo-sensitivity systems: (1) in the retina (RPR), (2) non-visual photoreceptors in the supraesophageal ganglion (brain) (BPR), and (3) caudal photoreceptors (CPR) in the sixth abdominal ganglion (AG) (Sullivan et al., 2009; Rodríguez-Sosa et al., 2008, Sánchez-Hernández et al., 2018). Previous studies using *Procambarus clarkii* crayfish have reported on two opposite locomotor responses induced by white light attraction at low intensities (<1.4 Lux) and withdrawal at high intensities (>5.6 Lux)) (Fernández de Miguel & Aréchiga, 1992a; Fernández de Miguel & Aréchiga, 1992b). Moreover, earlier studies using classical conditioning (i.e., light pulses of different colors) revealed color light-induced locomotor responses in crayfish (Krasne, 1973).

Marine species, such as cleaner shrimp (*Lysmata amboinensis*) that are sensitive to light-wavelength (480–540 nm) and capable of recognizing various color patterns, have shown behavioral changes due to light (Choi et al., 2018). Similarly, light-dark cycles using blue or green light pulses have induced locomotor activity changes in shrimp (*Stenopus hispidus*) (Esaka et al., 2016). Electroretinogram (ERG) studies, using white light, blue light (400–500 nm), and green light (500–600 nm), have assessed the spectral sensitivity in crayfish (aged one day to 16 weeks) (Fanjul-Moles & Fuentes-Pardo, 1998). Other reports revealed that the locomotor activity rhythm of young crayfish synchronizes to blue and red monochromatic lights when deprived of retina and *lamina ganglionaris* (Miranda-Anaya & Fanjul-Moles, 1997; Fanjul-Moles & Prieto-Sagredo, 2003). This finding aligned with the extraretinal photoentrainment mechanisms of circadian rhythm and motor activity in adult organisms of this crustacean decapod.

In a recent study on light transduction in *P. clarkii*, two opsin proteins in retinal and extraretinal photoreceptors had been established (with one opsin showing sensitivity to short-wavelength light (SWL, blue), whilst the other showed sensitivity to long-wavelength light (LWL, green)) (Kingston & Cronin, 2015). Furthermore, asymmetry in the firing rate between both CPRs was described after comparing spontaneous activity in response to white light and monochromatic lights (SWL and LWL) or the dark (Pacheco-Ortiz et al., 2018; Sánchez-Hernández et al., 2018; Rodríguez-Sosa et al., 2019).

Early studies often used locally manufactured automated devices, based on the use of an array of infrared photodiodes (940 nm), to study the effects of white light and food in the locomotor activity rhythm of the *P. clarkii* (Fernandez de Miguel et al., 1989; Fernández de Miguel & Aréchiga, 1992a; Fernández de Miguel & Aréchiga, 1992b; Fernández de Miguel & Aréchiga, 1994). Subsequently, some laboratory studies have made use of low-cost personalized software to record locomotor activity from animals (especially since commercial equipment is expensive and relies on closed architecture predefined software that restricts users to certain tools) (Heredia-López et al., 2013).

Today, open-source systems are available for use. The development of these highly flexible animal monitoring systems (with their wide range of compatible equipment) allows for more suitable operations. However, the development and operation of a system may come with its own set of limitations (e.g., the need for prior technical knowledge (Pennington et al., 2019; McElroy et al., 2020), the use of dated technologies like photodiodes and receivers (Shuranova et al., 2005, Rodríguez-Sosa et al., 2017), problems caused by a high sensitivity to light changes in the workspace (Valdés et al., 2012), and the necessity of manually analyzing obtained videos (Cha et al., 2012).

Open-source software has been utilized in various studies to monitor the behaviors of different animals under different conditions and represents in a more precise, less time-consuming, affordable, and more efficient method than the manual measurement of movement through rulers or grids. Specifically, this type of software has been used with mice in conditioned spaces (Kafkafi et al., 2003; York et al., 2013; Samson et al., 2015; Hong & Moon, 2018), pig herds in pens (Gronskyte et al., 2015; Nasirahmadi et al., 2015), and different marine species (Al-Jubouri et al., 2017; Miranda & Romero, 2017). This procedure also ensures the health of the animals by not being invasive, allows for the acquisition of quantifiable data that describe the behavior of the animals, and detects events such as molting (Tang et al., 2020) or sinking death (Ina et al., 2020). Yet, little research has been performed regarding the effect of different spectra light stimulation on crayfish locomotor activity.

The present work, therefore, aimed to develop an artificial vision system focused on the study of crayfish photosensitivity. The system would allow the acquisition of quick and exact information from 2D images and videos (Kanan & Cottrell, 2012; Rodríguez & Sossa, 2012) by using algorithms capable of multiple animal identification, monitoring, and performing measurements (Pajares & de la Cruz, 2001; García, 2008; Quintana et al., 2012). Development of the "Crayfish-Tracking Motion Vision" (CTMV) system, based on artificial vision and implemented in Python language, would prioritize an intuitive interface that requires minimal training and would allow for the simultaneous observation and measurement of locomotor activity in one to four crayfishes in a laboratory setting.

2. Materials and methods

2.1. Animals

The current study was approved by the Research Commission and the Research Ethics Committee of the Faculty of Medicine, UNAM (FM/DI/128/2019) according to the declaration of Helsinki. The *P. clarkii* crayfish used in this study were purchased from a local supplier and were cared for according to the Society for Neuroscience (2021) recommendations. A total of 15 adult crayfish were used (each weighing 20–30 g with 9–12 cm shell lengths) and were kept in the laboratory for two weeks. The animals were kept in water containers (with a water filter) at room temperature (23 ± 1 °C) under light-dark cycles (12:12 h) and had free access to vegetables and dried fish as food. For the test, the population will be divided in two groups, one placed in large containers and other in small containers.

2.2. Arrangement to study the crayfish locomotor activity

Locomotor tests were performed between 11:00 a.m. and 2:00 p.m. The crayfish were allowed to adapt to the darkness for 1 h, after which they were individually transferred to new water containers at room temperature (23 ± 1 °C). The latter were placed inside a white acrylic box with adhesive plastic on the inside and black color on the outside. Thereafter, the crayfish were stimulated using commercial lamps with blue light-emitting diodes (LED) (Max = 460 nm; Irradiance (I) = 0.089 W/m^2) or Green LED (Max = 524 nm; I = 0.12 W/m^2) having distinctive spectral regions. Calibration of the LED lamps was performed using an MSC15 photometer (Gigahertz-Optik GmbH, Germany). The pulses will be applied throughout the test, commonly of a 5 min duration, and the first and last minute will be taken as more significant.

