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Continuous noninvasive measuring of crayfish cardiac and behavioral activities

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Dear Editor-in-Chief,

On behalf of all co-authors I would like to introduce the manuscript entitled Continuous noninvasive measuring of crayfish cardiac and behavioral activities I am submitting to the JoVE.

The approach described in manuscript has good utility for water quality assessment and for laboratory etho-physiological studies. The biomonitoring system is inexpensive, has relatively few components and allows long-term continuous simultaneous monitoring of cardiac and behavioral activities of multiple crayfish. The method we proposed is now applied at the local brewery for controlling quality of water used for beverage production, we therefore believe that our method could be of interest not just for biologists or adjacent fields who could apply it for various laboratory investigations of large invertebrates, but for people dealing with industrial water quality issue too.

Thank you very much in advance for your consideration

TITLE:

Continuous Noninvasive Measuring of Crayfish Cardiac and Behavioral Activities

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KEYWORDS:

Bioindicator, biomonitoring, contactless, ethology, heart rate, invertebrate, locomotion, water quality

SUMMARY:

This article presents a noninvasive biomonitoring system for the continuous recording and analyses of crayfish cardiac and locomotor activities. This system consists of a near-infrared optical sensor, a video-tracking module, and software for evaluating crayfish heartbeats that reflects its physiological condition and characterizes crayfish behavior during heartbeat fluctuations.

ABSTRACT:

A crayfish is a pivotal aquatic organism that serves both as a practical biological model for behavioral and physiological studies of invertebrates and as a useful biological indicator of water quality. Even though crayfish cannot directly specify the substances that cause water quality deterioration, they can immediately (within a few seconds) warn humans of water quality deterioration *via* acute changes in their cardiac and behavioral activities.

In this study, we present a noninvasive method that is simple enough to be implemented under various conditions due to a combination of simplicity and reliability in one model.

This approach, in which the biological organisms are implemented into environmental evaluation processes, provides a reliable and timely alarm for warning of and preventing acute water deterioration in an ambient environment. Therefore, this noninvasive system based on crayfish physiological and ethological parameter recordings was investigated for the detection of changes in an aquatic environment. This system is now applied at a local brewery for controlling the water quality of the water used for beverage production, but it can be used at any water treatment and supply facility for continuous, real-time water quality evaluation and for regular laboratory investigations of crayfish cardiac physiology and behavior.

INTRODUCTION:

The subject of aquatic organisms' applications, both as model organisms for various laboratory investigations[1,2] and as tools for monitoring industrial and natural/environmental water quality[3,4], appears to be well studied. Nevertheless, this topic is still of noteworthy interest for humans, irrespective of whether they belong to the scientific community or to other occupations. In spite of the existence of a number of advanced methods for monitoring certain parameters (so-called "biomarkers")[5-8], the most important requirements for selecting an indicator consist of three simple factors: (i) simplicity, (ii) reliability, and (iii) general availability.

Crayfish, as an essential representative of freshwater fauna, distinguishes itself because it is found worldwide, is widespread, and, in most cases[9], has a sufficiently large and hard carapace suitable for manipulation. This crustacean belongs to the group of higher invertebrates that provide sufficient development of vital physiological systems and respective organs while, at the same time, maintaining a relatively simple organization[10].

Methods based on the assessment of the range of crayfishes' biological and/or behavioral parameters, as described in the scientific literature, have significantly contributed to the development of biomonitoring and crayfish studies in general. Most of the currently available invasive methods for crayfish heart rate measurements are based on electrocardiogram recordings that require a complex and precise surgical procedure[11-13]; such manipulations can cause significant stress to and may require prolonged adaptation by the crayfish. Also, it is not known how long a crayfish can carry such electrodes and whether it will successfully molt while carrying such an attachment. The described noninvasive methods are based on plethysmographic recordings, which are complicated by hardware complexity and require a conditioning circuit for signal filtering[14] and an amplification or precise and expensive optic components[15,16].

In this study, we described an approach that contributes to existing results and offers new alternatives for improving current crayfish heart rate measurement procedures. Among the advantages, there are (i) a fast and noninvasive attachment that does not require a prolonged physiological adaptation; (ii) crayfishes' capability to carry the sensor within a period of a few months from molting to molting; (iii) the software capable of monitoring real-time cardiac and behavioral activities and the evaluation of data obtained concurrently from multiple crayfish; (iv) a low manufacturing price and simplicity. The biomonitoring system that we describe permits the noninvasive and continuous monitoring of crayfish cardiac and locomotor activities based on changes in crayfishes' etho-physiological characteristics. This system can easily be applied in laboratory examinations of the crayfish cardiac physiology and/or ethology, in addition to industrial implementations for controlling water quality at water treatment and supply facilities.

PROTOCOL:

1. Crayfish Selection

1.1. In order to successfully apply the current approach to crayfish, select the respective adult specimens with sufficient carapace sizes (which is a carapace length of at least 30 mm) for sensor attachment, visually examine it for the absence of diseases, and check whether it lifts both chelae when it is touched. The above-mentioned parameters indicate an eligible state of crayfish health.

