Shape Invariant Modelling of Circadian Rhythms with Random Effects and Smoothing Spline ANOVA Decompositions

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Abstract

Medical studies often collect physiological and/or psychological measurements over time from multiple subjects in order to study dynamics such as circadian rhythms. Under the assumption that the expected response functions of all subjects are the same after shift and scale transformations, shape invariant models have been applied to analyze this kind of data. The shift and scale parameters provide efficient and interpretable data summaries, while the common shape function is usually modelled nonparametrically to provide flexibility. However, due to the deterministic nature of the shift and scale parameters, potential correlations within a subject are ignored. Furthermore, the shape of the common function may depend on other factors such as disease. In this paper we propose shape invariant mixed effects models. A second stage model with fixed and random effects is used to model individual shift and scale parameters. A second stage smoothing spline ANOVA model is used to study potential covariate effects on the common shape function. We apply our methods to a real data set to investigate disease effects on circadian rhythms of cortisol, a hormone that is affected by stress. We find that patients with Cushing's syndrome lost circadian rhythms and their 24-hour means were elevated to very high levels. Patients with major depression had the same circadian shape and phases as normal subjects. However, their 24-hour mean levels were elevated and amplitudes were dampened for some patients.

Key words: biological rhythm; repeated measures data; self-modeling nonlinear regression model; semi-parametric nonlinear mixed effects model.

1 Introduction

Biological rhythms are fundamental to living matter from subcellular particles to the human organism (Wever 1979). For example, it is known that hormone cortisol levels peak in the morning and decrease to substantially lower levels late at night. Recent advances have shown the importance of these rhythms for everyday life in health as well as in disease (Kupfer, Monk and Barchas 1988, Reilly, Atkinson and Waterhouse 1997). Information on biological rhythms has been applied to almost every field of clinical and laboratory medicine (Touitou and Haus 1992, Hayes, Pauly and Reiter 1990, Redfern and Lemmer 1997).

Medical researchers are often interested in circadian rhythms: a cyclical variation in the intensity of a metabolic or physiological process with a period of about 24 hours. Experiments are typically conducted in such a way that variables of interest are measured several times during a time period, say 24 hours, from a group of normal (or sick) human subjects (or animals). The problems of interest are: (1) do circadian rhythms exist? and (2) do demographic (e.g. age, sex), environmental (e.g. time zone transition, work load) and medical, physiological or psychological (e.g. depression, stress) variables affect circadian rhythms, and if so how?

In an experiment to study immunological responses in humans, blood samples were collected every two hours for 24 hours from 9 healthy normal volunteers, 11 patients with major depression and 16 patients with Cushing's syndrome. These blood samples were analyzed for parameters that measure immune functions and hormones of the HPA axis (Kronfol, Nair, Zhang, Hill and Brown 1997). In this paper we will concentrate on cortisol. Figure 1 presents the observed concentrations (ug/dl) of the cortisol on a log scale from nine healthy subjects.

It is well-known that many hormones vary in a circadian pattern (Kronfol et al. 1997, Wang and Brown 1996). Since the 24-hour periodicity is entrained, the cycle length is fixed. The common practice is to fit a single sinusoidal function to each subject (Monk 1982, Cugini, Leone, Palma, Battisti, Wilson, Kawasaki, Tamura, Halberg and Cornelissen 1990, Prins and Hecquet 1992). Problems with this approach are: (a) the pattern over time may not be symmetric. That is, the peak and nadir may not be separated by 12 hours and/or the amplitude and width of the peak may differ from those of the nadir; (b) sometimes there are local minimum and maximum points (Wang and Brown 1996). It has been recognized that the sinusoidal function is too restrictive and "rhythms with a shape closely approximating a cosine curve are uncommon" (Reilly et al. 1997, p. 151). Thus "other statistical methods must have preference which do not precondition any definite rhythm shape" (Wever 1997, p. 20).

Although adding harmonics can improve the fit, it is difficult to decide how many harmonics to include in the model and the results are difficult to interpret. From Figure 1 one can see that the data are noisy and it is difficult to identify patterns among subjects. Average measurements of all subjects are often plotted against time to find a common feature. Such a procedure may produce artifacts when subjects have different phases, means or/and amplitudes (Reilly et al. 1997).

The goal of this article is to propose a new class of flexible methods and demonstrate their applications. In Section 2, we review the shape invariant models and introduce shape invariant mixed effects models. Analyses of the cortisol data are presented in Section 3. We conclude with discussions in Section 4.

