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TITLE:

Analyzing Long-term Electrocardiography Recordings to Detect Arrhythmias in Mice

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Arrhythmia, telemetry, long-term ECG, mouse, data analysis, Ponemah 6.42, Data Insights

SUMMARY:

Here we present a step-by-step protocol for a semiautomated approach to analyze murine long-term electrocardiography (ECG) data for basic ECG parameters and common arrhythmias. Data are obtained by implantable telemetry transmitters in living and awake mice and analyzed using Ponemah and its analysis modules.

ABSTRACT:

Arrhythmias are common, affecting millions of patients worldwide. Current treatment strategies are associated with significant side effects and remain ineffective in many patients. To improve patient care, novel and innovative therapeutic concepts causally targeting arrhythmia mechanisms are needed. To study the complex pathophysiology of arrhythmias, suitable animal models are necessary, and mice have been proven to be ideal model species to evaluate the genetic impact on arrhythmias, to investigate fundamental molecular and cellular mechanisms, and to identify potential therapeutic targets.

Implantable telemetry devices are among the most powerful tools available to study electrophysiology in mice, allowing continuous ECG recording over a period of several months in freely moving, awake mice. However, due to the huge number of data points (>1 million QRS complexes per day), analysis of telemetry data remains challenging. This article describes

a step-by-step approach to analyze ECGs and to detect arrhythmias in long-term telemetry recordings using the software, Ponemah, with its analysis modules, ECG Pro and Data Insights, developed by Data Sciences International (DSI). To analyze basic ECG parameters, such as heart rate, P wave duration, PR interval, QRS interval, or QT duration, an automated attribute analysis was performed using Ponemah to identify P, Q, and T waves within individually adjusted windows around detected R waves.

Results were then manually reviewed, allowing adjustment of individual annotations. The output from the attribute-based analysis and the pattern recognition analysis was then used by the Data Insights module to detect arrhythmias. This module allows an automatic screening for individually defined arrhythmias within the recording, followed by a manual review of suspected arrhythmia episodes. The article briefly discusses challenges in recording and detecting ECG signals, suggests strategies to improve data quality, and provides representative recordings of arrhythmias detected in mice using the approach described above.

INTRODUCTION:

Cardiac arrhythmias are common, affecting millions of patients worldwide[1]. Ageing populations show a growing incidence and thus a major public health burden resulting from cardiac arrhythmias and their morbidity and mortality[2]. Current treatment strategies are limited and often associated with significant side effects and remain ineffective in many patients[3-6]. Novel and innovative therapeutic strategies that causally target arrhythmia mechanisms are urgently needed. To study the complex pathophysiology of arrhythmias, suitable animal models are necessary; mice have been proven to be an ideal model species to evaluate the genetic impact on arrhythmias, to investigate fundamental molecular and cellular mechanisms, and to identify potential therapeutic targets[7-9]. Continuous ECG recording is a well-established concept in the clinical routine of arrhythmia detection[10].

Implantable telemetry devices are among the most powerful tools available to study electrophysiology in mice as they allow continuous recording of the ECG (a common approach is to implant the leads in a lead-II position) over a period of several months in freely moving, awake mice[11,12]. However, due to the huge number of data points (up to more than 1 million QRS complexes per day) and limited knowledge of murine standard values, the analysis of telemetry data remains challenging. Commonly available telemetry transmitters for mice last up to 3 months, leading to the recording of up to 100 million QRS complexes. This means that pragmatic analysis protocols are much needed to reduce the time spent with each individual dataset and will allow researchers to handle and interpret this huge amount of data. To obtain a clean ECG signal upon recording, transmitter implantation needs to be optimal—the lead positions should be as far apart as possible to allow higher signal amplitudes.

The interested reader may be referred to a protocol by McCauley et al.[12] for more information. Further, to minimize noise, cages and transmitters must be placed in a silent environment not prone to any disturbance, such as a ventilated cabinet with controlled environmental factors (temperature, light, and humidity). During the experimental period, lead positioning must be checked regularly to avoid loss of signal due to lead perforation or wound healing issues. Physiologically, there is a circadian alteration in ECG parameters in rodents as in humans, generating the need for a standardized approach to obtaining baseline

ECG parameters from a continuous recording. Rather than calculating mean values of ECG parameters over a long period, analysis of a resting ECG similar to that in humans should be performed to obtain basic parameters such as resting heart rate, P wave duration, PR interval, QRS duration, or QT/QTc interval. In humans, a resting ECG is recorded over 10 s, at a normal heart rate of 50–100/min. This ECG includes 8 to 17 QRS complexes. An analysis of 20 consecutive QRS complexes is recommended in the mouse as "resting ECG equivalent". Because of the above-mentioned circadian alteration, a simple approach is to analyze two resting ECGs per day, one at daytime and one at night time. Depending on the light on/off cycle in the animal facility, suitable times are selected (*e.g.*, 12 AM/PM), and basic parameters are obtained.

Next, a heart rate plot over time is used to detect relevant tachy- and bradycardia, with consecutive manual exploration of these episodes to get a first impression. This heart rate plot then leads to the important parameters of maximum and minimum heart rate over the recorded period as well as heart rate variability over time. After that, the dataset is analyzed for arrhythmias. This article describes a step-by-step approach to obtain these baseline ECG data from long-term telemetry recordings of awake mice over a recording period of up to three months. Further, it describes how to detect arrhythmias using the software, Ponemah version 6.42, with its analysis modules, ECG Pro and Data Insights, developed by Data Sciences International (DSI). This version is compatible with both Windows 7 (SP1, 64 bit) and Windows 10 (64 bit).

PROTOCOL:

1. Prearrangements

1.1. Start Ponemah 6.42 software, and confirm the username and serial number of the software license on the following screen by clicking on **Continue.**

1.2. Load the experiment containing the ECG of interest

1.2.1. If Ponemah is started for the first time, note that the **Ponemah Get Started** dialog opens, offering three options: 1) Create Experiment, 2) Load Experiment, 3) Import Experiment.

1.2.1.1. Select **Load Experiment** to open a file. Once the **Browse for Folder** dialog opens, select the experiment file with the extension (".PnmExp"), and load the file by clicking on **Open**.

1.2.1.2. To open a data set recorded in Ponemah 5.x or Dataquest ART, use the **Import Experiment** function.

NOTE: If the software is reopened, the last experiment is loaded automatically within the main window for further review. In the menu under **Experiment**, the same three options as in the **Ponemah Get Started** dialog are offered: 1) Create Experiment, 2) Open Experiment, 3) Import Experiment.

1.3. Click on **Actions/Start Review** from the toolbar, and go to the **Load Review Data** dialog box, which provides an overview of all the mice subjects and the respective signals recorded within the loaded experiment (**Figure 1A**).

1.3.1. Select the recording referring to the mouse that will be analyzed by clicking on the checkbox next to the mouse number in the left panel **Subjects**.

1.3.2. Select the checkbox next to **ECG** in the middle panel **Signal Types**.

1.3.3. Determine the duration of the signal that will be analyzed with the extreme right panel **Time Range**. Observe the following three options: **Entire Experiment**, which will load all the ECG data from the selected mouse; **Parser Segments**, which will only load data contained within Parser Segments added during a previous review session; **Time Range**, which allows the loading of a specific time range either by entering a specific start and end date or by entering a time duration.

1.3.4. To save the selection, use the **Loading Definitions** dialog in the upper left corner, which also allows loading of previously saved selections.

NOTE: The size of the selected data will be indicated by either a green or red bar based on the file size in the upper right corner under **Data Size**. Currently, the software allows loading up to 3 GB of data for Review; 3 GB data can be equivalent to a continuous 24 h recording of 3–4 days.

1.3.5. Click on **OK** to load the selected data set into **Review**.

1.4. After clicking **OK**, observe that the **Ponemah Review** window opens along with several separate windows. Although the **Events** and **Parameters** windows are opened and shown by default, manually select other necessary windows based on graphs of interest under the **Graphs/Graph Setup** toolbar.

NOTE: If **Events** and **Parameters** do not open by default, they can be activated by **Window/Parameters** and **Window/Events**.

1.4.1. Make note of the **Graph Setup** dialog, which allows setting up to 16 graphical windows providing both raw data (*e.g.*, ECG signals) and derived parameters (*e.g.*, XY loop) (**Figure 1B**).

1.4.2. Select the **Enable Page** checkbox to show the ECG tracing. In the list below, choose the line including the desired mouse (under **Subject**) and data type (under **Presentation**) by clicking on the respective checkbox on the left. Use the following settings: **Type**, Primary; **Label**, up to 11 characters displayed in the title bar of the window; **Time**, 0:00:00:01 indicating seconds as the unit used.

1.4.2.1. Enter the appropriate information in the **Label**, **Unit**, **Low**, and **High** text boxes.

NOTE: Enable two more pages, **Heart Rate Trend** and **Template**, which are helpful for the analysis of basic ECG parameters and for arrhythmia detection.

1.4.3. In the **Heart Rate Trend** page, activate another graph page and define as a trend to plot the heart rate (HR) over time. Use the following settings to plot the HR for the entire data that are loaded in **Review**: **Type**, Trend; **Input**, ECG; **Presentation**, HR; **Label**, HR Trend; **Unit**, bpm; **Low:** 50; **High:** 1000.

NOTE: Templates are ECG cycles with accurately placed marks that can be used as representative ECG cycles for pattern recognition analysis. They allow the selection of a small number of representative cycles and the matching of these templates to the entire ECG, thereby annotating all other cycles accordingly.

1.4.3.1. To use the template function, create a Template Library (a file in which the **Templates** are stored) for each subject. Do so by selecting the **Template Setup/Template library** option (**Figure 2A**).

1.4.3.2. Select **New…** from the dropdown menu under **Template library** to create a new **Template Library**.

NOTE: There are a few more options in the dropdown menu: **No Binding** disassociates any previous configured **Template Library** from the Subject. **Browse** associates an existing Template Library that was configured during a previous Review session.

1.4.3.3. Next, configure a **Template graph**, select **Setup/Experiment Setup/Graph Setup**, and select a Page to use as a Template graph page. Check the Enable Page check box, select Template for the Type, and ensure that Input reflects the users Subject/Channel selection. Type the appropriate information in the Label, Unit, Low, and High text boxes, and click on the OK button to display a graphic window for each graphic page that was configured under Graphic Setup as shown in Figure 2B.

NOTE: A graph setup page for Template settings will appear as shown in **Figure 2B**. According to the page selected in the **graph setup dialog**, the title bars of the windows are labeled from **pages 1–16**, based on the number of pages enabled (examples for pages 1, 2, 3 are shown in **Figure 3A**, **Figure 3B**, and **Figure 3C**, respectively).

1.5. Make some important adjustments in the ECG tracing window (**Figure 3A**).

1.5.1. Adjust the Y-axis representing the ECG amplitude by **double-clicking** within the ECG tracing window to select **Scaling**. Here, select **Autoscale** or adjust manually by using **High Axis Value** and **Low Axis Value**.

1.5.2. To adjust the X-axis representing the time, click on the respective toolbar icons: **Zoom In** to expand the time span (*i.e*., fewer QRS complexes are shown), **Zoom Out** to compress the time span (*i.e*., more QRS complexes are shown).

1.5.3. To show **DT** (**Delta Time**) and **RT** (**Real Time**) in the lower left corner, left-click on the ECG tracing with the cursor (a vertical black line) to position and see real-time information at the cursor location under **RT**.

1.5.4. As **DT** shows a time interval of the user's choice, right-click on the window, to both position the cursor and to select **Reset Delta Time** within the dialog that appears. Left-click to another position within the ECG tracing to measure the time interval between the selected time intervals shown as **Delta Time** (**DT**).

