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Niya Xiong Date

Assessing Impacts of Strain and Bedding type of Mice on Ambulation in Circadian Rhythm Test by Applying Linear and non-Linear Mixed Model, Poisson Mixed Model and Zero-Inflated Poisson Mixed Model

By

Niya Xiong

Master of Science in Public Health

Biostatistics and Bioinformatics

_____

Renee H. Moore, PhD
Committee Chair

_____

Shraddha I. Cantara, DVM, MS
Committee Member

_____

Vanessa Lee, DVM, DACLAM
Committee Member

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By

Niya Xiong

M.Eng

University of Electronic Science and Technology of China

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Thesis Committee Chair: Renee H. Moore, PhD

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Abstract

Assessing Impacts of Strain and Bedding type of Mice on Ambulation in Circadian Rhythm Test by Applying Linear and non-Linear Mixed Model, Poisson Mixed Model and Zero-Inflated Poisson Mixed Model

By Niya Xiong

Introduction: Circadian rhythm is the 24h biological cycle to facilitate an organism for daily environmental changes. It influences a broad range of biological processes, including neuronal, metabolic, and behavioral function. Circadian rhythm disruption may lead to acute and chronic impacts in behavior, wellbeing and health. Healthy laboratory animals with regular circadian rhythm are very crucial as appropriate models for research. In this study, the main goal is to examine whether mice strain type or cage bedding type will influence circadian rhythm behavior.

Methods: Number of ambulations was utilized to quantify mice activity. Linear and non-linear mixed model, Poisson mixed model and zero-inflated Poisson mixed model were introduced to assess effects of strain type, bedding type, time on mice ambulations in three different time periods, 0 ~ 23 h, initial three hours and dark phase.

Conclusions: For entire data (23 hours) analysis, B6 mice had higher activity compare to 129 mice, while when placing in 1/8 inch corn cob bedding cage would increase mice ambulations in contrast to 1/4 inch. Trends of strain type was similar when analyzing initial three hours data, however, 1/8 inch corn cob bedding type would decrease mice activity in this case. Results of dark phase analysis was unlike that of 23 hours or initial three hours. B6 mice still had higher activity while bedding type is not a significant risk factor of mice ambulations.

Keywords: Circadian Rhythm, Strain Type, Bedding Type, Linear or non-linear Mixed Model, Poisson Mixed Model, Zero-Inflated Poisson Mixed Model

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1. Introduction

The circadian rhythm is regulated by circadian time-keeping system which evolved from cyanobacteria to humans. Circadian rhythms are 24 hours rhythms to facilitate creatures to optimally adjust their behavior, metabolism, and physiology with the external world. At the molecular level, cell autonomous transcription-translation feedback loops (TTFLs) regulates circadian rhythm which contains the transcription factors CLOCK and BMAL1 (1). Abnormal circadian rhythm is associated with many medical conditions including increased risk for lung tumorigenesis (2), behavioral despair (3) and Parkinson's disease (4). In order to get accurate study conclusions, healthy laboratory animals are very crucial as appropriate models to study such medical conditions affecting humans.

Bedding type directly affects the health and wellbeing of these animals as it is an important component of laboratory rodent housing. For example, corncob bedding has features of high absorbency, ability to minimize detectable ammonia, and low cost. However, mice eat the corn cob which lead to inaccurate conclusion when conducting dietary studies (5). Corncob, cellulose, recycled paper, and Nestpak bedding are common bedding types available for lab rodent housing (6). Different types of bedding may lead to changes in experimental results and health status of animals which could be induced by dust and particulates. Besides, Buddaraju *et al.* (7) investigated that bedding alters drug metabolism and aspects of endocytosis of rats. Ammonia is generated in cages when bacteria break down the urea in rodent urine to NH_3 and CO_2. High intra-cage ammonia levels can cause subclinical degeneration and inflammation of nasal passages and olfactory epithelial necrosis in exposed mice (8). Ammonia levels are considered the single most important factor in determining the frequency of cage cleaning (9). Choosing appropriate

bedding will provide sufficient urine absorption and bacterial regulation to minimize ammonia production thereby reducing costs and improving animal welfare. Few studies have been conducted about finding the association between bedding type and behavioral assays like circadian rhythm monitoring in the past. Moehring *et al.* (10) revealed bedding material affects baseline mechanical paw withdrawal thresholds, noxious responses to a needle stimulus, and heat sensitivity of mice two weeks after housing the animals on the bedding. Increased aggressive behavior was observed on cardboard-based bedding compare to corncob bedding due to high level of estrogens in the cardboard-based bedding (11). Mice have a characteristic nocturnal pattern and sleep more during photophase (light phase) than during scotophase (dark phase) (12). C57BL/6J and 129S1/Svlm are commonly primary strains used in these behavioral tests. Male mice were used in this study because they are much more commonly used in behavior studies than females, and they have more urine which lead to higher ammonia levels in cages.

The goal of this study is to check if there is any circadian rhythm behavior difference for mice housed on 1/4 inch versus 1/8 inch corn cob bedding. Another variable that is considered in this study is the strain of mouse, as different strains of mice can have variable behavior patterns. . Linear and non-linear mixed model, poisson mixed model and zero-inflated poisson mixed model were employed to assess the relationship between bedding type, strain type and circadian rhythm behavior in different period of time within a day. The ultimate goal is to compare different models and find the most appropriate model for precise estimates and interpretability in the context of this study.

2. Methods & Materials

2.1 Animals and housing conditions

There were ten male mice C57BL/6J (B6 mice) (JAX stock #000664) and ten male mice 129S1/Svlm (129 mice) (JAX stock #002448) conducted circadian rhythm test. All of them were obtained at four weeks of age from Jackson Labs (Bar Harbor, Maine) and Charles River Laboratories (Wilmington, Massachusetts). All animals were housed in an AAALAC, International accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All animal procedures were reviewed and approved by Emory University's Institutional Animal Care and Use Committee. Mice were housed in cages (Lab Products, Zyfone 750-Super Mouse cage, model no. 75031) with either 1/4 inch or 1/8 inch corn cob bedding (Bed-O-Cobs, The Andersons, Maumee, OH). Cages were changed once a week and were kept in rooms maintained at 30-70% relative humidity, 72º F temperature and 12:12 hour light:dark cycle.

2.2 Circadian Rhythm Test

Before conducting circadian rhythm test, animals were kept in their original groups and acclimated to their environment for six weeks. Animals were placed in plexiglass activity cages with either 1/4 inch or 1/8 inch corn cob bedding and unlimited supply for food and water for 23 hours. To evaluate ambulation, consecutive beam breaks from infrared photobeams (San Diego Instruments) were counted by a computer for 23 hours (10am-9am, dark phase 7pm-7am) and number of ambulations were recorded.

2.3 Data Summary

This study was a longitudinal study that followed animals from 10am through next day 9am. The movement of each mice was recorded as the number of beam interruptions and

reported as ambulation. Higher number of ambulation indicated higher activity of mice. Mice are nocturnal rodents, therefore if we detected abnormal high ambulation in daytime, then we consider circadian rhythm might be disrupted. Number of ambulation of each mouse were obtained every 30 minutes, and we considered 7pm to 7am was dark phase. Two independent variables of strain and bedding type were treated as categorical variables and detection time was regarded as a continuous variable in models. Mice were place in cages for real-time observation at 10am, then first record would be obtained at 10:30 am. In order to change detection time to continuous variable, 10:30 am was converted as 0.0 hour, 11:00 am was converted as 0.5 hour, and so on. 129 and B6 were two strain types for mice, 1/4 inch and 1/8 inch were two different size of corn cob bedding. The outcome of this study was number of ambulations for mice, which was incorporated as a continuous variable or a count variable in corresponding models.

2.4 Statistical Analysis

Figuring out the potential factors that affect the mice activities in day and night is the main goal in this study. We aim to identify models which predict accurately and interpret easily. Before conducting regression analyses, we checked dispersion of data by computing coefficient of variation.

2.4.1 Fitting Models for Cross-Sectional Data

To estimate cross-sectional determinants of ambulation, we computed means or medians of ambulation in each time point as dependent variable and applied linear and non-linear models with or without three-way interaction of strain, bedding type and time. ANOVA was performed to access the necessity of interaction.