Note: If several crayfish are expected to be used in the trial and are exposed to the same conditions, the experimental group should be formed based on several parameters: (i) similar weight and length; (ii) comparable heart rate; (iii) pronounced nocturnal activity; (iv) regular food consumption; (v) inter-molting period[17]. Sometimes, it is hard to define whether a crayfish is near to molting by the heart rate measurements or visual or tactile examinations only; therefore, the analyses of the crayfish's hemolymph total protein content can be helpful. Protein content is expected to be higher when the crayfish is closer to molting than in the inter-molting state[18].

2. Recording of Crayfish Cardiac Activity and Behavior

2.1. In order to noninvasively measure crayfish heart rates, preliminarily prepare the sensor for this procedure. Before this, put a crayfish into the tank with water and let it acclimate there for a few days as the preparation of the sensor[19] will also take a few days.

2.1.1. Axially couple an IR light-emitting diode (LED) with a phototransistor. Attach the optical sensor circuit onto a board; it will require a power supply of 5 V. For the LED connection, place a 200-Ω resistor on the IR sensor board; in order to connect the phototransistor, place a 220-Ω resistor on the board.

2.1.2. When attached to the crayfish, the sensor output is modulated by the amount of hemolymph filling the crayfish cardiac muscle and scatters an incident light from the LED. In order to avoid reciprocal interference of the illuminated IR light by the LED and the reflected IR light from the crayfish heart, which is received by the phototransistor, place a small wall (0.5 x 1.5 x 4 mm, thickness x height x width) made of black antistatic plastic between the LED and the phototransistor.

2.1.3. Place the LED in a waterproof package, and cover the surface of the sensor with the waterproof dielectric gel from the side adjacent to the carapace for the protection of the electronic components from potential damage (**Figure 1**). Let the gel dry for 3 d in order to gain its best protective properties.

2.1.4. For an analog signal, attach thin flexible cables (about 3 m long) to the sensor and connect to the analogue-to-digital converter (ADC); from this, a digitized signal will be transferred to a personal computer over a USB interface, at which point the information about the crayfish cardiac activity is saved, analyzed in real-time with special software (see **Table of Materials**), and stored for further detailed analyses.

2.2. As soon as the sensor is prepared, attach it to the crayfish. In order to do this, switch the computer on and run the software. Determine the number of crayfish to be fixed to the sensors and displayed on the screen and set the sampling rate from 1 to 500 samples per second (sample/s).

2.3. Remove the crayfish from the water and wipe its dorsal carapace side with a paper towel. Wrap the chelae and abdomen of the crayfish in the paper towel in order to avoid any damages by human hand and to eliminate additional stress on the crayfish caused by warm human hands.

Note: Do not use a previous cooling of the crayfish on ice or in the freezer for its immobilization before manipulations with the sensor attachment. The difference in temperatures leads to crayfish dorsal surface weeping which, in turn, leads to unreliable sensor fastening and quick adhesive detachment from the crayfish's carapace.

2.4. Prepare a surface (*i.e.*, take a small flat piece of plastic or tear a piece of sticky tape and fix it to a table) and a stick for mixing the glue. Press out two small drops (of a diameter of about 0.5 cm) from tubes A and B containing epoxy glue and quickly mix them.

2.5. Attach the sensor to the crayfish dorsal carapace and try to find the place in which the cardiac signal amplitude would be maximal. Hold the crayfish with the sensor in one hand and, using the other free hand, put a drop of mixed glue on each of the four auxiliary wires located on the sensor (fix them in between steps 2.1.1 and 2.1.4.). Do not move the sensor at least 5 min until the glue hardens (the glue hardening depends on the ambient temperature and humidity).

Note: When fixing the sensor to the crayfish carapace, examine thoroughly the whole cardiac area from the carapace side in order to define the area with the best (maximal) cardiac signal amplitude. That will help the software to provide more precise heart rate calculations.

2.6. Touch the glue using a free hand, and if it is not sticky, put the unwrapped crayfish with the attached sensor (**Figure 2**) to the box without water for few more minutes until the glue is completely dry.

Note: An optimal temperature for crayfish and glue manipulation varies from 18 to 22 °C. At these temperatures, the glue hardens within 5 to 7 min and is completely dry within 8 to 10 min. At lower temperatures, the stress in the crayfish is less pronounced; however, the glue needs more time to harden, about 15 and 20 min under 15 °C and 10 °C, respectively. At higher temperatures, particularly above 25 °C, the glue hardens within 3 min, but the crayfish undergoes much more stress; therefore, try to minimize the exposure of the crustacean to extreme conditions without water.

2.7. Before moving the crayfish back into the tank, dip its cephalothorax into the water several times with short intervals of a few seconds in order to allow a discharge of the air that has accumulated in the gills. Then, put the crayfish into the water for approximately 1 h to remove any excess chemicals. After this process is complete, release the crayfish into the water and allow it to acclimate for one to two weeks under experimental conditions, depending on observed physiological indices. Optimal water exchange during the acclimation periods is every other day.

Note: Characteristics of crayfish that have acclimated and are in a healthy state include pronounced circadian cardiac and locomotor activities, regular food consumption, and spending most daylight in a specialized shelter (if provided).

3. Camera and Software Setup

3.1. Start the video camera; the software will automatically switch on.

3.2. Select an option of movement detection and the software will start tracking the behavior and linking it with the cardiac activity recordings.

