Comprehensive ECG reference intervals in C57BL/6N substrains provide a generalizable guide for cardiac electrophysiology studies in mice

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Introduction

Reference ranges are a powerful tool for diagnostic decision-making in clinical medicine and their use has become increasingly common[1]. Reference ranges are derived intervals containing a defined subset of values from a large and comparable population dataset. These values, designed to delineate the expected range of a given parameter, are used clinically to identify outlier values. Individuals presenting with values outside of a clinically defined reference range are considered abnormal and flagged for follow up clinical investigation.

Looking beyond clinical applications, reference ranges are of enormous value in pre-clinical, basic scientific research using *in vivo* modelling[1]. They are used to define "normality" for a given genetic background, sex, and age of animals, such as inbred mouse strains. To our knowledge, there are no published reference ranges for electrocardiography (ECG) in the laboratory mouse. Such reference ranges would provide the research community with the information necessary to evaluate the consequences of pharmacological, environmental, or genetic perturbations, the latter opening up the opportunity to uncover genotype*phenotype associations.

We used ECG data collected under the auspices of the International Mouse Phenotyping Consortium (IMPC[2], https://www.mousephenotype.org) to generate the first mouse-specific cardiac physiology reference ranges. Here, data were collected from over 26,000 conscious or anesthetized C57BL/6N wildtype control mice stratified by sex and age. The unprecedented scale of this data resource yields a robust reference range for a broad and commonly studied set of ECG parameters that are clinically important to assess myocardial electrical processes and cardiac function.

Results

ECG data collected by IMPC contributing centers (data release, DR, 15.0) were available from 26,706 wildtype control mice, stratified as presented in **Table 1** and summarized below. All the mice were from a C57BL/6N inbred substrain. ECG was performed on conscious mice, or mice anesthetized with either isoflurane or tribromoethanol. The majority of mice (90.6% or 24,194) were tested at a mean age of 12 weeks (designated as “early adult” or EA), while the remaining 9.4% (2,512) of mice were tested at a mean age of 62 weeks (designated as “late adult” or LA). Sex was evenly distributed at both EA and LA time points. Raw data can be downloaded using the following link: https://www.mousephenotype.org/data/previous-releases/15.0. The total number of reported parameters varied slightly between mice and can be accessed in ***Supplemental Table 1***.

Variability Assessment

A panel of 15 output parameters were collected from ECG, namely heart rate (HR), RR-, PR-, PQ-, ST-, and QT-interval, and QT corrected (QTc) using the Mitchell formula[4], QRS complex, coefficient of variation of R-R intervals (CV), heart rate variability (HRV), pNN50, rMSSD (Root Mean Sum of Squared Distance), mean R-amplitude, mean SR-amplitude and QT corrected (QTc) dispersion (parameter definition in ***Supplemental Table 2***).

In multi-center, large-scale, high-throughput programs such as the IMPC, variability in the measured values was to be expected. However, the extent of this variability dictates the sensitivity and robustness of each parameter.

Variability testing was performed on all DR 15.0 ECG data from the IMPC, independently of anesthetic agent in this analysis. For each sex, individual ECG parameters were tested for variability in EA and LA populations. The following standard metrics for assessing distribution variability were calculated:

1) Coefficient of variation (COV) (100*standard deviation/mean) assumes a parametric distribution and normalizes the variability to the most typical score (mean) but is sensitive to outliers. To support the parametric COV test, we applied a 2) “Quartile-based CV” (QCV), defined as interquartile range (IQR) (75-25%) relative to the median (100*IQR/median). QCV is a similar metric to COV but uses non-parametric measures of variability, therefore makes no assumptions of normality but is still readily influenced by outliers[3,4].

Based on this analysis, exclusion criteria were defined as any parameter with acceptable variability based on Eurachem guidelines (https://www.eurachem.org/index.php/publications/guides) of ≥30 for COV (**Figure 1**) and a QCV ≥30 for EA and LA mice (***Supplemental Figure 1***). Figure 1 shows that the retained parameters are all clustered closely together, however the excluded parameters show a wide range of variability. Specifically, seven ECG-parameters (CV, HRV, pNN50, rMSSD, mean R-amplitude, mean SR-amplitude and QTc dispersion) exceeded the variability criteria in both sexes (male and female) and ages (EA and LA) and were excluded from further analysis (**Figure 1**). The variability threshold was exceeded least for QTc dispersion in EA and mean R- and SR-amplitude for LA, however, for the remaining parameters that were excluded, variability was in excess of 2-7 times the threshold.

A PQ-interval reference range is provided for conscious EA and LA mice (***Supplemental Figure 2)*** however, PQ-interval was excluded from further analysis in this study because data points were only captured in EA and LA mice from one of the ten data contributing centers. The remaining seven ECG-parameters [heart rate (HR), RR-, PR-, ST- and QT-interval, QRS complex, and QT corrected (QTc) using the Mitchell formula[5]] consistently presented with low variability across the whole IMPC dataset thereby giving high confidence to establish robust, generalizable reference ranges for EA and LA populations on the C57BL/6N inbred genetic background.

Despite the exclusion of several parameters, the electrical conduction phases of a cardiac cycle were entirely captured by the robust parameters included herein (**Figure 2**). The lengths of PR-interval and QRS complex covered the atrial and ventricular depolarization phases (e.g. contraction), whereas lengths of QT- and ST-intervals implied the ventricular repolarization (e.g. relaxation) in voltage over time.

Assessment of Data Distribution

The distribution of data was assessed via histograms for the seven selected ECG parameters stratified by sex, age, and anesthetic regime (**Figure 3**). This visual representation of the frequency of occurrence per value in the data was useful for revealing conformity to- and deviations from- a normal distribution, for each parameter. Visual inspection of the histograms showed that the data appeared practically normal for parameters PR, QT and QTc Mitchell, and modestly skewed for HR, QRS, ST and RR. To assess normality mathematically, we applied the Shapiro-Wilk test which revealed statistically significant deviation from a normal distribution for some, but not all, ECG parameters. **Table 2** presents data as median and 95% reference range (2.5th and 97.5th

percentile) to account for the lack of normal distribution of some parameters and to provide a consistent data presentation[4]. For the sake of completeness, mean, standard deviation and sample size are provided for the seven selected ECG parameters stratified by sex, age, and anesthetic regime in ***Supplemental Table 1.***

Interestingly, male, and female data showed similar distributions by visual inspection (**Figure 3**). To test the hypothesis that there is no difference between each sex, a simple two-tailed t-test was performed independently for each anesthetic regime and age group, and Cohen's *d* was calculated as an effect size measure (***Supplemental Figure 3, 4 and 5 - Panels a and b, stratified by age***).

For some parameters, p-values reached significance <.001, for others we found no evidence of a difference. However, for all parameters the corresponding Cohen's *d* value revealed small to negligible effect sizes. We therefore considered the possibility that the large group sizes could be overstating the biological differences between the sexes for some parameters.

To address this, we applied a bootstrap analysis stratified by age group. In brief, random sampling (1000x randomized) of different sub-sample sizes, ranging from 5 to 100 mice, were applied to test the robustness of the effect for each parameter comparing females and males. The sub-sample group sizes were chosen to more closely approximate standard experimental groups. The proportion of significant t-tests ($p<.05$), from the 1000 comparisons, indicates the power to find the sex difference, for that sub-sample size. If the proportion of significant tests remains near 5% regardless of sub-sample size, then this indicates the influence of the Type 1, i.e. false positive, error.

Recordings from both conscious (***Supplemental Figure 3)*** and isoflurane anesthetized mice (***Supplemental Figure 4)*** show that the ECG parameters consistently have very low proportions of significant tests for sexual dimorphism, with most parameters fluctuating around 5% of tests. Tribromoethanol anesthesia (***Supplemental Figure 5)*** however, reveals weak sexual dimorphism for a subset of parameters. This may be due to a bias from drawing bootstrap samples from a much smaller "population" than the other conditions, but we cannot exclude the possibility that this anesthetic has a small but significant impact on the sexes.

Effect of Anesthetic Agent

To investigate the effect of different anesthetic agents on cardiac conduction function and ECG profiles, conscious data stratified by sex and age are displayed for comparison with those of isoflurane or tribromoethanol data (**Figure 4).** Female data are placed directly above male for ease of visualization. **Figure 4** shows distinct distribution clusters for conscious, isoflurane and tribromoethanol groups split by EA (**Figure 4 – Panels A to G**) and LA (**Figure 4 – Panels H to N**). As before, no data were available for tribromoethanol anesthesia in LA mice.

As expected, the physiological benchmark of highest heart rate in conscious mice compared to anesthetized animals was observed (**Figure 4 – Panels A and H**). To assess the differences between EA anesthetic states, we tested conscious versus isoflurane and conscious versus tribromoethanol groups, by a one-way ANOVA with planned comparisons, and observed highly significant differences between those groups (**Table 3**). These data clearly show differences in ECG parameters that can be attributed to the anesthetic regime; therefore, it is essential to establish reference ranges separately

by condition (conscious or anaesthetized) and by anesthetic (isoflurane or tribromoethanol).

