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TITLE:

Impedance Pneumography for Minimally Invasive Measurement of Heart Rate in Late Stage Invertebrates

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SUMMARY:

Measuring heart rate during a thermal challenge provides insight into physiological responses of organisms as a consequence of acute environmental change. Using the American lobster (*Homarus americanus*) as a model organism, this protocol describes the use of impedance pneumography as a relatively noninvasive and nonlethal approach to measure heart rate in late stage invertebrates.

ABSTRACT:

Temperatures in oceans are increasing rapidly as a consequence of widespread changes in world climates. As organismal physiology is heavily influenced by environmental temperature, this has the potential to alter thermal physiological performance in a variety of marine organisms. Using the American lobster (*Homarus americanus*) as a model organism, this protocol describes the use of impedance pneumography to understand how cardiac performance in late stage invertebrates changes under acute thermal stress. The protocol presents a minimally invasive technique that allows for real-time collection of heart rate during a temperature ramping experiment. Data are easily manipulated to generate an Arrhenius plot that is used to calculate Arrhenius break temperature (ABT), the temperature at which heart rate begins to decline with increasing temperatures. This technique can be used in a variety of late stage invertebrates (i.e., crabs, mussels, or shrimps). Although the protocol focuses solely on the impact of temperature on cardiac performance, it can be modified to understand the potential for additional stressors (e.g., hypoxia or hypercapnia) to interact with temperature to influence physiological performance. Thus, the method has potential for wide-ranging

applications to further understand how marine invertebrates respond to acute changes in the environment.

INTRODUCTION:

In recent decades, increased input of greenhouse gases (i.e., carbon dioxide, methane, and nitrous oxide) into the atmosphere has resulted in widespread patterns of environmental change[1]. The world's oceans are rapidly warming[2,3], a trend that may have severe impacts on organismal physiology. Temperature heavily influences physiological rates, and organisms have an optimal temperature range for performance[4,5,6]. As such, individuals may encounter difficulties in maintaining proper oxygen delivery to tissues as temperatures stray outside of this range. This has the potential to lead to declines in aerobic performance in the face of warming ocean temperatures[5,7].

In a laboratory setting, a method to understand the physiological impacts of environmental change is to examine cardiac performance in the context of thermal stress. This provides insight into how exposure to predicted warming conditions may alter performance curves[5,6] as well as the potential for acclimation plasticity[8]. A variety of methods have been successfully implemented to previously measure heart rate in marine invertebrates. However, many of these techniques involve surgical removal or major manipulation of the exoskeleton and prolonged implantation of measurement devices[9-11], which introduces additional stress to the test subject and increases the time needed for a successful recovery prior to experimentation. Moreover, less invasive techniques (e.g., visual observation, videography) may be restricted to early life history stages when organisms may be fully or semi-transparent[12]. Furthermore, additional challenges may be presented to researchers who are not well-versed in more technologically advanced methodologies (e.g., observations via infrared transducers or Doppler perfusion[8,11]).

This protocol uses the American lobster (*Homarus americanus*) as a late stage marine invertebrate model to demonstrate the use of impedance pneumography for assessing changes in heart rate during a temperature ramping experiment. Impedance pneumography involves passing of an oscillating electrical current (AC) across two electrodes positioned on either side of the pericardium to measure changes in voltage as the heart contracts and relaxes[13,14]. This technique is minimally invasive, as it employs the use of small electrodes (i.e., 0.10–0.12 mm diameter) that are gently implanted just beneath the exoskeleton. Finally, it provides real-time assessments of both heart rate and water temperature during the ramp through the use of a data logger.

The protocol also provides instructions for calculating Arrhenius break temperature (ABT), the temperature at which heart rate begins to decrease with increasing temperatures[13,15]. The ABT serves as a nonlethal indicator of the thermal limit of capacity in test subjects that may be favored over measuring the critical thermal maximum (CT_{max}, the upper limit of cardiac function[5,6]), as lethal limits are often extreme and rarely encountered in the natural environment[5].

PROTOCOL:

1. Equipment setup

1.1. Wrap clear, malleable tubing around itself to create a heat-exchanging coil that is approximately 8–10 cm in diameter and has extensions 40–70 cm long. Secure the coil using electrical tape.

1.2. Attach the heat-exchanging coil to the external supply and return fittings of a cooling/heating circulating water bath. Ensure the connection is secure using hose clamps.

1.3. Fill the well of the cooling/heating circulating water bath with reverse osmosis (RO) water and plug the power cord into an outlet. Turn the water bath on and make sure there are no leaks in its connection to the heat exchanging coil.

1.4. Set up the impedance convertor by plugging in the black BNC cable to the **AC** output on the unit and connecting it to the data logger (**Table of Materials**) using the **Channel 1** port.

1.5. Plug the thermocouple probe (temperature recorder) into the T-type pod, then plug the T-type pod into the **Channel 2** port of the data logger.

1.6. Plug the power cord of the data logger into a power supply and connect the data logger to a PC computer using the USB cable connector.

1.7. Fill the acclimation chamber and experimental arena with 7.5 L of artificial sea water (salinity = 35 ppt, pH = 8.1, temperature = ~12 °C).

NOTE: The volume, temperature, and chemistry of the water needed for the acclimation chamber and starting conditions in the experimental arena are dependent upon experimental design. Importantly, these containers must be large enough to comfortably submerge the test subject.

2. Implantation of electrodes

2.1. Place the lobster on a plastic grate that fits easily into the experimental arena such that the body comfortably makes a Y-shape at one end of the rectangle.

2.2. Carefully secure the lobster's claws and abdomen to the plastic grate using small cable ties. The cable ties should be tight enough to prevent movement but allow room for surgical scissors to remove them upon completion of the experiment.

2.3. Dry off the carapace with a paper towel and clean it with a cotton ball soaked in 70% ethanol.

2.4. Create the holes for the electrodes.

2.4.1. Using a small drill bit (e.g., 1.6 mm), slowly and carefully hand-drill two small holes (nearly) through the carapace on either side of the pericardium.

2.4.2. Finish each hole by gently inserting a sterile dissecting needle.

2.4.3. If the needle does not easily go through the carapace, continue to slowly hand-drill before trying the needle again.

NOTE: To minimize stress in experimental animals, practicing this technique prior to experimentation is highly recommended. Over time, users can easily determine by feeling when the drill bit is nearly though the carapace and switch to the needle. Hand-drilling is appropriate for lobsters and crabs, especially if the exoskeleton is soft (i.e., animal has recently molted). However, if the test subject has a thicker exoskeleton or shell (i.e., a bivalve), a Dremel tool is more appropriate.

2.5. Obtain the electrodes (36–38 G magnetic wire, 0.10–0.12 mm diameter) and scrape off a small bit of insulation at the wire's tip using a dissecting knife blade. Carefully bend the tip of each wire into a small hook using forceps and insert one into each of the newly drilled holes.

2.6. Secure each wire lead using a small drop of cyanoacrylate glue and allow it to dry for 5–10 min.

NOTE: It is crucial to use the glue sparingly, as adding too much will reinsulate the wire and prevent the signal from being recorded.

2.7. Once the glue is dry, attach the wire leads to the impedance convertor and turn it on. Place the lobster into the acclimation chamber and allow it to acclimate to the implanted electrodes for 15–20 min.