2.3. CTMV system features

The Crayfish-Tracking Motion Vision (CTMV) system was developed using Python (an open-access coding language) and by applying the Tkinter 2.7 and OpenCV libraries. The software interface allowed for parameter configuration relevant to the crayfish locomotor test. Operating characteristics (Table 1) and a flow chart describing the design of the CTMV system (Fig. 1) were established.

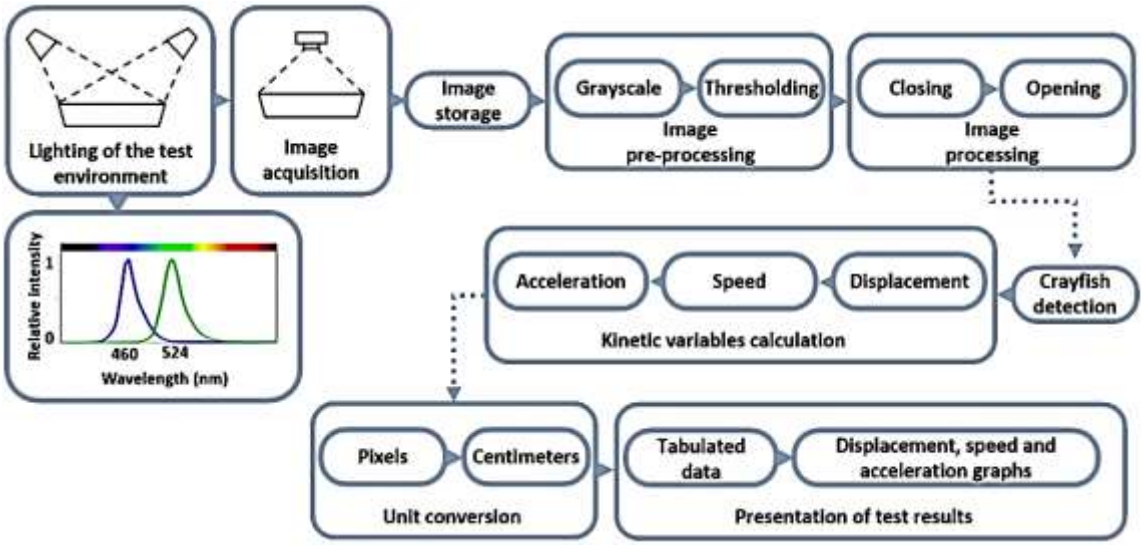

Fig. 1. Block diagram of the CTMV system that was designed to characterize crayfish locomotor activity.

Table 1. CTMV system features

1.	Capture images at one frame per second.
2.	Definition of the number, duration, and intervals of the test.
3.	Programming the start time of the tests.
4.	Set height of the video camera.
5.	Selection of the area of interest inside the image.
6.	Simultaneous measurement of locomotor activity from one to four crayfishes placed in separate compartments.
7.	Simple user interface.
8.	Analysis of the trajectory, distance, speed, and acceleration of the locomotor activity of the animal, from the captured images. Generation of documents with tabulated results and graphic representation.
9.	Lighting control system for the change of monochromatic lights during tests.

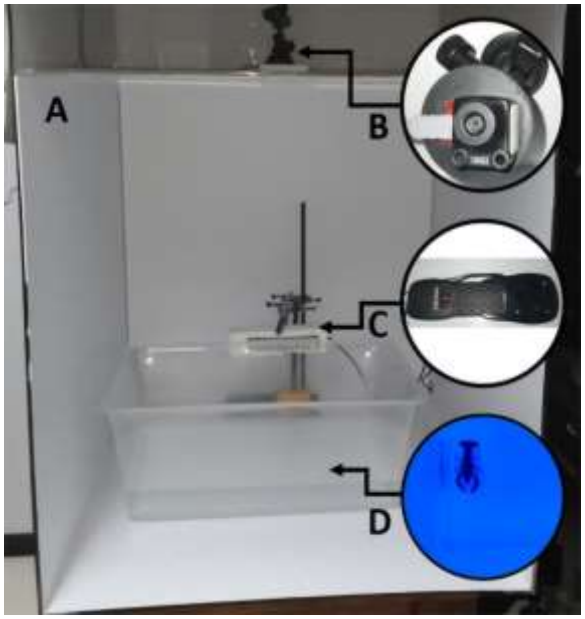

Fig. 2. Artificial vision system physical assembly.
A) Work area setup. B) IDS camera. C) Lighting system.
D) Container for *P. Clarkii*.

Crayfish locomotor behavior was monitored under different lighting settings using an artificial vision system that located objects of interest as well as acquired and automatically stored 2D images for later pre-processing and processing, as shown assembled with all its components in Fig. 2. For object detection a First Input First Output (FIFO) procedure was implemented and complemented using the First Moment of Area Theorem (FMAT) for the estimation of centroids. Starting from these, basic kinetic functions were implemented to calculate the position, speed, and acceleration of the locomotor activity, which were presented as tabulated data and with a graphic representation.

2.3.1. Lighting of the test environment

To establish lighting of the test environment, an area completely devoid of ambient light was controlled using two 127 V AC lamps (i.e., one with blue LED and another with green LED). To regulate the lighting and establish communication with the CTMV, a commercial V2500 regulator (with a capacity of 1500 W) was modified to activate and/or deactivate up to four contacts using an Arduino Nano microcontroller (https://store.arduino.cc/products/arduino-nano) (Fig. 3). This allowed for lighting configuration during the test (i.e., green or blue) and coordination with movement monitoring.

Fig. 3. Lighting system diagram for the synchronization of green and blue light during the monitoring stage. Dotted arrows are internal to the lighting system and the solid arrows indicate external communication.