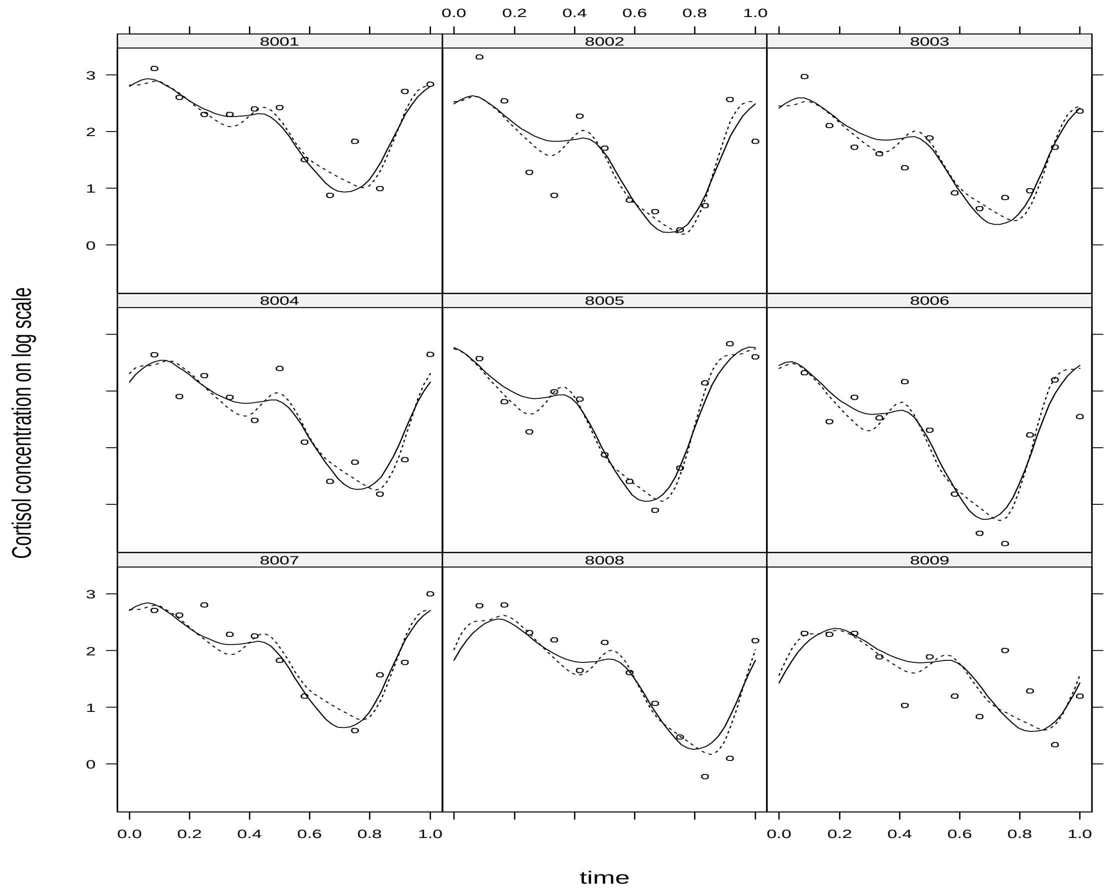

Figure 1: Plots of cortisol concentration on log scale and fitted curves for normal subjects. Circles are observations. Solid lines represent fits from model (10). Dotted lines represent fits from model (7). Subjects' IDs are shown in the strip.

2 Shape Invariant Mixed Effects Models

2.1 Shape Invariant Model for Circadian Rhythms

Wang and Brown (1996) developed a flexible Shape Invariant Model (SIM) using a periodic spline function as the common curve and attributed individual variations by 24-hour means, phases and amplitudes. More precisely, they assumed that

$$y_{ij} = \mu_i + \alpha_i f(t_{ij} - \tau_i) + \epsilon_{ij}, \qquad i = 1, \cdots, m; \quad j = 1, \cdots, n_i, \tag{1}$$

where m is the total number of subjects, n_i is the number of observations for subject i, y_{ij} is the response of ith individual at the jth time point t_{ij}, μ_i is the 24-hour mean of the ith individual,

$\alpha_i > 0$ is the amplitude of the ith individual, and $0 \leq \tau_i < 1$ is the phase (horizontal shift) of the ith individual. For simplicity, the 24-hour period is transformed into [0,1]. ϵ_{ij}'s are random errors and $\epsilon_{ij} \overset{iid}{\sim} \mathrm{N}(0, \sigma^2)$.

The function f in (1) is the common circadian shape function. It is a periodic function with period 1. For identifiability of model (1), it is also assumed that the integral of f equals zero and $\sup_{t\in[0,1]} |f(t)| = 1$. Specifically, let

$$\begin{aligned} W_2^0(per) \;=\; \{\, f: \;\; & f \text{ and } f' \text{ are absolutely continuous}, \;\; \int_0^1 (f''(t))^2 dt < \infty, \\ & \int_0^1 f(t)dt = 0,\; f(0) = f(1),\; f'(0) = f'(1) \,\} \end{aligned} \tag{2}$$

be the reproducing kernel Hilbert space of cubic splines on the circle without the constant term (Wahba 1990). The model space for f in (1) is

$$\mathcal{M} = \{f \in W_2^0(per): \sup_{t\in[0,1]} |f(t)| = 1\}. \tag{3}$$

The SIMs have also been applied to areas such as human growth (Stutzle, Gasser, Molinari, Largo, Prader and Huber 1980), spirometry, spectrophometric and sensitometric analyses (Lawton, Sylvestre and Maggio 1972), bioassay (Guardabasso, Rodbard and Munson 1987), curve registration (Ramsay and Li 1998), and metabolism (Altman 1996). The advantage of model (1) is that no specific form has been assumed for the common function; it is only assumed to be periodic and smooth and its shape is decided by the data. Therefore the model is very flexible. Note that the sinusoidal function is a special case of model (1) with $f(t) = \sin 2\pi t$. The periodic spline function in model (1) reduces to a sinusoidal function if the extra flexibility is not warranted by data. Observations will be pooled from all subjects to estimate this shape function. Parameters of interest to scientists (24-hour mean, amplitude and phase) remain the same as those in a sinusoidal model. However, model (1) has the following drawbacks: (a) the parameters μ_i, α_i and τ_i in model (1) were assumed to be fixed effects. As a consequence, observations from the same subject were assumed to be independent; (b) besides the nonparametric function f, the number of parameters equals $3m+1$, which increases with the number of subjects. This may adversely affect the estimation and inference for the parameters under the common experimental situation that the number of subjects is large and the number of observations for each subject is small; (c) it is difficult to investigate covariate effects on parameters and/or the common curve. Ad hoc second stage analyses are often used to investigate covariate effects. In the next section we use mixed effects models for the parameters and smoothing spline ANOVA models for the common curve to overcome these problems.