NOTE: Quick or jarring movements, as well as incompletely dried glue, may cause the electrodes to become detached from the carapace. If this happens, return to step 2.6.

2.8. Turn the data logger on and open the LabChart software on the computer. Click **New Experiment** and leave the **Chart View** screen open.

2.9. In **Chart View**, locate the **Channel Function** menu for Channel 1 from the right-hand section of the screen. Choose **Input Amplifier** from the menu and select **AC Coupling**. The incoming signal from the test subject will now appear on the screen in real-time.

NOTE: The sensitivity of the channel can be adjusted by selecting the **Range** pop-up menu. Adjust the range until the signal peaks are 25%–75% of the full scale. Close the **Input Amplifier**

by clicking **OK**.

2.10. On the impedance convertor, adjust the **Gain** (size) and **Balance** until a strong signal is observed on the data logger output, aiming to keep the **Balance** near zero.

2.11. On Channel 2, select T-Type pod to record real-time temperature data.

2.12. When both channels are set up properly, click the **Start** button, and the data logger will begin logging data.

3. Temperature ramping

3.1. After the acclimation period, place the plastic grate with the attached lobster carefully into the experimental arena and set the heat-exchanging coil on top of the grate.

3.2. Place the thermocouple probe near the lobster, ensuring it is fully submerged before placing the lid on the experimental arena to reduce visual stress to the test subject.

3.3. Adjust the balance as needed and place a comment on the output stating that the trial has begun.

3.4. The output can and should be saved periodically throughout the experiment.

3.4.1. Click **File** and select **Save As** to initially save the output to the computer.

3.4.2. When saving during the experiment, click **File** and select **Save**.

NOTE: Although the LabChart software can recover files in the event of an accidental program shutdown (e.g., a power outage), it is recommended to save active files every 15–20 min during the experiment to prevent data loss.

3.5. Increase the water temperature of the experimental arena at a rate of ~1.5 °C every 15 min to achieve a ramp from 12 °C to 30 °C over a 2.5 h period by adjusting the temperature of the recirculating water bath.

NOTE: The geographic distribution of the American lobster spans a 25 °C thermal gradient, and individuals can acclimate to and survive at temperatures of up to 30 °C[16]. As such, 30 °C was chosen as the upper limit for this temperature ramp, as it ensures that lobsters experience a stressful scenario that does not reach the critical thermal maximum[13], which could lead to mortality. The specific rate of warming was selected because it falls within a range of warming rates implemented in studies using other species[8,14] as well as previous research on the American lobster[13,27]. Prior to implementing this protocol, it is important to 1) determine the appropriate range of temperatures for a given experiment and 2) conduct a pretrial temperature ramp with an empty experimental arena, as this will help to determine the

necessary temperature adjustment of the water bath to achieve the desired ramp. This may also differ depending on the volume of water in the arena.

3.6. Throughout the temperature ramp, record whenever an adjustment that may impact the output occurs.

3.6.1. Note that the balance on the impedance convertor will likely need to be adjusted throughout the experiment, and doing so may cause an unintentional spike in the output.

3.6.2. As the temperature in the experimental arena begins to reach levels outside of the preferred thermal range of the test subject, involuntary muscle contractions may result in an erroneous “spike” in the output. If this occurs, make a comment to identify areas of the output that should be removed during the data conversion process.

3.7. When the ramp is completed, remove the lobster from the experimental arena and place it into a recovery bath (12 °C) for ~20 min. If desired, continue to monitor the lobster’s heart rate until it returns to basal levels.

3.8. After 20 min, hit the **Stop** button on the PowerLab output and save the file. Carefully remove the electrodes and cut the cable ties with surgical scissors before returning the test subject to its holding tank.

NOTE: Rather than placing a lobster directly into the recovery bath, another option is to slowly return the experimental arena to its starting temperature. This is accomplished by cooling the experimental arena by ~1.5 °C every 15 min over the course of an additional 2.5 h.

4. Data conversion

4.1. Open **Data Pad**. Set Column A to time by double-clicking on **Column A** and clicking on **Selection & Active Point** on the left-hand side of the **Data Pad Column A Setup** menu. Select **Time** from the right-hand side of the menu and close the window by clicking **OK**.

4.2. Set Column B to the average temperature by double-clicking on **Column B** and selecting the **Statistics** option from the left-hand side of the **Data Pad Column B Setup** menu. Select **Mean** from the right-hand side of the menu and **Channel 2** as the **Calculation source** at the bottom of the menu’s window. Click **OK** to close the window.

4.3. Converting the voltage recorded to beats per minute

4.3.1. Double-click on **Column C** and select **Selection & Active Point** on the left-hand side of the menu. Select **Selection Duration** from the right-hand side of the menu and click **OK** to close the window.

4.3.2. Double-click on **Column D** and select **Cyclic Measurements** on the left-hand side of the

menu. Select **Event Count** from the right-hand side of the menu, and **Channel 1** as the **Calculation source**. Click **OK** to close the window. This will count the peaks of the data to determine heart rate across a selected portion of data.

NOTE: If needed, select the **Options** button from the bottom of the menu and adjust the **Detection Settings** to more accurately read the data. Scan through the data file and determine if the "Sine" or "Spikey" shape options result in counts of only the major peaks of the heartbeat output. Additionally, adjust the **Detection Adjustment** threshold on the right-hand side of the menu to ignore noise in the output file.

4.3.3. Double-click on **Column E** and select **Cyclic Measurements** on the left-hand side of the menu. Select **Average Cyclic Rate**, and **Channel 1** as the **Calculation source**. Adjust the **Detection Settings** and **Detection Adjustment** to match the settings for Column D (if manipulated in step 4.4.2). Click **OK** to close the window. This provides the final estimation of heart rate (as beats per minute) over a selected portion of data.

4.4. When the columns are set up, return to the data file and highlight the desired sections of the output, omitting areas of erroneous data as identified by comments in section 3.6.

4.4.1. Select **Commands** and **Multiple Add to Data Pad**.

4.4.2. Select **Time** from the **Find using** drop-down menu and pull data every 30 s by checking the **Every** box and entering "30" under the **Select** menu.

4.4.3. Click the **Current selection** option from the **Step through** menu and click **Add**.

4.5. Return to the Data Pad screen and select **File** and **Save As** to save the output as an Excel file.

NOTE: Here, heart rate is reported (in beats per minute) every 30 s as opposed to every minute based on previous research[8,27]. This also helps to more accurately capture changes in the real-time collected voltage data. It is possible to select data at shorter or longer time intervals based on individual preference.

5. Calculation of Arrhenius break temperature

5.1. Open the data file in Excel and manipulate the output from the LabChart software.

5.1.1. Convert the temperature from Celsius to the reciprocal of Kelvin using the following equation: [1000/(temperature °C + 273.15 K)].

5.1.2. Obtain the natural log of heart rate: ln(BPM).

5.2. Generate an Arrhenius plot by plotting heart rate as a function of temperature, expressed

as ln(BPM) vs. reciprocal (K)[13,15].

5.3. In SigmaPlot, fit the data with a piecewise regression and determine the intersection point, which is the ABT.

5.3.1. Copy and paste the transformed data into a new workbook. Select the **Statistics** option from the main menu and **Regression Wizard** from the drop-down list.

5.3.2. In the **Equation** window, select **piecewise** from the **Equation Category** menu and **2 segment linear** under the **Equation Name** box. Click **Next**.