2.4.2 Fitting Models for 23 Hours Longitudinal Data

Linear and non-linear mixed models and zero-inflated Poisson mixed models were employed to fit 23 hours longitudinal data. Fit statistics were identified as Akaike information criterion (AIC), Bayesian information criterion (BIC), Log likelihood and deviance. Degree of freedom (df), deviance and R-squared could not be assessed for zero-inflated models. Diagnostic plots of Model 2(c) were generated by using SAS (Version 9.4; SAS Institute Inc, Cary, NC) (Figure S3). Pearson correlation was computed to assess the accuracy of prediction.

2.4.3 Fitting Models for Initial 3 Hours Longitudinal Data

Linear mixed models and Poisson mixed models were utilized to fit initial 3 hours longitudinal data. R-square from each Poisson mixed model were conditional which described the proportion of variance explained by both the fixed and random factors. Diagnostic plots of Model 4(a) and Model 5(b) (Table 1) were generated by using SAS (Figure S4, S5). Pearson correlation was computed to assess the accuracy of prediction.

2.4.4 Fitting Models for Dark Phase Longitudinal Data

Original data with detection time from 7pm to 7am was extracted to create new data to conduct analyses. 7pm was converted as 0.0 hour, 7:30 pm was converted as 0.5 hour, and so on. The largest value of time should be 12 hours. 3:30 am was the turning point from Figure 3, that is 8.5 hours in this circumstance. Linear and non-linear mixed models and poisson mixed models were performed to analyze dark phase longitudinal data. Diagnostic plots of Model 5 were generated by using SAS (Figure S6). Pearson correlation was computed to assess the accuracy of prediction.

2.5 Statistical Consideration

Diagnostic plots of residuals were used to evaluate model assumptions for linear regression models. Statistical analyses were conducted by R and SAS. Tables and graphs were created by R except diagnostic plots that we specifically mentioned. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Cross-Sectional Data Analysis of the Association between Strain type, Bedding type, Time and Number of Ambulation

$$\text{Model 1(a)}: y_{median} = \beta_0 + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \epsilon_i \quad \epsilon_i \overset{iid}{\sim} N(0, \sigma^2)$$

$$\text{Model 1(b)}: y_{median} = \beta_0 + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 x_{strain} * x_{bedding} + \beta_5 x_{strain} * x_{time} + \beta_6 x_{bedding} * x_{time} + \beta_7 x_{strain} * x_{bedding} * x_{time} + \epsilon_i \quad \epsilon_i \overset{iid}{\sim} N(0, \sigma^2)$$

In this study, ten mice were detected in real time for 23 hours, and number of ambulations were recorded for every 30 minutes. To check the repeatability of these ten mice, we computed coefficient of variations for each time point and found most of coefficient of variations were less than 100% which implied repeatability was good (Figure S1). First, we analyzed the data as cross-sectional by utilizing median or mean of ten mice ambulations as the outcomes at each time point. No distinct difference between scatter of mean and median ambulations could be observed in Figure 1; therefore, we chose median ambulations as the dependent variable in cross-sectional analysis since it was more robust. In Table 2, when not considering three-way interaction among strain, bedding and time (Model 1(a)), mice would have 304.25 times (95% CI: 241.20 ~ 367.31, P-value < 0.001) more ambulations with B6 strain as compared to 129 strain, while no significant effect of

bedding type. Ambulation also decreased 15.37 times (95% CI: -20.12 ~ -10.62, P-value < 0.001) as one hour went on which was reasonable since mice were more adaptable in the cage over time. P-values of all interactions were greater than 0.05 in Model 1(b) which suggests there was no interaction between strain, bedding type and time. Adjusted R-squared of Model 1(a) was 0.41, which room for improvement in goodness of fit. This combined with the possibility of non-linearity of time from Figure 1 drew us to consider:

$$\text{Model 1(c)}: y_{median} = \beta_0 + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 (x_{time} - 8.5)_+ + \beta_5 (x_{time} - 9.5)_+ + \epsilon_i \quad \epsilon_i \overset{iid}{\sim} N(0, \sigma^2)$$

Generalized addictive model was used to assess the non-linearity of time points and observed apparently non-linear trend over time from Figure 2. Piecewise spline with knots at 8.5 hours and 9.5 hours were included in Model 1(c). These two knots were turning points from Figure 3. The expected mean difference in number of ambulations between the B6 and 129 mice was about 304.25 times (95% CI: 259.90 ~ 348.60, P-value < 0.001) in Table 2. Similar with Model 1(a) and 1(b), bedding type was a not significant factor of ambulations. In first 8.5 hours, time was negatively associated with ambulations with 86.05 (95% CI: -99.90 ~ -72.20, P-value < 0.001) and then positively related to ambulations in subsequent one hour with 650.90 (95% CI: 550.24 ~ 751.56, P-value < 0.001). At last 13.5 hours, number of ambulations decreased again with 602.23 (95% CI: -697.67~ -506.79, P-value < 0.001) per hour. Adjusted R-squared was dramatically escalated to 0.69 and diagnostics plots were good (Figure S2), suggesting piecewise spline non -linear model is a better fit than the linear models (1a, 1b).

3.2 Longitudinal Data Analysis of the Association between Strain type, Bedding type, Time and Number of Ambulation (23 hours)

$$\text{Model 2(a)}: y_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

$$\text{Model 2(b)}: y_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 x_{strain} * x_{bedding}$$
$$+\beta_5 x_{strain} * x_{time} + \beta_6 x_{bedding} * x_{time} + \beta_7 x_{strain} * x_{bedding} * x_{time} + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

For longitudinal data analysis, random intercept linear mixed model (Model 2) was selected to examine the effect of strain and bedding type on number of ambulations. In Table 3, without interaction terms (Model 2(a)), number of ambulations increased 313.50 times (95% CI: 289.53 ~ 337.48, P-value < 0.001) with B6 strain as compared to 129 strain and 1/8 inch bedding type was associated with 28.44 (95% CI: 4.46 ~ 52.41, P-value = 0.02) in estimated ambulations compared to 1/4 inch. Similarly, for each increasement of an hour, number of ambulations reduced 15.59 times (95% CI: -17.40 ~ -13.79, P-value < 0.001). When adding three-way interaction terms of strain type, bedding type and time in Model 2(b), trends of strain and time were similar, while bedding type was not significant related to mice activity. Interaction terms except bedding type and time were all significant (P-value < 0.01), which indicated the interaction between strain and bedding type was dependent on time. P-value of ANOVA between Model 3a and Model 3b was less than 0.01 which suggested necessary of three-way interaction in model. R-squared values of Model 2(a) and (b) were 0.34 and 0.37 respectively (Table S1), therefore it might indicate piecewise spline should be added to model.

$$\text{Model 2(c)}: y_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 (x_{time} - 8.5)_+$$
$$+\beta_5 (x_{time} - 9.5)_+ + \epsilon_{ij} \quad \theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

We still utilized 8.5 hours and 9.5 hours as piecewise knots in Model 2(c). Since we already had five independent variables in Model 2(c), interaction would not be considered in case model would be too complicated. The estimated difference in number of ambulations between the B6 and 129 mice was about 313.50 times (95% CI: 293.38 ~ 333.63, P-value < 0.001) (Table 3). 1/8 inch bedding type was associated with 28.44 (95% CI: 8.31~ 48.56, P-value = 0.006) times in estimated ambulations compared to 1/4 inch. These two estimated coefficients were exactly same with Model 2(a). Mice activity significantly reduced at first 8.5 hours with 84.25 times per hour (95% CI: -90.34 ~ -78.15, P-value < 0.001), escalated until 9.5 hours with 621.86 times per hour (95% CI: 577.57 ~ 2666.14, P-value < 0.001) and then reduced again with 573.51 times per hour (95% CI: -615.50 ~ -531.53, P-value < 0.001). AIC, BIC, log likelihood and deviance of Model 2(b) and Model 2(c) were close to each other (Table S1), however R-squared of Model 3c was 0.54. Also the distribution of studentized residual was approximately normal distributed and most of dots in QQ plot were align on the diagonal line (Figure S3). These evidences implied the Model 2(c) was appropriate for this data.