2.2 Shape Invariant Mixed Effects Models

We will construct a two-stage model. At the first stage, we assume the same model as (1) and rewrite it as

$$y_{ij} = \phi_{1i} + \exp(\phi_{2i}) f(t_{ij} - \text{alogit}(\phi_{3i})) + \epsilon_{ij}, \quad i = 1, \cdots, m, \quad j = 1, \cdots, n_i. \tag{4}$$

Note that the exponential transformation is used to force the amplitude to be positive and the inverse logistic transformation, $\text{alogit}(x) = \exp(x)/(1 + \exp(x))$, is used to force the phase to be inside interval [0,1]. We will allow random errors within a subject to be correlated: $\boldsymbol{\epsilon}_i = (\epsilon_{i1}, \cdots, \epsilon_{in_i})^T \sim N(0, \sigma^2 \boldsymbol{\Lambda}_i)$. Thus model (4) itself is more general than the classical SIM (1).

Let $\boldsymbol{\phi}_i = (\phi_{1i}, \phi_{2i}, \phi_{3i})^T$. Denote $\boldsymbol{z}_i$ as the covariate vector of subject i. At the second stage, between-subject differences are modelled using covariate information by the following linear model (Lindstrom and Bates 1990):

$$\boldsymbol{\phi}_i = \boldsymbol{A}_i \boldsymbol{\beta} + \boldsymbol{B}_i \boldsymbol{b}_i, \quad \boldsymbol{b}_i \stackrel{iid}{\sim} \mathrm{N}(0, \sigma^2 \boldsymbol{D}), \tag{5}$$

where $\boldsymbol{\beta}$ is a p-vector of fixed population parameters, $\boldsymbol{b}_i$ is a q-vector of random effects associated with subject i, and $\boldsymbol{A}_i$ and $\boldsymbol{B}_i$ are $3 \times p$ and $3 \times q$ design matrices determined by the covariate vector $\boldsymbol{z}_i$. More complicated models such as the nested model in Section 3 may be used.

Covariate effects on the circadian shape function can be modelled using a smoothing spline ANOVA (SS ANOVA) decomposition

$$f(\boldsymbol{z}, t) = \mu + f_1(\boldsymbol{z}) + f_2(t) + f_{12}(\boldsymbol{z}, t), \tag{6}$$

where μ is a constant, f_1 and f_2 are the main effects of covariates $\boldsymbol{z}$ and t respectively, and f_{12} is the interaction between $\boldsymbol{z}$ and t. Note that $\boldsymbol{z}$ could be a vector of covariates. Thus further SS ANOVA decompositions may be constructed for f_1 and f_{12}. Which model space and SS ANOVA decomposition to use for f depends on the domains of $\boldsymbol{z}$ and t, prior knowledge, constraints for identifiability, and purpose of the analysis. See Wahba (1990) and Gu (2002) for details about SS ANOVA decompositions.

Equations (4), (5) and (6) altogether define a shape invariant mixed effects model (SIMM). Interpretations of the parameters remain the same. Correlation within a subject is modelled by random effects and random errors. Covariate effects on parameters and/or the common curve are part of the model. The awkward constraint for identifiability, $\sup_{t \in [0,1]} |f(t)| = 1$, can be dropped by removing a constant term from the fixed effect of ϕ_{2i}.

Lindstrom (1995) extended the SIM by including random shift and scale parameters. She used a free-knot spline with a fixed number of knots to model the common curve. Our second stage models are more general. Especially, the second stage SS ANOVA model (6) for the common function is new. Our procedure allows data to decide the shape of the common curve. For simplicity, our methods are presented using periodic splines. It is straightforward to generalize these methods to the setting of general smoothing splines (Wahba 1990).

2.3 Estimation, Inference and Software

Because f interacts with parameters (fixed or random) in a complicated (nonlinear) manner, estimation and inference are complicated. Ke and Wang (2001) developed estimation methods for Semi-parametric Nonlinear Mixed effects models (SNM). Since the SIMM is a special case of the SNM model, the methods developed for SNM models can be used.

We now briefly describe these methods for SIMMs. Let $N = \sum_{i=1}^m n_i$, $\boldsymbol{y}_i = (y_{i1}, \cdots, y_{in_i})^T$, $\eta(\boldsymbol{\phi}_i, f; t_{ij}) = \phi_{1i} + \exp(\phi_{2i}) f(t_{ij} - \text{alogit}(\phi_{3i}))$, and $\boldsymbol{\eta}_i(\boldsymbol{\phi}_i, f) = (\eta(\boldsymbol{\phi}_i, f; t_{i1}), \cdots, \eta(\boldsymbol{\phi}_i, f; t_{in_i}))^T$. We estimate $\boldsymbol{\beta}$, $\boldsymbol{b}_i$'s and f as minimizers of the following double penalized log-likelihood