2.3.2. Image acquisition

An uEye xs video camera (IDS company) with a 0.25-inch sensor was used for image acquisition and could capture 2D images at a rate of up to 15 frames per second (FPS), in the RGB format, and a resolution of 640 x 480 pixels (px) (Shutter, 2018). The video camera was placed at a height of 68 cm (obtaining a 62 x 46 cm field view) which allowed for observation of all the crayfishes in their containers, at the same time, a criterion was respected that all objects of interest exceeded the size of 3x3 pixels. Additionally, the radial distortion of the camera was reviewed to see if it would be necessary to apply a correction, this was achieved using the "Camera calibration" application of MATLAB, providing a radial distortion of 0.2 px at 40 cm and 0.1 px at 70 cm, which was considered the recommended and

within the acceptable range, so no correction was necessary (MathWorks, 2013).

Image acquisition was configured with an active and an inactive stage which automatically alternated according to the number of tests programmed by the user. This resulted in the generation of non-consecutive test sets to observe the evolution of behavior over time. The images, captured at 1 FPS, were identifiable based on the root name and were automatically stored on a hard drive.

2.3.3. 2D Image processing

Open CV libraries were used for 2D image processing and continually collected information on the animal's locomotion until completion of the monitoring stage (allowing images to be used as a set). Since information on the color of the crayfish or the type of environment was not relevant to the experiment, processing was simplified by using a light background with grayscale animal images (Fig. 4A) (Kanan & Cottrell, 2012). The grayscale images were subsequently segmented to identify crayfish from the bottom of the image (Quintana et al., 2012).

The Otsu method was used to determine the most efficient threshold value for all expected conditions (Rodríguez & Sossa, 2012). Its implementation was achieved during an optional calibration stage (establishing a constant threshold value) considering variations in lighting, camera height, number of crustaceans, etc. The established threshold value was compared against the grayscale value of each pixel, and data below the threshold value were classified as white or black pixels (García, 2008). The results corresponded to a binary image of the crayfish (with the program representing elements of interest in white). To allow for better visual representation, the tone scale for all binary images was inverted to illustrate animals in a dark tone against a white background (Fig. 4B).

2.3.4. 2D Image post-processing

After segmentation of the crayfish image, it was common to find errors regarding the separation of objects of interest (e.g., partial reflection of the animal on the container's translucent wall) (Fig. 5A). Morphological operations for 2D images were, therefore, applied to minimize noise and unwanted elements (García S., 2008). First, the opening operation was applied, which involved sequential dilation and erosion operations where the area of each object was first reduced (until unwanted objects disappeared) and then later partially recovered (resizing the objects using the erosion) (Fig. 5).

Thereafter, the closing operation (i.e., the opening operation in reverse) was applied. In doing so, incomplete objects, or those that showed holes with erosion, were completed (Fig. 6A). An approximation of the original shape of the crayfish was recovered by applying a dilatation operation (Fig. 6B) (Pajares & de la Cruz, 2001).

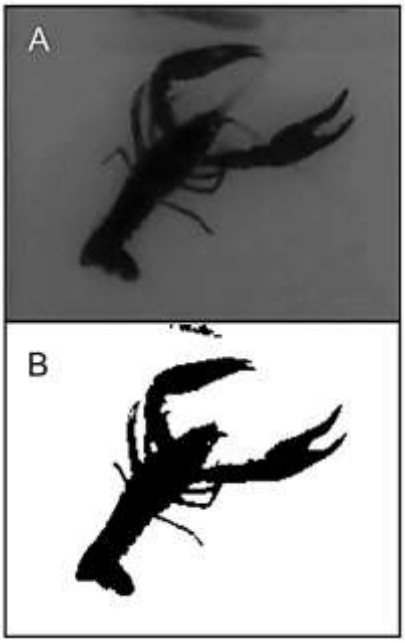

Fig. 4. Segmentation of a crayfish image.
A. Crayfish in grayscale.
B. Result of crayfish image segmentation.

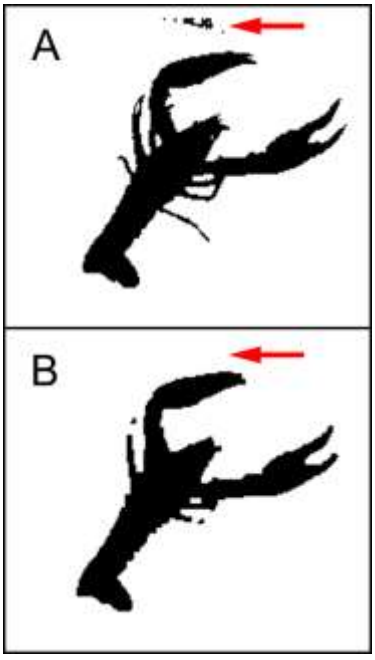

Fig. 5. Opening operation effect on a crayfish image. A. Crayfish image with unwanted elements (such as partial 202 reflection of the animal).
B. Crayfish image post-opening operation.
Arrows indicate areas with notable differences.

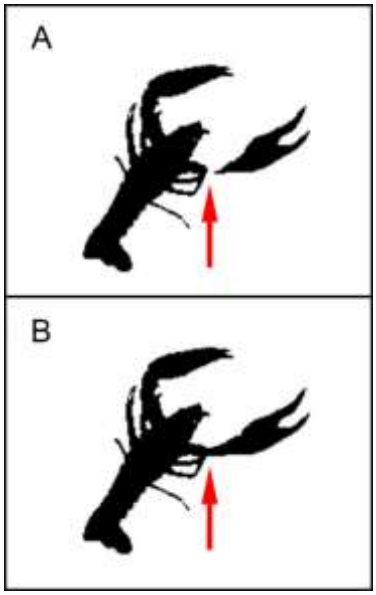

Fig. 6. Post-processing of a crayfish image. A. Incomplete detection of the animal. B. Crayfish image post-closing operation. Arrows indicate locations of notable differences.

2.3.5. Crayfish detection

The CTMV artificial vision system was developed to simultaneously study the locomotor activity of multiple crayfish that were placed in separate compartments under the same environmental conditions. For crayfish detection, it was necessary to identify the number of animals in the processed images. For this purpose, a "first-in-first-out" (FIFO) buffer was implemented (Willenz & Kamoon, 1997), where data were stored in a specific order (later to be read in the same order). For the crayfish count, an image was reviewed pixel by pixel whilst looking for white pixels. Once located, they were stored in the FIFO buffer, and the surrounding pixels were reviewed to detect all the white pixels that make up the object. Once all the white pixels belonging to an animal had been located, they were eliminated from the image. The rest of the image was subsequently reviewed (i.e., searching for animals that have not yet been detected). The CTMV system was also able to delimit the work area (i.e., restricting the analysis to areas of interest) (Fig. 7) and thereby minimize processing time.