Now we treat number of ambulations as a count variable in Poisson models. First the linear zero-inflated Poisson mixed model 3(a):

$$\text{Model 3(a)}: P(y=j) = \begin{cases} \pi_i + (1-\pi_i)exp(-\mu_i) & if \quad j=0 \\ (1-\pi_i)\dfrac{\mu_i^{y_i} exp(-\mu_i)}{y_i!} & if \quad j>0 \end{cases}$$

$$log\lambda_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \epsilon_{ij}$$

$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

The response variable in this study was number of ambulation which was a count variable, therefore we considered Poisson regression would be more appropriate to fit the model. However, a large number of zeros were observed in ambulation for each mouse

(Figure 3(a)), zero inflated poisson mixed model (ZIP) might be a way to solve problem. Response variable was separated into two groups, one group with zero probability of a count greater than 0 (Zero-Inflation model) and another group whose counts were generated by the standard Poisson regression model (Count model). Observed values of zero could generate from either group (13).

$$\text{Model 3(b)}: P(y=j) = \begin{cases} \pi_i + (1-\pi_i)exp(-\mu_i) & if \quad j=0 \\ (1-\pi_i)\dfrac{\mu_i^{y_i} exp(-\mu_i)}{y_i!} & if \quad j>0 \end{cases}$$

$$log\lambda_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 x_{strain} * x_{bedding}$$
$$+\beta_5 x_{strain} * x_{time} + \beta_6 x_{bedding} * x_{time} + \beta_7 x_{strain} * x_{bedding} * x_{time} + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

$$\text{Model 3(c)}: P(y=j) = \begin{cases} \pi_i + (1-\pi_i)exp(-\mu_i) & if \quad j=0 \\ (1-\pi_i)\dfrac{\mu_i^{y_i} exp(-\mu_i)}{y_i!} & if \quad j>0 \end{cases}$$

$$log\lambda_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 (x_{time} - 8.5)_+$$
$$+\beta_5 (x_{time} - 9.5)_+ + \epsilon_{ij} \quad \theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

In count model of Model 3(a), among those whose strain was B6, number of ambulations significantly increased 3.90 times (95% CI: 3.87 ~ 3.92, P-value < 0.001) compared with 129 strain type (Table 4). The expected mean of ambulations was multiplied by 1.18 times (95% CI: 1.18 ~ 1.19, P-value < 0.001) when strain was 1/8 inch compared to 1/4 inch. When increased one hour, ambulations would decrease incident rate as 0.9514 times (95% CI: 0.951 ~ 0.952, P-value < 0.01). Likewise, three-way interactions were included in Model 3(b), the trends of strain type and time were similar, but bedding type switched to negatively associated with mice activity. P-values of all interaction terms were less than 0.001 which suggested necessity of interactions. Trends of strain and bedding type were same with Model 3(a), and time was positively related to mice activity during 8.5 hours to 9.5 hours, and negatively related in other time. AIC and BIC of Model 3(a) were relatively

smallest among all three zero-inflated poisson mixed models (Table S1). In order to further chose better one between non-linear mixed model 2(c) and zero-inflated poisson mixed model 3(a), we plotted relationship between predicted outcome and actual outcome (Figure 5). We found Model 2(c) was more accurate when ambulation count was large while Model 3(a) was better when count was close zero. The Pearson correlations of Model 2(c) and 3(a) were 0.73 and 0.61 which implied non-linear mixed model 2(c) was preferable in general.

3.3 Longitudinal Data Analysis of the Association between Strain type, Bedding type, Time and Number of Ambulation (Initial three hours)

It is known that mice are nocturnal and more active in night time. However, Figure 3 showed mice were in apparently more active condition in initial three hours compared to dark phase. The investigators believe this is because once the mice were placed into a new cage, they immediately started real-time exploration of their new environment. As they became more familiar with the environment, their decreased activity. Therefore, we sought to determine whether strain and bedding type would influence ability of mice adapting to new environment.

Linear and non-linear mixed model 4(a) and 4(b) were applied to analyze the potential relationship between strain, bedding, time and ambulation in initial three hours. Model 4(a) and 4(b) were same with Model 2(a) and 2(b) except for using initial 3 hours data instead of 23 hours. 610.83 (95% CI: 562.41 ~ 660.24, P-value < 0.001) was the average difference in number of ambulations between 129 while B6 strain type and bedding type was not significant factor of mice activity (Table 5). Time was strongly negatively related to number of ambulations with 189.98 (95% CI: -218.92 ~ -161.05, P-value < 0.001) per hour during initial three hours. Bedding type changed as a significant factor when introducing

three-way interaction, whereas only strain and bedding interaction term was significant. Piecewise spline was not necessary for initial three hours data, since no obvious turning point from Figure 3. Although Model 4(b) had better fit statistics compare to Model 4(a), most of interaction terms were not significant (Table S2). Diagnostic plots of Model 4(a) suggested data met model assumption and it could be utilized to conduct analysis (Figure S4).

$$\text{Model 5(a)}: y_{ij}|\theta_i \sim Poisson(\lambda)$$
$$log\lambda_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{phase} + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_i \overset{iid}{\sim} N(0, \sigma^2)$$

$$\text{Model 5(b)}: y_{ij}|\theta_i \sim Poisson(\lambda)$$
$$log\lambda_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 x_{strain} * x_{bedding}$$
$$+\beta_5 x_{strain} * x_{time} + \beta_6 x_{bedding} * x_{time} + \beta_7 x_{strain} * x_{bedding} * x_{time} + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_i \overset{iid}{\sim} N(0, \sigma^2)$$

Similarly, Since the outcome was a count variable, we tested the possibility of Poisson mixed regression. From Figure 6, only two mice had small proportion of zeros in outcome during initial three hours, which suggested regular Poisson mixed regression should be adequate. Poisson models with (Model 5(a)) or without (Model 5(b)) three-way interactions were conducted. We found that all two-way interaction terms but not the three-way, bedding and time were significant from Table 6. Rate ratio between B6 and 129 strain was expected as 1.51 times (95% CI: 1.47 ~ 1.54, P-value < 0.001). 1/8 inch compared to 1/4 inch were expected to have a rate 0.76 times (95% CI: 0.73 ~ 0.78, P-value < 0.001) greater for number of ambulations. The estimated mean of ambulations was multiplied by 0.44 times (95% CI: 0.34 ~ 0.35, P-value < 0.001) when we increased time by 1 hour. Interaction term of strain and bedding type was significant correlated with ambulation and it might suggest the effect of strain was different between 1/8 inch and 1/4 inch bedding

type. Model 5(b) had notably smaller AIC and BIC (Table S2), and distribution of conditional studentized residuals was approximately normal (Figure S5). Therefore, Model 4(b) was more preferable in contrast to Model 5(a). To further explore the better one from linear mixed model 4(a) and Poisson mixed model 5(b), we checked predictive accuracy by plotting (Figure 7). Both models performed high accuracy; Pearson correlations of Model 4(a) and 5(b) were 0.88 and 0.91. Model 5(b) is appropriate to assess influence of strain and bedding type on ability of mice adapting new environment in initial three hours.

3.4 Longitudinal Data Analysis of the Association between Strain type, Bedding type, Time and Number of Ambulation (Dark Phase, 8.5h ~20.5h)

Circadian rhythm is known dramatically different between daytime and night and mice are nocturnal rodents, accordingly, we desired to investigate the effects of strain and bedding type on mice activity during the night. 7pm to next day 7am was identified as dark phase, that is 8.5 hours to 20.5 hours in this study.

First, we performed linear mixed model with (Model 6(a)) or without interaction (Model 6(b)) and found all interaction terms were not significant except for strain and time. Model 6(a) and 6(b) were same with Model 2(a) and 2(b) except for using dark phase data instead of 23 hours. In Model 6(a), being B6 mice increased the number of ambulations by 330.51 (95% CI: 302.55 ~ 358.48, P-value < 0.001) in contrast to 129 strain type, meanwhile, bedding type would not significantly change mice activity (Table 7). Time was negatively linked with ambulation as 27.25 (95% CI: -31.13 ~ -23.38, P-value < 0.001) per hour. R-squared values of Model 6(a) and 6(b) were 0.43 and 0.44 (Table S3).