$$\sum_{i=1}^m (\boldsymbol{y}_i - \boldsymbol{\eta}_i(\boldsymbol{A}_i\boldsymbol{\beta} + \boldsymbol{B}_i\boldsymbol{b}_i, f))^T \boldsymbol{\Lambda}_i^{-1} (\boldsymbol{y}_i - \boldsymbol{\eta}_i(\boldsymbol{A}_i\boldsymbol{\beta} + \boldsymbol{B}_i\boldsymbol{b}_i, f)) + \sum_{i=1}^m \boldsymbol{b}_i^T \boldsymbol{D}^{-1} \boldsymbol{b}_i + \\ N\lambda_1 J_1(f_1) + N\lambda_2 J_2(f_2) + N\lambda_{12} J_{12}(f_{12}),$$

where $J_1(f_1)$, $J_2(f_2)$ and $J_{12}(f_{12})$ are penalties for nonparametric components in (6), and λ_1, λ_2 and λ_{12} are smoothing parameters. σ^2 and variance-covariance parameters in $\boldsymbol{\Lambda}_i$'s and $\boldsymbol{D}$ are estimated based on an approximation to the profile likelihood (equation (14) in Ke and Wang (2001)).

Wang and Ke (2002) developed a user friendly S-Plus package for fitting many spline based models. One function, `snm`, is designed for fitting general SNM models. Thus this function can be used to fit SIMMs. This package is available at `http://www.pstat.ucsb.edu /faculty/yuedong/research`. Programs used for fitting the hormone data are listed in the Appendix.

Inference for f was described in Ke and Wang (2001). Inference for parameters are conditional on the estimate of f with degrees of freedom adjusted to reflect the cost for estimating f. Specifically, when f is fixed, the SIMM reduces to a nonlinear mixed effects model. Thus the standard methods such as the t-test based on equation (7.20) in Pinheiro and Bates (2000) can be used.

3 Data Analyses

The data set obtained from the experiment described in Section 1 contains the following variables: `ID` of subjects, `time` when measurements were taken, cortisol concentrations on log scale (named as `horm`), and subject `group` (normal, depression, Cushing's syndrome). For simplicity, the `time` variable of 24-hour period is transformed into [0,1]. Observations are shown in Figures 1, 2 and 3 as circles.

We first fit the following SIMM for each group

$$y_{ij} = \beta + b_{1i} + \exp(b_{2i}) f(t_{ij} - \text{alogit}(b_{3i})) + \epsilon_{ij}, \quad i = 1, \cdots, m, \quad j = 1, \cdots, n_i, \tag{7}$$