1.6. Ensure that each segment of the tracing (P, Q, R, T wave) is recognized and correctly annotated for the ECG analysis. To achieve this, define and analyze **Attributes** by a right click within the ECG window, and click on the **Analyze/Attributes** option.

NOTE: The **ECG Analysis Attributes dialog** opens as shown in **Figure 4A**. At the top of this dialog, several options (**QRS**, **PT**, **Advanced**, **Noise**, **Marks**, **Notes**, **Precision**) allow for the adjustment to various settings (explained below).

1.6.1. Click on the **QRS tab** to adjust R and QS identification.

1.6.1.1. **QRS Detection Threshold**: Apply the entered percentage to the largest derivative peak illustrated within the **waveform window**.

NOTE: Define an optimal value to eliminate undersensing (*i.e*., some R waves may be not detected) and oversensing of peaks (*i.e.*, other peaks, such as T waves, may be misinterpreted as R waves). The threshold (region highlighted in pink in **Figure 4A**) should intersect with the derivative of the ECG. Ideally, the attribute values, which help to identify QRS complexes and to distinguish between clear cycles and noise events, should be maintained at constant (or almost constant) levels between all recordings from one project to allow comparability over different animals per project. After establishing optimal values, maintain the attribute settings for the entire recording.

1.6.1.2. **Min R Deflection**: Ensure that the R amplitude change (based on minimum/maximum signal values and not isoelectric levels) exceeds this value before annotating it as an R wave.

NOTE: Min R Deflection should be ideally higher than noise and lower than the expected deflection of R wave. A low value may result in noise sensing and therefore oversensing, a high value may result in undersensing.

1.6.1.3. **Maximum Heart Rate**: Ensure that the value entered here is higher than the maximum heart rate expected.

NOTE: A low value may result in undersensing, a high value may result oversensing as noisy cycles have a greater chance of getting marked as R waves.

1.6.1.4. **Minimum Heart Rate**: Ensure that the value entered here is close to the lowest heart rate expected.

NOTE: Adjust heart rate limits for each recording individually depending on the signal amplitude and the degree of noise. Researchers must be aware that a wide range of heart

rates may result in failure to detect arrhythmias; a narrow range of heart rate, however, may result in extreme oversensing (*e.g*., thousands of episodes identified as “tachycardia”, which no longer allow a meaningful analysis).

1.6.1.5. Adjust **Peak Bias** to detect positive and negative R waves.

NOTE: A positive Peak Bias favors detection of positive R waves; a negative Peak Bias favors detection of negative R waves.

1.6.1.6. **Intra Cardiac**: Use this setting in cases where the P wave rapidly changes and when its derivative may exceed the derivative of the R wave resulting in false annotation of the P wave as an R wave.

1.6.1.7. **Baseline Recovery Threshold**: Set this value, which represents a “blanking period“ around the R wave, to prevent the software from searching for Q or S waves as small artefacts might otherwise result in false annotation of Q or S waves.

NOTE: For example, a value of 0 will result in searching for Q/S waves from the peak of the R wave, a value of 70 will result in searching for Q/S waves only after 70% recovery of the R wave height.

1.6.2. Click on the **PT tab** for settings for the detection of P and T waves.

1.6.2.1. **Max QT interval**: Adjust this interval to define the interval at which a detected T wave will be accepted.

1.6.2.2. **T window from S**: Adjust this setting to define the search interval for a T wave starting from S wave to the right.

1.6.2.3. **T window from R**: Adjust this setting to define the search interval for a T wave starting from R wave to the left.

1.6.2.4. **P Window from R**: Adjust this setting to define the search interval for a P wave starting from R wave to the left.

1.6.2.5. **T Direction**: Set **Both** as default to search for both positive and negative T waves as this setting defines if only positive, only negative, or both positive/negative T waves are searched.

1.6.2.6. **P Direction**: Set **Both** as default to search for both positive and negative P waves as this setting defines if only positive, only negative, or both positive/negative P waves are searched.

1.6.2.7. **P Placement**: Adjust this setting to shift the P mark towards (high value) or away (low value) from the peak of the P wave.

1.6.2.8. **T Placement**: Adjust this setting to shift the T mark towards (high value) or away (low value) from the peak of the P wave.

1.6.2.9. **Alternate End of T**: Adjust this setting to search for an alternative T wave beyond the first potential T wave. Enter a lower value to select the first T wave and a higher value to select the alternative T wave.

1.6.2.10. **Peak Sensitivity**: Adjust this parameter to eliminate small peaks when identifying P and T waves. Use this in conjunction with Peak Identification.

NOTE: A value of 0 defines maximum sensitivity; a value of 100 defines minimal sensitivity. The minimal Peak Sensitivity value depends on the quality of the signal. If the level of noise is low and/or the P and T waves are clearly distinguishable, these waves are well triggered, even when the Peak Sensitivity is 100. Generally, the Peak Sensitivity and Peak Identification do not need adjustment unless the signal is noisy, and the analysis algorithm is encountering issues with the detection of P and T waves. If so, the best results are achieved by adjusting the parameter in steps of 25.

1.6.2.11. **Peak Identification**: Use this parameter in conjunction with Peak Sensitivity to define the threshold for the identification of P and T waves. Lower up to 0 Peak Sensitivity if small P/T waves are not identified. If P/T waves are not identified even when Peak Sensitivity is set to 0, then lower Peak Identification, adjust in steps of 25.

1.6.2.12. **High ST Segment**: Use this attribute if the T wave is very close to the QRS complex resulting in a high ST segment.

NOTE: As mice lack a distinct ST segment, with a T wave occurring directly after the QRS complex, this setting should not be used in mice.

1.6.3. Click on the **Advanced Attributes tab** to set low/high pass filters, to define the J point to determine ST elevation/depression (not useful in mice), to set correction factors for QT measurement, and to define arrhythmic QRS complexes by the height of the R wave and the duration of the QRS complex.

NOTE: Use the default settings predefined within this tab. If the signal is affected, *e.g.*, by electromagnetic interference, adjust the filter settings here, which may help to improve signal quality. Definition of “arrhythmic QRS complexes” does not improve the accuracy to detect premature ventricular capture beats over the method suggested here (each PVC will also result in a pause and is therefore detected by this approach). The other settings are only relevant to very specific research questions and are therefore not described in detail here.

1.6.4. Use the **Noise Tab** to adjust attributes to identify noise.

1.6.4.1. Click on the checkbox **Enable Noise Detection** to identify noise, and set **Bad Data Marks**.

1.6.4.2. Click on the checkbox **Enable Dropout Detection** to set **Bad Data Marks** around data defined as dropout based on the maximum/minimum signal value. Adjust **Min Good Data Time**, which defines the time between two dropout segments also considered as dropout even if the signal is good.

1.6.4.3. Adjust the **Bad Data Threshold** to define the level of noise above which the ECG signal cannot be properly analyzed.

NOTE: This noisy segment of data will be included between **Bad Data Marks** and will not be analyzed. No ECG-derived parameters will be reported for these segments of "bad data".

1.6.4.4. Specify the **Min Noise Heart Rate** below which heart rates are considered as noise.

1.6.5. Use the **Marks tab** to turn on and off validation marks.

NOTE: It is recommended to always turn on **Mark Cycle Numbers**, which will add a continuous number to each R wave identified. This will help to navigate through the ECG recording.

1.6.6. Use the **Notes tab** to enter notes that will appear in the experimental log file.

1.6.7. Use the **Precision tab** to define the precision at which parameters are reported.

1.6.8. Set attributes and click on **Recalculate** to see the effects of the adjustments made in the **Waveform window** as a preview.

1.6.9. If (in an ideal situation) all ECG waves are correctly annotated, click on **OK** to confirm the attributes settings, which opens the **Effects and Scope of Changes** dialog. To analyze the ECG, click on the checkboxes **Reanalyze the channel** and **The entire channel** and confirm by clicking on **OK**.

1.7. Depending on the input settings in the **Attributes dialog,** make a note of the validation marks that are displayed in the ECG tracing. Go through the recording manually, and check if **validation marks** as well as **bad data marks** are set correctly. Use Data Insights for checking the R marks and ECG Pro for checking P and T marks.

1.7.1. If many marks are incorrect, modify **Attributes** and reanalyze the recording.

NOTE: Specific settings can be applied to specific segments of data when the ECG morphology is different from the rest of the recording. The Ponemah software manual provides standard values for ECG Analysis Attributes for different species under **Ponemah Software Manual/Analysis Modules/Electrocardiogram/Attributes Dialog**. To start with, these values can be used and then adjusted manually, until enough or (in an ideal situation) all ECG waves are marked.

1.7.2. Perform manual clean-up if only a few marks are incorrect. Move each validation mark (except for R wave marks) to the correct position by left-clicking, holding, and moving the

respective mark. Right-click within the ECG recording to add additional validation marks or mark arrhythmic R waves. Right-click on an incorrectly set mark to delete this mark.

1.8. Click on **Actions/Logging Rate** (or press **F8**) to set the **Logging Rate**, which defines how often derived data is logged to the **Derived Parameter List View** or plotted to graphs that use the derived parameters. For analysis of basic ECG Parameters and Arrythmia, use **Epoch 1** as the standard setting, which sets logging rate to each cycle.

NOTE: The Logging Rate can be augmented at any time during Acquisition or Review.

2. Analysis of basic ECG parameters

NOTE: In addition to validation/bad data marks, the software also automatically measures and calculates a large variety of derived parameters which are then reported in the **Derived Parameter List**.

2.1. Click on **Subject Setup/Channel Details** to select any of the derived parameters.

NOTE: In the **Derived Parameter List**, each parameter is linked to the number of the respective QRS complex.

2.1.1. Double-click on a row in the **Parameter table** to display the corresponding ECG cycles in the center of the primary ECG graphic window and easily find and visualize the morphology of the ECG cycles that correspond to the derived parameters in the selected raw data.

NOTE: It is possible to synchronize in both directions: from the table to the graphic and also from the graphic to the table. When the logging rate is 1 Epoch, the synchronization is done for each individual cycle. This is easy to check from the cycle number (NUM) in the Parameters table and in the graphic. Especially in long recordings, this synchronization feature between the tables and the graphics is very useful.

2.2. To account for the circadian alteration in ECG parameters, rather than calculating mean values of ECG parameters over a long period, analyze a resting ECG similar to that in humans to obtain basic ECG parameters such as resting heart rate, P wave duration, PR interval, QRS duration, or QT/QTc interval. Analyze 20 consecutive QRS complexes in the mouse as “resting ECG equivalent”.

NOTE: In humans, a resting ECG is recorded over 10 s at a normal heart rate of 50–100 /min. This ECG includes 8 to 17 QRS complexes.

2.2.1. As mice follow a circadian rhythm, analyze two resting ECGs per day, one at day time and one at night time to control for circadian effects. Select suitable times depending on the light on/off cycle in the animal facility, *e.g.*, 12 AM/PM.

2.2.2. Select a section of the ECG with good signal quality and stable heart rate in the HR Trend graph within a defined reasonable timeframe around this time point (*e.g.*, ±30 min).

2.2.3. Confirm the accuracy of the validation marks or adjust manually in 20 consecutive QRS complexes. Add missing validation marks.

2.2.4. For further calculations and visualizations, mark the lines containing the values of these 20 consecutive QRS complexes in the **Derived Parameter List**, and copy to a spreadsheet or statistics software.