There was an obvious turning point for B6 type mice during 8.5 hours to 20.5 hours, hence piecewise spline might be needed in this circumstance. The reason we chose 8.5 hours as knot was explained in Methods and Materials.

$$\text{Model 6(c)}: y_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 (x_{time} - 8.5)_+ + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

Trends of strain and bedding of Model 6(c) were same as Model 6(a) (Table 7). Mice activity significantly reduced at first 8.5 hours with 38.91 times per hour (95% CI: -44.64 ~ -33.18, P-value < 0.001) and then increased with 54.11 times per hour (95% CI: -34.28 ~ 73.94, P-value < 0.001). Fit statistics of Model 6(c) was still not improved after introducing piecewise spline (Table S3). However, we considered Model 6(c) to be a better fit as compare to others because of smaller AIC and BIC, and normally distributed residual plots (Figure S6).

$$\text{Model 7(c)}: P(y = j) = \begin{cases} \pi_i + (1 - \pi_i) exp(-\mu_i) & if \quad j = 0 \\ (1 - \pi_i) \dfrac{\mu_i^{y_i} exp(-\mu_i)}{y_i!} & if \quad j > 0 \end{cases}$$
$$log\lambda_{ij} = (\beta_0 + \theta_i) + \beta_1 x_{strain} + \beta_2 x_{bedding} + \beta_3 x_{time} + \beta_4 (x_{time} - 8.5)_+ + \epsilon_{ij}$$
$$\theta_i \overset{iid}{\sim} N(0, \tau^2), \quad \epsilon_{ij} \overset{iid}{\sim} N(0, \sigma^2)$$

Figure 8a displayed a large proportion of zeros existed in outcome during dark phase, so we considered zero-inflated Poisson mixed model. Model 7(a) and 7(b) were same with Model 3(a) and 3(b) except for using dark phase data instead of 23 hours. In Model 7(a), rate ratio between B6 and 129 strain was expected as 4.22 times (95% CI: 4.19 ~ 4.27, P-value < 0.001). The expected mean of ambulations was multiplied by 1.15 times (95% CI: 1.14 ~ 1.16, P-value < 0.001) when strain was 1/8 inch compared to 1/4 inch. Number of ambulations would decrease incident rate as 0.916 times (95% CI: 0.915 ~ 0.917, P-value < 0.001) per hour. Interaction terms were significant in Model 7(b) which might implied

the necessity for inclusion. Similarly, piecewise spline was applied in Model 7(c). Trends of strain and bedding type were the same with Model 7(a), mice activity was declined before 8.5 hours and escalated consequently. AIC and BIC of Model 7(a) were lowest compare to others (Table S3). Finally, similar with Figure 5, linear mixed model 5 had higher accuracy when number of ambulations was larger, and zero-inflated Poisson mixed model was adept at predicting smaller number of ambulations (Figure 9).

4. Discussion

This study illustrated effects of strain and bedding type were dissimilar in different period of time in circadian rhythm test. Non-linear piecewise model 1(c) would be the best to assess determinants of median of ambulations. B6 mice were more likely to be active compare to 129 mice, while bedding type was not a significant factor for mice activity. Mice tended to decrease activity in initial 8.5 hours (10am ~ 7pm), rise in next hour (7pm ~ 8pm) and then decrease again after that (8pm ~ 9am). Treating the data longitudinally, non-linear mixed model 2(c) with random intercept was more preferable. Trends of strain type and time were similar, while 1/8 inch corn cob bedding would increase mice ambulation in contrast to 1/4 inch when applying 23 hours data. For the purpose of figuring out risk factors of ability to adapt to new environment, Poisson mixed regression with three-way interaction (Model 5(b)) was performed. B6 mice would adapt to new environment quicker than 129 mice. Among mice who were placed in 1/8 inch bedding cage, lower activity could be observed compared to 129 mice. The effect of strain on mice activity in 1/8 inch bedding was different than 1/4 inch. Results of dark phase analysis was unlike that of initial three hours. Results from non-linear mixed model 6(c) indicated bedding type was not a significant indicator for mice ambulation while B6 mice still had

higher activity. Mice were more active in first 8.5 hours (7pm ~ 3:30am) in contrast to other time in dark phase (3:30am ~ 9am).

There were several limitations in study design and statistical analysis. First, ambulations of one mouse with B6 strain and 1/8 inch bedding type were zero from 22pm to 9am. The reason might be the instrument was broken. Since we could not assure whether it was the fault of instrument or the mouse was actually static from 22pm to 9am, original data was still used in this study. However, changing zeros to missing values or refitting models without this mice to see if results differ as presented might be another ways to solve the problem and prevent us to make wrong conclusion. It would be interesting to investigate whether impacts of strain and bedding type were similar in light and dark phase. Nevertheless, light phase data was not adequate to conduct statistical analysis because only data from 2pm to 7pm and 7am to 9am could be applied and the data was not successive. If we included initial three hours data, incorrect deduction might be inferred since high activity in the beginning was abnormal, not the typical behavior for mice in the daytime. Prolonging the time of real-time observation would be great if assessing bedding and strain type effects were a potential study question. A large proportion of coefficient of variations were greater than 100%, although the distribution was left skewed (Figure S1). This might indicate the repeatability of the study was not sufficient and more mice should be involved in study.

Statistical models could be improved in some points. Piecewise knots were selected based on Figure 3, but one might consider there to actually be four turning points. Only two knots 8.5h (7pm) and 9.5 h (8pm) were included in the model for simplifying analysis translation, but it could have been at the sacrifice of predictive accuracy. Fit statistics of

Figure S3 to S6 were generated from SAS. They were slightly different with Table S1 to S3 which were calculated by R. Therefore, all models should be refit in SAS to check if impacts of factors were same. In this case, it was not a big issue since fit statistics were close to each other. There were obvious lines in diagnostic plots of Figure S3 and Figure S6 but not in Figure S4 and S5. This may be due to the many zeros that existed in dark phase over a day but very few in initial three hours. Figure 4b and 8b depicted distribution of ambulations greater than zero, while Figure 4c and 8c depicted distribution of entire data. No distinct difference could be observed no matter whether deleting zeros or not, and that might explain why zero-inflated Poisson regression did not improve goodness of fit. Subset data of ambulations greater than zero still not met assumption of Poisson regression which was mean was equal to variance. To further check the influence of zeros, binomial mixed model could be applied. Besides, variances were heteroscedasticity (Figure S3 and Figure S6) when fitting models for dark phase data and entire data and it violated assumptions of linear or non-linear mixed model. Generalized estimating equation (GEE) could be further used to deal with heteroscedasticity problem.

In conclusion, our study has confirmed a significant effect of strain, bedding type and time on the mice activity. We could choose corresponding strain and bedding type according to the requirements of studies based on this study.

5. Reference

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6. Tables and Figures

Table 1. Model Description

Number	Data	Description
Model 1a	0 ~ 23 hours	Linear Model
Model 1b	0 ~ 23 hours	Linear Model with Three-Way Interaction
Model 1c	0 ~ 23 hours	Non-Linear Model
Model 2a	0 ~ 23 hours	Linear Mixed Model
Model 2b	0 ~ 23 hours	Linear Mixed Model with Three-Way Interaction
Model 2c	0 ~ 23 hours	Non-Linear Mixed Model
Model 3a	0 ~ 23 hours	Linear Zero-Inflated Poisson Mixed Model
Model 3b	0 ~ 23 hours	Linear Zero-Inflated Poisson Mixed Model with Three-Way Interaction
Model 3c	0 ~ 23 hours	Non-Linear Zero-Inflated Poisson Mixed Model
Model 4a	0 ~ 3 hours	Linear Mixed Model
Model 4b	0 ~ 3 hours	Linear Mixed Model with Three-Way Interaction
Model 5a	0 ~ 3 hours	Linear Poisson Mixed Model
Model 5b	0 ~ 3 hours	Linear Poisson Mixed Model with Three-Way Interaction
Model 6a	Dark Phase (8.5h ~ 20.5h)	Linear Mixed Model
Model 6b	Dark Phase, (8.5h ~ 20.5h)	Linear Mixed Model with Three-Way Interaction
Model 6c	Dark Phase (8.5h ~ 20.5h)	Non-Linear Mixed Model
Model 7a	Dark Phase (8.5h ~ 20.5h)	Linear Zero-Inflated Poisson Mixed Model
Model 7b	Dark Phase (8.5h ~20.5h)	Linear Zero-Inflated Poisson Mixed Model with Three-Way Interaction
Model 7c	Dark Phase (8.5h ~20.5h)	Non-Linear Zero-Inflated Poisson Mixed Model

Table 2. Linear and non-linear regression model to estimate cross-sectional determinants of median mice ambulations (23 hours).