During crayfish counting, the centroid of each crayfish was determined using the center of mass principle (Boothroyd & Poli, 1980). For example, each animal may be considered as a set of smaller objects (corresponding to pixels). In knowing all the pixels, and their location, the centroid can be calculated through the First Moment of Area Theorem, which can be applied through equations 1 and 2, where m is the object's mass which takes the value of one, and the coordinates (X, Y) correspond to the pixel's location (Fig. 8).

$$X_c = \sum_{i=0}^{n} m_i X_i \,/\, \sum_{i=0}^{n} m_i \quad (1)$$

$$Y_c = \sum_{i=0}^{n} m_i Y_i \,/\, \sum_{i=0}^{n} m_i \quad (2)$$

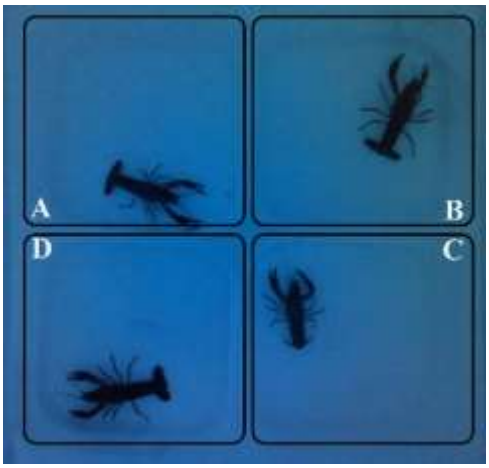

Fig. 7. Observation of four crayfish that were stimulated with monochromatic blue light and placed in individual 224 containers (labeled as A, B, C, and D). During the analysis of the tests the user establishes the regions of interest 225 depicted as rectangles

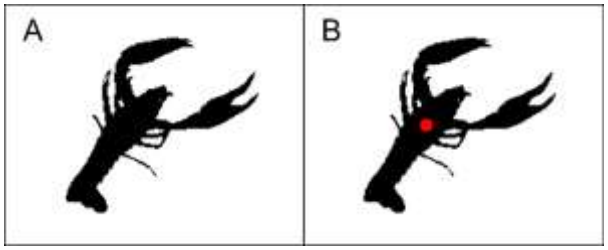

Fig. 8. Location of the crayfish centroid. A. Segmentation of the animal. B. The crayfish's centroid (red circle) was in 235 the anterior region, on the midline of the cephalothorax, and close to the animal's second pair of legs.

Analysis of crayfish locomotor activity was based on a series of processed images. Two consecutive images of the animal were taken, having two respective centroids, indicating the same animal at different time points (Fig. 9A and B). A straight line was subsequently drawn between both centroids (Fig. 9C) which represented the superposition of the two centroids. Then, based on their displacement, the Euclidian distance (D) traveled by the crayfish was calculated using equation 3 (Hibbeler, 2010) where (X, Y) are the Cartesian coordinates of the same animal's centroids in each captured image. Data were stored in a text file.

$$D = \sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2} \quad (3)$$

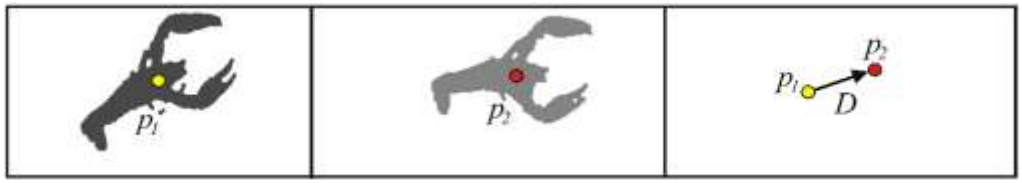

Fig. 9. For the displacement measurement of the crayfish, p_1 is taken as the initial position, while p_2 is the position of the same animal 1 s later, through the centroid positions the displacement is obtained as a vector (D).

Where D is the euclidean distance, p_1 is the coordinate (X_1, Y_1) and the point p_2 is the coordinate (X_2, Y_2).

A series of tests were performed to obtain the error in the displacement calculation, i.e., a synthetic object was placed, with rectangular shape of known dimensions, and was monitored while performing displacements of 1cm to be compared to the measure data. This activity was performed 25 times to obtain an average of 2%, which was deemed acceptable in the current study.

The crayfish speed had to be calculated from the first derivative of the position. However, since there is no continuous function that describes the crayfish position, the instantaneous speed of the displacement was calculated for each pair of consecutively processed images by using equation 4 (Beer et al., 2010).

$$V = \Delta D/\Delta t \tag{4}$$

In this equation, V is the speed, ΔD is the rate of change in the position of the crayfish (displacement), and Δt is the rate of change over time, being this a

constant of 1 s due to the constant acquisition speed of 1 FPS. This was considered as a constant since the capture speed was taken as the elapsed time. In this manner, an approximation of the crayfish speed was obtained and stored in a text file.

A similar approximation was made to calculate crayfish acceleration. Variation of the speed (ΔV) and time elapsed between the captured images were considered. Using equation 5, instantaneous acceleration (A) was calculated and stored in a text file.

$$A = \Delta V/\Delta t \tag{5}$$

2.3.7. Converting the pixel unit to cm

Measurements of kinetic variables were obtained in pixel units. To allow data conversion to the Metric Decimal System, the video camera was placed on a Kaiser base and was perpendicularly positioned to a horizontal grid surface (Fig. 10). However, a constant conversion factor could not be considered as it would vary according to the working distance. Therefore, surface images were captured at different distances to analyze the relationship between pixels and centimeters.

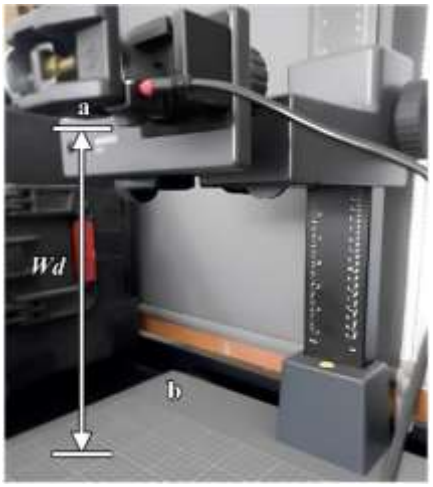

Fig. 10. Mounting of the video camera (A) on the Kaiser base grid in centimeters (B) for calibration (pixel-cm) tests. Captures were taken at different working distances as indicated by Wd.