3. Arrhythmia detection using pattern recognition (ECG PRO module)

NOTE: Ponemah's ECG PRO module uses selected QRS complexes as templates for further analysis. The ECG patterns of the templates are compared to all QRS complexes within the recording to calculate the percentage of similarity ("match") and to recognize arrhythmias (*e.g.*, atrial or ventricular premature capture beats). The number of QRS complexes needed to be marked depends on the variability of the QRS-amplitude within the recording. In certain cases, selecting and marking one QRS complex gives a similarity of 80 percent with the respective recording, marking the majority of QRS cycles. However, this is an ideal case and during analysis, the number of QRS complexes that need to be marked as templates is usually higher.

3.1. Mark QRS complexes as templates until at least a match of 80 percent or higher is achieved. Furthermore, use template matching to mark P, Q, S, and T waves if these are not or inadequately recognized after attributes settings (section 1.7).

NOTE: **R marks** must be identified for cycles prior to analyzing with ECG PRO. This requires that either the **R marks** are preserved from acquisition or the attribute-based analysis has to be executed prior to performing ECG PRO analysis. The other marks (**P, Q, S,** and **T**) need not be present for ECG PRO analysis.

3.2. After completing **Template** setup (as described in 1.4.4), select a desired ECG wave (with marked R). If necessary, adjust the **Validation Marks** to accurately reflect the appropriate positions of the **ECG Marks** of interest. Right-click on the cycle in the **Display Panel** in the **ECG Tracing** window, select **Add Cycle and Analyze [Single Template]**, and make a note of the cycle that appears in the **Template** window.

NOTE: An **Autoscale** may need to be performed for both the X- and Y-axes to see the full Cycle**. ECG Marks** may be moved within the **Template** graph page.

3.3. Right-click on the **Display Panel** of the **Template** window, and select **Add Cycle and Analyze (Single Template)** to launch the **Template Analysis** dialog shown in **Figure 4B**. Select the desired **Template Match Region** to which all other ECG cycles will be compared. If needed, change the advanced settings for the desired Match Region.

NOTE: Multiple **Match Regions** may be selected depending on the desired output from the analysis (the **Derived Parameters** of interest).

3.4. Select a **Data Range** on which to perform the analysis.

NOTE: The **Data Range** allows the reanalysis of the data visible in the graph, the data from the left edge of the visible region from the primary graph forward to the end of the loaded data set, the data within the **Parser Segments**, or the entire channel.

3.5. Select the type of **Cycles to Analyze.**

3.5.1. Select **All** to compare the Template Library to **All** cycles with a valid R mark.

3.5.2. Select **Unmatched** to skip previously matched cycles and compare the **Template Library** to only the unmatched cycles.

NOTE: This is useful when adding additional **Templates** to the **Template Library** for greater match coverage, as the processing time is shorter.

3.6. Select the desired **Match Method**. When selecting multiple **Match Regions** and **Whole Cycle**, use the **Template** that, on average, matches the cycle best to place the marks. When **Region** is used, for the best match for each **Match Region**, place the marks from different **Templates**.

3.7. Select **OK** to execute the analysis.

NOTE: Additional **Template Cycles** can be added to the **Template Library**, and the **Template analysis** can be re-run until the desired **Dialog Match %** is achieved. Doing this readjusts the waves in all the cycles that match the template.

3.8. Save **Template Libraries** through **Templates/Save** when the Review Session is closed.

3.9. To detect arrhythmia using template match, tag templates that have morphology different from that of the physiological waves after doing the template match (as described in section 3.1.) by right-clicking and selecting **Add Template Tag**, and select a type of cycle (*e.g.*, atrial ectopic, ventricular ectopic). Analyze these Tags using Data Insights.

4. Arrhythmia detection: a simplified manual approach using Data Insights

NOTE: For arrhythmia analysis, a correct annotation of P and R waves is necessary. However, even if clear P waves are visible within the ECG tracing, these P waves are sometimes not adequately identified even after adjusting the **Attribute** settings. As R waves are usually adequately recognized and annotated, a practical approach for further arrhythmia analysis using Data Insights is proposed below. For a general overview on arrhythmia detection using Data Insights and its predefined species-specific searches, the interested reader may be referred to Mehendale et al.[13].

4.1. Open **Data Insights** by clicking on **Experiment/Data Insights**.

4.1.1. Observe the **Search** panel at the top of the **Data Insights** dialog.

NOTE: On the left of the panel, it shows which search rule is applied to which channel/subject and the number of hits using this search rule. In the middle, all the search rules are listed, and on the right, the specific definition of a selected search rule is displayed.

4.1.2. Observe the **Results** panel displayed in the lower part of the **Search** panel.

NOTE: For each search hit, the corresponding ECG section is shown (top) along with a table indicating the time within the recording and the results of each search parameter (middle).

4.1.3. Observe the number of search hits displayed as a histogram in the bottom of the panel.

4.2. Given that the normal heart rate of a mouse is 500–724/min[14], define a search rule **bradycardia** to detect bradycardia.

4.2.1. Right-click within the search list, and select **Create New Search** to open the **Search Entry** dialog.

4.2.2. Right-click within the white box, and select **Add New Clause**.

4.2.3. Using the dropdown menus and text fields, define the search rule **Bradycardia-single** as **Value(HR_{cyc0}) < 500**. Click on **OK** to add this search rule to the list. Apply this search rule by clicking and dragging it to the channel of interest on the left.

NOTE: The search rule **Bradycardia-single** identifies every individual RR interval that is longer than 120 ms (= less than 500/min.).

4.2.4. As bradycardia requires more than one long RR interval, define an additional search rule **Bradycardia** as **Series(Bradycardia-single, 1)>=20**. Click on **OK** to add this search rule to the list. Apply this search rule by clicking and dragging it to the channel of interest on the left.

NOTE: In the **Results panel,** each section within the ECG recording consisting of at least 20 QRS complexes with a heart rate less than 500/min. is displayed.

4.2.5. To confirm bradycardia and to reject false results (*e.g.*, due to R wave undersensing), review each result manually. Left-click on the waveform, and press STRG+R to reject the selected result, which will disappear from the list of results.

NOTE: The rejected results are saved under **Result/Rejects**.

4.3. To detect tachycardia, define a search rule **tachycardia**.

4.3.1. Right-click within the search list, and select **Create New Search** to open the **Search Entry** dialog.

4.3.2. Right-click within the white box, and select **Add New Clause**.

4.3.3. Using the dropdown menus and text fields, define the search rule **Tachycardia-single** as **Value(HR_{cyc0})>724**. Click on **OK** to add this search rule to the list. Apply this search rule by clicking and dragging it to the channel of interest to the left.

NOTE: The search rule **Tachycardia-single** identifies every individual RR interval that is shorter than 82 ms (= more than 724 /min).

4.3.4. As tachycardia requires more than one short RR interval, define an additional search rule **Tachycardia** as **Series(Tachycardia-single, 1)>=20**. Click on **OK** to add this search rule to the list. Apply this search rule by clicking and dragging it to the channel of interest on the left.

NOTE: The **Results panel** displays each section within the ECG recording consisting of at least 20 QRS complexes with a heart rate of more than 724/min.

4.3.5. To confirm tachycardia and to reject false results (*e.g.*, due to R wave oversensing), review each result manually. Left-click on the waveform and use the shortcut STRG+R to reject the selected result, which will disappear from the list of results.

4.4. To detect sinoatrial and atrioventricular blocks, define a search rule **Pause**.

4.4.1. Right-click within the search list, and select **Create New Search** to open the **Search Entry** dialog.

4.4.2. Right-click within the white panel, and select **Add New Clause**.

4.4.3. Using the dropdown menus and text fields, define the search rule **Pause** as **Value(RR-I_{cyc0})>300**. Click on **OK** to add this search rule to the list. Apply this search rule by clicking and dragging it to the channel of interest to the left.

NOTE: The **Results panel** displays each section within the ECG recording with a pause of at least 300 ms.

4.4.4. To confirm a pause, to decide if the pause is a sinoatrial or atrioventricular block, and to reject false results (*e.g.*, due to R wave undersensing), review each result manually. Left-click on the waveform, and press STRG+R to reject the selected result, which will disappear from the list of results.

4.5. To detect Ectopic Rhythm, run the template match to these rhythms first (*e.g.*, ventricular ectopic), and then search for all the matched cycles to this template in **Data Insights**.

4.5.1. Right-click within the search list, and select **Create New Search** to open the **Search Entry** dialog.

4.5.2. Right-click within the white box, and select **Add New Clause**.

4.5.3. Click on **Value** using the dropdown menu, and select **Template**. On the right side, select the tag of the previously created template.

NOTE: The **Results panel** displays each section within the ECG recording with the cycle matching the Template.

4.5.4. To confirm the results and to reject false results (*e.g.*, due to R wave undersensing), review each result manually. Left-click on the waveform and press STRG+R to reject a particular cycle, which will disappear from the list of results.

NOTE: All search statements created can be imported and saved with suitable file names. All result tables can be saved and exported in spreadsheet/ASCII output format for further statistical analysis.

REPRESENTATIVE RESULTS:

Recording long-term ECGs results in huge data sets. The options for further analyses are manifold and depend on the individual research project. This protocol provides a description of some very basic readouts that can be used by most researchers, especially for screening experiments, *e.g.*, when characterizing a transgenic mouse line or when investigating the effects of a specific treatment in a disease model. A previous project involved the study of a novel drug candidate to determine whether it possessed cardiotoxic effects by analyzing ECG parameters over time. Telemetry transmitters were implanted 20 days before treatment, and ECG recordings were started 10 days before treatment to allow sufficient wound healing and acclimation of the mouse. Before treatment, the ECG was studied every three days; within the first week after treatment, the ECG was studied every day, after which the ECG was analyzed every seven days until the end of recording three weeks after treatment.

This approach allowed the detection of periods of reduced heart rate, increased atrioventricular (PR interval) and ventricular (QRS duration) conduction, as well as altered repolarization (QTc interval) in mice treated with the new drug as shown in **Figure 5**. This first step served as a “screening” that allowed the identification of time periods within the recording that potentially contained arrhythmias. A more detailed examination of the ECG revealed sinus pauses causing reduced heart rate two days after treatment and various degrees of atrioventricular (AV) blocks causing reduced heart rate six days after treatment. The latter finding was further supported by the prolonged PR intervals at this time point. To obtain these ECG parameters, 20 QRS complexes should be analyzed per time point and may therefore not be able to detect paroxysmal arrhythmia episodes at other time points.

To address this issue, it is advisable to specifically search for bradycardia and tachycardia episodes as well as for pauses using the ECG Pro module followed by manual review of detected episodes. This approach allows the detection of all relevant arrhythmias and the determination of the specific type of arrhythmia within the whole recording. For example, a tachycardia episode was detected in this study, which was identified as an atrial fibrillation.
As previously demonstrated, this approach further allows the determination of the time course of arrhythmia occurrence, *e.g.*, the time to first AV block after macrophage depletion[14]. Representative traces, as shown in **Figure 6**, are obtained as described above (**Figure 6A**: normal sinus rhythm; **Figure 6B**: sinus pause; **Figure 6C**: AV-block I°, **Figure 6D**:

AV-block II° type Mobitz 1; **Figure 6E**: AV block II° type Mobitz 2; **Figure 6F**: AV block III°; **Figure 6G**: atrial fibrillation).

FIGURE LEGENDS:

Figure 1: **Loading and reviewing data in Ponemah.** (**A**) **Load Review Dat** dialog providing an overview of all the mice and signals recorded within the loaded experiment. (**B**) **Graph Setup Dialog** to set graphical windows providing both raw data (*e.g.*, ECG signals) and derived parameters.