Model 1(a)				Model 1(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	247.16	38.72	<0.001	Intercept	201.55	61.07	0.001
Strain (B6)	304.25	31.96	<0.001	Strain (B6)	358.45	86.36	<0.001
Bedding (1/8)	31.34	31.96	0.33	Bedding (1/8)	-47.08	86.36	0.59
Time	-15.37	2.41	<0.001	Time	-9.24	4.68	0.05
Model 1(c)				Strain*Bedding	230.89	122.13	0.06
Intercept	467.44	39.00	<0.001	Strain*Time	-8.96	6.61	0.18
Strain (B6)	304.25	23.18	<0.001	Bedding*Time	2.83	6.61	0.67
Bedding (1/8)	31.34	23.18	0.18	Strain*Bedding *Time	-12.23	9.35	0.19
Time (< 8.5 h)	-86.05	7.02	<0.001				
Time (8.5 ~ 9.5 h)	650.90	51.01	<0.001				
Time (>9.5 h)	-602.23	48.36	<0.001				

Table 3. Linear and non-linear mixed regression model with random intercept to estimate longitudinal determinants of mice ambulations (23 hours).

Model 2(a)				Model 2(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	266.69	16.21	<0.001	Intercept	230.90	24.52	<0.001
Strain (B6)	313.50	12.24	<0.001	Strain (B6)	360.24	33.36	<0.001
Bedding (1/8)	28.44	12.24	0.02	Bedding (1/8)	-52.54	33.36	0.12
Time	-15.59	0.92	<0.001	Time	-10.49	1.81	<0.001
Model 2(c)				Strain*Bedding	211.64	47.18	<0.001
Intercept	482.83	18.79	<0.001	Strain*Time	-8.01	2.55	0.002
Strain (B6)	313.50	10.28	<0.001	Bedding*Time	3.35	2.55	0.19
Bedding (1/8)	28.44	10.28	0.006	Strain*Bedding *Time	-11.11	3.61	0.002
Time (< 8.5 h)	-84.25	3.11	<0.001				
Time (8.5 ~ 9.5 h)	621.86	22.61	<0.001				
Time (>9.5 h)	-573.51	21.44	<0.001				

Table 4. Linear and non-linear zero-inflated poisson mixed regression model with random intercept to estimate longitudinal determinants of mice ambulations (23 hours).

Model 3(a)				Model 3(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	5.10	0.004	<0.001	Intercept	5.56	0.007	<0.001
Strain (B6)	1.36	0.004	<0.001	Strain (B6)	0.76	0.008	<0.001
Bedding (1/8)	0.17	0.003	<0.001	Bedding (1/8)	-0.23	0.01	<0.001
Time	-0.05	0.0002	<0.001	Time	-0.09	0.001	<0.001
Model 3(c)				Strain*Bedding	0.52	0.01	<0.001
Intercept	5.64	0.005	<0.001	Strain*Time	0.05	0.001	<0.001
Strain (B6)	1.38	0.004	<0.001	Bedding*Time	0.02	0.001	<0.001
Bedding (1/8)	0.15	0.003	<0.001	Strain*Bedding *Time	-0.02	0.001	<0.001
Time (< 8.5 h)	-0.29	0.001	<0.001				
Time (8.5 ~ 9.5 h)	2.38	0.008	<0.001				
Time (>9.5 h)	-2.22	0.007	<0.001				

Table 5. Linear mixed regression model with random intercept to estimate longitudinal determinants of mice ambulations in initial 3 hours.

Model 4(a)				Model 4(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	507.36	31.97	<0.001	Intercept	642.03	44.13	<0.001
Strain (B6)	610.83	25.27	<0.001	Strain (B6)	438.09	58.72	<0.001
Bedding (1/8)	47.68	25.27	0.06	Bedding (1/8)	-155.78	58.72	0.01
Time	-189.98	14.80	<0.001	Time	-247.00	27.43	<0.001
				Strain*Bedding	213.70	83.04	0.01
				Strain*Time	36.75	38.79	0.34
				Bedding*Time	61.33	38.79	0.12
				Strain*Bedding *Time	31.90	54.86	0.56

Table 6. Linear poisson mixed regression model with random intercept to estimate longitudinal determinants of mice ambulations in initial 3 hours.

Model 5(a)				Model 5(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	6.00	0.030	<0.001	Intercept	6.59	0.03	<0.001
Strain (B6)	1.13	0.006	<0.001	Strain (B6)	0.41	0.01	<0.001
Bedding (1/8)	0.08	0.005	<0.001	Bedding (1/8)	-0.28	0.01	<0.001
Time	-0.32	0.003	<0.001	Time	-0.81	0.01	<0.001
				Strain*Bedding	0.32	0.02	<0.001
				Strain*Time	0.55	0.01	<0.001
				Bedding*Time	0.01	0.01	<0.001
				Strain*Bedding *Time	0.13	0.02	0.33

Table 7. Linear and non-linear mixed regression model with random intercept to estimate longitudinal determinants of mice ambulations in dark phase.

Model 6(a)				Model 6(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	258.29	19.60	<0.001	Intercept	225.33	29.00	<0.001
Strain (B6)	330.51	14.28	<0.001	Strain (B6)	406.55	38.71	<0.001
Bedding (1/8)	21.60	14.28	0.131	Bedding (1/8)	-29.01	38.71	0.45
Time	-27.25	1.98	<0.001	Time	-19.62	3.91	<0.001
Model 6(c)				Strain*Bedding	81.01	54.75	0.14
Intercept	297.92	20.79	<0.001	Strain*Time	-16.96	5.53	0.002
Strain (B6)	330.51	14.08	<0.001	Bedding*Time	4.15	5.53	0.45
Bedding (1/8)	21.60	14.08	0.13	Strain*Bedding *Time	-4.93	7.82	0.53
Time (< 8.5 h)	-38.91	2.93	<0.001				
Time (> =8.5 h)	54.11	10.13	<0.001				

Table 8. Linear and non-linear zero-inflated poisson mixed regression model with random intercept to estimate longitudinal determinants of mice ambulations in dark phase.

Model 7(a)				Model 7(b)			
Coefficients	Estimates	SE	P-value	Coefficients	Estimates	SE	P-value
Intercept	5.05	0.006	<0.001	Intercept	5.58	0.010	<0.001
Strain (B6)	1.44	0.005	<0.001	Strain (B6)	0.82	0.011	<0.001
Bedding (1/8)	0.14	0.004	<0.001	Bedding (1/8)	-0.21	0.014	<0.001
Time	-0.09	0.001	<0.001	Time	-0.19	0.002	<0.001
Model 7(c)				Strain*Bedding	0.35	0.016	<0.001
Intercept	5.12	0.006	<0.001	Strain*Time	0.12	0.002	<0.001
Strain (B6)	1.44	0.005	<0.001	Bedding*Time	0.04	0.003	<0.001
Bedding (1/8)	0.14	0.004	<0.001	Strain*Bedding *Time	-0.03	0.003	<0.001
Time (< 8.5 h)	-0.11	0.001	<0.001				
Time (>=8.5 h)	0.13	0.003	<0.001				