With this equipment and varying the height of the video camera between 10–70 cm, surface images of 640 x 480 pixels were acquired. Fig. 11A shows an example of a grid base image (obtained at the height of 10 cm where the conversion factor was 104 pixels = 1cm). Fig. 11B shows data for various working distances where each value represents the mean (N = 5) of the equivalence of pixels (C) to 1 cm. Non-linear regression was, furthermore, calculated using equation 6, which describes the conversion factor from pixel to cm (R2 = 0.99) (Fig. 11B).

$$C = [(131)/(1 + (Wd/20.3)^{2.5})] + 3 \tag{6}$$

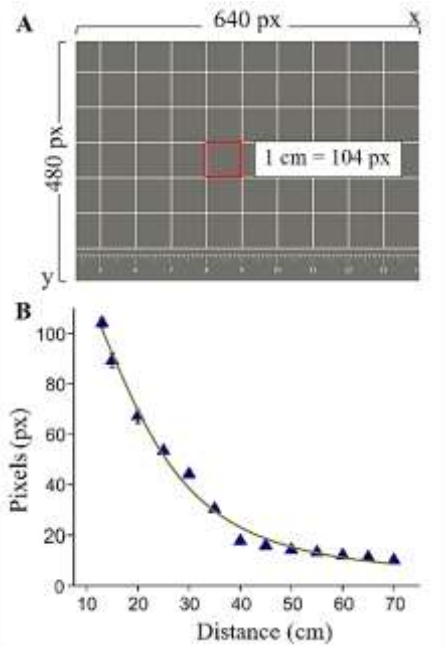

Fig. 11. Conversion from pixels to centimeters. A. Kaiser base image showing the grid surface obtained at a height of 20 cm. B. Equivalence of pixels at 1 cm. Triangles represent measurements obtained at different working distances. The solid line represents a non-linear fit.

2.3.8. Presentation of test results

The results obtained from each test were tabulated and represented in a graph for each crayfish. All tables contained position (X, Y), distance (mm), speed (mm/s), and acceleration (mm/s2) data. For the graphical representation of distance, speed, and acceleration, the matplotlib library was used. In addition, the CTMV software was programmed to superimpose the calculated crayfish path on the last analyzed image of each crayfish.

2.3.9. Statistical analysis

Crayfish locomotor activity, in response to light, was characterized by reaction time. Criteria for the latter included the time (s) needed for the animal to move 1 cm after applying a light stimulus, displacement (cm), speed (cm/s), and acceleration (cm/s2). Criteria values were expressed as mean values (alongside standard deviation (SD) and median (Me) values). Analysis included the test of normality (i.e., the Kolmogorov-Smirnov test) which compared the displacement during the first minute with that of the last minute. To achieve this, the accumulated displacement of the first minute (D_{fm}) was subtracted from all the values obtained in the last minute (D), resulting in a data set that excluded the first minute (Z), based on equation number 7 where i represents each of the measurements.

$$Z_i = D_i - D_{fm} \tag{7}$$

If the locomotor activity parameters did not have a normal distribution, two independent samples of these activities were compared with each other using the Mann-Whitney test. All statistical analyzes employed Origin (OriginLab, Northampton, MA) and PAST software (Hammer et al. 2001). Values of $p < 0.05$ were regarded as significant.

3. Results

3.1. User interface

The CTMV system has a simple and interactive graphical interface, in which the user configures the sequence of events to observe and measure crayfish locomotor activity during experimental tests (Fig. 12). CTMV software has been copyrighted (Secretary of Culture, National Institute of Copyright, 2020. México) and complemented with a user manual.

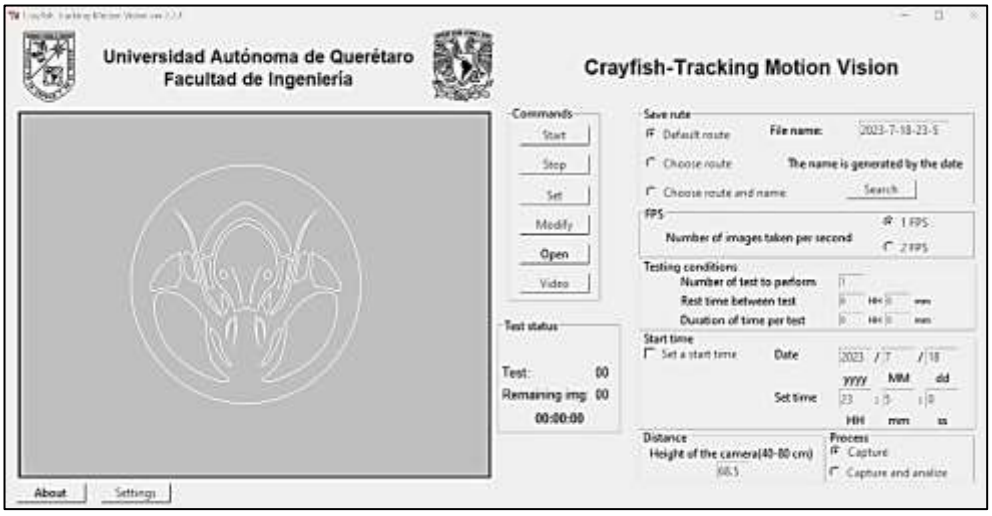

Fig. 12. Crayfish-Tracking Motion Vision software graphical interface.

3.2. Lighting system

The lighting system had been a modified commercial regulator that was controlled by the CTMV software to synchronize the lighting needed for crayfish monitoring. This system thus allowed the user to easily program the lighting (including green or blue color changes) needed for each test.

Table 2. Parameters of the crayfish locomotor activity measured with the CTMV system. Fragment of a data file imported into the spreadsheet.

Time (s)	Position X (pX)	Position Y (pY)	Distance (mm)	Speed (mm/s)	Acceleration (mm/s^2)
0	294	220	0	0	0
1	294	220	0	0	42.2
2	281	179	42.2	42.2	-4.2
3	288	141	80.1	37.9	-28.2
4	284	132	89.8	9.6	14.8
5	304	117	114.3	24.5	-7.6
6	318	107	131.2	16.9	1.2
7	336	103	149.3	18.1	-1.6
8	352	108	165.8	16.4	6.2
9	374	115	188.4	22.6	-5.8
10	391	117	205.2	16.8	-8.9

3.3. Analysis of the trajectory, distance, speed, and acceleration of crayfish locomotor activity using the CTMV system

Light stimulation with a pulse of blue or green monochromatic light, evoked locomotor activity in crayfish that had been transferred to a new container. Table 2 presents a sample of the parameters tabulated by the software (with information about the locomotor activity as calculated by the CTMV system).