Figure 2: Template setup in Ponemah. (**A**) **Template Setup** window to configure and select a new or browse already configured **Template Library**. (**B**) Graph setup page for Template settings.

Figure 3: ECG tracings. (**A**) Screenshot of the windows containing the ECG trace; (**B**) heart rate plot; and (**C**) Template window.

Figure 4: Analysis of attributes of an ECG tracing. (**A**) An **ECG Analysis Attributes dialog**. At the top of this dialog, several tabs (**QRS**, **PT**, **Advanced**, **Noise**, **Marks**, **Notes**, **Precision**) allow the adjustment of various settings. The settings are presented in the middle part of the dialog. At the bottom of the dialog, the ECG tracing is shown in the **waveform window**. At the top of the **waveform window**, the ECG tracing is shown; at the bottom, the derivative of the ECG tracing, including a visualization of the setting thresholds above, is shown. In the example presented here, a QRS Detection Threshold of 40% is defined, which is indicated by the pink background at the bottom. (**B**) **Template Analysis Dialog**: Select the desired **Template Match Region** to which all other ECG cycles will be compared. In this example, the T Wave is selected as the **Match Region** for analysis with a **Minimum Match** of 85%. This means that if the T Region does not match with at least 85% confidence, the cycle will not be marked as a match.

Figure 5: Basic ECG Parameters over time in a drug intervention cohort. Blue panel: night time, yellow panel: daytime. From left to right: Heart Rate, PR interval, QRS duration, QTc interval.

Figure 6: Representative ECG traces. (**A**) Normal sinus rhythm, (**B**) sinus pause, (**C**) AV-block I°, (**D**) AV-block II° type Mobitz 1, (**E**) AV block II° type Mobitz 2, (**F**) AV block III°, (**G**) atrial fibrillation. Scale bars = 100 ms. Abbreviation: AV = atrioventricular.

Figure 7: Analysis flowchart. Abbreviation: HR = heart rate.

DISCUSSION:

The surface ECG is the primary diagnostic tool for patients suffering from heart rhythm disorders, providing insights into many electrophysiological phenomena. Nevertheless, sufficient analysis of cardiac surface ECG pathologies requires knowledge and definition of normal physiologic parameters. Many years of epidemiological research have led to broad consent on what is physiologic in humans and thus enabled physicians worldwide to clearly distinguish the pathologic. However, the analysis of surface ECG data is a major challenge in murine models; distinguishing between physiological and pathological ECG results can be

difficult due to incomplete understanding and definition of basic ECG parameters[15,16]. In 1968, Goldbarg et al. were the first to describe ECG in healthy mice[17]. Besides showing heart rates and basic ECG patterns, such as PR interval and QRS duration, they described major differences between anesthetized and awake animals and differences between various anesthetics and different murine breeds, which was later confirmed by other groups[16,17].

These early data emphasize why interpretation of murine ECG data is delicate and complicated. With growing interest in murine models for arrhythmia research in the past decades, more research has been focused on mouse electrophysiology and has generated evidence on the patterns of activation and repolarization in the mouse heart. The interested reader may be referred to a recent article by Boukens et al. for a detailed review of the murine ECG and its underlying currents[15]. Kaese et al. provided an overview on murine ECG standard values and major differences between human and murine ECG traces[18]. The first major difference is heart rate: healthy awake mice have a heart rate of 550–725 beats per minute, PR intervals of 30–56 ms, a QRS duration of 9–30 ms, and a repolarization phase that is very distinct from that observed in humans[14]. Further, the murine ECG regularly shows the occurrence of J-waves and a small and less distinctive T-wave, making analysis of the ST-segment and QT interval difficult[18,19]. Overall, murine models have become the most widely used model organism for cardiovascular research, including arrhythmias[8].

Taking into consideration the above described interspecies differences that very likely also influence arrhythmogenesis, these models can provide valuable insights. The analysis of basic ECG parameters, such as heart rate and duration of different intervals, can be reliably done using software such as Ponemah, LabChart, or ECGAuto among many others with their respective analysis algorithms. Examples for data display are shown in **Figure 5**. Arrhythmia detection, however, is far more delicate, and there are no widely established approaches for murine long-term ECG analysis for arrhythmias. Different approaches have been used to overcome the technical and methodological difficulties associated with arrhythmia detection of long-term ECG recordings in mice. These approaches range from only using short recordings for the manual analysis for arrhythmias[20] to simple considerations accepting inaccuracy as described by Thireau et al.[21]. These researchers performed heart rate variability analysis by simply excluding all sections of their recording with R-R intervals not contained in the range of the mean R-R interval ± 2 standard deviations to exclude all arrhythmias, ectopic beats, and artefacts without any manual review. This is the reason for this semimanual approach using Ponemah and its consecutive analysis modules, ECG Pro and Data Insights. This software solution can be used to analyze a vast range of physiologic signals, ranging from ECG in large mammals to blood pressure or temperature data in very small species.

The software comes with many resources on how to analyze different types of data. Nevertheless, although working quite well with ECG signals from larger animals, the low signal amplitude and therefore, high noise of signals derived from species, such as living and awake mice, can lead to a number of difficulties using a common approach to analysis. Noise will often mask P or T waves and thus disable the use of most of the predefined search rules within Data Insights. Care must be taken to define optimal values of the QRS detection threshold and to keep the attribute values used to identify QRS complexes and distinguish between clear cycles and noise events. A high percentage of the QRS detection threshold may result in undersensing (*i.e.*, some R waves may be not detected), whereas a low percentage may result

in oversensing (*i.e.*, other peaks, such as T waves, may be misinterpreted as R waves). Further, specific questions in arrhythmia research in mice are understandably not the main topic of the materials provided by DSI, and finding specific information can be difficult. Within this protocol, a simple and pragmatic approach is used to define different arrhythmias extrapolating established human definitions.

For example, in human long-term ECG data, a pause longer than 3 s is considered significant[22]. This results in a human heart rate of 20/min., representing a third of the minimum physiologic heart rate of 60/min. As described by Kaese et al.[18], the murine minimum physiologic heart rate equals 550/min., making 200/min. approximately a third of that rate. According to the human definition, pauses of more than 0.3 s can be assumed to be significant in mice. Further, it is a simple and pragmatic approach to describe differences in baseline parameters as relative changes to the respective control. This takes into consideration the differences between individual mouse lines and is an elegant way to identify the probable pathologic without relying on (often lacking) established normal values. This simple approach, summarized in **Figure 7**, is suitable for all groups studying cardiac arrhythmias in murine models using implantable telemetry devices. It leads to the evaluation of general ECG parameters as well as data on heart rate over time and the detection of a wide variety of arrhythmias. Therefore, this article attempts to provide a step-by-step approach for ECG and arrhythmia analysis and adds significantly to the guidance and manuals that have already been published.

ACKNOWLEDGMENTS:

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DISCLOSURES:

None

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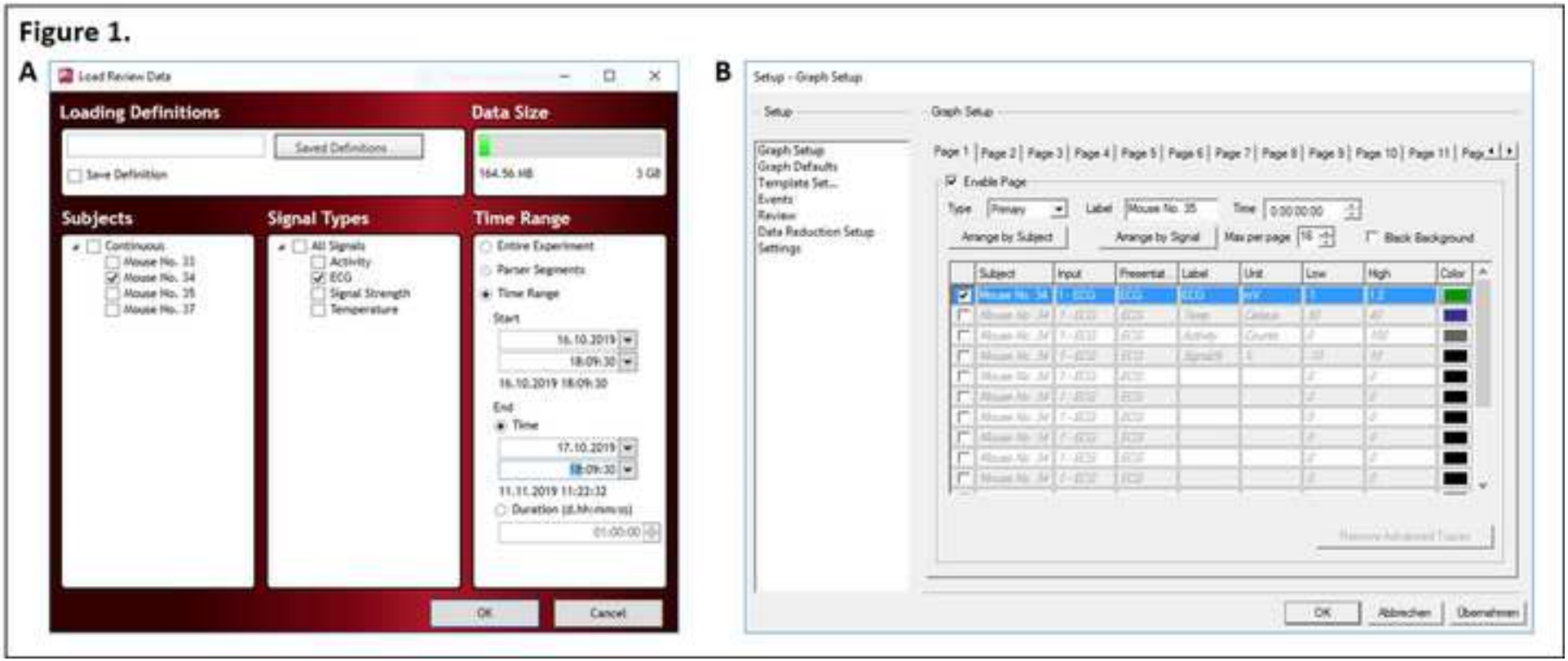

Figure 2

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Figure 2.

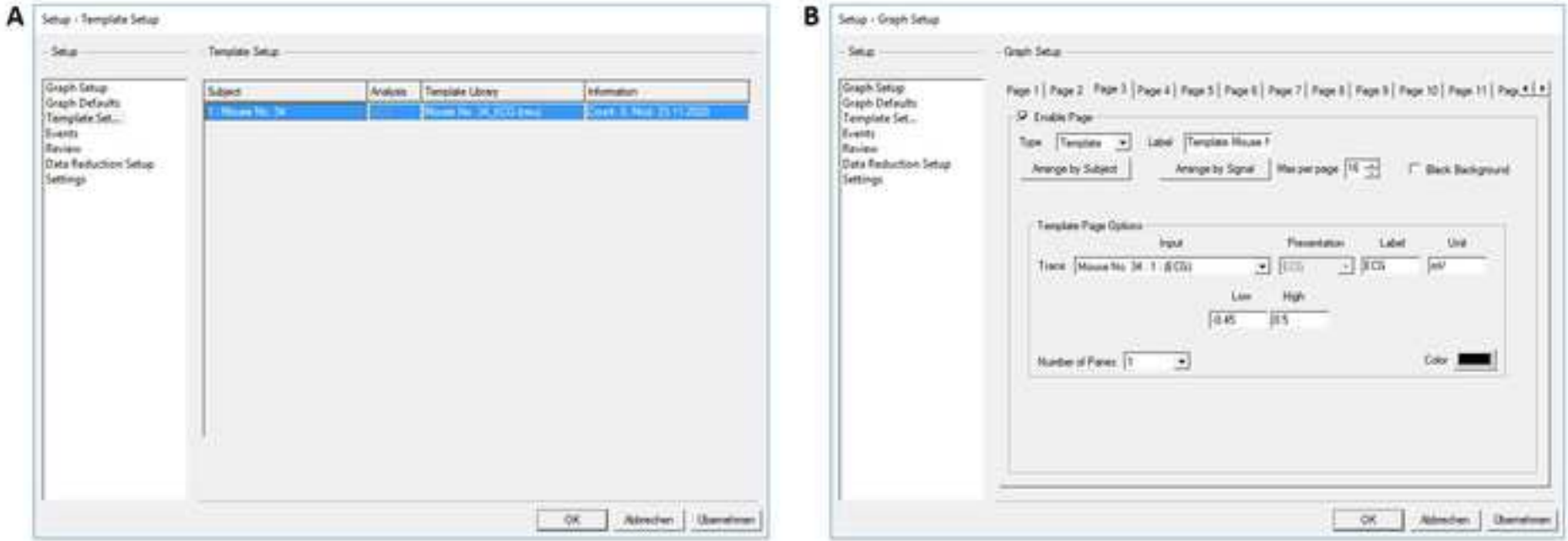

Figure 3

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Figure 3.