5.3.3. In the **Variables** window, select the transformed temperature data to be the **t** variable and the transformed heart rate data to be the **y** variable, using the drop-down options in the **Variable Columns** menu. Make sure that **XY Pair** is selected in the **Data From** menu before clicking **Next**.

5.3.4. After reviewing the **Fit Results** window, click **Next** and check the box for **Create Report** in the **Numeric Output Options** window. Click **Next**.

5.3.5. In the **Graph Options** window, check the **Create new graph** option under the **Fit Results Graph** section, and **Add equation to graph title** under the **Graph Features** Section. Click **Finish**.

5.3.6. On the **Results** output page, retrieve the equations and parameter values for the two regions of the piecewise regression, as well as the statistical output for the regression (e.g., R^2, F-statistic, and p-value).

5.3.7. Using the parameter values and equations generated, set the two segments equal to each other and solve for the variable "t" to determine the ABT. Convert this value back to Celsius using the following equation: °C = (1000/t) - 273.15.

NOTE: The ABT can also be calculated in the R statistical computing environment using the package "segmented"[17] in the program SAS[18], or using the "Segmental linear regression" routine in Prism8[19].

REPRESENTATIVE RESULTS:

This protocol describes the use of impedance pneumography to obtain real-time data for heart rate (in voltage) and temperature during a temperature-ramping experiment. When perforing this technique, the amplitude of the voltages and temperatures recorded will vary based on experimental design and focal species. However, the voltage output displayed in real-time follows a generic sine distribution when the protocol is implemented correctly (**Figure 1A**). As the temperature in the arena is increased, the real-time distribution of voltage changes to reflect an increased frequency of voltage peaks (i.e., heart beats; **Figure 1B**). As the arena temperature continues to increase to levels outside of the test subject's optimal performance

window, the distribution changes to depict a reduced frequency of voltage peaks with a sine-like shape interrupted by sporadic peaks and/or moments of "flat-lining" (**Figure 1C**).

Once raw data are converted using the Data Pad component of the LabChart software, the resulting distribution of heart rate (in beats per minute) over the course of the temperature ramp follows a parabolic distribution if the experiment is successful (**Figure 2**). As the temperature in the arena is increased, the heart rate of the test subject also increases to meet elevated energetic demands associated with warmer temperatures. However, as temperature continues to increase and the test subject begins to experience moderate to extreme thermal stress, heart rate begins to decline or becomes erratic as the subject begins to exhibit passive thermal tolerance (e.g., onset of anaerobic respiration, metabolic rate suppression, and reduced activity[5,7]). When heart rate and temperature data are transformed and an Arrhenius plot is generated, the point at which the heart rate begins to decline (ABT) can be calculated (**Figure 3**). The Arrhenius plot is then fit with a piecewise regression using statistical software in which the intersection of the two lines represents the ABT.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative output from LabChart data logger. Real-time change in voltage across electrodes of the test subject is displayed in red, and the concomitant real-time output of the arena temperature (°C) is displayed in blue. In the beginning of the experiment under cooler temperatures (e.g., 13.1 °C), voltage should follow a generic sine-like distribution (**A**). As temperature is increased (e.g., 23 °C), the frequency of voltage peaks should increase, but the distribution should remain sine-like (**B**). Finally, as the test subject is pushed outside of its optimal thermal performance window (e.g., 28.5 °C), the voltage peaks should become erratic as the frequency decreases (**C**).

Figure 2: Expected distribution of heart rate over the temperature ramp course. Voltage data collected by the data logger are converted to heart rate in beats per minute (BPM) using the Data Pad component of the software. When the ramp is conducted correctly, a parabolic distribution of heart rate over the temperature range tested is displayed.

Figure 3: Example of an Arrhenius plot. Once data have been converted in the Data Pad and exported, they are transformed to generate an Arrhenius plot. In this example, data are fit with a piecewise nonlinear regression in SigmaPlot, generating equations for the left- and right-hand segments (region 1 and region 2, respectively) of the regression line, as well as goodness-of-fit metrics. The intersection of the two regression lines is solved as the ABT (red star).

DISCUSSION:

This protocol describes the use of impedance pneumography to measure changes in heart rate of late stage invertebrates during a temperature ramping experiment. The primary benefit of this technique compared to other laboratory-based approaches[9-11] is that it is minimally invasive and does not involve major surgical manipulation of the exoskeleton, thus reducing the

amount of recovery time needed prior to experimentation. Moreover, the equipment is easy to use, and resulting data can be simply manipulated and interpreted in the suggested software program. While the American lobster is used here as a model subject, this technique has been successfully implemented in blue mussels (*Mytilus* spp.[14]) and can be easily modified for use in other late stage invertebrates (i.e., crabs, shrimps, and other bivalves).

An additional benefit of the protocol is that it focuses on calculating the ABT as a nonlethal indicator of thermal limits. Although numerous studies present the CT_{max} as the significant endpoint when determining thermal physiological performance[5,8,20-23], organisms rarely encounter temperatures in this range in the natural environment[5]. Moreover, as the CT_{max} is often a lethal temperature, using this metric as the preferred endpoint precludes the use of test subjects in additional or follow-on experimentation post-thermal stress[23]. When aiming to calculate the ABT using this protocol, it is crucial to increase the temperature in the experimental arena to the point of pushing the test subject to its physiological limit without inducing death. Therefore, it is recommended to determine the potential thermal limits of the focal species via a pilot study (when possible) prior to determining the full range of the experimental temperature ramp.

It is also recommended that researchers determine and observe natural variations in basal heart rate of a focal species when temperature in the experimental arena is maintained at a constant and non-stressful level prior to the ramping experiment. This is particularly helpful for focal species in which resting heart rate information is not available in the published literature. It also serves as ample practice of electrode implantation techniques. This may also help researchers determine the appropriate acclimation time required to ensure that no false spikes in heart rate are due to handling stress at the beginning of an experiment.

Although the protocol discusses the use of impedance pneumography in the context of thermal stress alone, it can also be utilized to explore the potential interactive effects of other stressors on thermal physiology. Organismal performance may be reduced in the presence of environmental stressors (i.e., hypoxia, hypercapnia, pollutants, and/or changes in salinity), which may also compress optimal temperature ranges for performance[7,24-26]. As such, this protocol can be modified to explore how exposure to various stressors prior to temperature ramping may impact performance.

For example, Harrington and Hamlin[27] exposed juvenile *H. americanus* to current or predicted end-century pH conditions (8.0 and 7.6, respectively) for 2 months prior to assessing cardiac performance during a temperature ramp. Lobsters pre-exposed to more acidic environments exhibited a significant reduction in mean ABT compared to those held under current pH conditions. This suggests that a low pH environment reduces thermal performance and may increase the risk of cellular damage due to heat stress at lower temperatures[27]. Future efforts could expand on the method presented here to include pre-exposure to any combination of environmental stressors prior to following this protocol. Moreover, this protocol can be modified to measure changes in cardiac performance during exposure to biotic stressors as well as how thermal limits can change according to ontogeny[4,5].