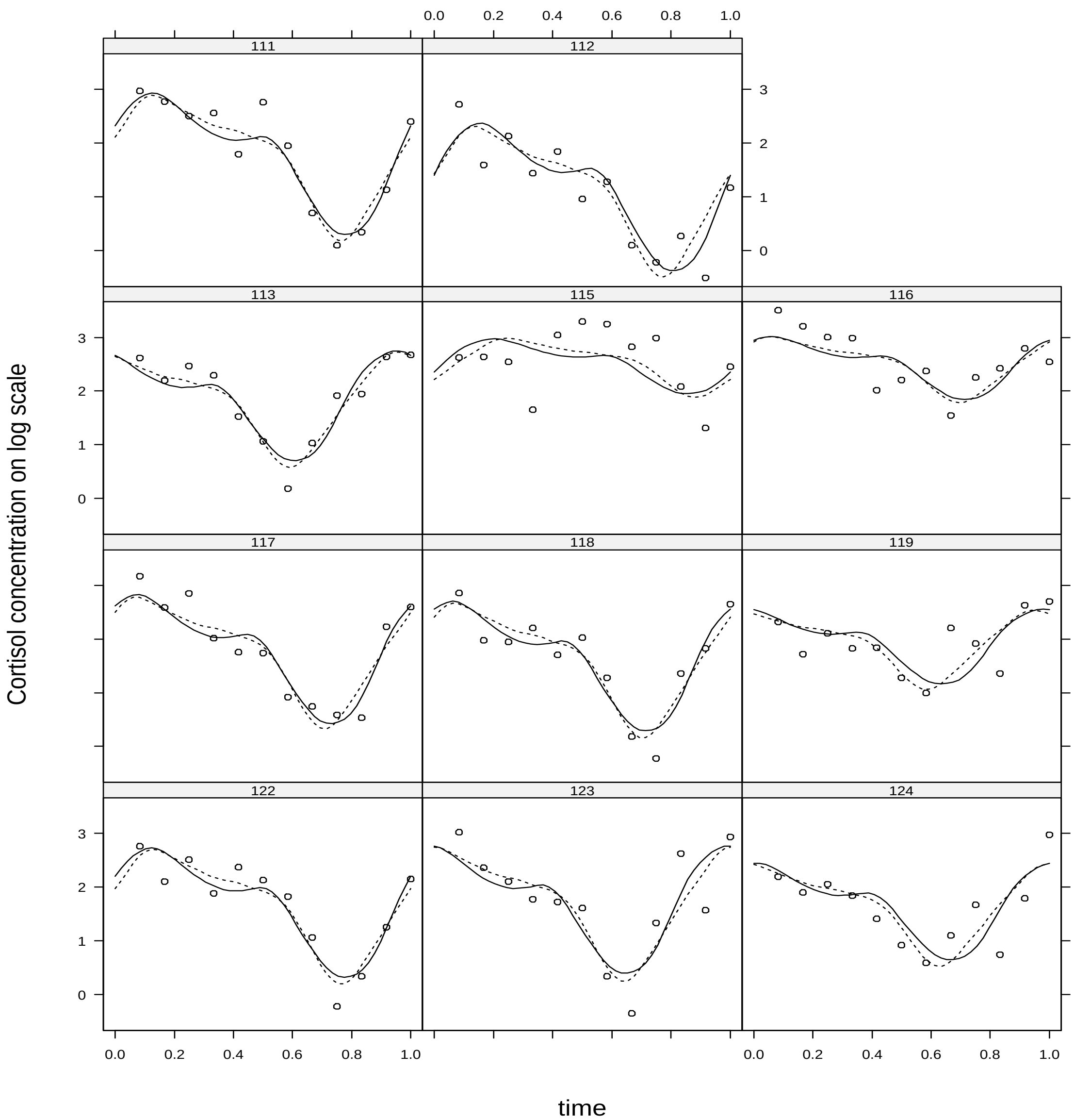

Figure 2: Plots of cortisol concentration on log scale and fitted curves for patients with major depression. Circles are observations. Solid lines represent fits from model (10). Dotted lines represent fits from model (7). Subjects' IDs are shown in the strip.

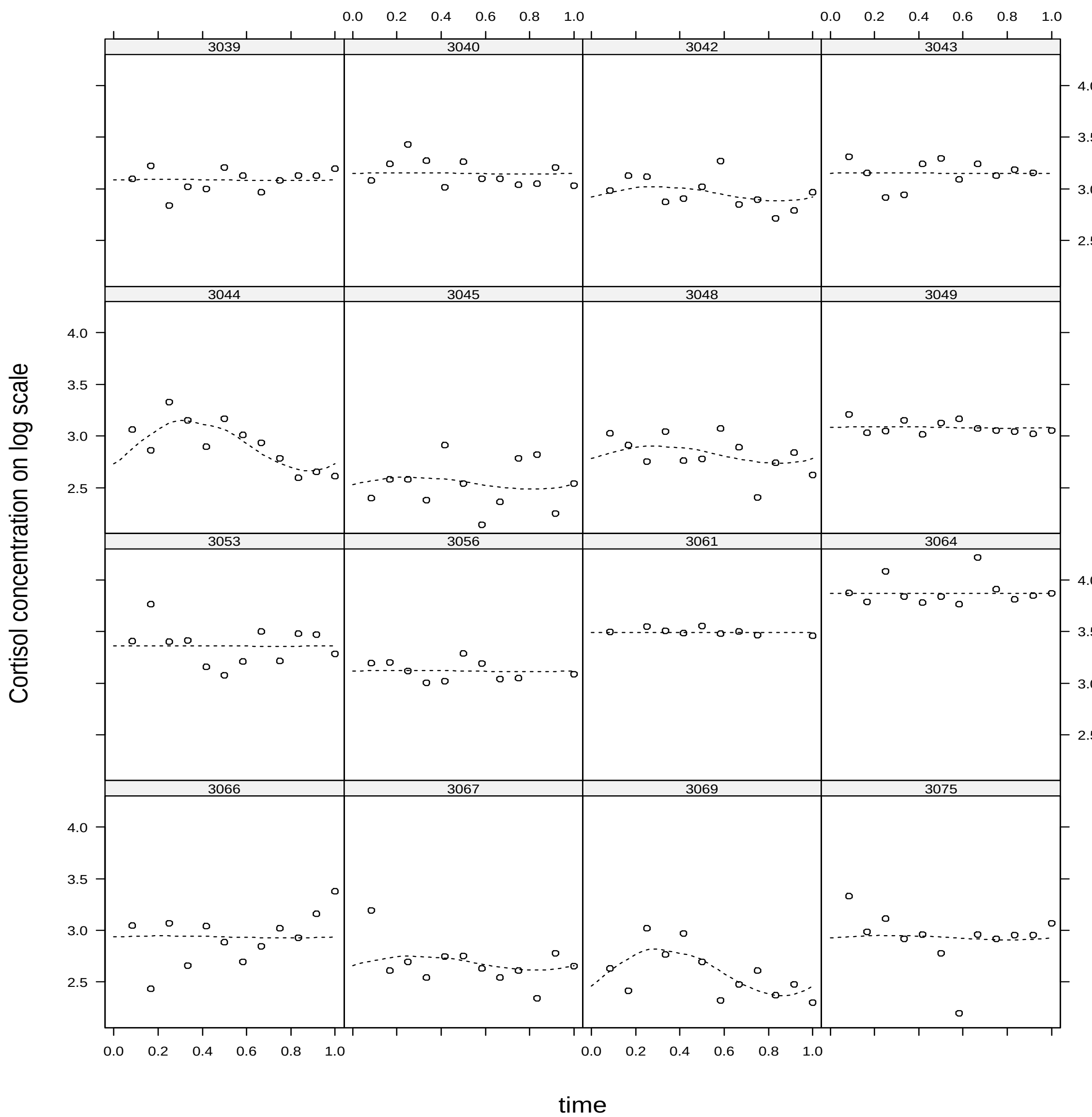

Figure 3: Plots of cortisol concentration on log scale and fitted curves for patients with Cushing's syndrome. Circles are observations. Dotted lines represent fits from model (7). Subjects' IDs are shown in the strip.

where the fixed effect β represents 24-hour mean of the population, the random effects b_{1i}, b_{2i} and b_{3i} represent the ith subject's deviation of 24-hour mean, amplitude and phase. We assume that $f \in W_2^0(per)$ and $\boldsymbol{b}_i = (b_{1i}, b_{2i}, b_{3i})^T \overset{iid}{\sim} N(0, \sigma^2 \boldsymbol{D})$, where $\boldsymbol{D}$ is an unstructured positive-definite matrix. The assumption of zero population mean for amplitude and phase parameters takes care of potential confounding between amplitude, phase and the nonparametric common function f in a natural way. In terms of notations in model (5), we have $\boldsymbol{A}_i = (1,0,0)^T$ and $\boldsymbol{B}_i$ equals a 3×3 identity matrix.