The CTMV software was also programmed to graphically represent crayfish locomotor activity based on accumulated distance, speed, and acceleration. For example, a pulse of monochromatic green light (during 1 min) was able to affect crayfish locomotion (plotted in Fig. 13). The accumulated distance traveled by the animal during the test resulted in a displacement of 658 mm (which had not been continuous due to stationary moments and short-duration movements) (Fig. 13A). In the first 5 seconds, a maximum speed of ca. 40 mm/s could be reached, whereafter the crayfish slowed to ca. 20 mm/s or kept still for a few seconds (Fig. 13B). Crayfish acceleration showed its maximum and minimum values in the first 5 s followed by fluctuations of several seconds (which correlated with the crayfish speed time course) (Fig. 13C).

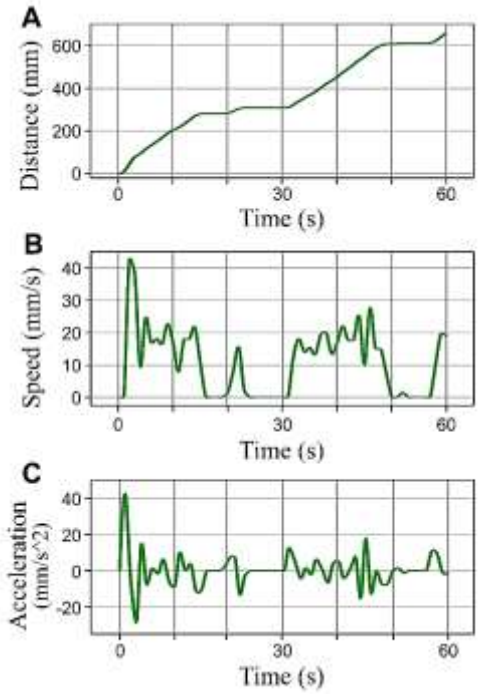

Fig. 13. Characteristic graphical representation of distance, speed, and acceleration of crayfish locomotor activity induced by light stimulation (using CTMV software). A. Cumulative distance (cm). B. Speed of movement (mm/s). C. Acceleration of movement (mm/s^2).

3.4. Crayfish locomotor activity due to blue or green light pulses

For crayfish groups monitored in a large container (LC, 43.5 cm x 33 cm), both light pulses caused the displacement of all crustaceans. However, with a reaction time of 3.8 s for the blue light pulse (N = 5) and a reaction time of 1.5 s for the green light pulse (N = 6), there had been a significant difference between the two light pulses. This variance was also seen in a typical CTMV software analysis image (Fig. 14), which showed two crayfish paths traveled (for five minutes) as a result of monochromatic blue or monochromatic green light pulses. Both light pulses caused the displacement of each crayfish towards the front wall, after which the animals both followed a course along the periphery of the container while walking either greater or shorter distances. First-minute trajectory representations for one crayfish after exposure to blue and green monochromatic lights, respectively, suggested differences in behavior (Fig. 14A and14C), whereas visual differences were less defined when the crayfish had been stimulated for a total of 5 minutes (Fig. 14B and 14D).

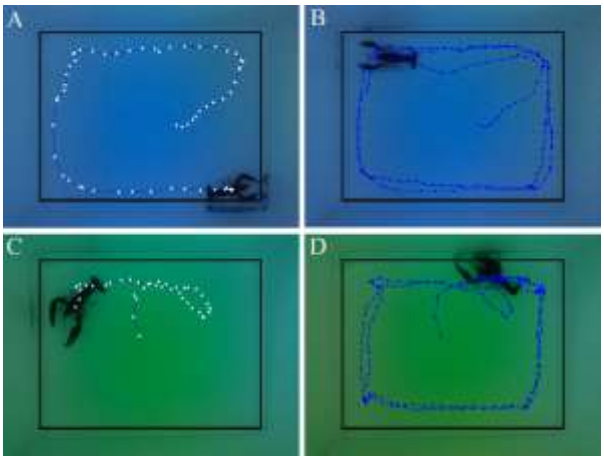

Fig. 14. Images of crayfish that had been separately stimulated with a blue or green monochromatic light pulse, respectively. A. Representation of the path followed by the crayfish during the first minute (post blue light). B. A 5 min trajectory of the same animal. C. Representation of the path followed by another crayfish during the first minute (post green light). D. A 5 min displacement trajectory for the same animal.

Using the CTVM system, statistical data were obtained for each of the crayfish groups. LC crayfish stimulated with blue light (N = 5) during the first minute showed (in Mean ± SD) a displacement of 42.9 (32.4) cm, a speed of 1.3 (1.1) cm/s, and an acceleration of 0.0 (0.8) cm/s2. During the last minute, they showed a displacement of 208.6 (86.9) cm, a speed of 1.2 (1) cm/s and an acceleration of 0.0 (0.8) cm/s2. The increment in crayfish locomotor activity was thus about fivefold. Meanwhile, LC crayfish stimulated with green light (N = 6) during the first minute showed a displacement of 40 (25.6) cm, a speed of 1.3 (1.2) cm/s and an acceleration of 0.0 (1) cm/s2, while data from the last minute showed a displacement of 285 (55) cm, a speed of 1.1 (1) cm/s and an acceleration of 0.0 (0.8) cm/s2.