Effect of Age on ECG Parameters

Two different age groups, i.e. mean of 12-weeks (minimum 8 and maximum 16 weeks) old EA and mean of 62 weeks (minimum 52 and maximum 78 weeks) old LA, have made it possible to explore the effect of age on ECG parameters in conscious and isoflurane anaesthetized mice. A two-tailed t-test was applied to test the difference between the means of EA and LA results in conscious mice (**Figure 5 - Panels a and b**). P-values <.001 were reached for all parameters, indicating high statistical significance and the corresponding Cohen's *d* effect size revealed negligible to medium standardized effect sizes (**Figure 5 – Panels a and b**).

To test the influence of unbalanced group sizes (i.e. large number of EA and smaller number of LA datasets), we applied a bootstrap analysis, this time stratified by sex (**Figure 5 – Panel c**). This bootstrap analysis demonstrated that parameters with even small to medium effect sizes required relatively large experimental group sizes to attain a conventional >80% value for power estimates[6,7], e.g. QRS and ST in conscious conditions required a group size of 50 mice to achieve >80% power with a p<.05 (**Figure 5 – Panel c, QRS (subpanel C) and ST (subpanel F)**). As expected, for parameters with negligible Cohen's *d* effect sizes, such as HR and RR, increases in sample size do not appreciably increase power (**Figure 5 – Panel c, HR (subpanel A) and RR (subpanel E)**). Parameters with less than 80% power even with up to n=100 animals, can be considered likely to be "similar in EA and LA" with no aging effect for most experimental

purposes. ***Supplemental Figure 6*** presents the equivalent t-test, Cohen's *d* and bootstrap analysis in EA and LA mice anesthetized with isoflurane.

In summary, **Figure 6** is a graphical representation of the median and 95% reference ranges (2.5th and 97.5th percentile) broken down by anesthetic regimen with the female data placed directly above equivalent male data for easy visual interpretation corresponding numeric values are presented in **Table 2**.

Validation of Reference Ranges Using Non-IMPC Data

Mice characterized by the IMPC are all substrains of one commonly used inbred genetic background, C57BL/6N. To test the validity of the reference ranges reported herein beyond C57BL/6N inbred mice, we used representative control animals from publicly available ECG data including: six founder strains from a collaborative cross study[8]; the Jaxwest1 project (https://phenome.jax.org/projects/Jaxwest1) with seven inbred strains of mice; and the Xing1: Aging study (https://phenome.jax.org/projects/Xing1)[9] with 29 inbred strains of which we have included herein the 26 strains with complete ECG data. An additional dataset was included using inbred, wildtype control animals from non-IMPC studies conducted at the German Mouse Clinic where data is available upon request. Validation was also carried out for LA population by using 12- and 20-month age groups of the Xing1 study. In each non-IMPC study, where suitable we presented the data split by sex and overlaid with the sex-specific 95% reference range calculated herein for conscious mice. Due to the small sample sizes in a subset of these comparator studies, however, the combined reference ranges for females and males are summarized in ***Supplementary Table 3*** for further comparison. **Figure 7** shows the founder strain data from the collaborative cross study overlaid with the reference ranges split by sex whereas

Supplemental Figure 7 illustrates data from the German Mouse Clinic, ***Supplemental Figure 8*** from the Jaxwest1 and ***Supplemental Figures 9-13*** depict LA data from the Xing1 study. Of note, HR is not presented throughout as it was not accessible for those studies yet it is indirectly visualized in the RR-interval plot due to the inverse correlation ship of HR and RR[10].

Remarkably, and true for all ECG parameters, most non-C57BL/6N values lay within our reference values. There is a subset of outliers that fall outside of the reference ranges which is to be expected with heterogeneity of small size and phenotypic differences seen between inbred mouse strains.

Discussion

Reference ranges for the assessment of abnormal electrocardiograms and cardiac conduction disorders in patients have long been established and are regularly adopted by expert bodies such as the North American Society of Pacing and Electrophysiology[11] and the European Society of Cardiology[12,13]. For mouse models, however, there are no such reference ranges.

In this multicenter study, we have established reference ranges using an exceptionally large ECG dataset comprising more than 26,000 wildtype control mice from the International Mouse Phenotyping Consortium (IMPC). The goal of the IMPC is to extend the functional annotation of the mammalian genome via the large-scale production and phenotypic characterization of single gene knockout mouse strains for all protein coding genes. The phenotypic pipeline used to characterize these knockout strains included cardiac electrophysiology assessment using ECG. For each knockout strain characterized, age, sex and genetic background matched wildtype control animals were also assessed. The ECG data from these C57BL/6N wildtype control mice hold extraordinary value and represent the focus of the current study.

Thus, this study represents a large mouse data set and allows the crucial understanding of the effects of sex, age, and anesthesia on electrocardiograms in mice. To this end, we introduced a stepwise refinement of the data analysis and started with an in-depth assessment of the variability of 15 ECG parameters gathered in the IMPC. We identified seven clinically relevant ECG parameters that were highly robust and had low variability. We excluded the remaining eight ECG parameters because of the excessive level of inter-mouse variability they displayed. Five of the eight excluded parameters were direct

measures of heart rate variability (HRV), or represented parameters derived from HRV (HRV, pNN50, rMSSD, mean R-amplitude and mean SR-amplitude). HRV depicts the change in the time interval between successive heartbeats and is an index of the parasympathetic nervous system[14,15]. HRV measurement is very sensitive to experimental methods (e.g. acclimation time, ECG sampling rate, and duration of recording), and has been shown to be incompatible with a high-throughput data collection set up such as that used by the IMPC[16,17]. Next, CV provides an indication of the function of the parasympathetic nerve and the autonomic nervous system through the physiological phenomenon of RR variation[18]. Such measurements, however, require stable and prolonged measurement times to be meaningful, which, as stated above for HRV, we do not have in the context of the high throughput testing paradigm used herein. Similarly, this susceptibility to broad variability in short duration measurements also applies to the parameter QT dispersion, which is defined as the difference between the longest and shortest QT interval in one of the surface ECG leads and quantifies the spatial inhomogeneity of ventricular repolarization. Mainly for methodological reasons, parameters with high variability were excluded here, but PR interval is the exception. This parameter was only collected by one center and therefore not included in the overall evaluation, but the values were made available in full in the supplemental materials. Despite the exclusion of those parameters, the robust ECG parameters that were included entirely captured the electrical conduction phases of a cardiac cycle and provided a comprehensive ECG evaluation.

Understanding the sex-related impact on ECG is crucial for ensuring robust reference values. In this study, we were able to show that the values for HR, RR-, PR-, ST- and QT-

interval, QRS complex, and QT corrected (QTc) using the Mitchell formula[5] are comparable in female and male mice with negligible sexual dimorphism. There may, however, be small sex differences for some parameters depending on the anesthetic agent. This observation is of key importance, and in part consistent with previous mouse data[19]. Whilst sexual dimorphism was not overtly apparent in inbred mice in the absence of any environmental, pharmacological or genetic perturbations, the literature clearly supports sex differences in heart health[20] and therefore our recommendation is that both sexes are included in any experimental design assuming that post-treatment we may detect sex differences.

Anesthetics cause a dose-dependent decrease in myocardial contractile force and associated ECG alterations with the most familiar landmark of decreased HR[21]. Our observations are that presence of anesthesia matters, we confirm a decreased heart rate in anesthetized mice and go on to reveal distinctions in isoflurane inhalation anesthesia and intraperitoneal injected tribromoethanol induced anesthesia[22,23]. These distinctions are pivotal and to emphasize them we mapped the effects of three different states (conscious, isoflurane and tribromoethanol anesthesia) on seven ECG parameters in detail and present anesthesia-specific reference values.

HR is an important determinant of cardiovascular performance defined by the activity of the sinoatrial node, the so-called pacemaker of the heart. The dysfunction of the sinoatrial node increases with age, and HR decreases due to tissue, cellular, and molecular mechanisms that underlie the reduction in pacemaker activity with age[24-26]. Interestingly, we did not observe any strong age-related ECG changes in the absence of any pharmacological, environmental, or genetic challenges in inbred C57BL/6N mice. The

differences in the reference ranges of 12-week-old young adult mice compared to 62-week-old adult mice were negligible. Our step-by-step analysis of these data in bootstrapping showed that age-related ECG effects are more likely, if at all, to be detected in large group numbers (>50). This dependency on the group size can be used as a guide for experimental design when considering aging.

In the IMPC, we control for genetic diversity by using C57BL/6N inbred background substrains thereby focusing our comparison on the genetic perturbation of interest i.e. the single gene that is knocked out on this common genetic background. The transferability from the C57BL/6N background used here, however, was demonstrated by independently validating the ranges using data from a broad spectrum of non-IMPC C57BL/6N and C57BL/6J strains, and other inbred strains, including wild derived inbred strains. This validation indicates that C57BL/6N-based reference values represent a robust and comprehensive indicator of normality and can be used as a starting point for many experimental investigations of cardiac function in the mouse.