Figure 1. Distribution of median and mean ambulations

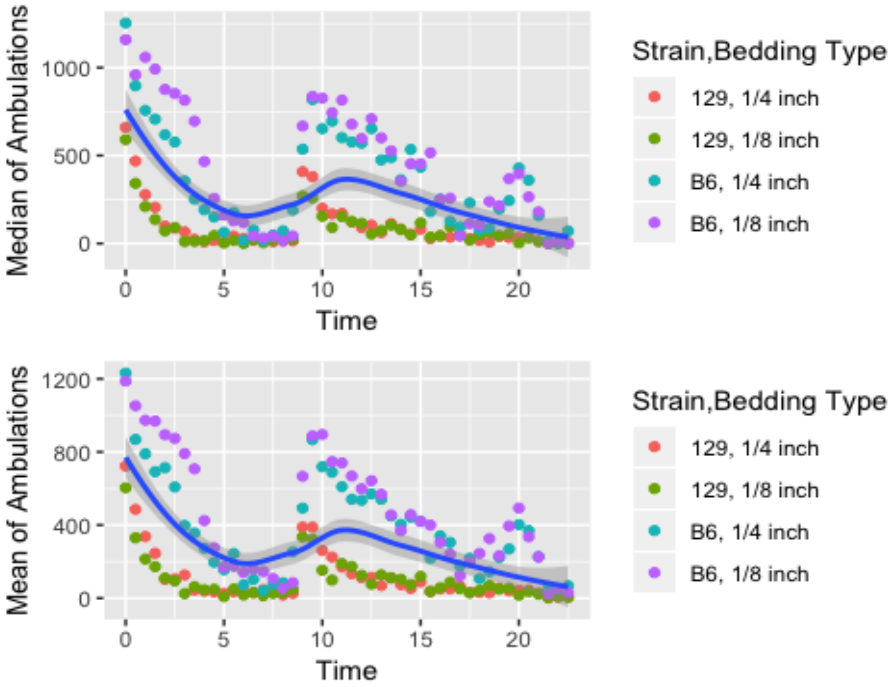


Figure 2. Association between time and time spline

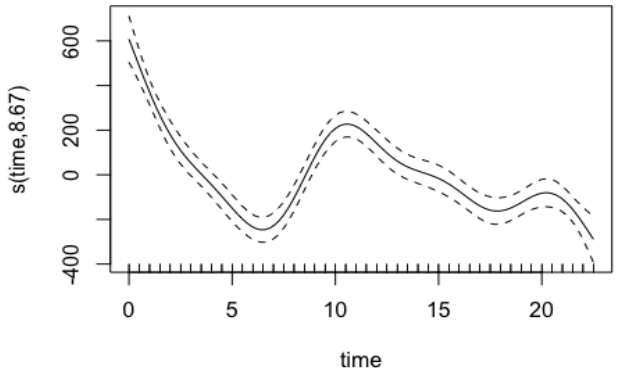


Figure 3. Distribution of median ambulations (+/- SE) over time

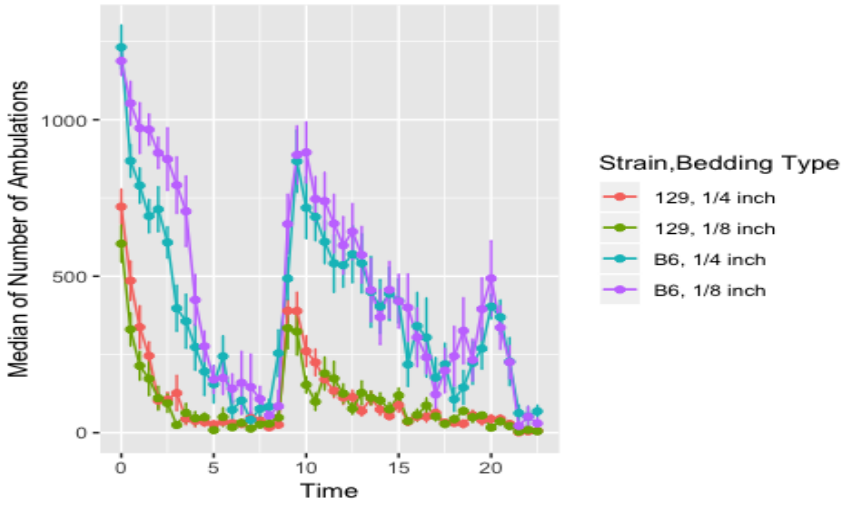


Figure 4. Visualization of zero and non-zero distribution (23 hours)

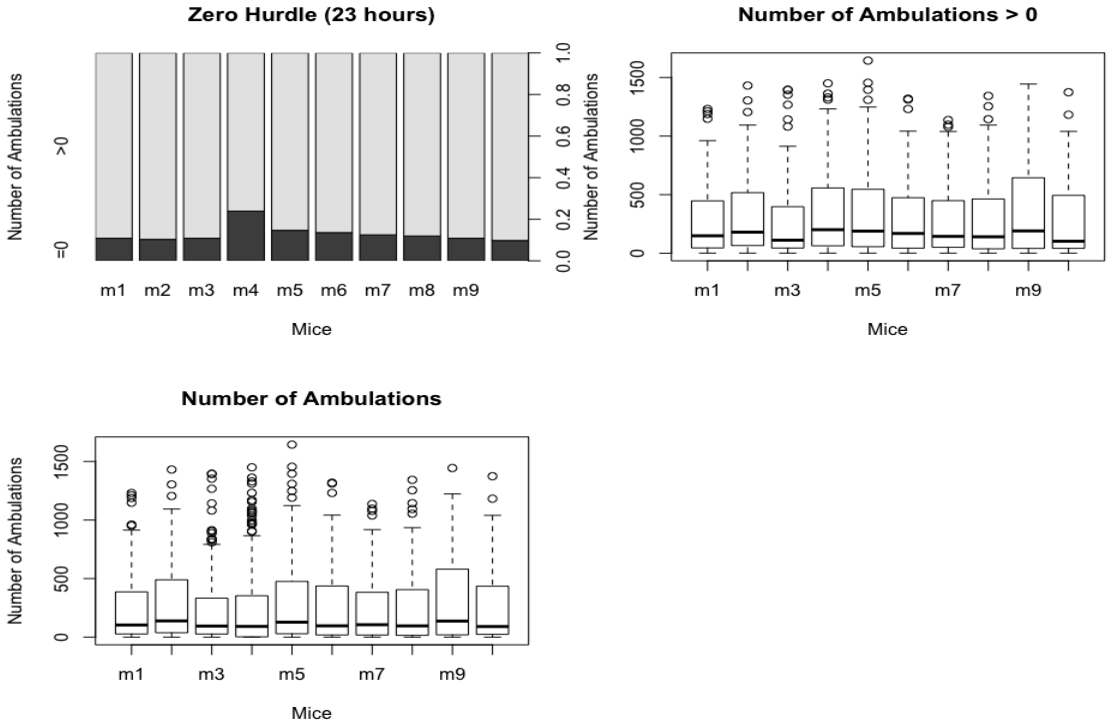


Figure 5. Actual vs. predictive number of ambulations (23 hours)

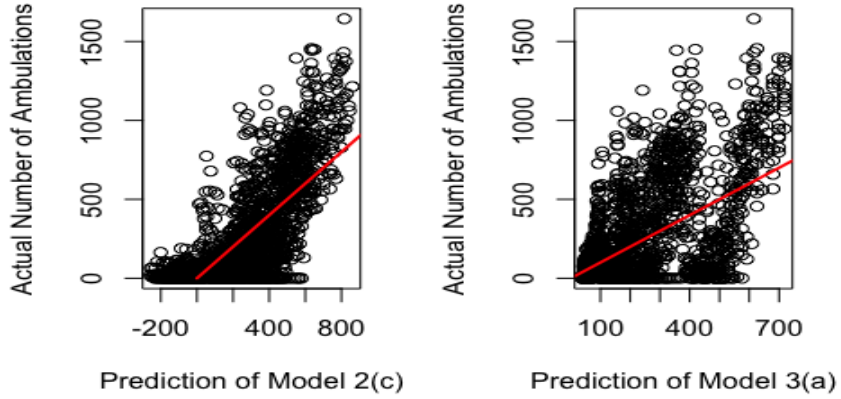


Figure 6. Visualization of zero and non-zero distribution (initial 3 hours)

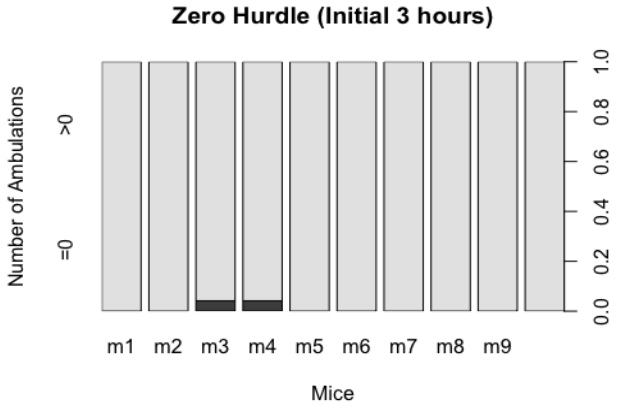


Figure 7. Actual vs. predictive number of ambulations (initial 3 hours)