A major limitation of this protocol is that the equipment as described is restricted for use in a laboratory setting, potentially limiting its applicability for field-based experiments that require more specialized equipment[8]. This technique also requires the restraint of highly motile test subjects (e.g., lobsters and crabs) to reduce the production of false data points resulting from non-cardiac muscle movements. Although this may restrict natural behaviors during a temperature ramp, the impact of restraints are consistent across all test subjects. Most importantly, there is the potential for tissue damage or death in test subjects if aggressive or careless drilling during electrode implantation is implemented. This contrasts sharply with infrared photoplethysmography, a truly noninvasive technique that utilizes an external infrared transducer to pass light through the pericardium and record heart function by converting reflected light energy to voltage[8,28].

Although infrared photoplethysmography reduces the risk of handling stress compared to impedance pneumography, correctly implanting electrodes using the described method results in minimal trauma, allows for a quick acclimation time, and leads to rapid recovery without inducing mortality in test subjects following the ramping experiment[27]. As there is no significant difference in the cardiac output recorded by both methods[28], it is concluded that impedance pneumography is a reliable and minimally invasive technique for assessing cardiac performance. Finally, the numerous benefits and flexibility of the protocol have the potential to elucidate how various environmental factors interact with temperature to impact physiological performance in late stage crustaceans.

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The authors have nothing to disclose.

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Figure1

Click here to access/download;Figure;Figure1.psd

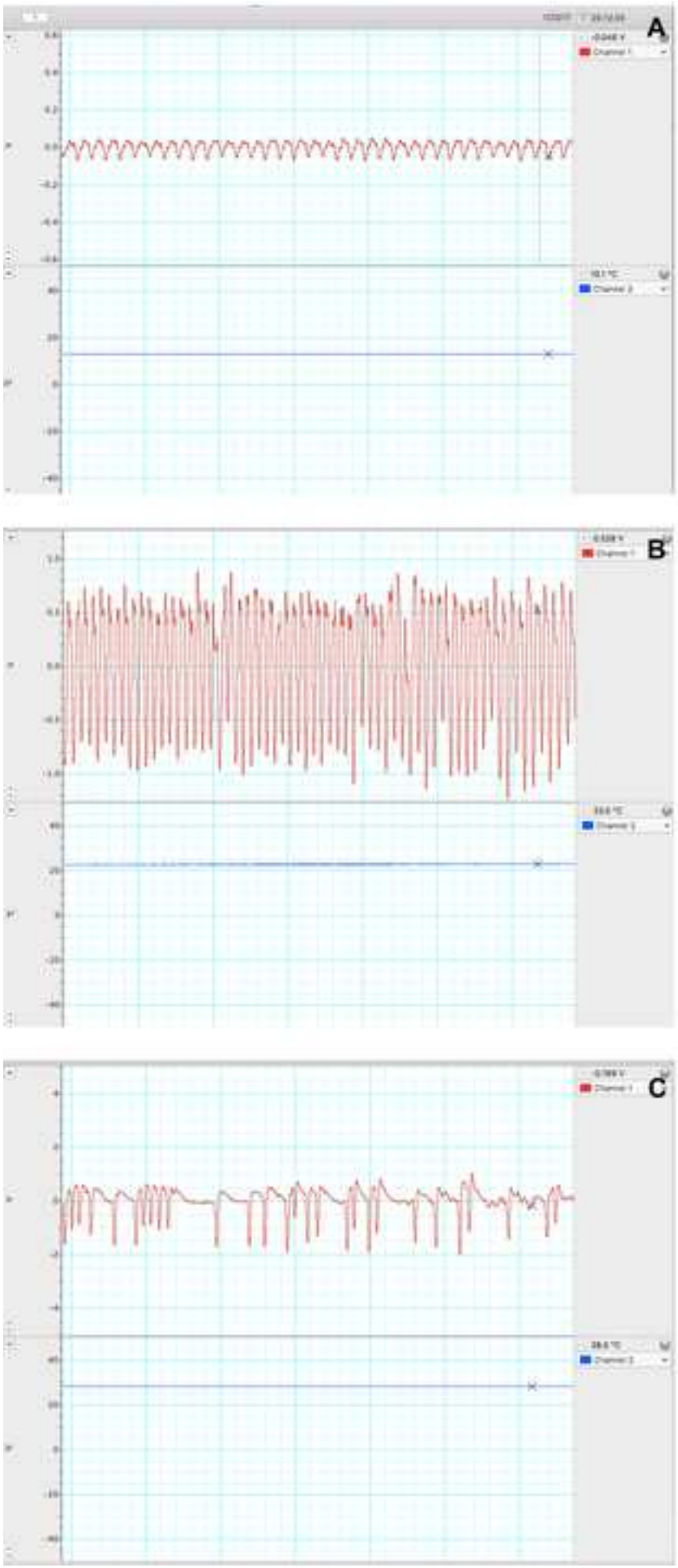
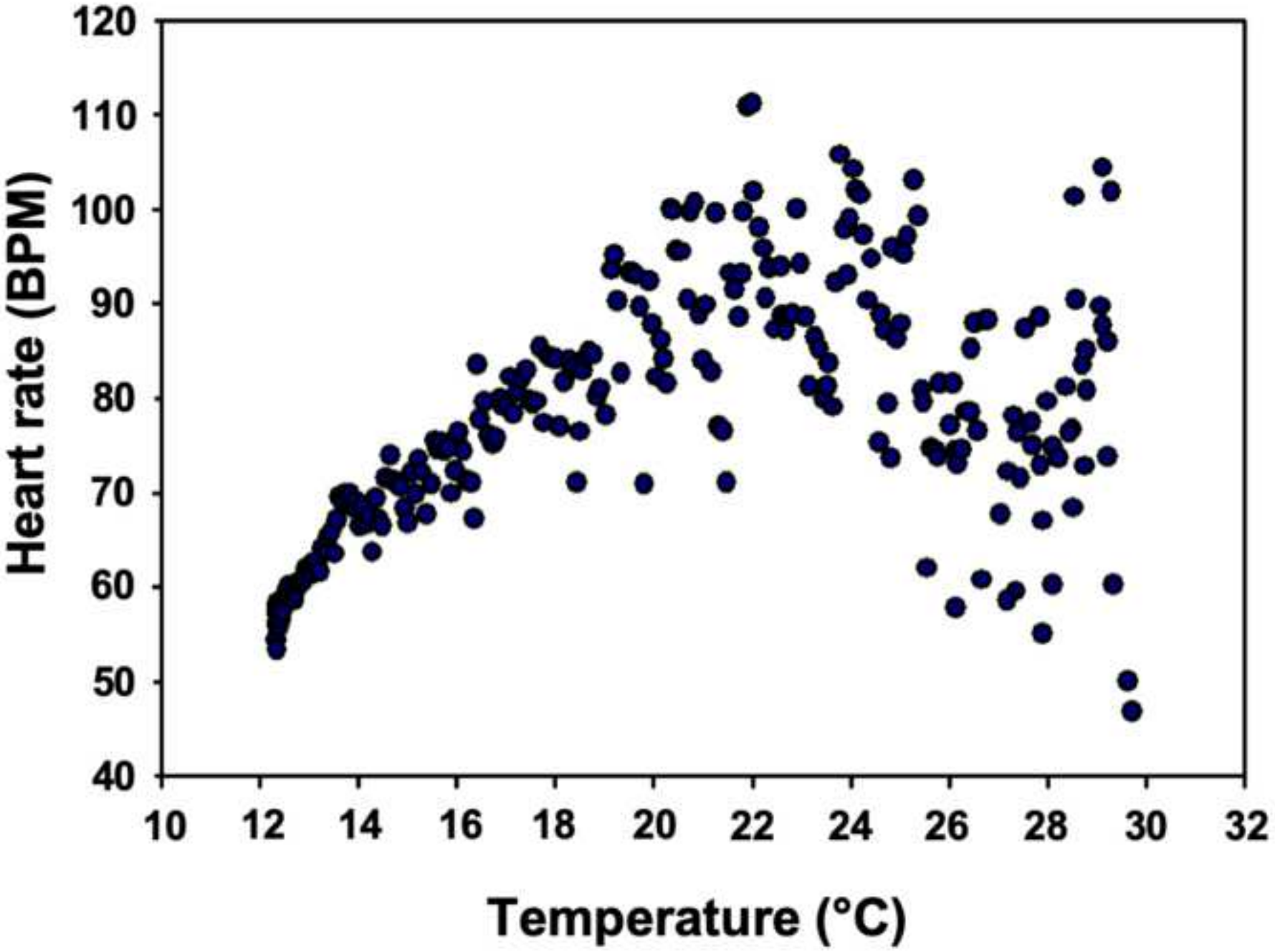
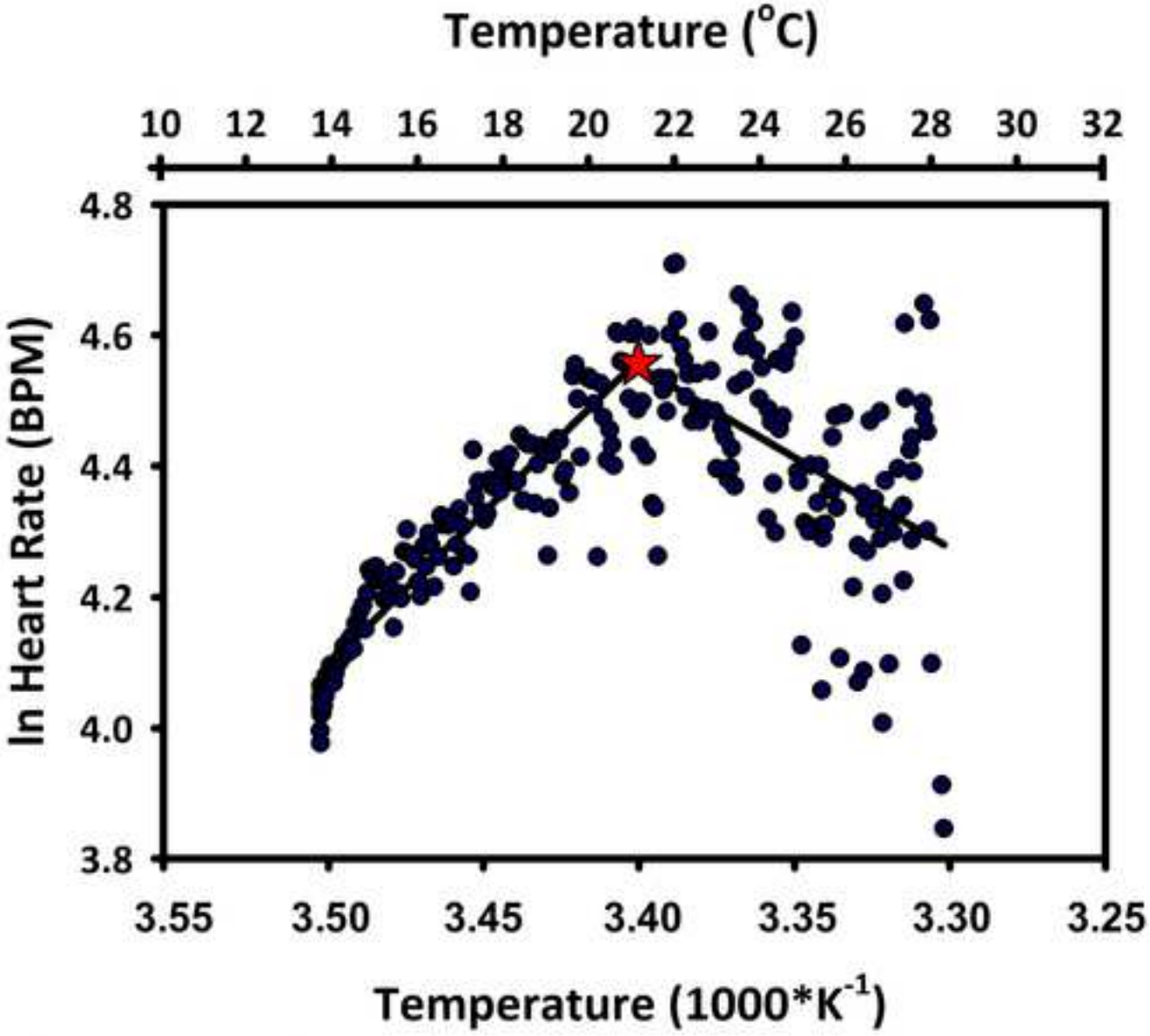