A comparative analysis of the accumulated displacement for the two LC crayfish groups (NBlue = 300 and NGreen = 360) is provided in Fig. 15. For the first minute, the displacement score of animals pulsed with blue light (B1) ($\boldsymbol{Me}$ = 41.5 cm) was similar to that of animals pulsed with green light (G1) ($\boldsymbol{Me}$ = 39.4 cm). A Mann-Whitney test indicated that this difference was not statistically significant ($\boldsymbol{U}$ = 52214, $\boldsymbol{p}$ = 0.46). In contrast, from minute 4 to 5, the blue light score (B5) ($\boldsymbol{Me}$ = 187.3 cm) was lower than that of the green light (G5) (Me = 274.1 cm) and a Mann-Whitney test indicated that this difference had been statistically significant ($\boldsymbol{U}$ = 25389, $\boldsymbol{p}$ < 0.001). Differences were not significant regarding the speed between both groups (first minute: $\boldsymbol{U}$ = 53150, $\boldsymbol{p}$ = 0.7; last minute: $\boldsymbol{U}$ = 50878, $\boldsymbol{p}$ = 0.2) nor the acceleration between both groups (first minute: $\boldsymbol{U}$ = 53457, $\boldsymbol{p}$ = 0.8; last minute: $\boldsymbol{U}$ = 53954, $\boldsymbol{p}$ = 1).

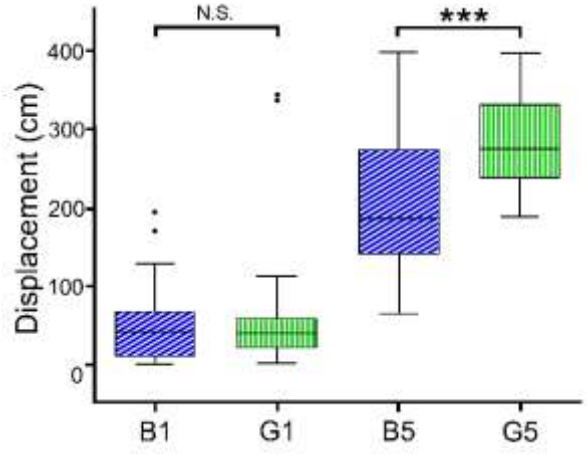

Fig. 15. Effects of monochromatic light pulses on the accumulated distance in two crayfish groups (thoghtout a 5 minutes test). B1. Group 1: distance traveled during the first minute (stimulated with blue light) (N = 5). G1. Group 2: distance traveled during the first minute (stimulated with green light) (N = 6). B5. Group 1: distance covered from minute 4 to 5 (stimulated with blue light). G5. Group 2: distance covered from minute 4 to 5 (stimulated with green light). NS = not significant, *** = $\boldsymbol{p}$ < 0.001.

Furthermore, a comparative analysis was performed for the displacement and speed of two groups of crayfish (stimulated with monochromatic blue light and monitored in small containers (SC) (N = 240) or LC (N = 300)), previously presented. Fig. 16A illustrates a first-minute evaluation (for SC1 and LC1, respectively) and a last-minute evaluation (SC5 and LC5, respectively). The SC1 crayfish displacement score ($\boldsymbol{Me}$ = 18.4 cm) was smaller than that of LC1 ($\boldsymbol{Me}$ = 41.5 cm) and, as indicated by a Mann-Whitney test, the difference was statistically significant ($\boldsymbol{U}$ = 23502, $\boldsymbol{p}$ < 0.001). Similarly, the SC5 crayfish displacement score ($\boldsymbol{Me}$ = 114.2 cm) remained lower than that of the LC5 crayfish ($\boldsymbol{Me}$ = 187.3 cm) and proved to be statistically significant ($\boldsymbol{U}$ = 9967, $\boldsymbol{p}$ < 0.001).

Lastly, speed (cm/s) for both crayfish groups in the two different containers had been significantly different (Fig. 16B) with SC1 crayfish ($\boldsymbol{Me}$ = 0.6 cm/s) having smaller speed values than LC1 crayfish ($\boldsymbol{Me}$ = 1.2 cm/s) ($\boldsymbol{U}$ = 26562, $\boldsymbol{p}$ < 0.001), and SC5 crayfish ($\boldsymbol{Me}$ = 0.4 cm) remaining lower in speed than LC5 crayfish ($\boldsymbol{Me}$ = 1.2 cm) ($\boldsymbol{U}$ = 22134, $\boldsymbol{p}$ < 0.001).

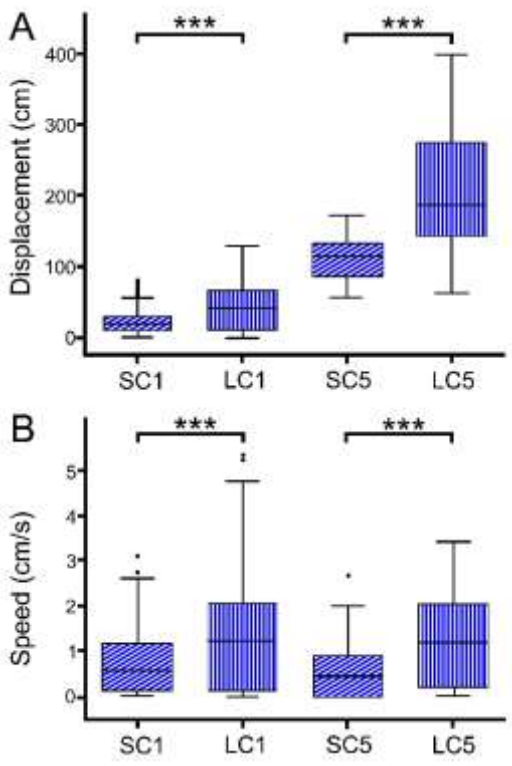

Fig. 16. Comparison of the displacement and speed of two crayfish groups (for 5 minutes) when stimulated with monochromatic blue light. Group 1 = placed in large containers (LC); Group 2 = placed in small containers (SC). A. Displacement of a crayfish group in SC (N = 4) vs. a group in LC (N = 5), evaluated during the first minute (SC1 and LC1, respectively) and minute 4 to 5 (SC5 and LC5, respectively). B. Locomotor activity speed of each crayfish group, (compared as described above). *** = $p < 0.001$.

3.5. Simultaneous measurement of crayfish locomotor activity in separate compartments

The CTMV system allowed for the simultaneous study of up to four crayfish. Firstly, the user subdivided the visual field into rectangular areas of interest (decreasing the processing and analysis time of captured images). To study up to four crayfish simultaneously, all crayfish had been placed in individual compartments which were separately examined using a single video camera connected to the computer. Using the same sequence of images, the displacement, respective trajectory, speed, and acceleration of every crayfish could be calculated. The data (automatically stored) could then be used to draw corresponding graphs for each identified crayfish.