Figure 4.

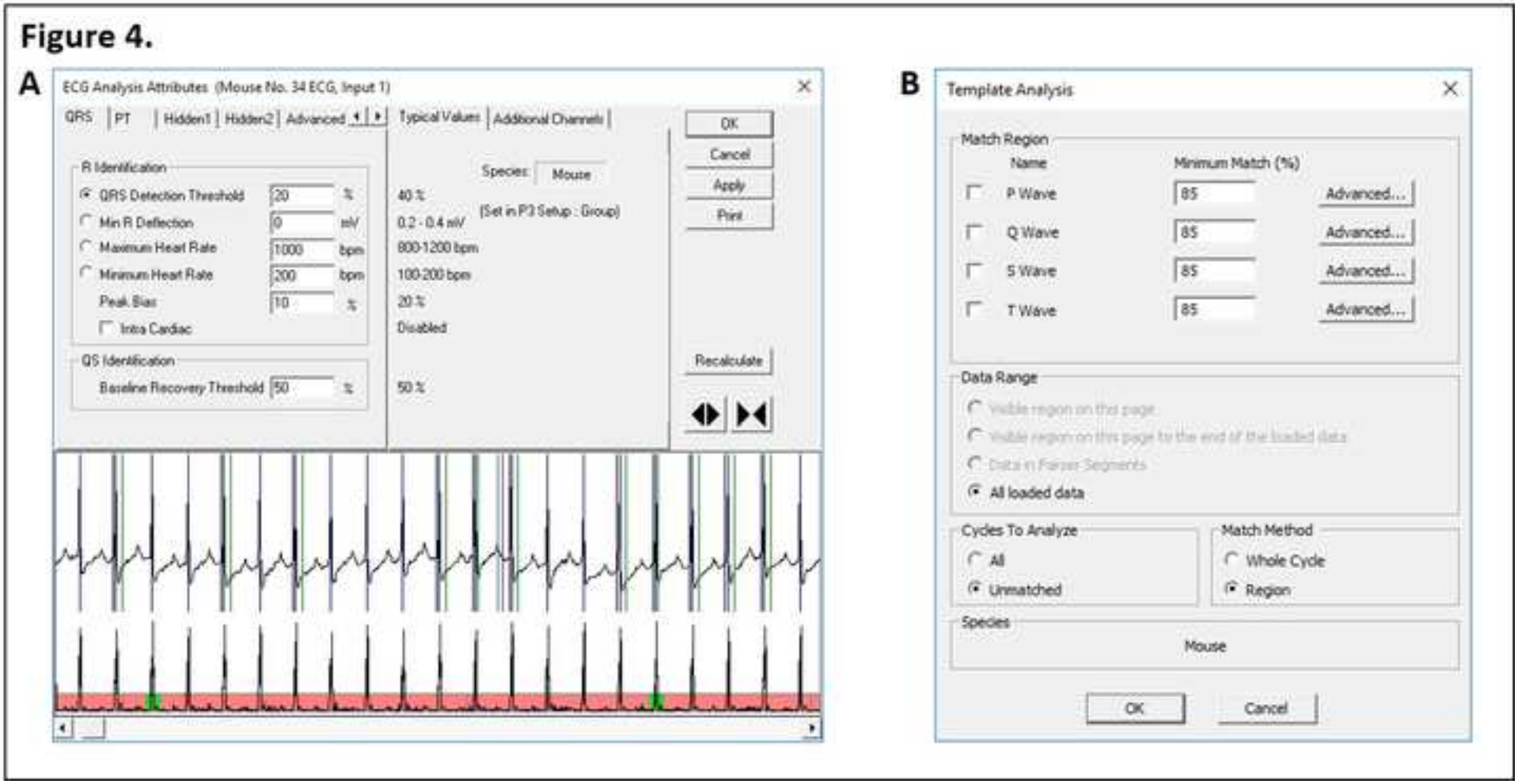


Figure 5

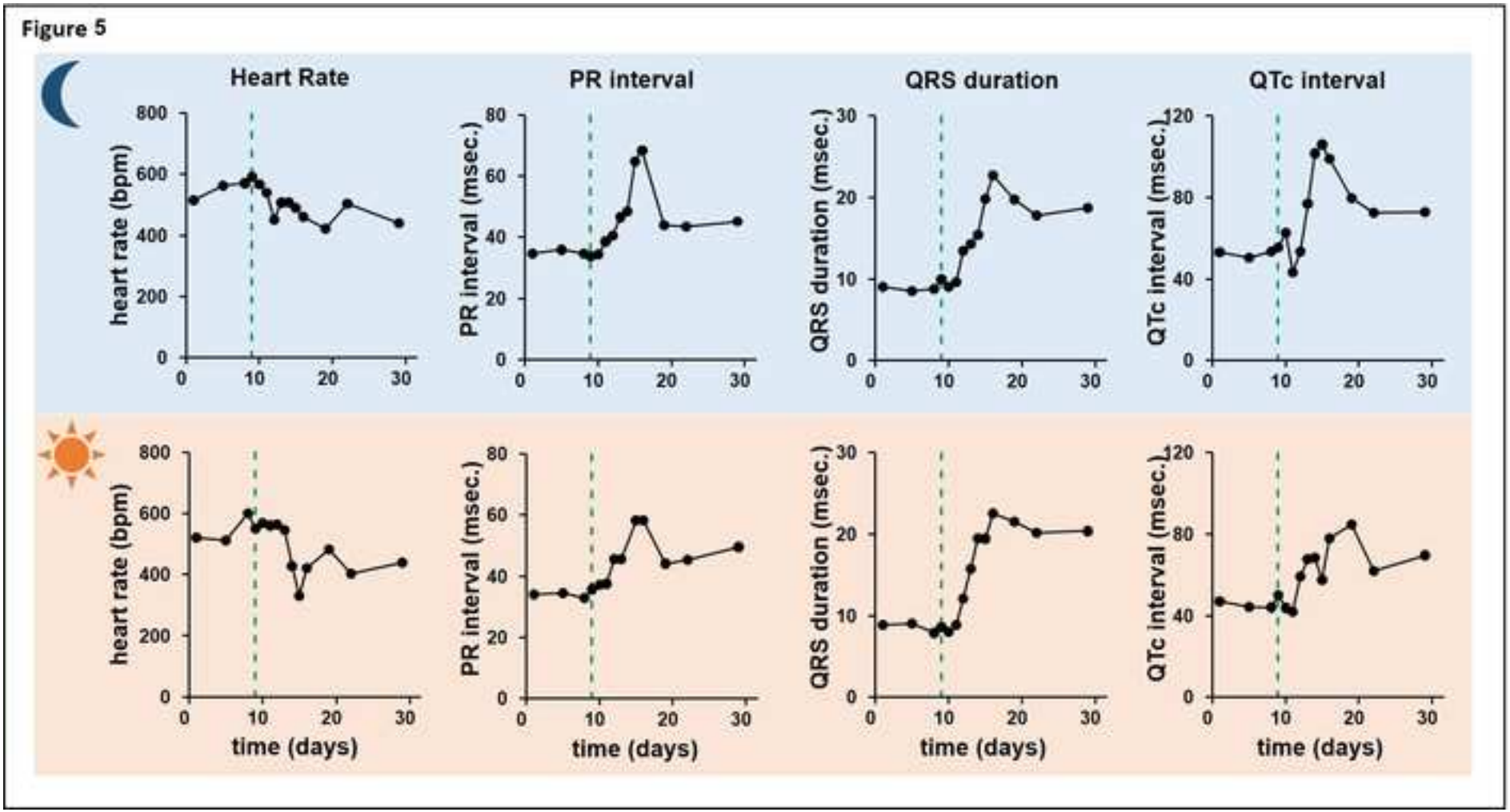


Figure 6

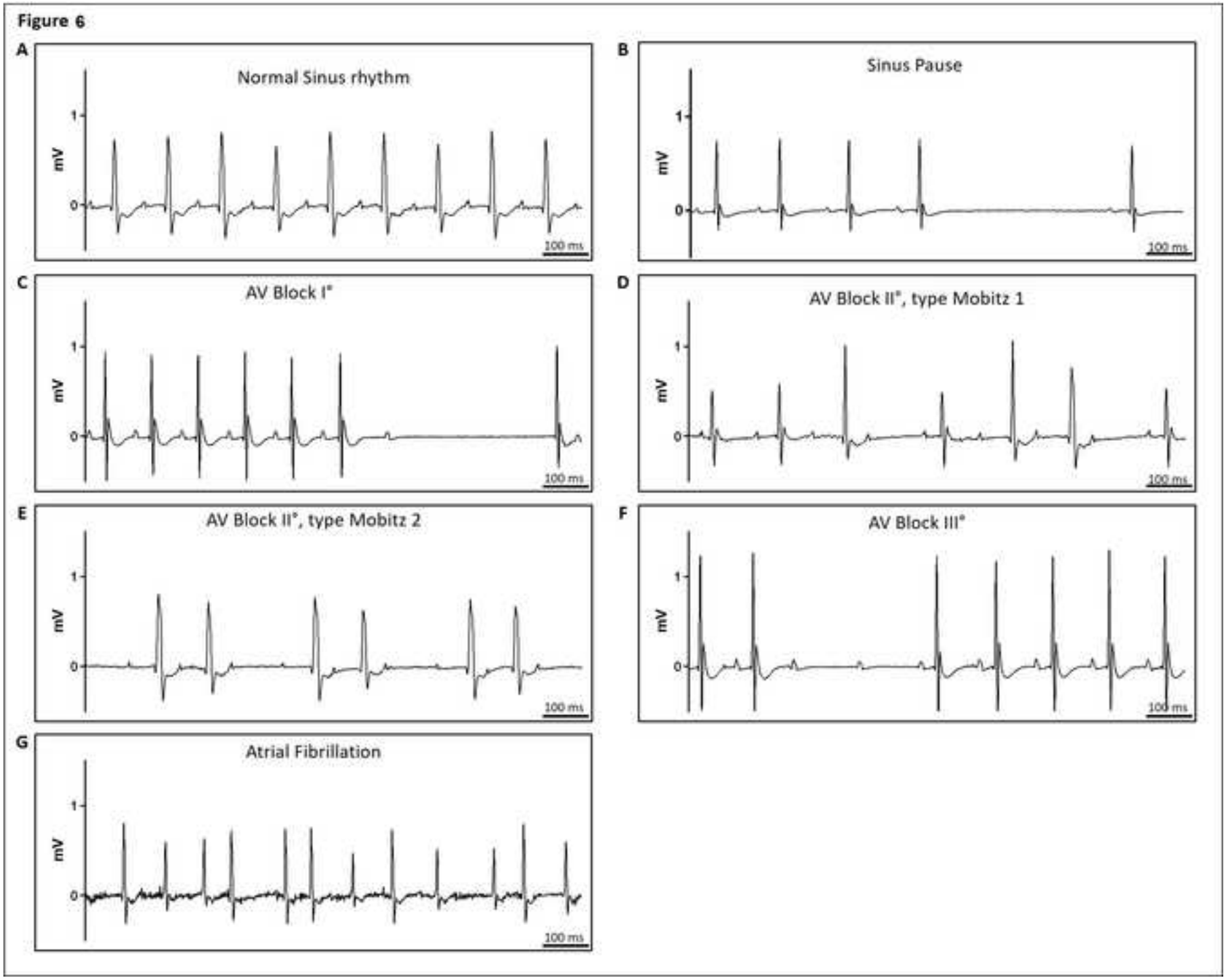
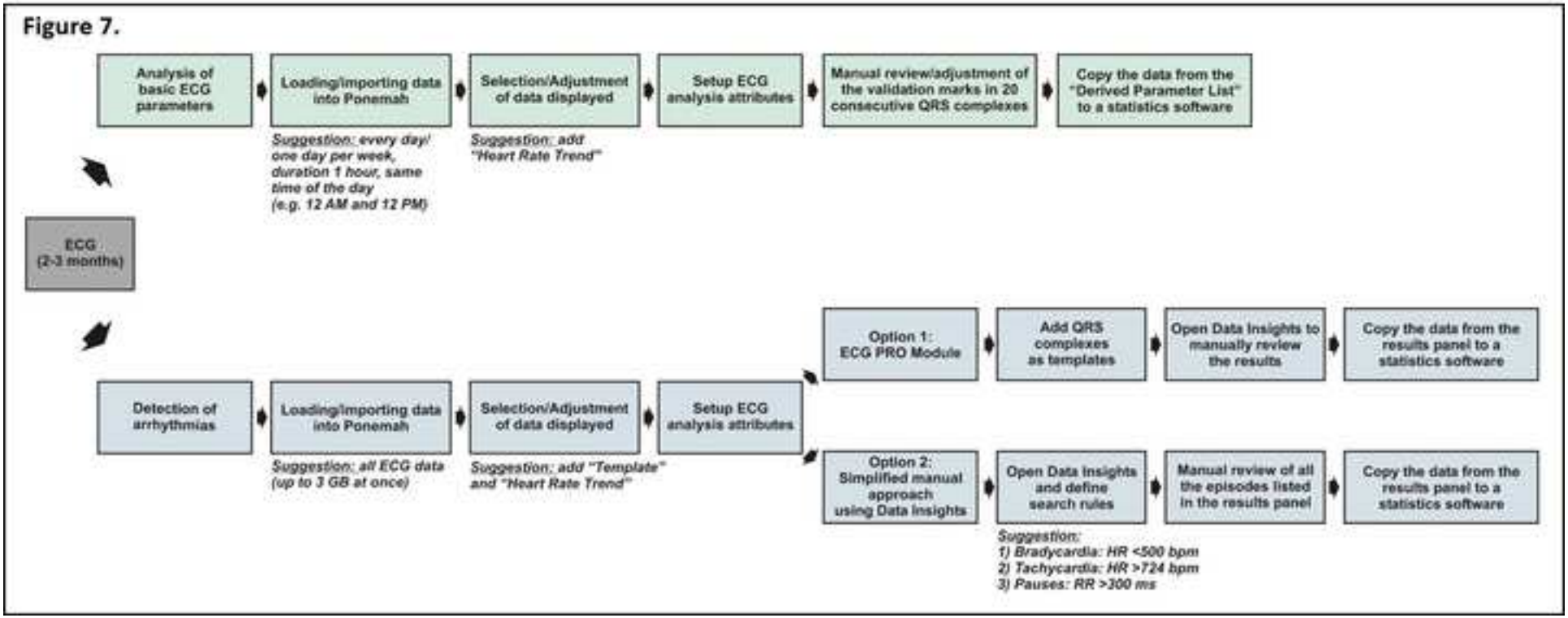


Figure 7



Name of Material/ Equipment	Company	Comments/Description
Ponemah Software	Data Science international	ECG Analysis Software

Dear editorial Board, dear Reviewers,

We want to sincerely thank you for taking the time to thoroughly proofread and comment on our work and we appreciate your comments and suggestions, which we feel will substantially improve our manuscript. Please find enclosed our point by point response.

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., etc.
Response: Thank you for this opportunity, thorough proofreading has been done by all authors independently and all grammar and spelling issues found have been corrected.

2. Please provide email addresses of all the authors in the manuscript.
Response: Email addresses of all authors have been added to the manuscript.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Response: The summary has entirely been rewritten according to your requirements. It now says:

"Here we present a step-by-step protocol for a semi-automated approach to analyse murine long-term ECG data for basic ECG parameters and common arrhythmias. Data is obtained by implantable telemetry transmitters in living and awake mice and analysed using Ponemah and its analysis modules ECG Pro and Data Insights from Data Science International (DSI)."

4. Please remove the < >, and italicization from the command labels. Instead, just boldface the command labels.
Response: All command labels were adjusted as suggested.

5. Include a single space between the quantity and its unit. E.g. "20 /min" instead of "20/min"
Response: single spaces were added between all quantities and units as required.

6. Please ensure that the protocol is written in an imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). 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". Also combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
Response: Thank you for this comment, we revised the entire protocol according to your suggestion, imperative tense was added wherever actions are described and new Notes were added when optional adjustments needed to be part of the protocol. Since these changes are multiple and throughout the entire text, please have a look into the track changes of the revised manuscript for a detailed view of what was changed.

7. Avoid the use of personal pronouns in the protocol. E.g. “we”, “our”, etc.
Response: Personal pronouns were eradicated from the protocol, however remained within other parts of the manuscript when necessary as for example within the summary where specific use of personal pronouns was asked by the editors (see third editorial comment)

8. Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all the figures. Data from both successful and sub-optimal experiments can be included.
Response: We have put a lot of thinking into this section of the manuscript. Our aim is to give researchers a set of methods to analyse their own murine long-term ECG recordings in the context of their own research. The final readout does always require this set of methods, but the readout itself primarily depends on the researcher’s specific topic and experimental design and this incorporates uncountable parameters. That is, why we decided to keep this section rather short. But we do understand the disadvantages of this approach and therefore added a specific example of how we analysed some of our own data and which parameters we chose as final readouts. Find new results within Figure 6. The correspondent section of the manuscript now reads:

“Recording long-term ECGs result in huge data sets. The options for further analyses are manifold and depend on the individual research project. Our protocol provides a description of some very basic readouts that can be used by the majority of researchers, especially for screening experiments, e.g. when characterizing a transgenic mouse line or when investigating the effects of a specific treatment in a disease model.
In one of our previous projects, we studied if a novel drug candidate has cardiotoxic effects by analyzing ECG parameters over time. We implanted telemetry transmitters 20 days before treatment and started ECG recordings 10 days before treatment to allow sufficient wound healing and acclimatisation of the mouse. Before treatment, we studied the ECG every three days, within the first week after treatment we studied the ECG every day and switched then to analyses every seven days until the end of recording three weeks after treatment. This approach allowed to detect periods of reduced heart rate, increased atrio-ventricular (PR interval) and ventricular (QRS duration) conduction, as well as altered repolarization (QTc interval) in mice treated with the new drug.
This first step served as a “screening” that allowed to identify time periods within the recording potentially containing arrhythmias. We had a more detailed look at the ECG and could observe sinus pauses causing reduced heart rate two days after treatment and various degrees of AV blocks causing reduced heart rate six days after treatment. The latter finding was further supported by the prolonged PR intervals at this time point.