Figure2

Click here to access/download;Figure;Figure2.psd





SigmaPlot piecewise nonlinear regression:
$F_{3,256} = 197.3$, $p < 0.001$, $R^2 = 0.70$

Region 1(t) = 6.428 – 0.586t
Region 2(t) = 5.612 – 0.344t

Table of Materials

Click here to access/download;Table of Materials;JoVE61096_Materials_Harringtonetal_REVISED.xlsx

Name of Material/ Equipment	Company	Catalog Number
1.6 mm (1/16 in) drill bit	Milwaukee Tool at Home Depot	1001294900
38 AWG Copper Magnet Wire	TEMCo	MW0093
Cyanoacrylate glue	Loctite	852882
Ethanol, 70% Solution, Molecular Biology Grade	Fisher BioReagents	BP82931GAL
Excel	Microsoft	N/A
Fisherbrand 8-Piece Dissection Kit	Fisher Scientific	08-855
Fisherbrand Isotemp Refrigerated/Heated Bath Circulators: 5.4-6.5L, 115V/60Hz	Fisher Scientific	13-874-180
Fisherbrand Sterile Cotton Balls	Fisher Scientific	22-456-885

Fork Terminal, Red Vinyl, Butted Seam, 22 to 16 AWG, 100 PK	Grainger	5WHE6
Impedance converter	UFI	Model 2991
LabChart software	ADInstruments	N/A
LED Soldering Iron	Grainger	28EA35

PowerLab datalogger	ADInstruments	ML826
Prism8	GraphPad	N/A
R	R Project	N/A
Rosin Core Solder	Grainger	331856
SAS	SAS Institute	N/A

SigmaPlot	Systat Software, Inc.	N/A
T-type Pod	ADInstruments	ML312
T-type Thermocouple Probe	ADInstruments	MLT1401
UV Cable Tie, Black	Home Depot	295813

Comments/Description

This is for a 1.6 mm (1/16 in) diameter drill bit. This item can be found at most home-improvement stores.

This wire is used to make the wire electrode leads that are implanted into the test subjects. This listing is for a 4 oz coil of 38-gauge magnetic wire. TemCo also has 36-gauge magnetic wire that is also suitable for use in constructing wire electrodes.

This item includes a brush tip, which makes it easier to control the amount of glue used to secure electrodes to the carapace.