A software assay was developed to detect locomotor activity in a group of crayfishes (4), that were kept in separate small containers (SC, 19 cm x 19 cm) and were stimulated with a blue light pulse (5 min). A typical superposition example of each animal's path is shown in the last CTMV software photogram (Fig. 17). Each trajectory (constructed by the images from a delimited locomotor activity region) was of short movements or quiescence. Also, for each crayfish, the crossing event through the central area of the container had been achieved on more occasions.

The SC crayfish group (stimulated with blue light) showed a reaction time of 4 (± 2.4) s. Data during the first minute (N = 240) showed (Mean ± SD): displacement of 22 (17.6) cm, speed of 0.7 (0.7) cm/s, and acceleration of 0.0 (0.6) cm/s2, whereas that of the last minute (N = 240) showed: displacement of 112 (29.8) cm, speed of 0.6 (0.5) cm/s and acceleration of 0.0 (0.4) cm/s2.

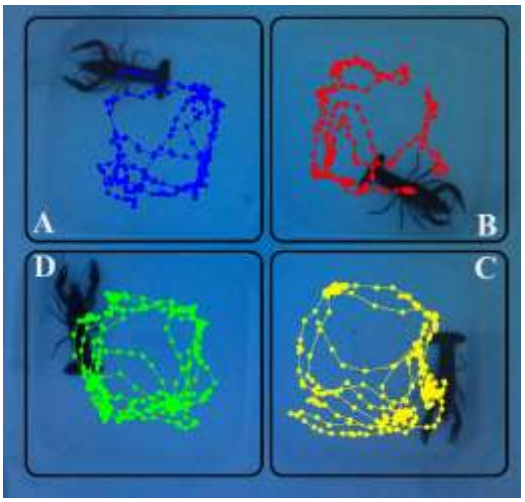

Fig. 17. Effect of blue monochromatic light pulse through out a 5 min test on locomotor activity of four crayfishes (monitored separately but simultaneously). In panels A, B, C, and D, the CTMV system automatically represented the path for each identified crayfish.

4. Discussion

A crayfish-tracking motion vision system (CTMV) was developed in the current study. This static motion vision system is composed of a camera, four LED lamps, and a lighting control system which, in conjunction with its respective software, provides a user-friendly interface. The system can follow test configurations according to scheduled testing hours and allows for changes in parameters (e.g., monitoring and resting time programming). Furthermore, the system can focus on certain areas of interest and can change the environmental light from blue to green. Finally, possible features include 2D image processing parameters and object detection; calculation of displacement, speed, and acceleration; the generation of result tables and graphs; and the creation of a final time-lapse composite image.

The system was applied to simultaneously acquire locomotion activity of up to four crayfish (placed in different containers to avoid social interaction (Jiménez-Morales et al., 2018) and individual measurements were performed (allowing

for multiple tests under the same conditions). The CTMV system allowed for time management improvement and, as reported in other animal models, the system easily implemented procedures to effectively monitor locomotor activity in crayfish (York et al., 2013; Samson et al., 2015; Nasirahmadi et al., 2015; Hong & Moon, 2018).

Specifically, the effect of blue or green monochromatic lights pulses regarding crayfish locomotor activity was reported for the first time. Findings indicated that both light spectra could induce locomotor activity (Figs. 13 and 14). Such exploratory behavior in these crustaceans has previously been described by other experimental protocols (Shuranova et al., 2005; Drozdz et al., 2006) and different animal models (Kafkafi et al., 2003). Furthermore, the effects of the green and blue monochromatic lights were significantly different in crayfish locomotor activity, with the blue light having a reaction time about 2.5 times greater than the reaction time with blue light and the displacement had a significant difference of 37%. These differences in locomotor activity could be attributed to a differential distribution of the two opsins that participate in light transduction in the retinal and extraretinal photoreceptor system that has previously been described in *P. clarkii* crayfish (Kingston & Cronin, 2015; Sánchez-Hernández et al., 2018; Rodríguez-Sosa et al., 2019). Both crayfish groups showed a similar speed and acceleration.

Additionally, a relationship was observed between the size of the container and the crayfish locomotor activity (considering the accumulative displacement and speed). In the large container, the displacement and speed increments were about two times that of the small container (Fig. 16). Interestingly, the SC crayfish crossed through the central area of the container on more occasions than the LC crayfish (Figs. 14A, 14B, and 17) which suggested that the invertebrates may have displayed a form of anxiety-like behavior. For example, it has been reported that crayfish may avoid aversive illuminated areas within aquatic plus-mazes (Fossat et al., 2014).

Recently, Franke and Hörstgen-Schwark (2015) postulated that light regime regulation may be an approach to improve the efficiency of crayfish production. In line with this, Toyota et al. (2022) reported that, depending on the season, blue light stimulus using LED lamps may promote faster larval growth in female *P. clarkii*. From this context, it has been suggested that examination of monochromatic light effects (SWL and LWL) on locomotor activity due to an interplay between the photoreceptor system and the distributed circadian system in crayfish, may represent an interesting avenue for future research (Rodríguez-Sosa et al., 2008; Sullivan et al., 2009; Rodríguez-Sosa et al., 2017).

Different approaches have been developed to obtain video motion data as a plugin for Adobe After Effects commercial software (Koehnsen et al., 2020). Alternatively, software like the 28 free programs reviewed by Panadeiro et al. (2021) has been developed for animal tracking. Here, with a similar purpose, a simple, efficient, and inexpensive system for quantitative detection of crayfish locomotion is presented in the form of the CTMV system. Such a system allows for the accurate representation of animals (in images) and, based upon centroid calculations, supports quantitative analysis. The current study describes all the information necessary to introduce, evaluate, and reproduce the CTMV system's capabilities. Studies on crustacean locomotion have many applications in science and fisheries (with potential impacts on circadian rhythms and reproductive cycles). The final software is available through GitHub, in the repository called "CTMV", it is freely accessible (https://github.com/cmrogelio/CTMV).

Conclusions

In the current study, a crayfish-tracking motion system, founded on artificial vision, had been successfully developed. The CTMV software interface is user-friendly for performing test configuration and simultaneously acquiring locomotion activity of up to four crayfish by performing individual measurements. Moreover, monochromatic blue vs. green light showed a significant difference in light-induced activity of *P. clarkii* crayfish. These findings encourage the use of the CTMV system for further investigations regarding the role of monochromatic light in crustacean physiology.

Conflict of interest

The authors have no conflict of interest to declare.

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