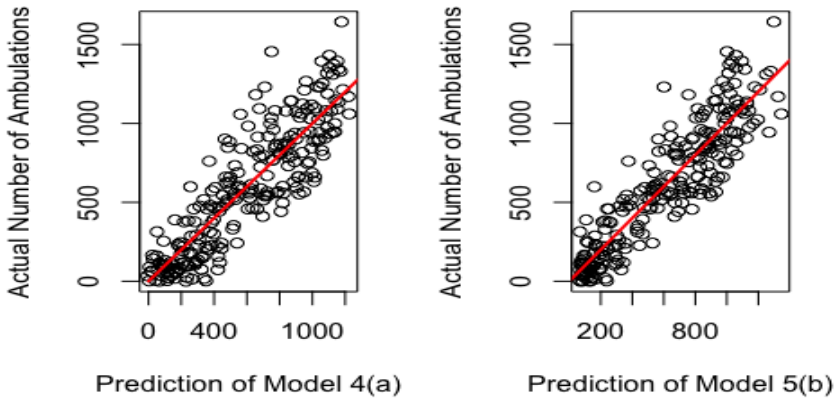


Figure 8. Visualization of zero and non-zero distribution (dark phase)

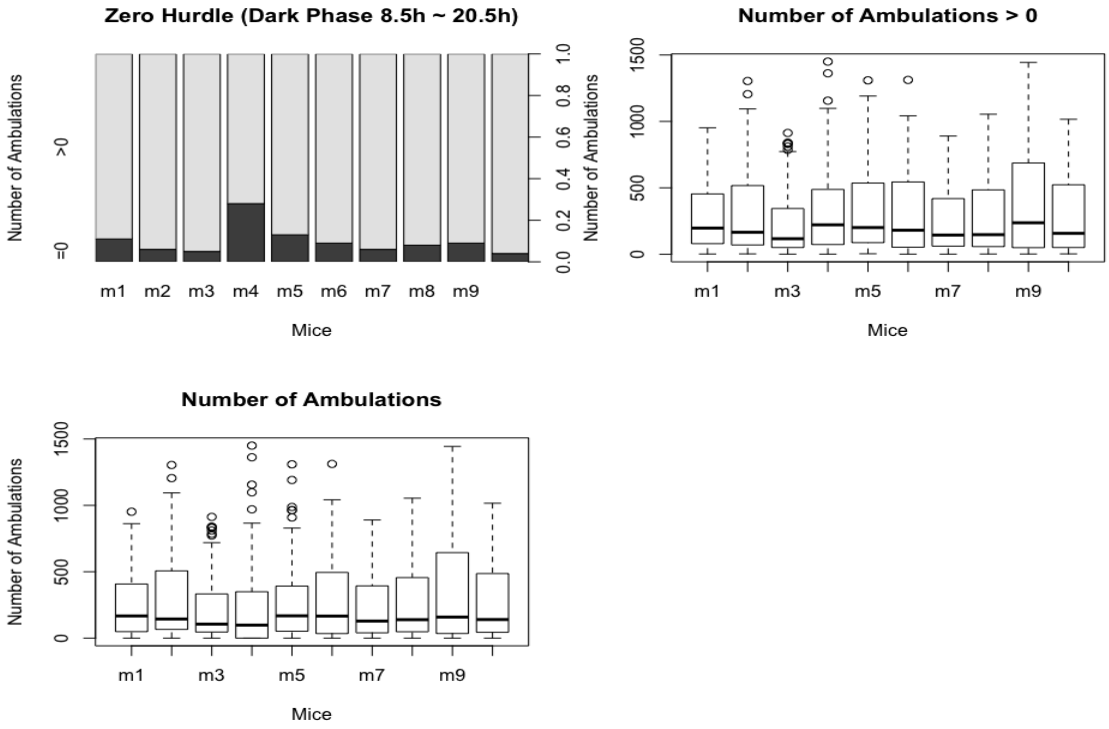
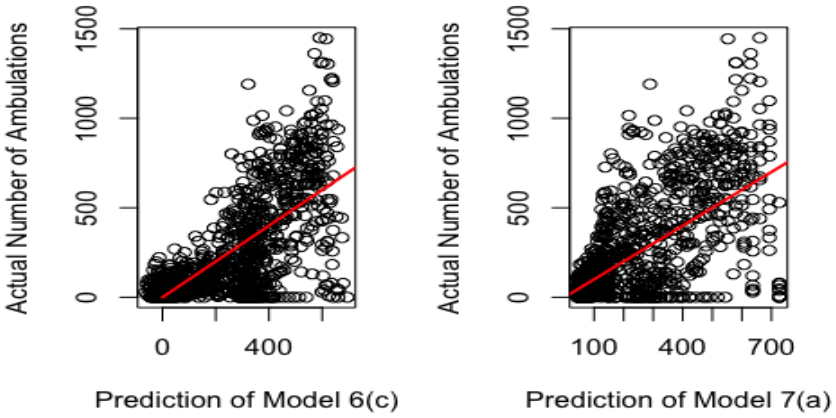


Figure 9. Actual vs. predictive number of ambulations (dark phase)



7. Supplement Tables and Figures

Table S1. Fit statistics of models fitting 23 hours data

	Df	AIC	BIC	Log Likelihood	Deviance	R-squared
Model 2(a)	6	25735	25768	-12861	25723	0.34
Model 2(b)	10	25664	25719	-12822	25644	0.37
Model 2(c)	8	25098	25142	-12541	25082	0.54
Model 3(a)	-	13891.81	13894.53	-6936.91	–	–
Model 3(b)	-	13907.81	13912.95	-6936.91	–	–
Model 3(c)	-	13899.81	13903.75	-6936.91	–	–

Table S2. Fit statistics of models fitting initial 3 hours data

	Df	AIC	BIC	Log Likelihood	Deviance	R-squared
Model 4(a)	6	3230.1	3250.9	-1609.0	3218.1	0.76
Model 4(b)	10	3199.0	3233.8	-1589.5	3179.0	0.80
Model 5(a)	5	24571	24588	-12280	24561	0.996
Model 5(b)	9	16212	16243	-8097	16194	0.998

Table S3. Fit statistics of models fitting dark phase data

	Df	AIC	BIC	Log Likelihood	Deviance	R-squared
Model 6(a)	6	13694	13724	-6841.2	13682	0.43
Model 6(b)	10	13674	13723	-6827.0	13654	0.44
Model 6(c)	7	13668	13703	-6827.1	13654	0.44
Model 7(a)	-	13886.01	13888.73	-6934.003	-	-
Model 7(b)	-	13902	13907.15	-6934.002	-	-
Model 7(c)	-	13890.01	13893.33	-6934.003	-	-

Figure S1. Distribution of coefficient of variation

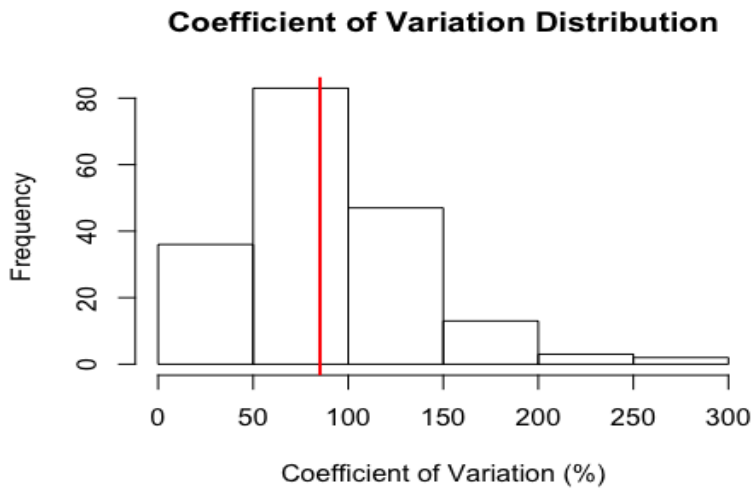


Figure S2. Diagnostic plots of non-linear model 1(c)