Note: A crayfish motion detection module consists of a video camera that tracks crayfish behavior from below the tank and the software that combines the behavior with cardiac activity. The data from the module are used to facilitate more precise cardiac activity data processing by eliminating periods in which the crayfish demonstrates high locomotive activity. Sudden crayfish movements (*i.e.*, an escape reaction or feeding initiation) can result in fluctuations or short-time spikes in cardiac signals that may reduce the precision of cardiac interval calculations.

REPRESENTATIVE RESULTS:

As a result, we obtained a combination of crayfish cardiac and behavioral activities, recorded and saved in a txt-format file (**Figure 3**). Besides the number of experimental crayfish, the date, and the sampling rate, the file consists of three columns: (1) the continual time in hh:mm:ss format; (2) the heart rate automatically calculated in beats per minute; (3) the locomotion registered as absence (0) or presence (1) of any movement. When the crayfish was inactive, zero was assigned to the cell responsible for movement, and when it moved, then number one appeared in the respective cell. When continuously recording, the data file was automatically created every day at 00:00 hours (12:00 AM). It was crucial to include locomotion since it could have caused changes in the heart rate (**Figure 4**). After 10 s, a food odor (milled, filtered, and diluted Chironomidae larvae) was delivered into the tank containing the crayfish, using a peristaltic pump. At 14 s, the crayfish recognized the stimulus, and its heart rate slightly decreased due to the so-called orienting response. After 20 s, the heart rate increased, thus resulting in a decrease in cardiac intervals. At 26 s, the crayfish moved toward the stimulus source, and both the physiological excitation caused by the food odor and the locomotion initiation resulted in a substantial heart rate increase. At 37 s, there was also evidence of abrupt crayfish motion. Additionally, locomotion could have substantially contributed to the heart rate growth during the crayfish's reactions to certain stimuli (**Figure 5**). A disturbed crayfish typically has an increase in heart rate, as seen during the 30- to 40-min interval with occasional locomotion. However, during the 45- to 50-min interval, the locomotion is much more pronounced. This locomotion contributed to a heart rate that is significantly higher than that seen during the period with decreased locomotion. If the data from the file is transferred to another application or the above programming algorithm is used, the data containing just the cardiac activity of the crayfish could be obtained and subsequently processed if necessary (**Figure 6**). The heart rate of undisturbed crayfish is characterized by a monotonic amplitude of the heartbeat curve and by approximately equal cardiac intervals between each cardiac peak.

In order to analyze crayfish behavioral patterns (such as passed distance, preference of a certain area in the tank or arena, and locomotion velocity), it would be possible to exchange the current camera with a standard video camera with a flat wide-angle lens, as the currently used camera does not make a recording but just tracks locomotion. Alternatively, a recording with any of the online applications for catching a video from the screen could be used.

FIGURE AND TABLE LEGENDS:

Figure 1: Noninvasive infrared optoelectronic sensor.

Figure 2: Signal crayfish, *Pacifastacus leniusculus*, holding the sensor on its carapace.

Figure 3: An example of the data file.

Figure 4: Crayfish heartbeat during the change from normal to disturbed conditions when exposed to food odors.

Figure 5: Heart rate and locomotion activities of a crayfish in undisturbed (0 - 30 min) and disturbed (30 - 60 min) conditions.

Figure 6: Undisturbed crayfish heart rate.

DISCUSSION:

It has been widely suggested that the measurement of certain physiological parameters (such as heart or ventilation rate or both) is a more reliable method for recording crayfish reactions than the evaluation of behavioral responses that do not always occur immediately[11]. However, it is evident that the most efficient approach for assessing real crayfish reactions to environmental changes is the combination of cardiac activity and behavior recordings since that makes it possible to see the reason(s) for the crayfish heartbeat changes and whether or not they occur as a result of chemical alterations in the ambient environment or because of locomotion initiation. During water quality monitoring, it is crucial to eliminate all outside influences on the changes in crayfish physiological markers, including abrupt movements that have increasing effects on the heart rate but do not present an alarm for the biomonitoring system.

Another possibility for facilitating a more precise and informative heartbeat evaluation are the chronotropic and inotropic parameter analyses of crayfish cardiac activities mainly related to specific shapes in crayfish cardiac signals[19]. Such analyses confirmed that even when the heartbeat changed only a few beats per minute, some of the secondary parameters can indicate significant changes in crayfish cardiac activities[19].

Despite the number of benefits in using the described approach, research around monitoring crayfish has moved toward an absolute minimization of tactile crayfish manipulations. In the recently developed contactless system[20], the elimination of sensors and their respective wires means that crayfish of any size can be used for the monitoring procedure. It is also possible to keep multiple crayfish in one experimental area since the absence of any wires prevents wire tangling and crayfish movement restrictions. The crayfish will carry just two tiny pieces of a highly reflective tape that indicates its cardiac area. These pieces of tape can be attached to the crayfish even after a few post-molting days. Crayfish cardiac activities and behaviors are recorded by the video camera and analyzed in real-time by the coordinating software. Along with other technical advances, the modified approach will cause a significant decrease in the price of the monitoring system due to limited hardware.

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DISCLOSURES:

The authors have nothing to disclose.