In summary, we have created a unique and comprehensive map of ECG reference ranges that will be foundational for future mouse studies. While based on inbred mouse substrains that are C57BL/6N in origin, these reference ranges have utility across different mouse strains and are important guides in studies of electrical conductivity disorders.

Methods

The International Mouse Phenotyping Consortium

The International Mouse Phenotyping Consortium (IMPC) represents a multi-institutional and collaborative research initiative encompassing twenty-four major research organizations and funding agencies, distributed globally. The IMPC seeks to generate and phenotype a knockout mouse line for every protein-coding gene in the mouse genome (www.mousephenotype.org)[27,2]. Phenotyping is carried out under the uniform operating procedures detailed in IMPReSS (International Mouse Phenotyping Resource of Standardized Screens; www.mousephenotype.org/impress/index), which were developed and validated during the pilot programs EUMORPHIA and EUMODIC[28].

IMPC Centers Contributing Electrocardiography Data

IMPC data release (DR) 15.0 was used herein (https://www.mousephenotype.org/data/previous-releases/15.0). The following subset of ten IMPC data-contributing centers provided electrocardiography (ECG) data in DR 15.0 (ethical approval details are included in parenthesis after each contributing center):

1. Baylor College of Medicine (BCM) (Institutional Animal Care and Use Committee approved license AN-5896).

2. German Mouse Clinic Helmholtz Zentrum München (GMC) (#144-10, 15-168)

3. Medical Research Council (MRC) – Harwell (HAR) (Animal Welfare and Ethical Review Body approved licenses 70/8015 and 30/3384).

4. Institute Clinique de la Souris, Mouse Clinical Institute (ICS) (#4789-2016040511578546v2).

5. The Jackson Laboratory (JAX) (Institutional Animal Care and Use Committee approved licenses 14004, 11005, and 99066. JAX AAALAC accreditation number was 000096, NIH Office of Laboratory Animal Welfare assurance number was D16-00170).

6. RIKEN BioResource Research Center (RBRC) (Animal Care Committee approved animal use protocols 0153, 0275, 0277, and 0279).

7. University of California – Davis (UCD) (Institutional Animal Care and Use Committee approved animal care and use protocol number was 19075. UCD AAALAC accreditation number is 000029, and the NIH Office of Laboratory Animal Welfare assurance number is D16-00272 # (A3433-01).

8. Seoul National University, Korea Mouse Phenotyping Center (KMPC) (KRIBB-AEC-19189).

9. Czech Centre for Phenogenomics (CCP) (AV CR 62/2016, Academy of Sci., Czech Rep.).

10. The Centre for Phenogenomics, Toronto (TCP) (22-0275 and 22-0279).

ECG data was collected from mice at one of two possible time points. For the Early Adult (EA) Pipeline data were collected at a mean of 12 weeks with the minimum of 8 and maximum of 16 weeks of age. For the Late Adult (LA) Pipeline data were collected at a mean of 62 weeks with the minimum of 52 and maximum of 78 weeks of age. Animal welfare was assessed routinely for all mice involved.

Animals

This study includes data collected from inbred wildtype control animals tested as part of the IMPC goals. These mice, both males and females, were on a C57BL/6N genetic background of substrains: C57BL/6NCrl (CCP, HMGU, ICS, TCP and UCD); C57BL/6NJ (JAX and BCM); C57BL/6NJcl (RBRC) and C57BL/6NTac (KMPC, HMGU, ICS and HAR). Non-IMPC mice were from four different studies: (1) The founder strains animals from a study titled "The Collaborative Cross: A Recombinant Inbred Mouse Population for the Systems Genetic Era"[8] with A/J, C57BL/6J, 129S1/SvlmJ, NOD/ShiLtJ, NZO/HlLtJ, CAST/EiJ, PWK/Ph, and WSB/EiJ inbred strains (https://phenome.jax.org/projects/GMC13); (2) The Jaxwest1 project, a multi-system analysis of physiology on seven inbred strains of mice: 129S1/SvImJ, A/J, BALB/cJ, C57BL/6J, DBA/2J, NOD/ShiLtJ and SJL/J (https://phenome.jax.org/projects/Jaxwest1); (3) Wildtype control animals from three non-IMPC studies performed at the German Mouse Clinic (https://www.mouseclinic.de/) with a standard sample size (20-30 control animals per study). The mouse backgrounds were: (i) An independent repeat of strain C57BL/6NJ (Jackson Laboratory strain #:005304) that is used by some of the IMPC contributing centers; (ii) C57BL/6J (JAX strain #:000664), the most commonly used inbred mouse strain and the first to have its genome sequenced; and (iii) FVB (JAX strain #:001800), a widely used multipurpose inbred line. For more information on these inbred strains, visit: https://www.jax.org/strain; and (4) The Xing1, Aging study: Electrocardiogram for 29 inbred strains of mice (https://phenome.jax.org/projects/Xing1)[9]. Xing1 recorded ECG characteristics in the following 26 inbred mouse strains: 129S1/SvImJ, A/J, BALB/cByJ, BTBR-T+tf/J, BUB/BnJ, C3H/HeJ, C57BL/10J,

C57BL/6J, C57BLKS/J, C57BR/cdJ, C57L/J, CBA/J, DBA/2J, FVB/NJ, KK/HIJ, LP/J, MRL/MpJ, NOD.B10Sn-H2/J, NON/ShiLtJ, NZO/H1LtJ, NZW/LacJ, P/J, PL/J, RIIIS/J, SM/J, and SWR/J. AKR/J, PWD/PhJ and SJL/J were excluded herein due to incomplete ECG data.

Data Collection

The IMPC standard operating procedure provides an overview of the conscious and anesthetized ECG procedures used by contributing centers (https://www.mousephenotype.org/impress/ProcedureInfo?action=list&procID=1415&pipeID=7). In brief, conscious ECG was collected using ECGenie equipment (Mouse Specifics, Inc.) as detailed previously by Spielmann et al.[29]. Based on availability of equipment and local expertise, some contributing centers opted to perform anesthetized EGC using Power Lab recording equipment and LabChart software (ADInstruments). Mice were anesthetized either with inhaled isoflurane (anesthesia was induced using 2.5-4% isoflurane in oxygen then maintained using 2-2.5% isoflurane in oxygen) or injected tribromoethanol (Sigma, stock concentration 20 mg/ml, dose calculated as 0.5 g/kg body weight). Anesthetized mice were positioned supine on a warming pad apparatus that maintained the animal's core temperature at 37^{o}C. Needle electrodes were placed subcutaneously as follows: the negative electrode in the right forelimb; the ground electrode in the right hindlimb; and the positive electrode in the left hindlimb. ECG data was collected for up to 120 seconds and the resulting data analyzed using LabChart8 software (ADInstruments).Regardless of the methodology, ECG was recorded in a dimly lit, quiet procedure room. In order to eliminate circadian influences ECG was recorded during the morning when the resting phase of a mouse begins.

Data Quality

Standard protocols for ECG signal analysis were used to analyze the data. For this the software uses a so-called peak detection algorithm, which finds the peak of the R waves and calculates the heart rate (HR).

In anesthetized mice and for each cardiac cycle, the P, Q, R, S and T peaks were defined and from these the HR as well as the duration of the QRS complex, PQ interval, PR interval, QT interval and ST interval were automatically measured whereas in conscious ECG the peaks were automatically determined and averaged over multiple cardiac cycles. Only unique P, Q, R, S and T peaks have been included in the automatic calculations. Furthermore, the software automatically defined the end of the T-wave of each signal as the point where the signal intersects the isoelectric line. If any peaks were not selected correctly by the software, the position of this marker was corrected manually. Some subtle differences in annotation placement were observed across IMPC contributing centers. Heart rate variability (HRV) was calculated as the mean of the differences between successive heart rates for the entire set of ECG signals. The QT intervals were frequency corrected by applying the following equation (QTc) Mitchell et al.[14]. Noise and movement artefacts were automatically eliminated by the software.

Data were curated and subject to quality control at the IMPC prior to Data Release 15 (August 11th, 2021) and we excluded one additional mouse from the analysis due to a biologically implausible QRS value.

Statistical Methods

Bespoke methods were developed to assess ECG reference ranges and are independent of the methodologies implemented on the IMPC portal.

Data analysis was conducted using R (version 4.0.4, R Core Team 2022[30]) with figures and tables produced in ggplot2, embedded in RMarkdown HTML files. Variability of all the data was assessed with two metrics a) coefficient of variation (COV) and b) "Quartile-based CV" (QCV), defined as interquartile range (IQR) (75-25%) relative to the median (100*IQR/median).

Visual methods, as well as formal statistical tests were applied to test whether the scores of the individual parameters were normally distributed. Data were separated by age, sex and anesthesia regime and histograms for each parameter were plotted. Shapiro-Wilks tests were conducted to assess normality. Reference ranges were calculated based on median, 25th percentile and 75th percentile. In addition, the mean, standard deviation, and parameter sample size were provided to reflect the distribution of data. To reflect the distribution of each parameter, the 95% confidence intervals can be calculated by mean±1.96*standard deviation for each parameter.