The S-Plus program for fitting model (7) to normal subjects is shown in the Appendix. The fits are shown in Figures 1, 2 and 3 as dotted lines. The estimated common functions are shown in Figure 4 together with their 95% Bayesian confidence intervals.

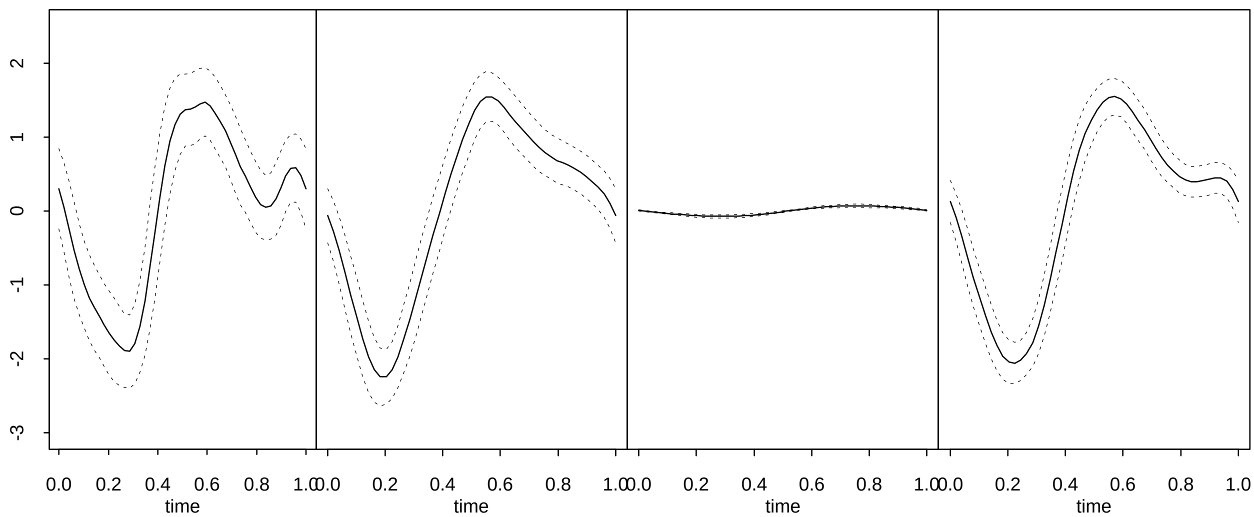

Figure 4: Solid lines are estimates of the common functions and dotted lines are 95% Bayesian confidence intervals. The left three panels are estimated common curves for the normal, depression and Cushing's syndrome groups respectively. The right panel is the estimated common curve in model (10).

Observations close in time from the same subject may be correlated. We fit model (7) with random errors within each subject modelled by a first-order autoregressive structure. The estimated lag 1 autocorrelation coefficients are small. Estimates of other parameters remain the same. Therefore random errors are assumed to be independent in the remaining of this section.

From Figures 7 and 4 it is apparent that the common function for the Cushing's syndrome group is almost zero, which indicates that circadian rhythms were lost for patients with Cushing's syndrome. The absence of a circadian rhythm has been considered as hallmark of the diagnosis of Cushing's syndrome (Boyar, Witkin, Carruth and Ramsey 1979, Liu, Kazer and

Rasmussen 1987). Unlike normal subjects, patients with Cushing's syndrome fail to decrease cortisol secretion in the late evening. Therefore, the measurement of elevated late evening cortisol is a very simple and useful way to screen patients for Cushing's syndrome (Raff, Raff and Findling 1998, Castro, Elias and Quidute 1999). It has also been noticed that some patients with Cushing's syndrome still demonstrate circadian rhythms (Refetoff, Van Cauter, Fang, Laderman, Graybela and Landau 1985, Tourniaire, Chalendar and Rebbatu 1986). Figure 3 suggests that cortisol levels of patients 3044 and 3069 may still have circadian rhythms.

We now compare patients with major depression with normal subjects. We will first investigate potential effects of depression on the shape function. We find that these two groups had the same shape function which allows us to further investigate potential effects of depression on the parameters.

The shape functions for the normal and depression groups are similar (Figure 4). We now test the hypothesis that the shape functions of these two groups are equal. We achieve this by fitting data from these two groups simultaneously. Consider the following model

$$\begin{aligned} y_{ijk} &= \beta_k + b_{1i(k)} + \exp(b_{2i(k)}) f(k, t_{ijk} - \text{alogit}(b_{3i(k)})) + \epsilon_{ijk}, \\ & \quad i = 1, \cdots, m, \quad j = 1, \cdots, n_{ik}, \quad k = 1, 2, \end{aligned} \tag{8}$$