To obtain these ECG parameters we suggest to analyse 20 QRS complexes per time point and may therefore not be able to detect paroxysmal arrhythmia episodes at other time points. To address this issue we suggest to specifically search for bradycardia and tachycardia episodes as well as for pauses using the ECG Pro module followed by manual review of detected episodes. This approach allows to find all relevant arrhythmias and to determine the specific type of arrhythmia within the whole recording. For example, we detected a tachycardia episode which turned out to be atrial fibrillation.
As we have previously demonstrated, this approach further allows to determine the time course of arrhythmia occurrence, e.g. the time to first AV block after macrophage depletion[1].”

9. Please include a comparison of this method with other methods in use in the discussion.
Response: Thank you for this comment. The need to develop this approach aroused from the lack of established ways to deal with the massive amount of data generated by continuous ECG recording over three months in combination with the low signal quality and amplitudes of murine ECGs in living animals as well as the differences between murine and human ECG parameters in the context of cardiac arrhythmias. There are multiple workarounds used by researchers like using shorter recording periods to subsequently manually analyse the data for arrhythmias or just use simple readouts as heart rate variability analyses which only depend on reliably annotating the R wave with some analysis software. A new paragraph and two references[2,3] have been added to the discussion to further embed our approach into the published methods:

"Analysis of basic ECG parameters such as heart rate, and duration of different intervals can up to date reliably be done using software such as Ponemah, LabChart or ECGAuto among many others with their respective analysis algorithms. Arrhythmia detection, however is way more delicate and there are no widely established approaches for murine long-term ECG analysis for arrhythmias. Different approaches have been used to overcome the technical and methodological difficulties coming along with arrhythmia detection of long-term ECG recordings in mice. These approaches range from only using short recordings to be able to manually analyse them for arrhythmias as for example done by Toib et al.[2] up to simple considerations accepting inaccuracy as performed by Thireau et al.[3] with their approach to heart rate variability analysis by simply excluding all sections of their recording with R-R intervals not contained in the range of the mean R-R interval ± 2 standard deviations to exclude all arrhythmias, ectopic beats and artefacts without any manual review. This is why we settled with our semi-manual approach using Ponemah and its consecutive analysis modules ECG Pro and Data Insights."

10. Please include a single line space between successive steps, and highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Response: single space lines have been added between individual steps and 3 essential pages of the protocol have been highlighted for the visualization.

11. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Full Source Title. Volume (Issue), FirstPage – LastPage (YEAR).]. Do not use "&" in the citations.
Response: All references have been adjusted to your standards. We would like to emphasize that we initially used the Endnote template provided by JOVE and therefore suggest adjusting the template if it no longer meets JOVE's citation requirements.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
Dr. Philipp Tomsits et al. describe the analysis way of long-term ECG recordings to detect arrhythmias in mice with the software Ponemah with its analysis modules ECG Pro and Data Insights developed by Data Sciences International (DSI). The analysis is that after detection of heart rate, P wave duration, PR interval, QRS interval, or QT duration, automatic screening for individually defined arrhythmias within the recording is analyzed with Data Insights module. The content of the manuscript as a method is very fancy.

Major Concerns:
I do not have any major comments.

Minor Concerns:
JoVE is a method journal. Therefore, I do not suggest the novelty of the manuscript. However, if the manuscript has a little expansion of both protocol and analysis of the ECG data that is not written an official manual in the Ponemah with modules ECG Pro and Data Insights, it may be perfect.
Response: We thank the reviewer for her/his kind words and her/his suggestion. The amount of information provided by DSI is massive, but it failed to solve the problems we were facing when analysing our recordings for arrhythmias. This is why we developed the simplified manual approach described in this protocol. This approach is not part of the manuals provided by DSI and has to our best knowledge not been published before. But we do agree with you that expanding this section by suggesting specific readout parameters and how to obtain them does add to this manuscript and have therefore rewritten part of the result section and added a new figure (Figure 6).