Investigation of Anesthesia, Sex and Age Effects

To investigate the effect of anesthesia on the different parameters, we calculated a one-way Analysis of Variance (ANOVA) with planned comparisons of "Conscious versus Isoflurane" and "Conscious versus Tribromoethanol", separated by sex whereas "Isoflurane versus Tribromoethanol" was not tested. These planned comparisons were used to compare conscious vs unconscious. When looking for differences between

groups we tested the null hypothesis. P-values and F-values with degrees of freedom were calculated.

The effects of sex (female vs male) and age (EA vs LA) were compared using the same statistical analyses. In each case a simple two-tailed t-test was performed and the Cohen's *d* effect size calculated from the "effsize package" (R library). Due to the central limit theorem (CLT)[31], the large sample sizes allowed parametric statistical testing of these effects.

These large group sizes provide overwhelming statistical power and may overestimate the importance of the effects. Bootstrapping tests were done to verify the biological significance of any differences in a range of more realistic experimental group sizes.

Data availability

All data used are available to the public for download at the IMPC(https://www.mousephenotype.org/data/previous-releases/15.0).

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Conflict of interest

There is no conflict of interest for any of the authors listed above.

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Table and Figure captions

Table 1: ECG data were available from a total of 26,706 mice, stratified by sex, age at testing (EA = 12 weeks of age; LA = mean of 62 weeks of age), and conscious state (conscious, anesthetized using isoflurane, or anesthetized using tribromoethanol).

Table 2: Median and 95% reference ranges of HR, PR-, QRS complex, QT-, RR- and ST-intervals, and QT corrected (QTc) using the Mitchell formula. Data are stratified by sex, age (EA and LA) and conscious state. **Panel a.** Conscious, isoflurane and tribromoethal anesthetized female mice. **Panel b.** Conscious, isoflurane and tribromoethal anesthetized male mice. Note, there were no data for LA mice anesthetized using tribromoethanol.

Table 3: Significant differences between the statistical comparison of conscious versus isoflurane ($p<.001$) and conscious versus tribromoethanol ($p<.001$ to $p=.004$) in female (Panel a) and male (Panel b) mice for HR, PR-, QRS complex, QT-, RR-, ST-interval and QTc Mitchell. Test: p-value and F-value of one-way ANOVA with planned comparison.

Figure 1: Coefficient of variation (COV) analysis of data split by sex (female and male) and age (EA and LA) identified parameters with excess variability (COV >30%) that were excluded from further analysis (white bars). Parameters in blue were below the COV threshold of 30% and were retained for further analysis. These were in ascending COV percentage QTc Mitchell, PQ-, QT-, QRS-complex, ST-, RR- and PR-interval.

Figure 2: Representative averaged waveform of a C57BL/6N mouse in voltage over time reflecting the most commonly applied ECG annotations. Some subtle differences in annotation placement were observed across IMPC contributing centers. Figure 2 shows the depolarization and repolarization phases with prominent ECG parameters such as P, Q, R, S and T and their respective interval lengths as a function of voltage over time.

Figure 3: Histograms presenting the distribution of each selected ECG parameter for male and female mice separately. By visual inspection, no sexual dimorphism is apparent. **Panel a:** Recorded in the conscious state in early adult, EA (Sub-panels A-G) and late adult (LA) mice (Sub-panels H-N). **Panel b:** Recorded under isoflurane anesthesia in early adult, EA (Sub-panels A-G) and late adult (LA) mice (Sub-panels H-N). **Panel c:** Recorded under tribromoethanol anesthesia in early adult, EA (Sub-panels A-G). No late adult data are available for tribromoethanol anesthesia.

Figure 4: Comparison of the anesthetic regimes with the conscious state recordings. Distribution of the seven selected ECG parameters presented by histograms, stratified for female and male mice in early adult, EA (Sub-panels A-G) and late adult (LA) populations (Sub-panels H and N). Color code: Conscious, isoflurane and tribromoethanol anesthesia.

Figure 5: Testing age-differences in conscious mice.

T-test results when comparing conscious EA versus LA data show high significance for all parameters ($p<.001$) and negligible to medium Cohen´s *d* standardized effect sizes. **Panel a**: females, **Panel b:** males. **Panel c.** Bootstrap analysis of power estimates for sample sizes ranging from 5-100 mice, presented for each of the seven selected ECG parameters.

Figure 6: Reference ranges split by anesthetic regimen showing median, and 95% reference ranges (2.5th and 97.5th percentile). Female data are directly above the male data for EA (Sub-panels A-G) and late adult (LA) populations (Sub-panels H and N). No late adult data are available for tribromoethanol anesthesia. Color code: Conscious, isoflurane and tribromoethanol anesthesia.

Figure 7: Independent, non-IMPC study on six of the founder strain mice reported in "The Collaborative Cross: A Recombinant Inbred Mouse Population for the Systems Genetic Era"[8] study, including 129S1/SvlmJ, A/J, C57BL/6J, NOD/ShiLtJ, NZO/HlLtJ, and PWK/PhJ inbred strains show a close alignment to the reference ranges reported herein for PR-, QRS complex, QT-, RR-, and ST-interval based on multiple C57BL/6N substrains indicating good utility for those reference ranges. Data for HR was not available in this study. Mice were conscious, split by sex and ~12 weeks of age, equivalent to the IMPC EA time point. Red dotted lines depict the boundaries of the sex- specific reference ranges calculated herein, for each parameter.

Supplemental Table 1: Comprehensive overview of mean, standard deviation, and sample number for each of the seven selected ECG parameters stratified by conscious state (conscious, anesthetized with isoflurane, or anesthetized with tribromoethanol), age [early (EA) and late (LA) adult time point] and sex (**Panel a**. Females; **Panel b.** Males).

Supplemental Table 2: Definition and unit of measure for each ECG parameter reported.

Supplemental Table 3: Comprehensive overview of median and 95% reference ranges (2.5th and 97.5th percentile) for each of the seven selected ECG parameters, stratified by conscious state (conscious, anesthetized with isoflurane, or anesthetized with tribromoethanol) and age [early (EA) and late (LA) adult time-point]. Sex is combined (females plus males) for this analysis to generate a both-sex-combined reference range.

Supplemental Figure 1: "Quartile-based CV" (QCV), defined as interquartile range (IQR) (75-25%) relative to the median (100*IQR/median), analysis of data split by sex (female and male) and age (EA and LA) identified parameters with excess variability (QCV ≥30% for EA and LA time point) that were excluded from further analysis (white bars). Parameters in blue were below the QCV threshold and were retained for further analysis.

In the QCV analysis, but not in the COV, there were three parameters (light blue bars) in the LA population (HR, RR and PR) that marginally exceeded the limit of QCV ≥30 but were retained.

Supplemental Figure 2: Histograms presenting the distribution of PQ-interval data along with calculated ranges (mean ± SD and median and 95% reference range) for conscious EA and LA mice stratified by sex. These calculations are based on data from one contributing center (German Mouse Clinic).

Supplemental Figure 3: Testing sex-differences in conscious mice.

T-test results when comparing data from conscious male and female animals for each of the seven selected ECG parameters, stratified by age [early (EA) and late (LA) adult time point]) demonstrated that some parameters show high significance ($p<.001$), while for others there was no evidence of sexual dimorphism. Associated Cohen´s *d* standardized effect sizes were negligible. **Panel a** shows EA time point, **Panel b** shows LA time point, and **Panel c** shows bootstrap analysis of power estimates for female and male samples ranging from 5-100 mice, presented for each of the seven selected ECG parameters.

Supplemental Figure 4: Testing sex-differences in isoflurane anesthetized mice.

T-test results when comparing data from isoflurane anesthetized male and female animals for each of the seven selected ECG parameters, stratified by age [early (EA) and late (LA) adult time point]) demonstrated that some parameters show significance ($p<.05$) and negligible to small Cohen´s *d* standardized effect sizes. **Panel a**. shows EA time point. **Panel b.** shows LA time point, and **Panel c.** shows bootstrap analysis of power estimates for female and male samples ranging from 5-100 mice, presented for each of the seven selected ECG parameters.

Supplemental Figure 5: Testing sex-differences in tribromoethanol anesthetized mice.

T-test results when comparing data from tribromoethanol anesthetized male and female animals for each of the seven selected ECG parameters, stratified by age [early (EA) but **no** late (LA) adult time point] demonstrated that three parameters show high statistical significance (p<.001) namely HR, PR- and RR-interval whereas medium significance for ST- (p=.003) and QT-interval (p=.018) and no significance for QRS complex (p=.522) and QTc Mitchell (p=.283) were detected with negligible to medium Cohen´s *d* standardized effect sizes. **Panel a**. shows EA time point, and **Panel b.** shows bootstrap analysis of power estimates for female and male samples ranging from 5-100 mice, presented for each of the seven selected ECG parameters.

Supplemental Figure 6: Testing age-differences in conscious mice.