where k represents `group` with $k = 1$ and $k = 2$ correspond to depression and normal groups respectively, fixed effect β_k is the population 24-hour mean of `group` k, random effects $b_{1i(k)}$, $b_{2i(k)}$ and $b_{3i(k)}$ represent the ith subject's deviation of 24-hour mean, amplitude and phase. Note that subjects are nested within `group` which is reflected in our notations. We allow different correlation structures for the random effects in each `group`. That is, we assume that $\boldsymbol{b}_{i(k)} = (b_{1i(k)}, b_{2i(k)}, b_{3i(k)})^T \overset{iid}{\sim} N(0, \sigma^2 \boldsymbol{D}_k)$, where $\boldsymbol{D}_k$'s are unstructured positive-definite matrices. We assume different common functions for each `group`. Thus f is a function of both `group` (denoted as k) and `time` (denoted as t). We model the `group` effect using a one-way ANOVA model with model space R^2 and the `time` effect using a periodic spline model with model space $W_2^0(per)$, where R^2 is the Euclidean two-space. That is, we assume that $f \in R^2 \otimes W_2^0(per)$. Writing $R^2 = \{1\} \oplus \{g : \sum_{k=1}^2 g(k) = 0\}$ where $\{1\} = \{g : g(1) = g(2)\}$ is the subspace of constant functions, we have the following SS ANOVA decomposition

$$R^2 \otimes W_2^0(per) = W_2^0(per) \oplus \left[\{g : \sum_{k=1}^{2} g(k) = 0\} \otimes W_2^0(per) \right],$$

or equivalently,

$$f(k, t) = s(t) + ss(k, t), \tag{9}$$

where $s(t)$ is the main effect of `time`, and $ss(k, t)$ is the interaction between `group` and `time`. It is easy to see that the hypothesis $H_0: f(1, t) = f(2, t)$ is equivalent to $H_0: ss(k, t) = 0$.

The S-Plus program for fitting the SIMM (8) and (9) is shown in the Appendix. The estimated smoothing parameter for the interaction term $ss(k,t)$ is large, which means that the interaction is small. In fact, $ss(k,t)$ is essentially zero: the estimates are on the magnitude of 10^{-6} while the posterior standard deviations are on the magnitude of 10^{-4}. Therefore we conclude that normal subjects and patients with major depression have the same shape function. This result confirms the assumption made in Wong, Kling, Munson, Listwak, Prolo, Karp, McCutheon, Geracioti, DeBellis, Rice, Goldstein, Veldhuis, Chrousos, Oldfield, McCann and Gold (2000) which was based on plots rather than on a formal test. Note that the classical methods based on a sinusoidal function implicitly assume the same shape functions for different groups. Thus these methods can not be used to test such a hypothesis, and there is no test available in literature.

Under the assumption of one shape function for both groups, we now can investigate differences of 24-hour mean, amplitude, and phase between these two groups. For this purpose, we consider the following model

$$\begin{aligned} y_{ijk} &= \beta_k + b_{1i(k)} + \exp(b_{2i(k)} + d_1 \times I_{[k=2]}) \times f(t_{ijk} - \text{alogit}(b_{3i(k)} + d_2 \times I_{[k=2]})) + \epsilon_{ijk}, \\ & \quad i = 1, \cdots, m, \quad j = 1, \cdots, n_{ik}, \quad k = 1, 2, \end{aligned} \tag{10}$$

where $\beta_2 - \beta_1$, d_1 and d_2 measure the differences of 24-hour mean, amplitude and phase between the normal group and the depression group.

The S-Plus program for fitting model (10) is shown in the Appendix. Estimates of $\beta_2 - \beta_1$, d_1 and d_2 are -0.2724 (se = 0.1311, p-value = 0.0389), 0.2350 (se = 0.0767, p-value = 0.0024), and 0.0299 (se = 0.0916, p-value = 0.7441) respectively. Standarded errors and p-values are calculated based on the t-test described in Section 2. We conclude that the differences of 24-hour mean and amplitude are significant, while the difference of phase is not. We refit model (10) without the d_2 term. The fits are shown in Figures 1 and 2 as solid lines.

The right panel of Figure 4 shows the estimated common function and its 95% Bayesian confidence intervals from the final model. Data from these two groups are pooled to estimate the common function. Thus the Bayesian confidence intervals are narrower.

Figure 5 shows the estimated 24-hour mean levels plotted against the estimated amplitudes. The amplitudes for patients with Cushing's syndrome are adjusted to have the same scale as those for normal subjects and patients with major depression. We conclude that for patients with Cushing's syndrome, 24-hour means were elevated to much higher levels. Circadian rhythms were lost except for patients 3044 and 3069. For patients with major depression, 24-hour means were elevated. However, their amplitudes were similar to those of normal subjects, except for patients 115 and 116 whose 24-hour mean levels and amplitudes were closer to those of patients with Cushing's syndrome. The statistically significant differences of the amplitudes between the normal group and the depression group are mainly contributed by these two patients. The highly elevated cortisol levels and waning circadian rhythms in these two