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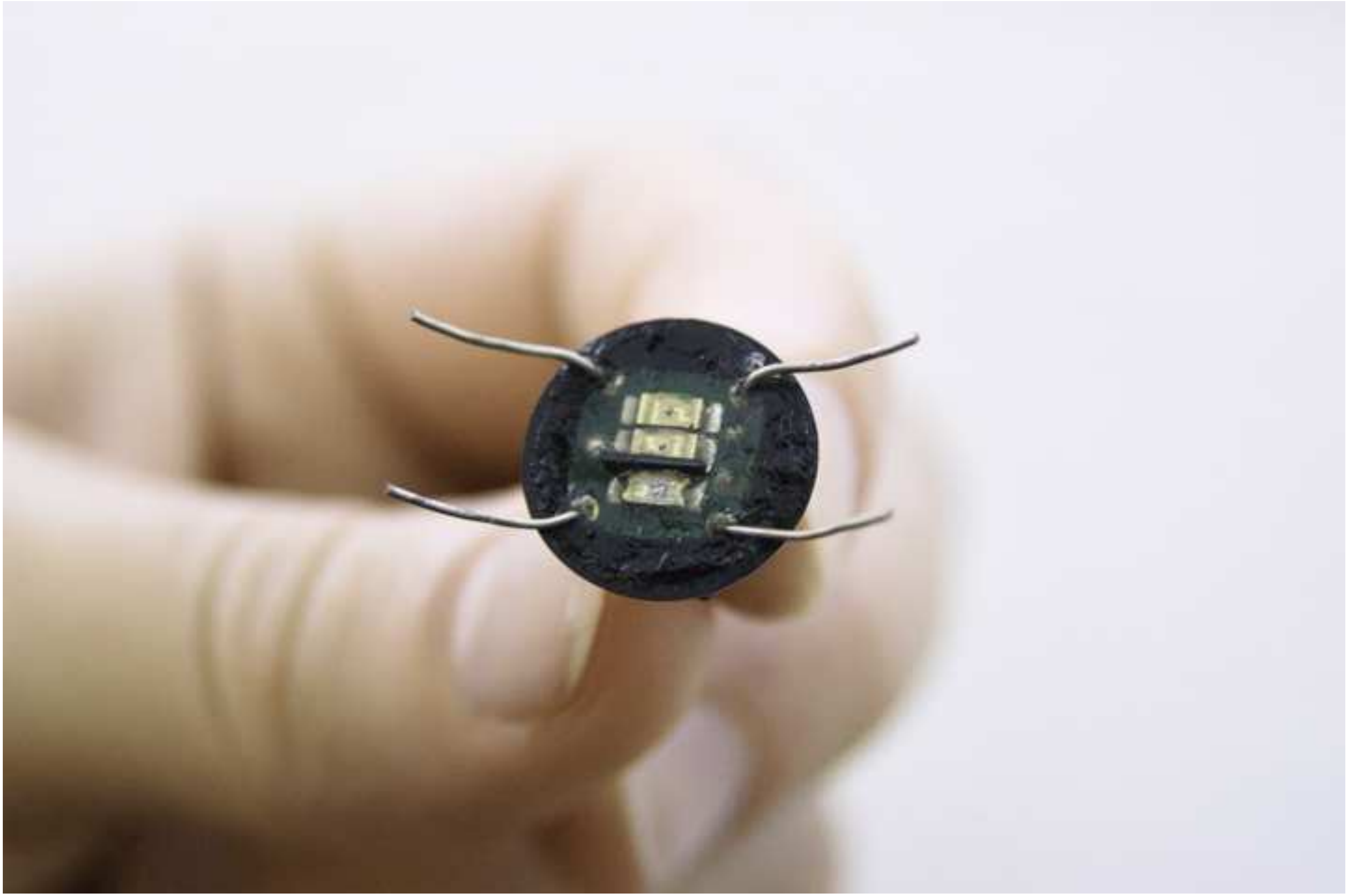

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Crayfish BPM (beats per minute) file
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Aquarium No. : 1
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Time : 00:00:00