There is little to no aging effect by equivalent t-test, Cohen's *d* and bootstrap analysis in EA and LA mice anesthetized with isoflurane. In females, high statistical significance is detected for three parameters (p<.001) namely HR, QRS complex and RR-interval and no significance for PR-, QT- and ST-interval and QTc Mitchell with negligible to small Cohen´s *d* standardized effect sizes. In males, high statistical significance is detected for five parameters (p<.001) namely HR, QRS complex, QT-, RR- and ST-interval and no significance for PR-interval and QTc Mitchell with negligible to small Cohen´s *d* standardized effect sizes. **Panel a**. shows females. **Panel b.** shows males and **Panel c.** shows bootstrap analysis of power estimates for EA and LA sample size ranging from 5-100 mice, presented for each of the seven selected ECG parameters.

Supplemental Figure 7: Wildtype control animals from three non-IMPC studies tested at the German Mouse Clinic (https://www.mouseclinic.de/) with a standard sample size (20-30 control animals per study) on the background strains C57BL/6NJ, C57BL/6J and FVB show a close alignment to the reference ranges reported herein for HR, PR-, QRS complex, QT-, RR-, and ST-interval, and QTc Mitchell values based on multiple C57BL/6N substrains indicating good utility for those reference ranges. Mice were conscious, split by sex and ~12 weeks of age, equivalent to the IMPC EA time point. Red

dotted lines depict the boundaries of the sex-specific reference range calculated herein, for each parameter.

Supplemental Figure 8: Seven inbred strains of The Jaxwest1 project 129S1/SvImJ, A/J, BALB/cJ, C57BL/6J, DBA/2J, NOD/ShiLtJ and SJL/J show a close alignment to the reference ranges reported herein for PR-, QRS complex, QT-, RR-, and ST-interval based on multiple C57BL/6N substrains indicating good utility for those reference ranges. Mice were conscious, split by sex and ~12 weeks of age, equivalent to the IMPC EA time point. Red dotted lines depict the boundaries of the sex-specific reference range calculated herein, for each parameter.

Supplemental Figure 9-13: The Xing1 Aging study includes 29 inbred strains of which 26 have been included here: 129S1/SvImJ, A/J, BALB/cByJ, BTBR-T+tf/J, BUB/BnJ, C3H/HeJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/cdJ, C57L/J, CBA/J, DBA/2J, FVB/NJ, KK/HlJ, LP/J, MRL/MpJ, NOD.B10Sn-H2/J, NON/ShiLtJ, NZO/H1LtJ, NZW/LacJ, P/J, PL/J, RIIIS/J, SM/J, and SWR/J. The data show a close alignment to the reference ranges reported herein for PR-, QRS complex, QT-, RR-, and ST-interval based on multiple C57BL/6N substrains indicating good utility for those reference ranges. Mice were conscious, split by sex and ~12 months or ~20 months of age, equivalent to the IMPC LA time ranges with a minimum of 52 and a maximum of 78 weeks of age. Red dotted lines depict the sex-specific boundaries of the reference range calculated herein, for each parameter.

Table 1:

	Conscious	Isoflurane	Tribromoethanol	Sum
EA female	9,240	2,672	226	12,138
EA male	9,238	2,598	220	12,056
LA female	620	693	0	1,313
LA male	589	610	0	1,199
Sum	19,687	6,573	446	26,706

Table 2:

a **FEMALE**

	Conscious		*Isoflurane*		*Tribromoethanol*	
	EA	LA	EA	LA	EA	
Parameter	median [95% range]	median [95% range]	median [95% range]	median [95% range]	median [95% range]	
Heart Rate [bpm]	760 [650;815]	748 [700;790]	415.8 [292.6;511.7]	440.5 [339.6;530.3]	440 [355.8;550.5]	No LA data available
PR [ms]	28.7 [20.5;35.8]	26.3 [18.2;35]	44.5 [29.6;72.7]	46.7 [30.5;58.9]	49.1 [41.4;57.7]	
QRS [ms]	10.7 [8.4;14.8]	11.8 [8.3;15.3]	10.1 [7;17.9]	10.1 [7.8;16.6]	13.2 [10.6;17]	
QT [ms]	42 [36.4;45.8]	43.3 [37.9;46.2]	52.1 [35.4;86.9]	52 [29.5;74.2]	53.5 [40.7;75.7]	
RR [ms]	79 [73.7;92.9]	80.2 [76;86.1]	144.4 [117.3;205]	136.2 [113.2;176.7]	136.2 [108.9;166.1]	
ST [ms]	30.8 [13.1;35.8]	32.3 [27;35.3]	40 [24.3;78]	38.5 [31.9;58.3]	42.8 [28.3;63.1]	
QTc Mitchell [ms]	47.4 [41.7;51.1]	48.5 [42.5;51.5]	42.4 [29.9;65.5]	45 [25;62.8]	45.2 [36.4;60]	

b **MALE**

	Conscious		*Isoflurane*		*Tribromoethanol*	
	EA	LA	EA	LA	EA	
Parameter	median [95% range]	median [95% range]	median [95% range]	median [95% range]	median [95% range]	
Heart Rate [bpm]	764 [661;819.7]	751 [702.3;796.3]	422.6 [298;534.9]	443.1 [325.1;553.7]	414.1 [329.9;573.1]	No LA data available
PR [ms]	28.6 [20.4;35]	25.8 [18.2;34.8]	43.2 [32.5;63.6]	44.3 [34;54.9]	47.7 [41.9;55.7]	
QRS [ms]	10.6 [8.3;14.6]	11.8 [8.6;16.1]	10 [7;19.7]	10 [7;17.3]	13.4 [10.8;16.8]	
QT [ms]	41.8 [36.6;45.5]	43.3 [38;46.6]	52.8 [37.9;89.2]	51.2 [34.6;64.8]	55.2 [42.6;76.6]	
RR [ms]	78.6 [73.2;91.4]	79.9 [75.4;86.1]	142 [112.2;201.6]	135.4 [108.4;184.6]	145 [104.7;181.7]	
ST [ms]	30.6 [12.6;35.6]	32.1 [27.6;35.3]	40.7 [26.9;79.3]	38.7 [35.8;41.9]	45.2 [32.4;65.9]	
QTc Mitchell [ms]	47.5 [41.9;51]	48.7 [42.8;51.8]	43.4 [32.6;64.9]	45.4 [32.7;58]	46 [39.3;62.8]	

Table 3:

a **FEMALE**

	Conscious vs Isoflurane	Conscious vs Tribromoethanol
Heart Rate [bpm]	F(1)=120674.4, p<.001	F(1)=2122.7, p<.001
PR [ms]	F(1)=21279, p<.001	F(1)=872.8, p<.001
QRS [ms]	F(1)=78.1, p<.001	F(1)=273.2, p<.001
QT [ms]	F(1)=1754.3, p<.001	F(1)=135, p<.001
RR [ms]	F(1)=72292.8, p<.001	F(1)=839, p<.001
ST [ms]	F(1)=3278.4, p<.001	F(1)=285.3, p<.001
QTc Mitchell [ms]	F(1)=221, p<.001	F(1)=8.3, p=.004

b **MALE**

Conscious vs Isoflurane	Conscious vs Tribromoethanol
F(1)=116569.4, p<.001	F(1)=2865.6, p<.001
F(1)=23952.9, p<.001	F(1)=1142.3, p<.001
F(1)=71.6, p<.001	F(1)=294.8, p<.001
F(1)=2067.7, p<.001	F(1)=217.7, p<.001
F(1)=64552.1, p<.001	F(1)=1468.3, p<.001
F(1)=4149.9, p<.001	F(1)=424.1, p<.001
F(1)=88.9, p<.001	F(1)=8.3, p=.004

Figure 1:

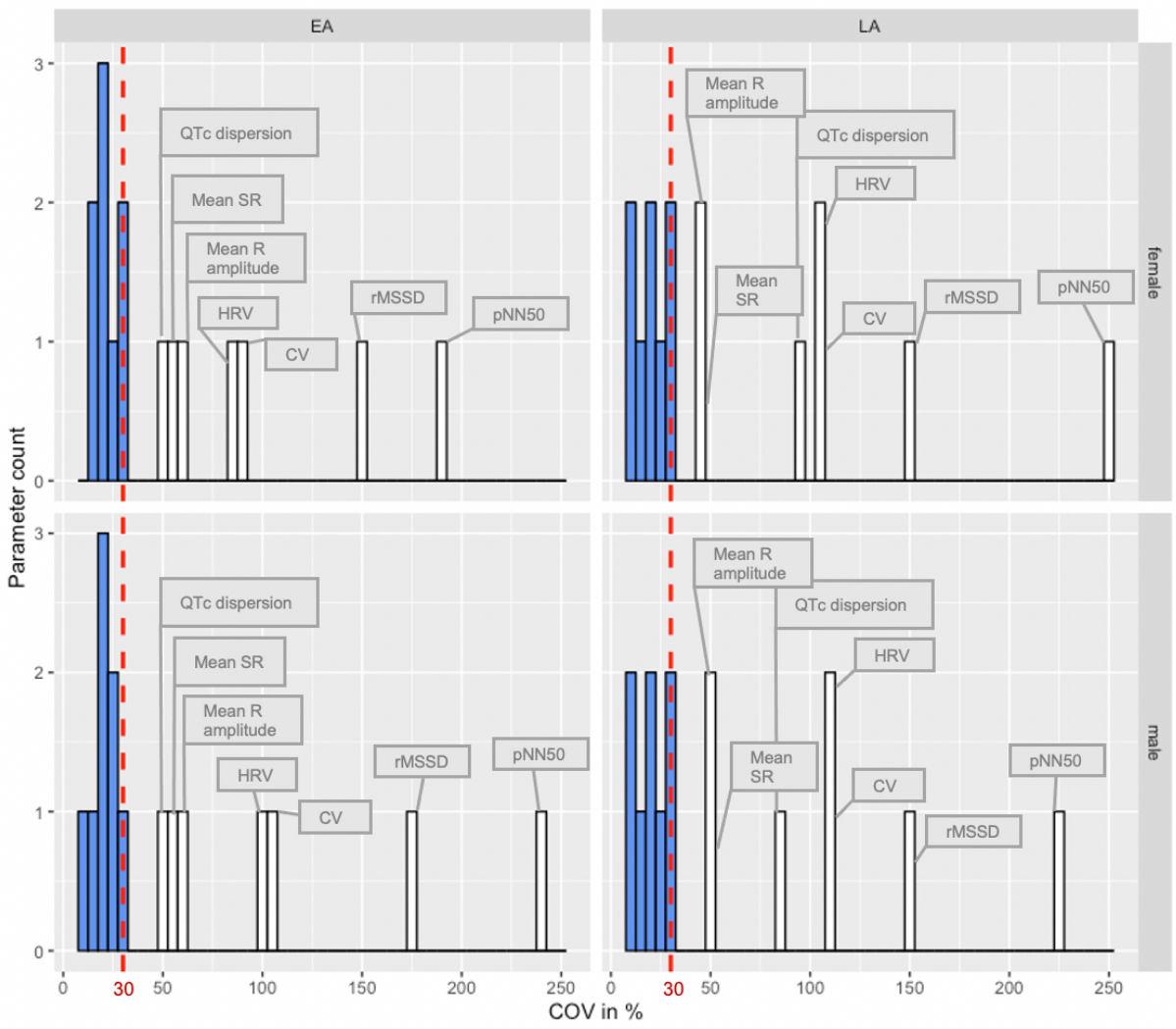


Figure 2:

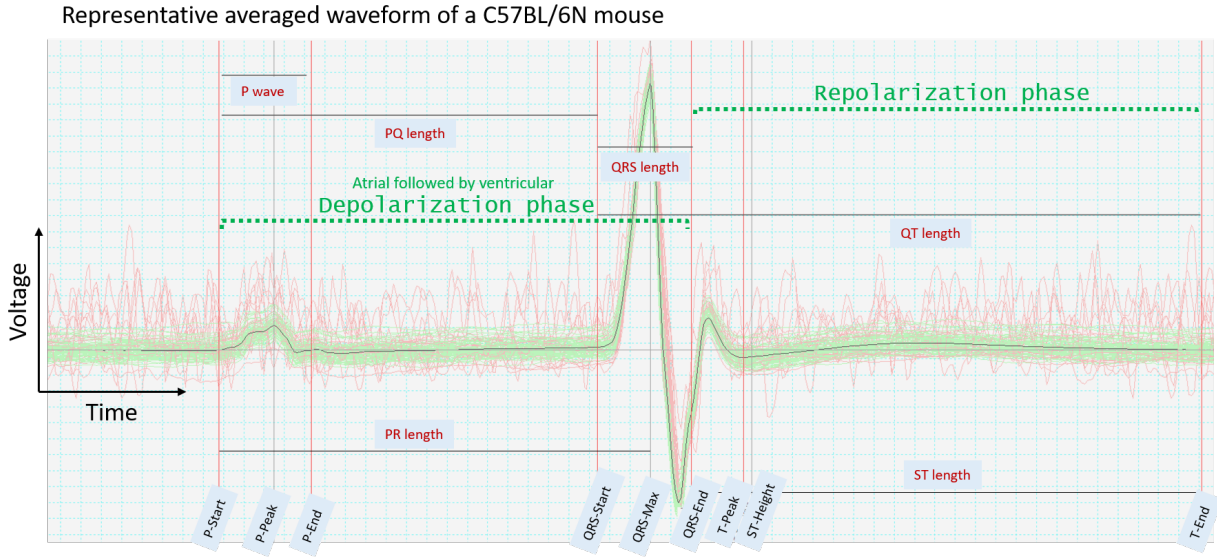
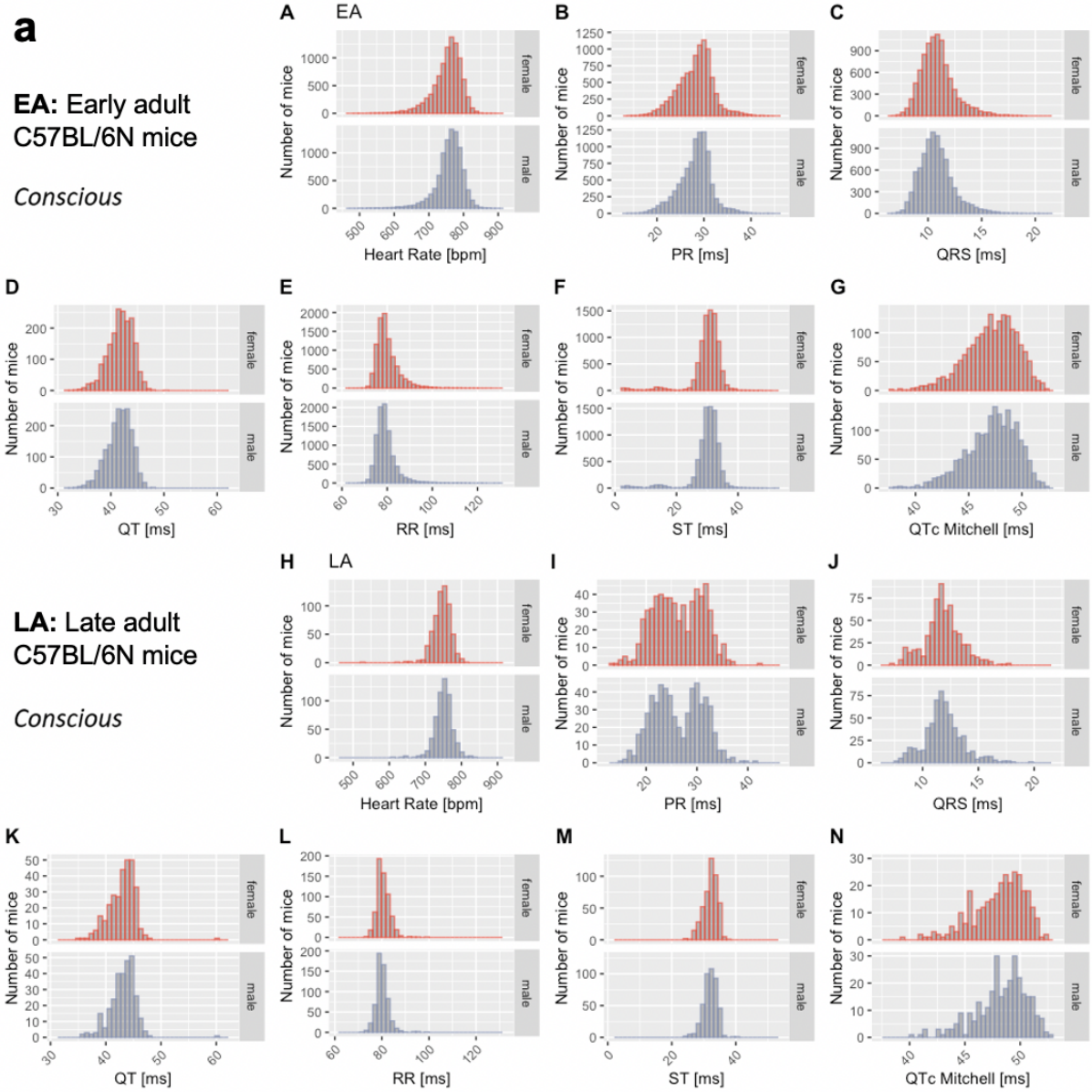


Figure 3:


a
EA: Early adult C57BL/6N mice
Conscious
LA: Late adult C57BL/6N mice
Conscious
A EA
B
C
D
E
F
G
H LA
I
J
K
L
M
N
Number of mice
female
male
Heart Rate [bpm]
PR [ms]
QRS [ms]
QT [ms]
RR [ms]
ST [ms]
QTc Mitchell [ms]