"Recording long-term ECGs result in huge data sets. The options for further analyses are manifold and depend on the individual research project. Our protocol provides a description of some very basic readouts that can be used by the majority of researchers, especially for screening experiments, e.g. when characterizing a transgenic mouse line or when investigating the effects of a specific treatment in a disease model.
In one of our previous projects, we studied if a novel drug candidate has cardiotoxic effects by analyzing ECG parameters over time. We implanted telemetry transmitters 20 days before treatment and started ECG recordings 10 days before treatment to allow sufficient wound healing and acclimatisation of the mouse. Before treatment, we studied the ECG every three days, within the first week after treatment we studied the ECG every day and switched then to analyses every seven days until the end of recording three weeks after treatment. This approach allowed to detect periods of reduced heart rate, increased atrio-ventricular (PR interval) and ventricular (QRS duration) conduction, as well as altered repolarization (QTc interval) in mice treated with the new drug.
This first step served as a "screening" that allowed to identify time periods within the recording potentially containing arrhythmias. We had a more detailed look at the ECG and could observe sinus pauses causing reduced heart rate two days after treatment and various degrees of AV blocks causing reduced heart rate six days after treatment. The latter finding was further supported by the prolonged PR intervals at this time point.
To obtain these ECG parameters we suggest to analyze 20 QRS complexes per time point and may therefore not be able to detect paroxysmal arrhythmia episodes at other time points. To address this issue we suggest to specifically search for bradycardia and tachycardia episodes as well as for pauses using the ECG Pro module followed by manual review of detected episodes.

This approach allows to find all relevant arrhythmias and to determine the specific type of arrhythmia within the whole recording. For example, we detected a tachycardia episode which turned out to be atrial fibrillation.
As we have previously demonstrated, this approach further allows to determine the time course of arrhythmia occurrence, e.g. the time to first AV block after macrophage depletion[1].”

Reviewer #2:
Manuscript Summary:
In this manuscript, the authors describe a protocol for the analysis of mouse ECG telemetry data using the Ponemah and Data Insights software in order to assess cardiac arrhythmias. The paper provides detailed instructions on the use of the software interface, including guidelines on setting the relevant parameters for data analysis. Overall, the text is well-written and clear. Nevertheless, the scope of the manuscript seems quite limited, and several points outlined below require further elaboration and clarification.

Major Concerns:
A major shortcoming of the protocol is the strict limitation to the Ponemah and Data Insights software, rendering it useful only for researchers using the Data Sciences International (DSI) telemetry setup. Even though this is likely the most widely used hardware/software combination, other solutions are available which might become more widespread in the future, potentially reducing the relevance of this protocol. In addition, any major update of the DSI software might also alter the user interface and severely diminish the usefulness of this protocol. An alternative would have been to develop a set of custom algorithms to analyze the raw data extracted from the ECG recordings, which will also provide more insight into the actual data filtering and analysis operations performed during the procedure, which is now mostly operating on a "black box" principle.
Response: Thank you for this comment. We do agree, it is a shortcoming to provide a protocol only for a specific software platform. Therefore, we present more a concept of telemetry data analysis by a practical approach combining semi-automated “screening” followed by manual review. Since the DSI telemetry setup is by far the most widely used setup, we demonstrated our concept by using DSI’s Ponemah software. However, our practical method can also be applied to other software such as AD Instrument’s LabChart requiring just some slight modifications. Therefore, the scope and focus of this article is more to provide a concept for arrhythmia detection in murine long-term ECG recordings rather than providing a step by step software manual for a specific Ponemah version. In order to demonstrate our suggested conceptual approach, a step by step explanation nevertheless seemed like the most feasible way and was therefore used within this protocol. We hope that our article motivates other researchers to adapt the concept and apply it to other software solutions.

I was also missing a section in the protocol providing some advice on the ideal conditions for data collection. As appropriately highlighted in the discussion, the mouse ECG can be influenced by many factors including genetics as well as environmental factors. It would be interesting to at least provide some recommendations for researchers on how to design ideal experimental conditions in order to maximize data quality.
Response: We do agree with the reviewer, a section on ideal experimental conditions significantly adds to the manuscript and was therefore enclosed to the discussion:

"To obtain a clean ECG signal upon recording, transmitter implantation needs to be optimal, meaning the lead positions should be as far apart as possible to allow higher signal amplitudes. The interested reader may be referred to a protocol by McCauley et al[4] for more information. Further, to minimize noise, cages and transmitters have to be placed in a silent environment not prone to any disturbance, such as a ventilated cabinet with controlled environmental factors as temperature, light and humidity. During the experimental period, lead positioning has to be checked regularly to avoid loss of signal due to lead perforation or wound healing issues."

Important technical considerations regarding sampling frequency and data filtering are also missing from the manuscript. While possible issues with noisy data are briefly mentioned, the authors fail to mention how to treat recordings affected by noise due to movement of the mouse.
Response: Thank you for this comment. Noise due to movement cannot be fully prevented in living mice. For the analysis of the baseline ECG parameters, we thus suggest to pick ECG tracings at a certain time of the day, e.g. 12 am to 1 pm and analyse a section with good signal quality within that time period. Sampling frequency – as far as we know – cannot be changed for ECG recordings upon acquisition and the default sampling frequency leads to a high enough time resolution to sufficiently analyse mouse ECG data, even episodes with fast and short arrhythmias.

It is also not clear what the final output of the proposed analysis method will be. Can any quantitative outputs regarding the amount and type of arrhythmias be extracted from the data in order to compare the characteristics of different experimental groups?
Response: We thank the reviewer for this comment. The output of our analysis method is detection of arrhythmia within the respective recording, the way to present these findings depends on the specific aim of the researcher and can range from simple outcomes as e.g. atrial fibrillation is present yes/no, to arrhythmia burden, number of episodes, mean duration of episodes and so on. Because the spectrum of final readouts is so divers and depends on the individual researcher's experimental design, we did not specify this section further previously. But we do see, that a representative example of a long term recording analysed as described within this manuscript might enlarge comprehensibility and therefore added an example of possible readouts using this approach to the results section:

"Recording long-term ECGs result in huge data sets. The options for further analyses are manifold and depend on the individual research project. Our protocol provides a description of some very basic readouts that can be used by the majority of researchers, especially for screening experiments, e.g. when characterizing a transgenic mouse line or when investigating the effects of a specific treatment in a disease model.
In one of our previous projects, we studied if a novel drug candidate has cardiotoxic effects by analyzing ECG parameters over time. We implanted telemetry transmitters 20 days before treatment and started ECG recordings 10 days before treatment to allow sufficient wound healing and acclimatisation of the mouse. Before treatment, we studied the ECG every three days, within the first week after treatment we studied the ECG every day and switched then to analyses every seven days until the end of recording three weeks after treatment. This approach allowed to detect periods of reduced heart rate, increased atrio-ventricular (PR

interval) and ventricular (QRS duration) conduction, as well as altered repolarization (QTc interval) in mice treated with the new drug.
This first step served as a "screening" that allowed to identify time periods within the recording potentially containing arrhythmias. We had a more detailed look at the ECG and could observe sinus pauses causing reduced heart rate two days after treatment and various degrees of AV blocks causing reduced heart rate six days after treatment. The latter finding was further supported by the prolonged PR intervals at this time point.
To obtain these ECG parameters we suggest to analyse 20 QRS complexes per time point and may therefore not be able to detect paroxysmal arrhythmia episodes at other time points. To address this issue we suggest to specifically search for bradycardia and tachycardia episodes as well as for pauses using the ECG Pro module followed by manual review of detected episodes. This approach allows to find all relevant arrhythmias and to determine the specific type of arrhythmia within the whole recording. For example, we detected a tachycardia episode which turned out to be atrial fibrillation.
As we have previously demonstrated, this approach further allows to determine the time course of arrhythmia occurrence, e.g. the time to first AV block after macrophage depletion[1]."

Considering the requirement for manual data review, what is the length of recordings that can be realistically analyzed using the described methods: is it suitable only for short-term (hours) measurements or are long-term follow-ups (days, weeks) also feasible? It would be important to comment on this aspect, since one of the major strengths of the telemetry approach is that mice can be followed up for long time periods.
Response: We thank the reviewer for this comment, we failed to emphasize this important point. The major strength of the telemetry approach indeed is the follow up over longer periods of time. We do use this approach in our experiments to analyse data obtained from telemetry recordings lasting an entire battery life of the transmitter. This equals to a time period of approximately three months. We have added clarification to the introduction:

"This article describes a step-by-step approach to obtain these baseline ECG data from long-term telemetry recordings obtained from awake mice over a recording period of up to three months."

Finally, it is also regrettable that no extra attention is given to other analysis modalities which will be very relevant to the ECG data, such as e.g. heart rate variability analysis.
Response: We thank the reviewer for pointing towards this issue. We agree with the reviewer that other measurements such as HRV are important for many researchers, but there are many other important readouts as well and it is impossible to address all of them in one manuscript. Thus, we decided to focus on the very basic long-term ECG analysis including assessment of basic ECG parameters and most common arrhythmia types since probably most researchers will need these analyses. With our practical approach, researchers will become familiar with such ECG data and will be able to start an initial analysis of their own data. Based on that, researchers will quickly become experienced enough to adjust our protocol to their individual needs and to add new analyses.

Minor Concerns:

- In Point 1.3.5 the authors mention that the maximum data size that can be analyzed by the Ponemah software is 3 GB. To what length in time of recording (at a suitable sampling frequency rate) does this correspond?
Response: Thank you for the question**.** The maximum amount of data which can be reviewed at one go corresponds to 3 GB. The 3 GB data usually corresponds to a continuous 24 hour recording of 3-4 days. However, the number of days of data equivalent to 3 GB can vary, for example if animals are removed from the transmitter platform for further experiments.

- Point 1.6.1.1: will these values be the same for the entire recording of the same mouse, or within one experiment where multiple mice are compared? Or does it need to be adjusted to the specific conditions within each recorded fragment?
Response: Thank you for this comment, we have added clarification to the protocol in section 1.6.1.1.:

*"**NOTE:** The attribute values help to identify QRS complexes and to distinguish between clear cycles and noise events. Ideally, these values are kept the same between all recordings from one project to allow comparability over different animals per project. However, in reality, this is not always possible and slight chances in attribute values have to be done. If necessary, we suggest keeping these changes as minimal as possible. After establishing optimal values, the attribute settings have to be maintained for the entire recording."*

- Points 1.6.1.3-4: will these settings not lead to unwanted filtering out of arrhythmic events?
Response: We thank the reviewer for this comment. Indeed, there is some risk for undersensing if the lower and/or upper heart rate limit is set too narrow. On the other hand, oversensing is also a relevant challenge when analysing these data which requires some balance. If a recording is somewhat noisy, a broader range of heart rate will result in extreme oversensing which will make any analysis impossible (in such cases we discovered several thousand episodes per 24 hours which were detected as "arrhythmias", i.e. a manual review of all these episodes is no longer feasible). Thus, we added a note to the paragraph emphasizing this issue:
*"**NOTE:** Heart rate limits have to be adjusted for each recording individually depending on the signal amplitude and the degree of noise. Researchers have to be aware that a wide range of heart rate may result in failure to detect arrhythmias, a narrow range of heart rate, however, may result in extreme oversensing (e.g. thousands of episodes identified as "tachycardia" which do no longer allow a meaningful analysis)."*

- Point 3.1: how many QRS complexes need to be marked as templates for reliable data analysis?
Response: There is no fixed number of QRS marks. It depends on percentage similarity achieved by marking a QRS as a template. Explanation has been added in the manuscript section 3:

"The number of QRS complexes needed to be marked depends uponon the variability of the QRS-amplitudes of the QRS complex within the recordingin ECG signal recorded. In certain cases , selecting and marking one QRS complex may give a similarity of 80 percent similarity with the rest of ECGrespective recording, marking the majority of QRS cycles. However, this is an ideal case and often during analysis, the number of QRS complexes that need to be marked

as templates depends on percentage similarity achievedis usually higher. In any case, this has to be done until at least a match of 80 percent or higher is achieved."