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0:00:15	98	0
0:00:20	99	1
0:00:25	99	1
0:00:30	97	0
0:00:35	99	1
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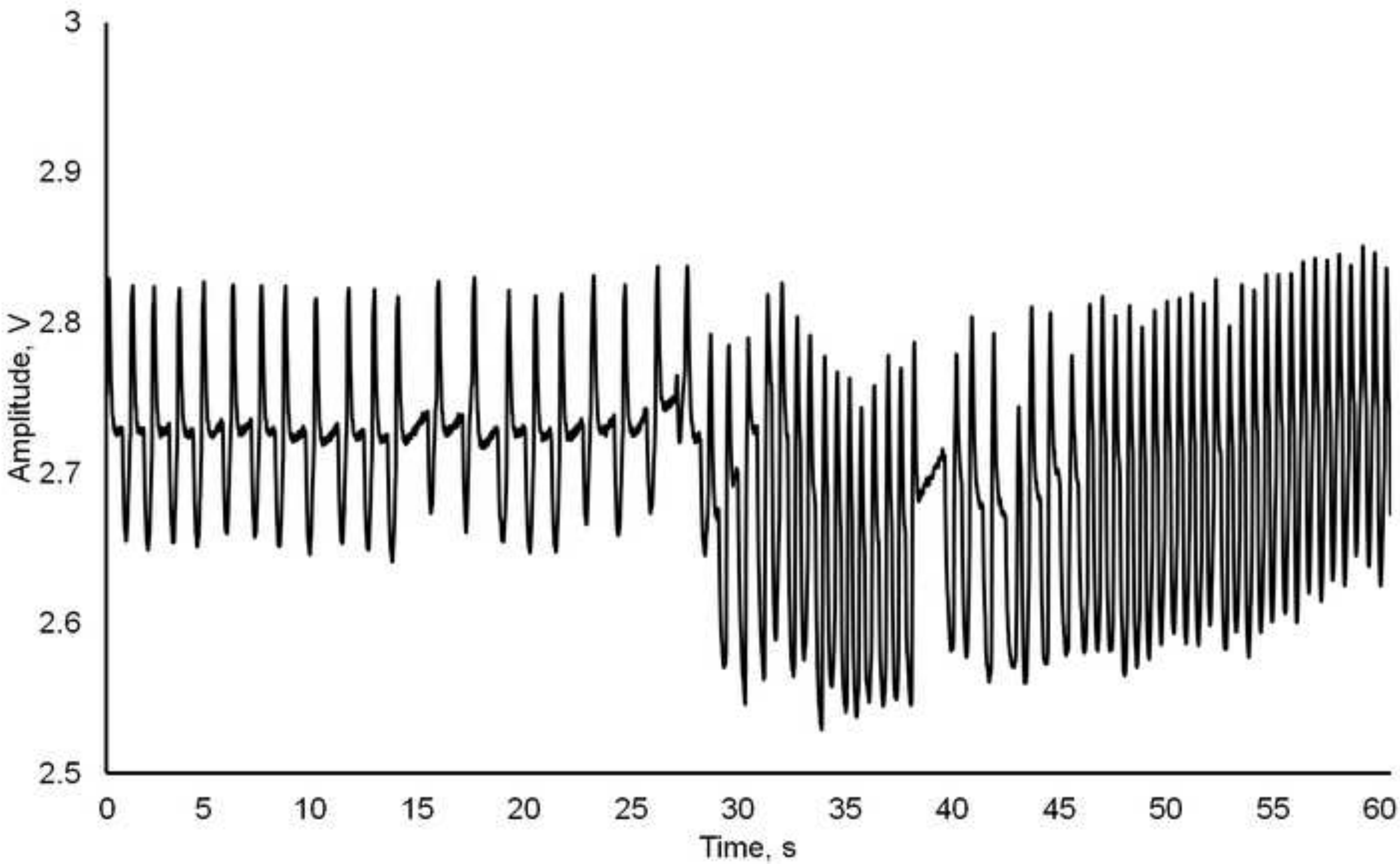
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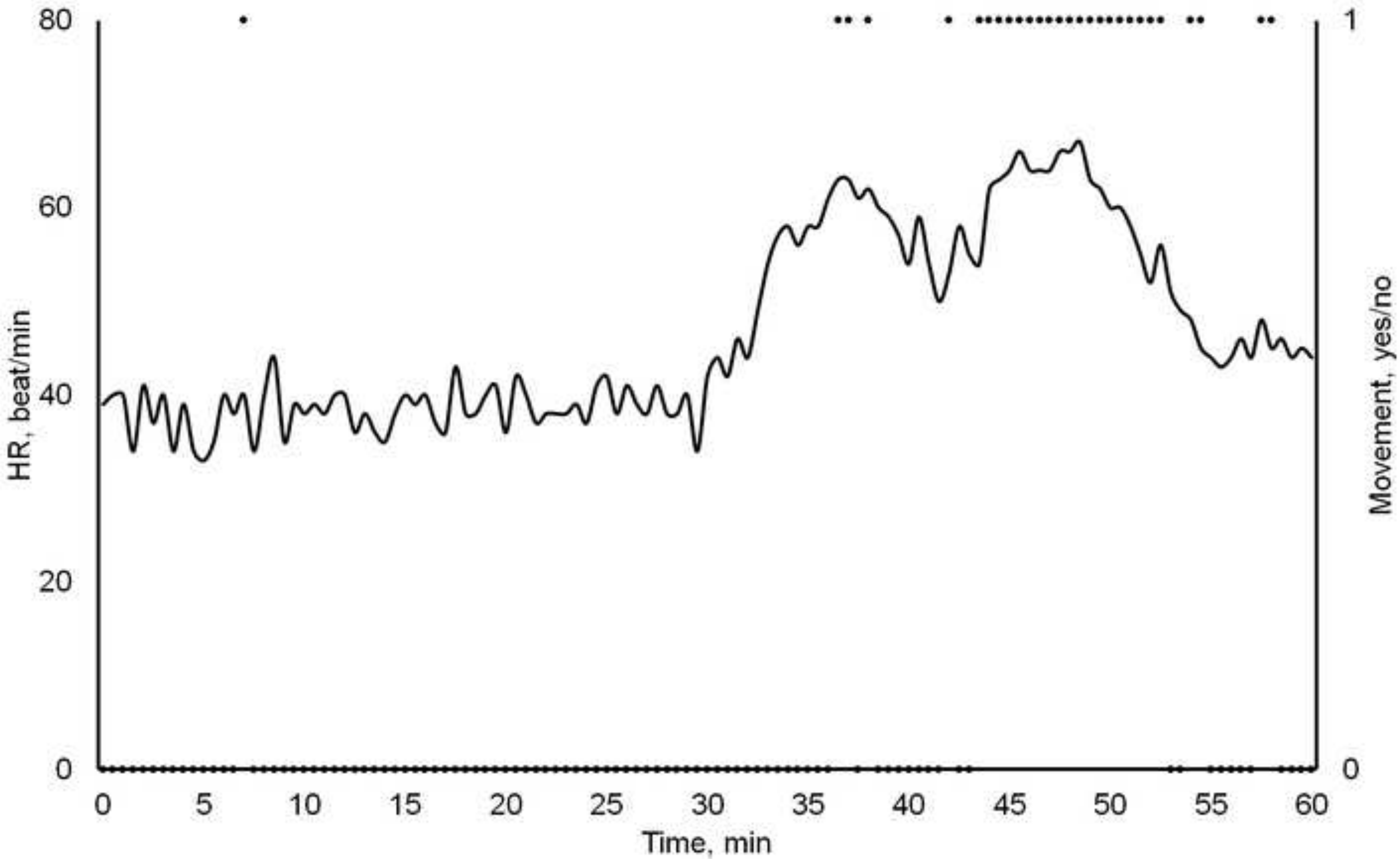
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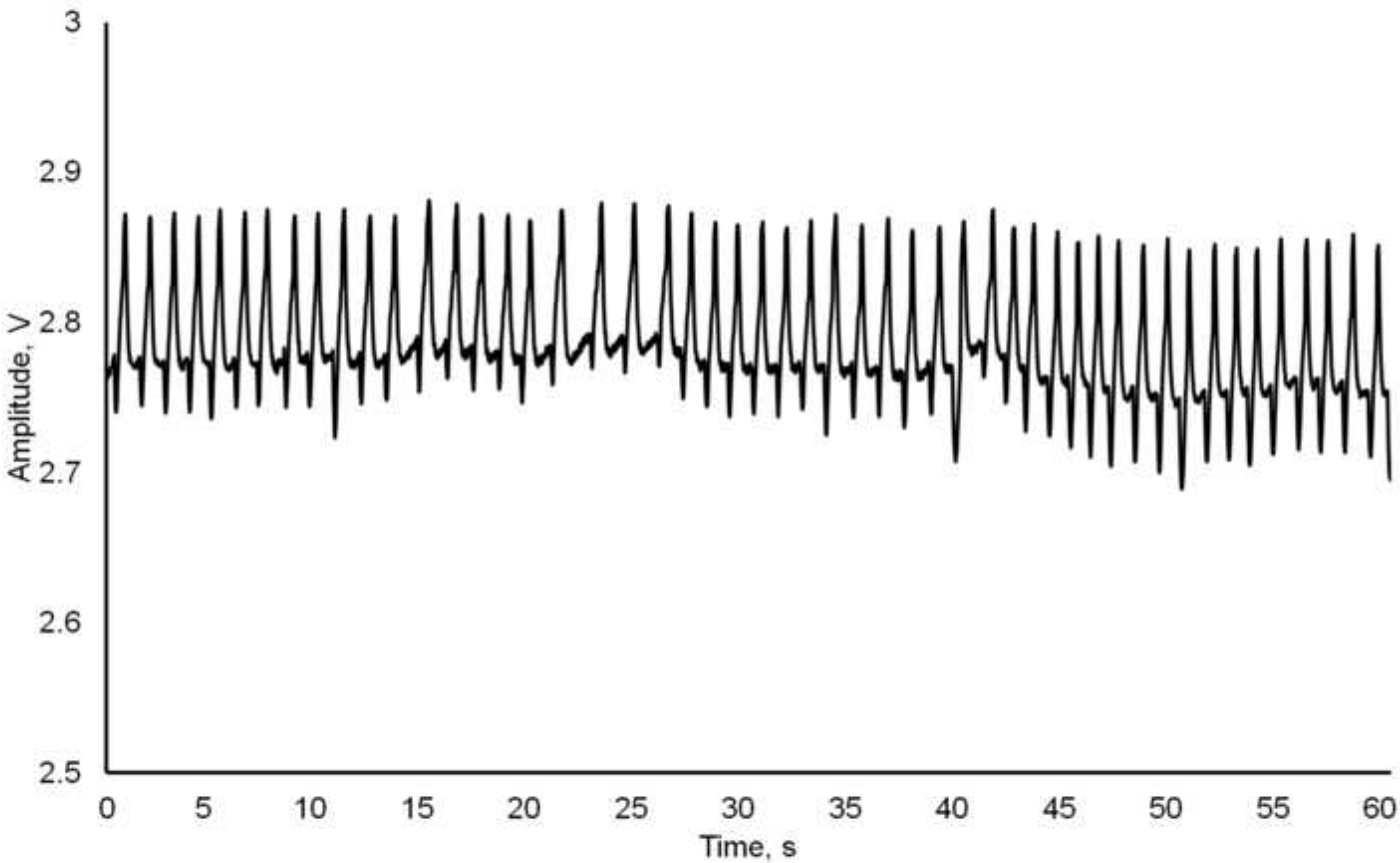
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
IR LED diode	KINGBRIGHT ELECTRONIC	KP-3216F3C	
Phototransistor	EVERLIGHT	ELPT15-21C	
Resistor	ROYAL OHM	0805S8J0201T5E	
Resistor	ROYAL OHM	0805S8F2200T5E	
Capacitor	KEMET	C0805C334K5RACTU	
Cable	TECHNOKABEL	FTP KAT.5E 4X2X0,14C	
Connector	HARTING	21348100380005	
Connector	HARTING	21348000380005	
Dielectric gel	KRAYDEN	Sylgard 535	
Analogue-to-digital convertor	TEDIA	UDAQ-1416CA	
Glue	KUPSITO.SK	7338723044	
Kinect video camera	ABCSTORE.CZ	GT3-00002	
Analysis software	University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, Institute of Complex Systems		Link to the software: www.frov.jcu.cz/crayfishmonitoring User name: frov Password: CF2018