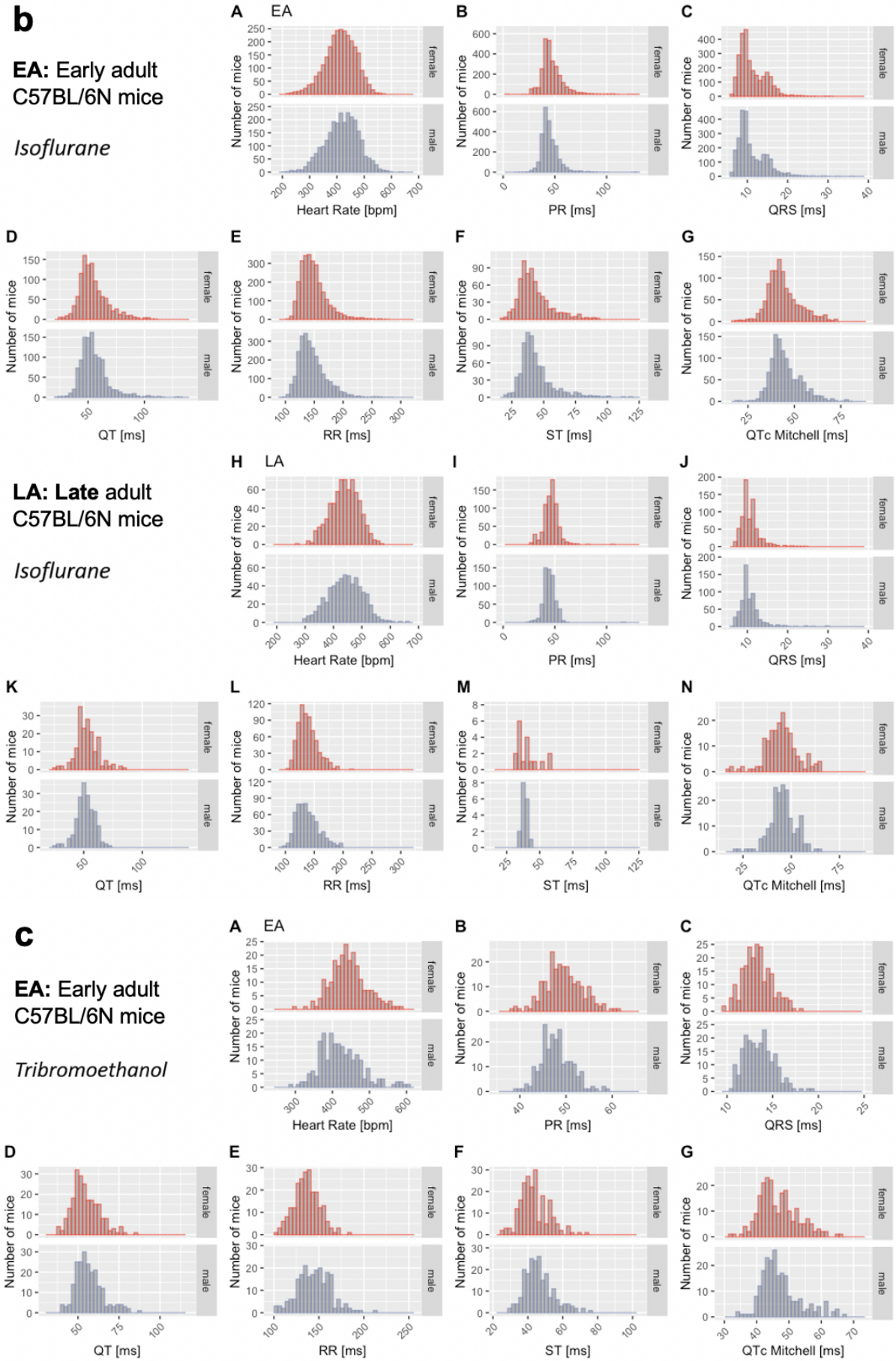

b
EA: Early adult C57BL/6N mice
Isoflurane
LA: Late adult C57BL/6N mice
Isoflurane
c
EA: Early adult C57BL/6N mice
Tribromoethanol
A EA
B
C
D
E
F
G
H LA
I
J
K
L
M
N
Number of mice
female
male
Heart Rate [bpm]
PR [ms]
QRS [ms]
QT [ms]
RR [ms]
ST [ms]
QTc Mitchell [ms]

Figure 4:

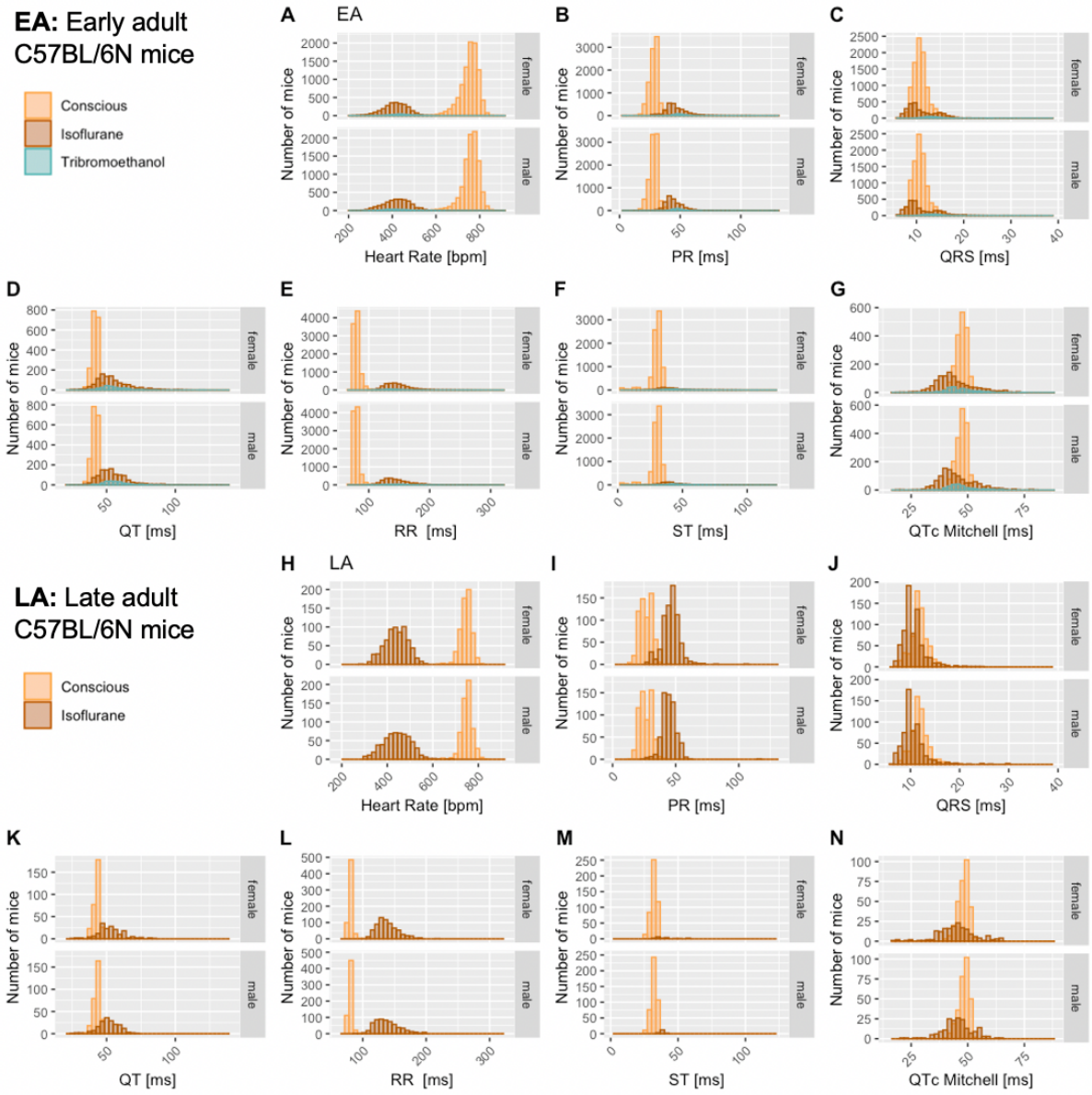

EA: Early adult C57BL/6N mice
Conscious
Isoflurane
Tribromoethanol
A EA
B
C
D
E
F
G
LA: Late adult C57BL/6N mice
Conscious
Isoflurane
H LA
I
J
K
L
M
N
Number of mice
female
male
Heart Rate [bpm]
PR [ms]
QRS [ms]
QT [ms]
RR [ms]
ST [ms]
QTc Mitchell [ms]