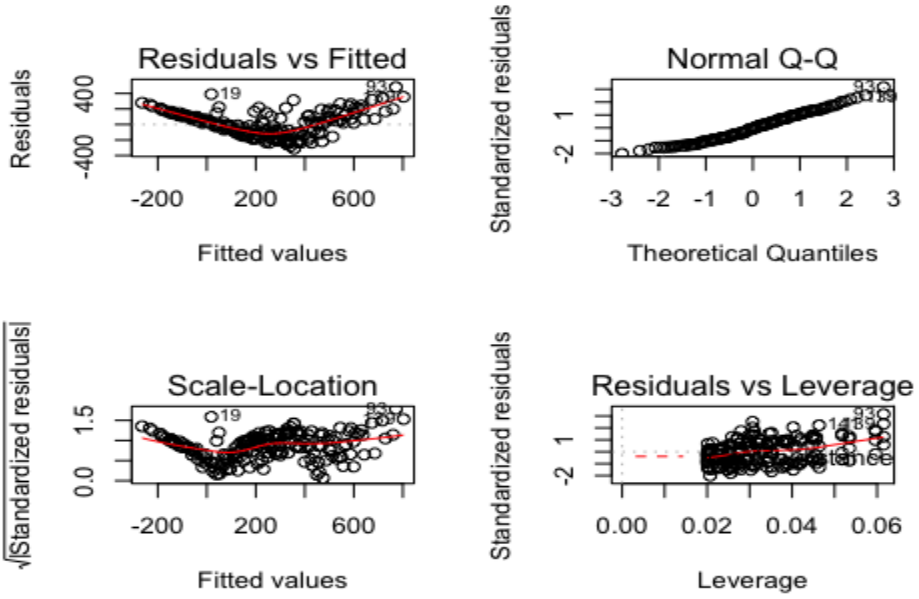


Figure S3. Diagnostic plots of non-linear mixed model 2(c) (23 hours)

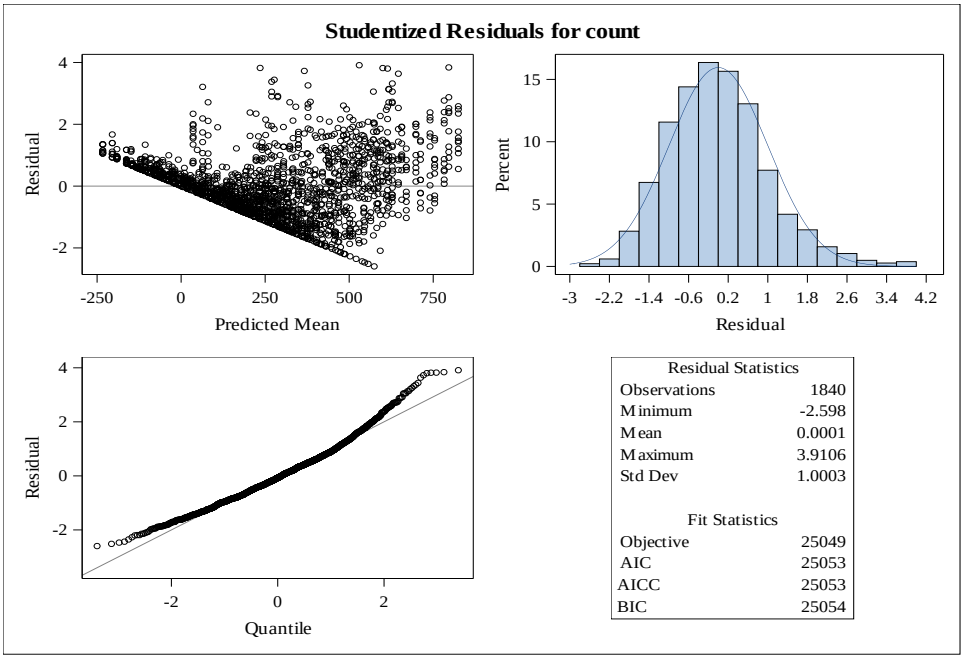


Figure S4. Diagnostic plots of linear mixed model 4(a) (initial 3 hours)

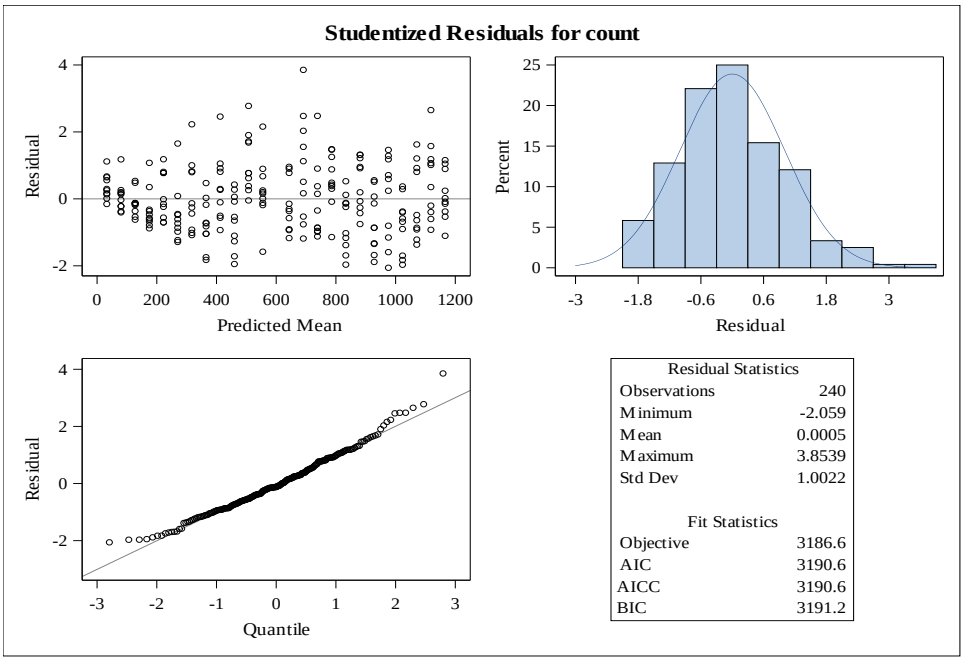


Figure S5. Diagnostic plots of poisson mixed model 5(b) (initial 3 hours)

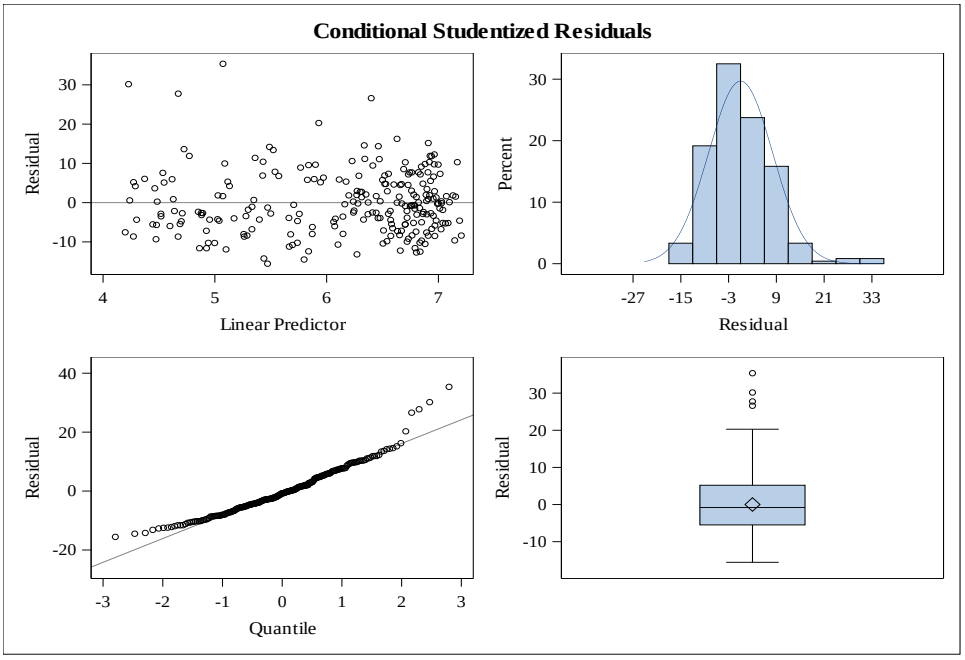


Figure S6. Diagnostic plots of non-linear mixed model 6(c) (dark phase)