This reagent is used in combination with the sterile cotton balls to disinfect the carapace prior to electrode implantation.

This program is used in the protocol for organizing, manipulating, and analyzing data. It is compatible with both PC and Mac operating systems.

This kit includes the forceps, scissors, dissecting knife (and blades), and dissecting needle needed to accomplish the electrode implantation steps in the protocol.

This is a complete system that consists of an immersion circulator and a bath. It can be used as a temperature controlled bath or to circulate fluid externally to an application. Temperature range of this water bath is -20 to +100 °C, and the unit heats/cools rapidly and is easy to drain upon conclusion of use.

These swabs should be soaked in 70% ethanol before being used to disinfect the carapace prior to electrode implantation.

Terminals are soldered to the magnetic wire to construct the wire electrodes. These can be purchased from a variety of home-improvement vendors.

Measures impedance changes correlated with very small voltage changes, ranging from 0.2 ohm to over 5 ohms. This model can convert impedance changes that stem from resistance, capacitance, or inductance variations, as well as a combination of all three.

Purchase of the PowerLab datalogger includes the LabChart software, but a license for the software can also be directly downloaded online. LabChart allows the user to record data, open and read LabChart files, analyze data, as well as save and export files. There is a free version of the software, LabChart Reader, but users can only open and read LabChart files and analyze them (i.e., it cannot be used to record, save, or export data files). One also has the option of selecting LabChart Pro, which includes LabChart teaching modules that can be used for educational purposes.

This is a generic soldering iron that can be used to solder the magnetic wire to the fork terminals to create the wire electrodes.

There are a variety of models of the PowerLab. This catalog number is for the 2/26 model that is a 2 channel, 16 bit resolution recorder with two analog input channels, independently selectable input sensitivities, two independent analog outputs for stimulation or pulse generation and a trigger input. The PowerLab features a wide range of low-pass filters, AC or DC coupling and adaptive mains filter. This unit has a USB interface for connection to Windows or Mac OS computers and a sampling rate of 100,000 samples/s per channel.

This program provides an additional option for calculating the Arrhenius Break Temperature through its “Segmental linear regression” data analysis option. This program does not require any programming and is compatible with both Mac and Windows operating systems.

This is free software for statistical computing that is compatible with UNIX platforms, as well as Windows and Mac operating systems. This program can also be used to calculate the Arrhenius Break Temperature using the “segmented” package. There are a number of tutorials and user guides available online through the r-project.org website.

This product has a diameter of 0.031 in (0.76 mm) and is ideal for use in soldering speaker wire (similar gauge as magnetic wire used for electrodes).

This program provides an additional option for calculating the Arrhenius Break Temperature. However, it does require programming and is not compatible with Mac operating systems.

This is the authors' preferred program for statistical determination of the Arrhenius Break Temperature. The "Regression Wizard" is easy to use and does not require any programming. One can obtain a free 30-day trial license before purchase. However, it is compatible only with PC computers.

Suitable for measurement of temperatures from 0-50 °C using T-type thermocouples.

Compatible with the T-type Pod for connection. Measures temperature up to 150 °C, and is suitable for immersion in various solutions, semi-solids, and tissue (includes a needle for implantation). This product is a 0.6 mm diameter isolated probe that is sheathed in chemical-resistant Teflon and a lead length of 1.0 m.

This is for a 100-pack of 8-inch (20.32 cm), black cable ties. However, based on the size of test subjects, smaller or larger cable ties may be needed. This item, and others like it, can be purchased at any home-improvement store.

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February 4, 2020

Dr. Lyndsay Troyer
Senior Science Editor
Journal of Visualized Experiments
1 Alewife Center Suite 200
Cambridge, MA, 02140

Dear Dr. Troyer,

I am writing to submit a revised version of the manuscript, "Impedance pneumography: a minimally invasive tool for measuring heart rate in late-stage invertebrates." We greatly appreciate the thoughtful reviews we received on our original submission (JoVE61096), which strengthened the manuscript. We have revised the manuscript to address the reviewers' concerns. On the following pages we have provided detailed responses to reviewer comments.

Thank you for your consideration and for the opportunity to submit this revised version of our manuscript. We look forward to your response.

Sincerely,

Amalia Harrington, Corresponding author

Authors: Amalia M. Harrington, Holland Haverkamp, and Heather J. Hamlin

Title: Impedance Pneumography: A Minimally Invasive Tool for Measuring Heart Rate in Late-Stage Invertebrates

Manuscript#: JoVE61096

Response to Editorial and Production Comments:

General Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly reviewed all components of the manuscript to correct any spelling or grammatical errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: PowerLab, LabChart, Excel.

Response: We have removed commercial language from the manuscript and have updated the Table of Materials and Reagents to include software items used (e.g., Excel, SigmaPlot, and R).

Protocol:

1. For each protocol step/substep, 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: We have reviewed the protocol focusing on answering the "how" question. Please see the tracked changes in the manuscript (lines 88 – 439) for specific revisions.

Specific Protocol steps:

1. 1: Please provide a header for this section.

Response: We added the header, "Equipment setup" for this section (line 88).

2. 3.4: How often, specifically, should the output be saved?

Response: We added a note to this section (lines 220 – 222) to explain how often we believe the data file should be saved. Although researchers do not need to save an active file throughout the experiment, it is good practice to save the output every 15 – 20 minutes to prevent data loss.

Figures:

1. Figure 3: Please provide equations for the regression lines as well as measures of the goodness of fit (e.g., R squared).

Response: We acknowledge this oversight from our first submission. We added the statistical results of the piecewise nonlinear regression (*F*-statistic, *p*-value, and R^2

value), as well as the equations for the two segments of the regression line as generated from the program SigmaPlot, to our figure. We also added an explanation of this output in our figure caption for Figure 3 (lines 445 – 447).

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: We reviewed the Table of Materials and added various software components (e.g., SigmaPlot, Excel, and R-studio) and materials previously not included.

2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

Response: We removed all commercial symbols from the Table of Materials.

Video:
1. Chapter Title Cards
a. @03:54, IV. Temperature ramping procedure: Consider capitalizing "ramping" and "procedure" to match the "Title Case" you've used in the previous chapter cards.

Response: We adjusted the capitalization of the chapter title cards to be consistent throughout the video.

b. @04:58, V. Data conversion: Consider capitalizing "conversion".

Response: We capitalized “conversion” to remain consistent with the title case throughout our video.

2. Graphics Placement
@05:03: The screen is a little titled here. Rotating or "corner-pinning" the corners may help with this distortion. It's not too noticeable, so leaving it as-is would be okay. You could also attempt to capture the software usage by using screen capture software, such as Open Broadcaster Studio.

Response: We applied the “corner-pinning” effect to all video content of the computer display to correct for tilt and skewness.

Response to Reviewer’s Comments:

Reviewer #1:
Manuscript Summary:
The manuscript is interesting as it describes a methodology that is minimally invasive and serves to determine the effect of a temperature ramp in relation to the elevation of the heart rate and its subsequent decrease, one of the important points to highlight is the use of Arrhenius Plot which is the intersection of the two regression lines and allows to calculate the point where the heart rate is maximum.

Major Concerns:
No comments

Minor Concerns:

1. I recommend to the authors that in the discussion they highlight the benefits of this methodology and compare it with Infrared photoplethysmogram is another methodology that has been widely used to determine the effect of temperature by measuring the heart rate of different aquatic organisms.