Figure 5:

a **FEMALE**

	p value	Cohen's D
Heart Rate [bpm]	p<.001	0.18 (negligible)
PR [ms]	p<.001	0.51 (medium)
QRS [ms]	p<.001	-0.58 (medium)
QT [ms]	p<.001	-0.48 (small)
RR [ms]	p<.001	-0.1 (negligible)
ST [ms]	p<.001	-0.42 (small)
QTc Mitchell [ms]	p<.001	-0.39 (small)

b **MALE**

	p value	Cohen's D
Heart Rate [bpm]	p<.001	0.22 (small)
PR [ms]	p<.001	0.51 (medium)
QRS [ms]	p<.001	-0.68 (medium)
QT [ms]	p<.001	-0.65 (medium)
RR [ms]	p<.001	-0.15 (negligible)
ST [ms]	p<.001	-0.42 (small)
QTc Mitchell [ms]	p<.001	-0.47 (small)

c

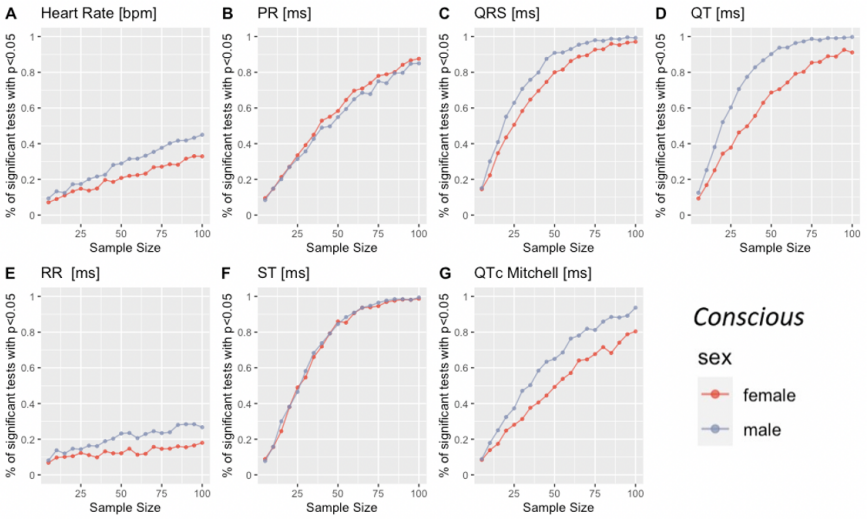


Figure 6:

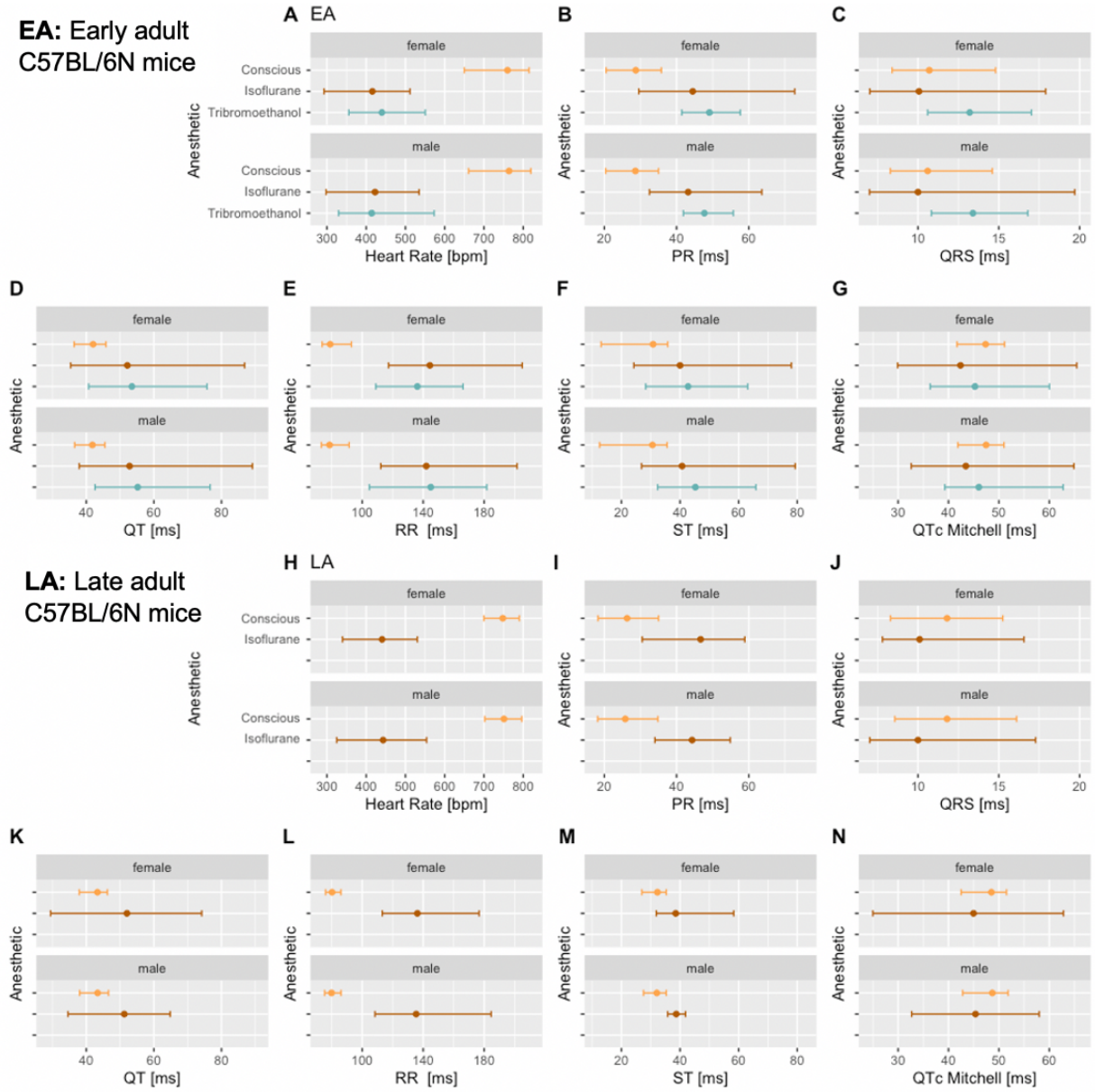
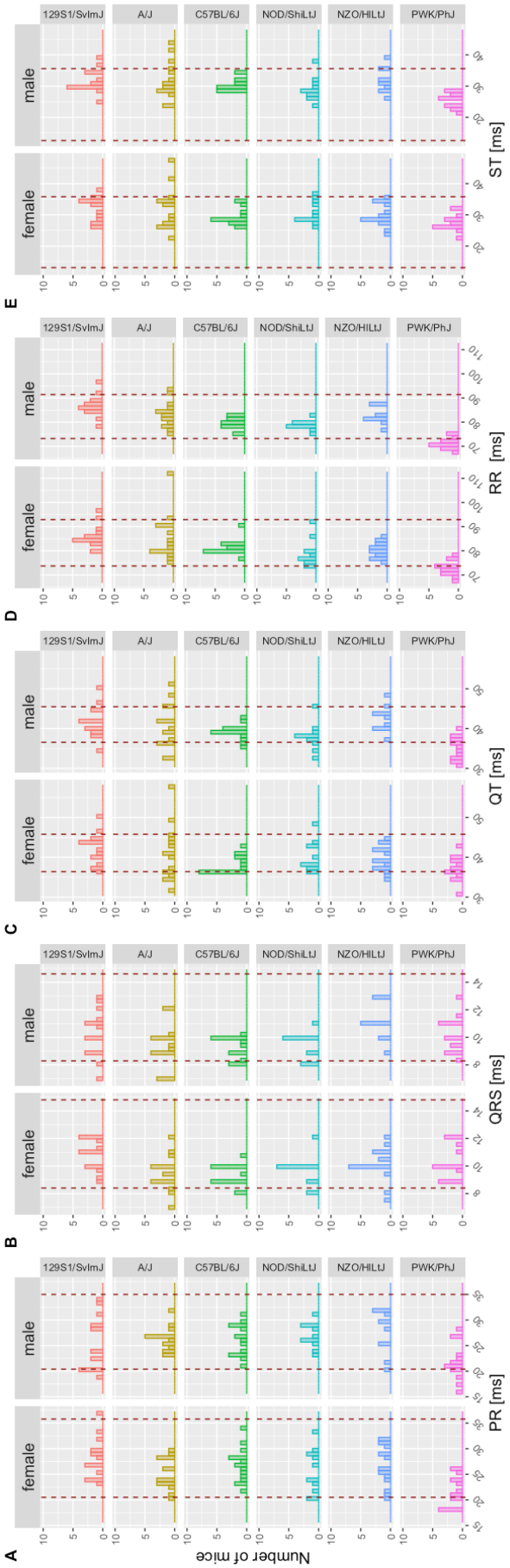

EA: Early adult C57BL/6N mice
LA: Late adult C57BL/6N mice
A EA
B
C
D
E
F
G
H LA
I
J
K
L
M
N
female
male
Anesthetic
Conscious
Isoflurane
Tribromoethanol
Heart Rate [bpm]
PR [ms]
QRS [ms]
QT [ms]
RR [ms]
ST [ms]
QTc Mitchell [ms]

Figure 7:


A
B
C
D
E
Number of mice
PR [ms]
QRS [ms]
QT [ms]
RR [ms]
ST [ms]
male
female
129S1/SvImJ
A/J
C57BL/6J
NOD/ShiLtJ
NZO/HlLtJ
PWK/PhJ

Supplementals