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Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
3. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
4. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
5. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
6. Figure legends: Please shorten the figure legends. The Discussion of the Figures should be placed in the Representative Results.
7. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Answers to editorial comments:
1. We carefully went through the manuscript to ensure grammar and spelling issues, they can be seen in tracking changes of the document.

2, 3, 4. Protocol was revised, simplified and clarified.

5. Essential steps and note were highlighted.
6. Figure legends were shortened and their description moved to results.
7. References were fixed.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a nice study in presenting another approach to monitor heart rate in crayfish and likely in other crustaceans as well. I feel people will benefit from seeing this procedure implemented in JoVE's format.

Major Concerns:
No major concerns

Minor Concerns:
Re: Continuous noninvasive measuring of crayfish cardiac and behavioral activities. JoVE58555

This is a nice study in presenting another approach to monitor heart rate in crayfish and likely in other crustaceans as well. I feel people will benefit from seeing this procedure implemented in JoVE's format.

A few points I think need to be addressed for the reader's sake:
1. Attach the sensor to the crayfish dorsal carapace and try find the place in which the
Line171- "cardiac signal amplitude would be maximal. Hold the crayfish with sensor in one hand and put a drop of mixed glue using the other free hand to cover each of the four auxiliary wires located on the sensor (include them between steps 2.1.1 and 2.1.4.). Do not move the sensor at least 5 min until the glue hardens (glue hardening depends on ambient temperature and humidity)."
Maybe it will be explained in the movie but I was not sure from the text if a small hold is made in the cuticle for the LED to reflect off the heart or if the LED is to penetrated through the cuticle.
If a hole is made, well I assume that is stressful to the crayfish just as previous procedures to implant 2 wires for impedance measures.
If a hole is not made, would not the penetration of the LED light depend on the color of the cuticle, thickness, molt stage as the new underlying cuticle is forming.
2. The weight of the entire apparatus was not listed and the potential limitations such as size of animal where the device becomes an impact on the animals ability to freely move, since the authors mentioned this could also be used for examining animals while they are behaving. The wires leading out of the animal and the black tube appear to be large, could they not be a thinner and less cumbersome for the crayfish to be able to move more freely?
3. Some crayfish/crustaceans have very dark pigmentation based on diet, could this also be a limitation?
4. Please list the weight and size (head to tail or postorbital to cephalothorax).

Answers to reviewers' comments:

1. It will definitely be demonstrated in the video, and there are no holes in the cuticle should be made to attach the sensor, as we would like to underline that the approach in noninvasive. Thus there is no a significant stress to crayfish from this manipulation.
   No, there is no effect of the cuticle color and thickness on the near infrared light penetration, however there could be a complication when recording a heart rate in the crayfish in premolting stage: with forming of a new cuticle, cardiac signal amplitude will decrease and with subsequent detachment of an old cuticle, the cardiac signal will be lost, as the sensor will stay attached to the old cuticle.
2. The weight of the sensor is approximately 5 g, the diameter of a wider measuring part is 10 mm and the diameter of coated wires is 2 mm. It could be thinner if remove an external coating, however, in that case it is not recommended to rely on it for a long time as crayfish can easily bite uncoated wires and damage one of them, and the signal will produce a significant noise or the sensor will stop working at all.
3. Color of experimental animal is not a problem for the NIR sensor as its light is strong enough to illuminate cardiac area, but at the same time NIR light is not harmful for crayfish or other large crustacean physiology.
4. Crayfish shown at the picture had 56.8 mm long carapace measured from the tip of the rostrum to the posterior edge of the cephalothorax.

Reviewer #2:
Manuscript Summary:
In the present study, a sensor was used to continuous monitoring of crayfish cardiac and locomotor activities. Method was explained about how the system work.

Major Concerns:
More info is needed about how long crayfish can carry the sensor?
How many crayfish were used to test cardiac and locomotor activities? If more than one is used, do author test if there is any significant difference between control groups?
How long sensor can record the activities?
Is it possible to change battery of the sensor?
Does sensor have negative effect on feeding and behavioral activities of crayfish?

Minor Concerns:
Abstract: Authors did not conduct an experiment to measure behavioral activities related to water quality deterioration. However, they studied and published it in another journal (Environmental Science and Pollution Research 25, 8396-8403). Thus, this sentence should be deleted from the text.