Response: We appreciate the feedback from the Reviewer. We have added additional text in the discussion (lines 502 – 555) to compare our method to efforts using Infrared photoplethysmography.

Reviewer #2:

Manuscript Summary:

The methods described here illustrate how to measure heart rate using impedance pneumography and then calculate Arrhenius Break Temperature on a crustacean. The main experimental protocol is described clearly, and for researchers who have the equipment available, this manuscript and video should make it easy to replicate the process, outside of the final data analysis steps.

Major Concerns:

1. I do take issue with the description of the method as not requiring surgical implantation, since it does require drilling through the exoskeleton of the animal (see comments below). A method such as photoplethysmography (infrared emitter/detector) can be argued to be completely non-invasive and thus non-surgical, but impedance pneumography isn't quite at that same level of minimizing disturbance of the animal.

Response: We acknowledge the Reviewer's concern and have added language to the manuscript to clarify our original text (lines 64, 75 – 78, and 454). The Reviewer notes drilling through the exoskeleton may cause trauma, which is true if done incorrectly. We recommend slowly hand-drilling with the smallest drill bit available, as well as finishing each hole with the gentle insertion of a dissecting needle (lines 138 – 151). In the protocol, we also added additional notes to clarify the importance of practicing this technique on non-test subjects prior to experimentation, and of moving slowly and with great caution (lines 153 – 156). When done correctly, there is very little damage to the exoskeleton, and we have observed full recovery (i.e., return to normal behavior) in test subjects within 24 hr of electrode removal. We also observed no mortality and no bacterial growth in the hemolymph (suggestive of infection) in individuals held up to three weeks post-experimentation (Harrington et al., *In prep*). However, we acknowledge that this technique is invasive when compared to photoplethysmography. Please see the response to Minor Concern #1 from Reviewer #1 for more details on how we contrasted our protocol with photoplethysmography in the discussion (lines 502 – 555).

2. The authors discuss the utility of this method for measuring heart rate responses to a variety of potential environmental stressors, and at the end mention that the need to restrain the animal could impact the results. Can the authors expand on how restraint might directly influence the heart rate response? Do the lobsters visibly struggle against the restraint during the trials, or do they eventually relax?

Response: During the development of this protocol, we found it was necessary to restrain the lobsters to reduce background "noise" that stemmed from the contraction/relaxation of other muscles while lobsters were moving. Restraining the animals also enables us to

minimally impact the animal though electrode placement as they are gently and shallowly placed inside of the animal and secured only with a small drop of cyanoacrylate glue (see response to Major Comment #1, above). Although restraining the animals does introduce the potential for non-natural behavior in response to warming (i.e., in the natural environment, lobsters move away from temperatures outside of their preferred thermal range), the potential impact is consistent across all test subjects (lines 506 – 507). In our experience, lobsters generally do not struggle against the restraints, but if they do it tends to occur during the acclimation period. However, as the temperature inside of the area begins to reach levels outside of the preferred thermal range (20 – 24 °C) and into extremely stressful temperatures (27 – 30 °C), some individuals do exhibit involuntary movements in response thus creating a "spike" on the output. In these instances, researchers can make a comment in the software and omit these data from analyses. We have included text in the protocol to explain this information as it will likely prove useful and should have been included in the original manuscript (see lines 240 – 266).

3. The methods in section 5 for calculating the piecewise regression and arriving at the Arrhenius Breakpoint Temperature are not sufficiently detailed. From what I can find, there is no piecewise regression function in current versions of Excel, so telling the reader to analyze the data in Excel and fit the piecewise regression isn't sufficient. This will likely lead inexperienced users to just arbitrarily fit two regression lines to two segments of the graph by choosing the breakpoint by eye, which of course will bias the ABT value (since the user just chooses it by eye). Ultimately it might be more appropriate to recommend the reader use an approach like the 'segmented' package in R or other available statistical software that will hopefully reduce the potential for user bias in the calculation of the ABT.

Response: This is an excellent point and we agree more detail is needed in the written protocol. We included additional information on how to arrive at the ABT using the piecewise nonlinear regression routine in SigmaPlot (lines 352 – 395). As the Reviewer suggests, we also included information on additional statistical resources available through R, as well as SAS and GraphPad, in both the manuscript (lines 393 – 395) and in the Table of Materials.

Minor Concerns:

1. Line 50: Other gasses besides CO2 contribute to warming, so it would be reasonable to broaden this sentence out to include those other greenhouse gasses.

Response: Our research focuses on understanding how organisms simultaneously respond to changes in carbonate chemistry and warming temperatures, and as such tend to focus on carbon dioxide as a major driver of environmental change. However, as this protocol has potential to be applied more broadly, we acknowledge the Reviewer's comment and have altered the text to include mention of other greenhouse gases that have contributed to warming trends (see lines 50 – 51).

2. Line 76: Here the text says that electrodes need not be surgically implanted, but the methods described in the paper certainly do require surgical implantation by drilling through the exoskeleton (or shell) of an organism to put the electrodes in contact with the tissue of the animal. This makes it a (minimally) invasive procedure that has the very real possibility of causing tissue damage if not done just right. Within the context of this paper and intended study

organisms (lobsters, crabs, bivalve molluscs), it's not fair to say that impedance pneumography doesn't require surgical implantation. It may be true for soft-bodied animals like humans, where a skin-surface attached electrode could work, but you're not dealing with soft-bodied animals here. Similarly, on Line 316 in the discussion, the claim is again made that this method doesn't require surgical implantation, despite the use of a needle and drill to pierce the exoskeleton of the animal. This introduces the chance of creating soft tissue damage and stress to the animal, and this risk shouldn't be ignored.

Response: We acknowledge the Reviewer's concern and have provided additional information to clarify our position and to address this comment. We initially interpreted "surgical implantation" to indicate the removal or manipulation of the exoskeleton, but realize the initial text requires additional information (see lines 64 and line 454). Please also see the response to your Major Concern #1 for additional details.

3. Line 100 step 1.5: The text refers to a thermistor here, but the temperature measuring device should be called a thermocouple (as used later in the paper). A thermistor relies on a fundamentally different method for measuring temperature via changing resistance.

Response: We acknowledge the misstep in our initial submission and have replaced the term "thermistor" with "thermocouple probe" in the protocol (lines 114 and 208).

4. Line 184-186, step 3.5: What is the reasoning behind the choice of 1.5C/hr heating rate and 30C maximum temperature? Was this choice made based on prior published studies, or perhaps average short-term heating rates found in the environment of the lobster?

Response: We stated that the rate of warming for the temperature ramp should be adjusted based on the desired range of the ramp for a given experiment in a note on this step (original lines 188 – 190). We added additional information clarifying the initial text in the protocol (lines 228 – 238). Specifically, we chose this range of temperatures as it extends to upper thermal tolerance range of American lobsters but does not extend to the critical thermal maximum of performance, thus preventing mortality (lines 228 – 232). Our rate of warming of 1.5°C per 15 minutes based on our previous work and research by others (lines 232 – 234). See also the original text in the discussion (lines 472 – 476) where we suggest researchers conduct a literature search and/or a pilot study to determine the potential thermal limits of the target species prior to determining the range of the experimental ramp.