- Point 4.3: "724" seems like a very arbitrary upper HR limit.
Response: We do agree with the reviewer, this arbitrary limit is a direct citation of a work by Boukens et al.[5] and reflects the lack of a general "norm" for mouse ECG parameters.

- Point 4.5.4: 500 ms seems like a high threshold to define a pause in the ECG which needs to be examined for a potential arrhythmic event. Blocked P-waves or episodes of sinus arrest can lead to RR-intervals shorter than 500 ms.
Response: Thank you for this comment, we do agree 500 ms is too long and this was a mistake, we use 300 ms as cut off for relevant pauses as elaborated in the discussion, the number was corrected.

Reviewer #3:
Manuscript Summary:
In this manuscript the authors address the issues of analyzing long-term telemetry data using the commercially available Ponemah software (DSI) which allows for semi-automated analysis. They provide a step-by-step guide as to how to use this software to analyse ECG recordings taken from conscious mice.

Major Concerns:
I do not have any major concerns with the manuscript but I do have a few recommendations/queries as listed below:

Minor Concerns:
1. For those who have not used or don't have Ponemah yet, it may be useful to list any technical requirements, for example, is it Mac compatible, system requirements (versions of windows etc).
Response: We do agree and have added this information to the introduction.

"This version is compatible with both Windows 7 (SP1, 64 bit), and Windows 10 (64 bit)."

2. In addition, most established researchers carrying out telemetry measurements are probably still using Dataquest ART to acquire data without the resources to upgrade to the latest acquisition system. From my experience with data acquired with Dataquest and analysed with an older version of Ponemah (v4 I think), the data files needed to be converted to Ponemah files before one could analyse them. Is this still the case with the newer version of Ponemah or are Dataquest files now automatically loaded?
Response: Thank you for this comment. Yes, older data can – as we briefly mention in section 1.2.1.2 – be converted and opened within this Ponemah version, inconveniently enough this cannot be done without conversion.

3. Is there a limit to the number of figures allowed in JOVE? The reason I ask is for those unfamiliar with the interface it would be useful to have images of all the tabs in the attributes

section. You have described these tabs and the parameters within but a visual aid would be useful.
Response: Thank you for this comment, as we do not think that there is a limit to the figures, we feel that the video will provide guidance for those not familiar with the software's interface and think that more figures will not significantly add to the manuscript but rather diminish readability.

4. Section 1.2 is a little confusing in that 1.2.1 and 1.2.2 are the same except for the opening paragraph in each (whether Ponemah is opened for the 1st time or not)- could this be reworded or written so it isn't repetitive.
Response: Thank you for this comment, we do agree and have rewritten the paragraph:

1.1. Load the experiment containing the ECG of interest
*1.1.1. If Ponemah is started for the first time, the **Ponemah Get Started** dialog openes offering three options: 1) Create Experiment, 2) Load Experiment, 3) Import Experiment*
*1.1.1.1. Select **Load Experiment** to open a file. The **Browse for Folder** dialog opens and the experiment file with the extension (".PnmExp") can be selected and loaded by clicking **Open**.*
*1.1.1.2. To open data set recorded in Ponemah 5.x or Dataquest ART, use the **Import Experiment** function.*

***NOTE:** If the software is reopened, the last experiment is loaded automatically within the main window for further review. In the menu under **Experiment** the same three options as in the **Ponemah Get Started** dialog are offered: 1) Create Experiment, 2) Open Experiment, 3) Import Experiment*

5. 1.3.3.2: Can you add parser segments at this stage or when can you do this?
Response: Parser segments can be added anytime to a recording.

6. 1.3.5: How long a recording constitutes 3GB? Also how long does the analysis take per mouse recording?
Response: Thank you for the question**.** The maximum amount of data which can be reviewed at one go corresponds to 3 GB. The 3 GB data usually corresponds to a continuous 24 hour recording of 3-4 days. However, the number of days of data equivalent to 3 GB can vary, for example if animals are removed from the transmitter platform for further experiments. The amount of time taken by analysis depends the individual aim as well as on the respective researcher's experience and can therefore not be estimated reliably. The provided practical approach however enables the researcher to be in control of the number of time points in need of manual review – which mainly determines the amount of time taken – by varying the respective search parameters as described in this protocol. An individual approach in the context of the specific research question has to be established, leading to a positive balance between labour and in depth analysis. Another important determinant that has to be considered is the amount of time needed for the software to safe changes made to the data file. This can take up to multiple hours, depending on the hardware used. Analysis of the baseline ECG parameters is quick and will only take approximately half an hour. Arrhythmia detection, however, is more delicate. Just to give an impression, we usually take around 2 to

3 hours, depending on the burden of arrhythmia within the dataset to analyse a 24 hour recording + plus the time for saving changes.

7. 1.6.3: I think this section is important and should be possibly more comprehensive.
Response: We thank the reviewer for mentioning this aspect. The term "Advanced Attributes tab" is a bit misleading since this is rather a "Additional Attributes tab". Here, you can adjust the J point important to determine ST segments, which, however, is not relevant for arrhythmia research. You can also set correction factors for QT measurement which we did not want to specifically mention since there is an ongoing debate among EP researchers if QT measurement in mice is useful or not. Also, you can define arrhythmic QRS complexes by the height of the R wave. This is highly dependent on the signal amplitude and – to our experience – this does not reliably allow to detect premature complexes. Finally, you can adjust the filter settings here. In our experience, we did not have to change any of these settings so far and thus we added a note:

***"NOTE:** We suggest to use the default settings predefined within this tab. If the signal is affected e.g. by electromagnetic interference, the filter settings can be adjusted here and may help to improve signal quality. Definition of "arrhythmic QRS complexes" does not improve the accuracy to detect premature ventricular capture beats over the method we suggest here (each PVC will also result in a pause and is therefore detected by our approach). The other settings are only relevant to very specific research questions and are therefore not described in detail here."*

8. I hope for the published version the graphics are better than what I am seeing, as the resolution is not great, at least on screen.
Response: Thank you for this comment. The low resolution you are facing may result from the conversion into one merged pdf file at submission. Since all our graphics meet the JOVE submission requirements we are confident that the resolution will be sufficient in the final article.

9. The output from the analysis are graphs and tables (numerical). Can the tables be saved as txt files or excel files for further analysis? I do not think this is mentioned anywhere.
Response: It is possible to save all result tables in excel/ASCII output format. We have added this in form of a NOTE at the end of the protocol.

***"NOTE:** all result tables can be saved and exported in excel/ASCII output format for further statistical analysis."*

10. Finally, does the information in this manuscript merely replicate what is in the Ponemah handbook/manual or does it add to it and make it clearer?
Response: We thank the reviewer for this comment. Our main goal is to provide a pragmatic approach to perform an initial analysis of large data sets of murine long-term ECG recordings by focussing on only the most important readouts: basic ECG parameters and most important arrhythmia features (tachycardia, bradycardia, pauses). It is true thatsome of the general information on how to use Ponemah can be found within the manufacturer's manual. However, we have added this information in a way to generate a step by step protocol that can be easily followed without additional reading of the manufacturer's manual which is a

very general manual describing every single feature of the software (e.g. also other transmitter signals) rather than focussing on arrhythmia research.
Thus, we think that our protocol does add a significant value over the pure manual and provides a more practical and clinically oriented approach to analyse long-term ECG data - which is very distinct from what DSI suggests - and will therefore be very helpful for a lot of researchers facing similar challenges with this software as we did.

11. Unless I am mistaken, figures 5E and 5F look like they are the same. I am assuming this is an oversight and not because the software cannot distinguish between 2nd degree AV block (type 2 Mobitz) and 3rd degree AV block. This needs to be addressed. Also within figure 5, the graphic for 1st degree block does not really fit the classical description. Maybe annotating with P-R intervals would be helpful.
Response: We thank the reviewer for this comment, you are absolutely correct, 5E and 5F are the same and we have overseen this upon initial submission, further what was 5D was supposed to be 5E and 5D was missing. These mistakes were corrected for resubmission. We further thought about annotating the PR intervals within the recordings, but to avoid overloading and keep good visibility we decided against it. Please find the new figure within resubmitted manuscript.

12. This is just a suggestion: would it be useful to compile a flow chart of the process from data acquisition to analysis in Ponemah?
Response: We thank the reviewer for this suggestion and after careful consideration decided to add a new Figure 7 (see manuscript).

13. The discussion and Introduction should mention how Ponemah compares to other commercially available software such as LabChart, for example.
Response: Again, thank you for this comment. The scope and aim of this article is not to compare commercially available software solutions and their analysis modules but rather to provide a pragmatic approach on how to deal with the large datasets resulting from three month long murine ECG recordings and consecutive search for arrhythmias within these data sets. Since Ponemah is the platform we use and since Ponemah is advertised as a high quality and simple to use software for this, this article focusses on this software. Nevertheless, we agree that at least mentioning other software and remarking some of the differences and similarities can be helpful and therefore added a paragraph to the discussion.

"Analysis of basic ECG parameters such as heart rate, and duration of different intervals can up to date reliably be done using software such as Ponemah, LabChart or ECGAuto among many others with their respective analysis algorithms. Arrhythmia detection however is way more delicate and there are no widely established approaches for murine long-term ECG analysis for arrhythmias. Different approaches have been used to overcome the technical and methodological difficulties coming along with arrhythmia detection of long-term ECG recordings in mice. These approaches range from only using short recordings to be able to manually analyse them for arrhythmias as for example done by Toib et al.[2] up to simple considerations accepting inaccuracy as performed by Thireau et al.[3] with their approach to heart rate variability analysis by simply excluding all sections of their recording with R-R intervals not contained in the range of the mean R-R interval ± 2 standard deviations to exclude all arrhythmias, ectopic beats and artefacts without any manual review. This is why we settled

with our semi-manual approach using Ponemah and its consecutive analysis modules ECG Pro and Data Insights."

1 Hulsmans, M. *et al.* Macrophages Facilitate Electrical Conduction in the Heart. *Cell.* **169** (3), 510-522.e520, (2017).

2 Toib, A. *et al.* Remodeling of repolarization and arrhythmia susceptibility in a myosin-binding protein C knockout mouse model. *American journal of physiology. Heart and circulatory physiology.* **313** (3), H620-h630, (2017).

3 Thireau, J. *et al.* Heart rate variability in mice: a theoretical and practical guide. *Experimental physiology.* **93** (1), 83-94, (2008).

4 McCauley, M. D. *et al.* Ambulatory ECG recording in mice. *Journal of visualized experiments : JoVE.* 10.3791/1739 (39), (2010).

5 Boukens, B. J. *et al.* Misinterpretation of the mouse ECG: 'musing the waves of Mus musculus'. *Journal of Physiology.* **592** (21), 4613-4626, (2014).