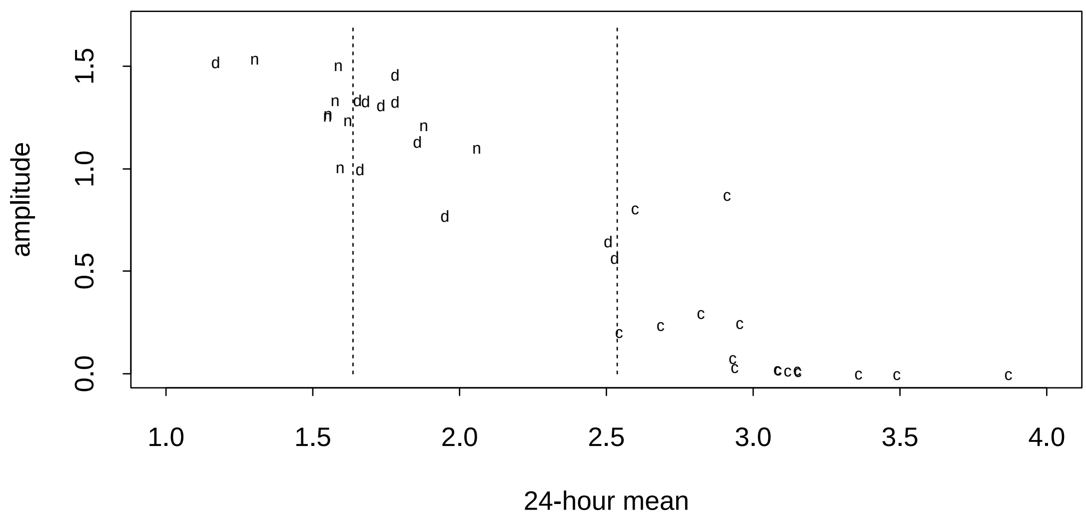

Figure 5: Plot of the estimated 24-hour mean levels against amplitudes. Normal subjects, patients with major depression and patients with Cushing's syndrome are marked as "n", "d" and "c" respectively. Dotted lines represent partition of three groups based on the tree method.

patients may suggest that their conditions were worse, and further medical tests are necessary to check if they have Cushing's syndrome.

Based on a different experiment, Wong et al. (2000) concluded that 30-hour mean cortisol levels were significantly elevated in patients with melancholic depression. They did not compare the differences of amplitudes. Our results are consistent with those in Wong et al. (2000).

Figure 5 shows a negative relationship between the 24-hour mean and amplitude. The estimated correlations between $b_{1i(k)}$ and $b_{2i(k)}$ are -0.786 for depression group ($k = 1$) and -0.531 for normal group ($k = 2$). This indicates that the negative correlation between the 24-hour mean and the amplitude exists not only at the `group` level, but also at the `subject` level. The difference in correlations may be used to investigate physiological changes (Tsaneva, Topalova and Beraha 1990).

4 Discussion

This article presents a flexible and coherent approach to investigate covariate effects on circadian rhythms. As in the classical ANOVA, we decompose the multivariate function $f(k, t)$ into main effects and interactions. The original hypothesis is transformed into one on the interaction component. This technique can be used in general to test differences between two functions (Härdle and Marron 1990, Wang and Ke 2002). The second stage SS ANOVA model (6) is defined for general $\boldsymbol{z}$. For example, letting $\boldsymbol{z} = (\text{gender}, \text{age})$, we can investigate gender and age effects on circadian rhythms (Cauter, Leproult and Kupfer 1996). For our specific application, we concentrated on circadian rhythms. The models and methods developed in this paper can be applied to ultradian and infradian rhythms too. If the period is not fixed and unknown, a scale parameter can be added to model (4).

It is noted that symptoms suggesting the presence of Cushing's syndrome are not pathognomonic. Thus diagnosis may be nonspecific (e.g. obesity, hypertension, menstrual irregularity, and glucose intolerance) (Orth 1995). Plots like Figure 5 may be helpful to improve the precision of diagnosis and/or to further cluster patients into groups. A simple classification by the tree method (Breiman, Friedman, Olshen and Stone 1984) is presented in Figure 5 as dotted lines. It uses the 24-hour mean only and classifies a subject as normal, depression and Cushing's syndrome if his 24-hour mean is below 1.64, above 1.64 but below 1.87, and above 1.87 respectively.

Besides cortisol, the experiment also measured many other variables such as CD4 and hormone ACTH. The methods in this paper can be used to investigate disease effects on circadian rhythms for all these variables. It can also be used to investigate possible association between variables such as ACTH and cortisol (Wang, Guo and Brown 2000), and disease effects on the association (Wong et al. 2000).

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5 Appendix

S-Plus program for fitting model (7) to normal subjects:

```
snm(horm~b1+exp(b2)*f(time-alogit(b3)),
    func=f(u)~list(periodic(u)),
    fixed=list(b1~1), random=list(b1+b2+b3~1),
    start=c(mean(cort.nor.dat$horm)), groups=~ID,
    control=list(prec.out=0.005,converg="PRSS"),
    spar="m", data=cort.nor.dat)
```

S-Plus program for fitting the SIMM (8) and (9):

```
snm(horm~b1+exp(b2)*f(group,time-alogit(b3)),
    func=f(g,u)~list(list(periodic(u),rk.prod(shrink1(g),periodic(u)))),
    data=cort.nordep.dat, fixed=list(b1~group),
    random=pdStrat(b1+b2+b3~1,strata=~as.factor(group)),
    weights=varIdent(form=~1|group),
    control=list(prec.out=0.005,converg="PRSS"),
    spar="m", start=c(1.8,-.2), groups=~ID)
```

S-Plus program for fitting model (10):

```
snm(horm~b1+exp(b2+d1*I(group=="normal"))*
    f(time-alogit(b3+d2*I(group=="normal"))),
    func=f(u)~list(periodic(u)),
    data=cort.nordep.dat, fixed=list(b1~group,d1+d2~1),
    random=pdStrat(b1+b2+b3~1,strata=~as.factor(group)),
    weights=varIdent(form=~1|group),
    control=list(prec.out=0.005,converg="PRSS"),
    spar="m", start=c(1.9,-0.3,0,0), groups=~ID)
```

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