5. Line 245, step 4.5.1: Why is the interval set to 30 seconds, rather than 1 minute (if the goal is to calculate beats per minute)? Is this a limitation of the software? Tepolt et al. 2014 insisted on using 30 second windows as well when using this method with green crabs, but without ever explaining why this choice was required (or sensible).

Response: The LabChart software reports a continuous collection of voltage data in real time. In order to more accurately report these data in terms of continues records of beats per minute, we opted to calculate heart rate at 30-second intervals as opposed to every minute. It is possible to convert these voltage data to beats per minute at even shorter time intervals over the course of the experiment, but we selected the 30-second interval based on previous work. We added a note in this section to clarify our point (lines 336 – 339).

6. Lines 261-264 (Sections 5.2, 5.3): Compared to the prior sections, this portion of the analysis protocol is lacking in detail. Is it self-evident in Excel how to fit a piece-wise regression and determine the intersection point? I would argue that it is not.

Response: We have added additional text to clarify this portion of the protocol. See response to your Major Concern #3 above for specific details of the edits made to the manuscript.

7. Table of Materials: There are a number of misspellings in the Comments/Description fields of the table (resolution, coupling, compatible, variations, magnetic)

Response: We have thoroughly reviewed the Table of Materials and have corrected all grammatical and/or spelling errors.

Reviewer #3:

Manuscript Summary:

This manuscript proposes a relatively straightforward and less invasive method for capturing heart rate from adult invertebrates, or species that are not transparent/mostly transparent where heart rate can be captured visually. The protocol can be used to address physiological effects of aquatic invertebrates under a variety of conditions, thus has broad applications for studies on environmental changes such as temperature, pH, water chemistry.

Major Concerns:

1. Overall I recommend this article for publication. One general concern is the minimal description on controls. The protocol mentions requiring a baseline recording at extended length so that the animal can settle and the recordings would not be affected by increase in heartbeat due to handling stress. With these electrophysiological recordings it would be good to describe a 'negative control' and 'positive control' to make sure your system is functioning properly. In the absence of capturing an archetypal heartbeat rhythm, it may be hard for a novice to separate a 'real' recording from noise or to feel assured that their setup is complete and they perhaps just have to work a bit more to place the electrodes properly in the pericardium. It would be also informative to add some tips or advice for troubleshooting in the event the participant is struggling with set up.

Response: In the representative results section, we do discuss how the general shape of the real-time heart rate should change over the course of the temperature ramp (lines 398 – 407, Figure 1). However, we agree with the Reviewer that additional information would be helpful to the reader and have added text to address this comment in the discussion section. Specifically, we expanded on the original text to include suggestions of running a pilot study to determine and observe natural changes in basal heart rates of test subjects where temperature in the arena is maintained (lines 476 – 483).

Minor Concerns:

General:

1. Could this protocol be modified or additionally explained in general terms so that it could be performed using other software?

Response: This protocol uses a PowerLab data acquisition system, which is designed to specifically work with the LabChart software. There are likely other data logger systems

that can be used in place of the PowerLab system, but we are unfamiliar with other potential options. However, we suggest using the PowerLab and LabChart system as it can be used in a variety of teaching applications in the context of human health, terrestrial systems, and marine organisms.

2. Provide versions of software on which this protocol was built.

Response: In our Table of Materials, we provide information on the LabChart program and statistical software (SigmaPlot and Excel) implemented in this protocol. Based on the Major Concern #3 from Reviewer #2 (see above), we also included information on statistical packages available in the R statistical computing environment, SAS, and GraphPad to conduct the piecewise/stepwise nonlinear regression analyses (lines 393 – 395).

3. Recommendations for best practices on how long to take a recording or duration to use for BPM and ABT estimations - e.g. number of minutes, number of heartbeats? What is the expected heart rate for Homarus americanus at the baseline temperature (12-13 C)?

Response: In response to Minor Concern #4 from Reviewer #2, we included additional information on why we selected the range of temperatures tested and the rate of the warming in our protocol, and we make suggestions on how other researchers could identify the appropriate parameters for their study question/species (lines 228 – 238; lines 472 – 483). Please also see our response to Minor Concern #5 from Reviewer #2 for clarification on why we chose to convert voltage data points to heart rate (as beats per minute) at 30-second intervals (lines 336 – 339). Although not reported here, the average resting heart rate for lobsters at 12 °C used in our research is between 50 – 70 BPM.

4. The animal is secured/physically restrained during recording to avoid movement-related signal. Would stomach movements affect or interfere with the recording?

Response: This is an interesting comment, and one that we cannot confidently confirm based on our previous research efforts. In lobsters, we have only observed disruptions in the voltage signal with noticeable body movements.

5. Line 47 - would be helpful to be clear that this measurement is on acute timescale rather than over an extended period of time

Response: We added additional text to the summary (line 27) and abstract to clarify the protocol is focused on short-term responses (lines 37 and 46 – 47).

6. Line 85 - start of protocol, would be helpful to describe general set up first: the arena, water bath for cooling/heating, acclimation tank, and supplies needed

Response: Although we acknowledge the Reviewer's opinion, we maintain that the order of the protocol as stated is the most efficient. It is important to set up the water bath first to ensure no leaks in the connections between it and the coil, and it may take several minutes for a potentially lose connection to be detected. We believe it is important to then set up all electronic equipment to ensure adequate power supply prior to starting the experiment. The last step before obtaining an experimental animal should be setting up the acclimation bath in order to eliminate the need of an additional recirculating water bath to maintain its temperature. In our experience, individuals new to electrode

implantation may take up to 20 minutes to complete this portion of the protocol. We therefore suggest filling the acclimation chamber as close to starting the acclimation period as possible to reduce the potential risk of the chamber becoming too warm.

7. Line 86- could there be a better way to describe the 'heat exchanging coil'? Its written as 'clear, malleable tubing' which to me implies a clear plastic tube, but the term 'heat exchanging coil' would suggest it's made of a conducting material such as a metal wire

Response: While we acknowledge the Reviewer's point, we maintain that calling this component a "heat exchanging coil" is appropriate even though it is not constructed of metal. It provides a similar function as a coil constructed of metal, and we cannot think of a better phrase to capture its purpose.

8. Line 93- define RO water

Response: We define "RO" water as "reverse osmosis" water (line 107).

9. Line 100 - how do you calibrate the thermistor(s), and depending on the size of the bath and the water flow it may be useful to have more than one thermistor

Response: In this protocol, we use the T-type Pod in conjunction with the thermocouple (previously stated as the "thermistor" – see response to Reviewer #2, line 114). According to the manufacturer, the Pod auto-calibrates the thermocouple probe and there is no need to perform additional calibration steps. While it may be useful to have more than one probe to record the temperature in the experimental arena, we believe placing the probe near the test subject (line 208) accurately captures the temperature it is experiencing throughout the ramping experiment.

10. Line 188-190 - might consider moving this description of a test on your set up earlier in the protocol

Response: While we acknowledge the Reviewer's comment and agree that having this information prior to implementing the protocol is crucial, we struggle to find a better place to include this information. However, we added additional information to this note to clarify how we selected the temperature range of this protocol's ramp and rate of warming (an increase of 1.5 °C per every 15 min) in response to a comment from Reviewer #2 (lines 228 – 238). We hope this additional information assists researchers in pilot work prior to implementing this protocol on test subjects.