Give more information about the waterproof dielectric gel i.e. name of the producer

Answers to reviewer's comments:

1. Theoretically, crayfish can carry the sensor if attached approximately one week post molting and up to the next molting, i.e. several month. Practically, we observed crayfish within 15 weeks before last of them lost the sensor, or better say glue lost its adhesive properties. Therefore, we assume that maximal period of sensor carriage strongly depends on the glue, as well as additional manipulations with crayfish, use of shelters and other individual conditions that can impact glue attachment and adhesiveness.
2. We did not count number of crayfish, as we had many experiment since approach development, but there were definitely hundreds of them.
3. The sensor can record cardiac activities as long as it attached to the crayfish. The duration of recording can also depend from capacity of internal or external hard disk where data are recorded and stored and number of crayfish as space of the disk decreases with number of experimental animals.
4. It is not possible change the battery as the sensor does not contain it, instead it is directly connected to analog-to-digital converter and needs continuous electrical supply.
5. According to our experience, the sensor does not negatively impact feeding or behavioral activities of crayfish. It can have minor effect during first day post attachment, but then crayfish is getting adapted very fast without any subsequent physiological or ethological disturbances.
6. In this manuscript, there is no data on crayfish ethological reactions during the water quality deterioration included, as it primarily presents an approach how to evaluate those parameters, regardless of ambient conditions. The results reporting crayfish reactions and behavior to water quality deterioration have been previously published by Pautsina et al., 2014 and Kuklina et al., 2018.
7. Krayden, Sylgard 535 waterproof dielectric gel.

Reviewer #3:

Manuscript Summary:

A noninvasive measuring system was developed for continuous recording and analyses of crayfish cardiac and locomotor activities. This system consisted of a near infrared optical sensor, a video tracking module, and software for evaluating crayfish heartbeats that reflected its physiological condition and for characterization of crayfish behavior during heartbeat fluctuations. This approach, in which the biological organisms are implemented into environmental evaluation processes, provides a reliable and timely alarm for warning of and preventing acute water deterioration in an ambient environment.

Minor Concerns:

What camera and special software is used for recording and analyses of crayfish cardiac and locomotor activities?

Lines 248-255. Figure 4. What kind of food is meant?

Answers to reviewer's comments:

1. There was used Kinect video camera for behavioral tracking.
2. Software provides calculation of the heartbeat frequency via analysis of cardiac signal based on detection of its minimums and maximums and on analysis of the signal frequency spectrum via Fourier analysis. For detection of locomotion, the method of dynamic background detection is used. Both methods are coupled in such way, that the system for locomotion detection provides the system for cardiac activity analysis with information on individual crayfish movements. Such combination enable to eliminate impact of crayfish locomotion on analysis of its cardiac activity in relation to environmental changes.
3. Odor of milled chironomidae larvae filtered through the filtration paper and diluted with water.

Reviewer #4:

Manuscript Summary:

This is a well-written paper containing very interesting results which merit publication. The data provided are very important for toxicology and aquaculture. It is demonstrated that signal crayfish can use as a bioindicator of water quality. Moreover, in addition to providing a better understanding of stereotypic crayfish behaviors induced by common and chemical stressors, the results of this study may serve as reference data for the evaluation of crayfish suitability for water quality tests.

For the benefit of the reader, however, a number of points need clarifying and certain statements require further justification. There are given below.

I made some comments regarding things I believe you could do to improve your work. It does not mean that you have to agree or rewrite in the same way. It is only a suggestion and different view, with the objective to contribute.

1) The reason for choosing signal crayfish, *Pacifastacus leniusculus*, as a model object in this study? Did other species, i.e. narrow-clawed crayfish *Astacus leptodactylus*, European crayfish *Astacus astacus*, etc., use in this study?

2) Which weight and length of signal crayfish, *Pacifastacus leniusculus*, are adequate and appropriate to use in this model test?
3) Which food odors were used in this model? What was the relationship between the intensity of food odor stimuli and the cardiovascular system responses?
4) Which any changes were observed in other systems and organs?
5) Were similar results obtained in the peculiarities of crayfish heartbeat using an electrocardiography approach and noninvasive biomonitoring system (a near infrared optical sensor, a video tracking module, and software for evaluating crayfish heartbeats)?
The manuscript is well written, objectives are clear, and discussion is properly addressed. The paper could be accepted after minor revisions.

Answers to reviewer's comments:

1. Noninvasive crayfish, *Pacifastacus leniusculus*, was chosen for this study due to its wide availability in Czech waters, while native crayfish (*Astacus astacus*, *Astacus leptodactylus*) are generally forbidden for using in such types of experiments because of their endangered status.
2. Crayfish of any species with sufficient carapace size (at least 30 mm CL) are appropriate for this type of study. However, it is evidently, that bigger crayfish is better to manipulate with.
3. There were used odor of chironomidae larvae in this model. We show here a reaction of crayfish to the very low food odor concentration, to see whether experimental animal would be able to immediately detect its entrance to water. Generally, crayfish reacted more intensively to higher food odor concentrations that reflected in higher heart rates increase. Intensity of cardiac reactions was also pronounced in prolonged maintenance of the heart rate at a high level, while at the low food concentration the heart rate stabilized to normal within shorter time.
4. Only effect on heart rate and locomotory activity were not tested in this study.
5. Depending on the intensity of the stimulus, crayfish demonstrated a decreasing heartbeat within approximately 60 s followed by the slightly delayed HR increase. Such peculiarity of crayfish cardiac reaction in presence of disturbance has been previously reported from analysis of electro-cardiograms.