A nonlinear model of stress hormone levels in rats - the interaction between pollution and parasites

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The impact of an infection with a parasite and a simultaneous cadmium exposure on the stress hormone levels of rats was studied. To this end, we introduce a nonlinear heteroscedastic model which is able to describe the temporal evolution of cortisol concentrations in groups of rats treated by cadmium or parasite infection. A thorough analysis gives strong evidence that parasitic infection and cadmium exposure affects the stress hormone level of rats in an additive manner. Therefore the host's response to environmental pollution should be studied in relation to parasite infections.

Key words: Nonlinear parametric model, mixed-effects model, smoothing spline model, simultaneous confidence bands, stress, pollution, parasites.

1 Introduction

Pollution induced impacts on the health of organisms is of increasing interest in recent years (e.g. Schüürmann and Markert [14]). Probably the most intensively studied pollution is the anthropogenic driven increase of heavy metal levels in the environment (Merian [8]). Especially toxic metals like Pb, Cd and Hg were investigated very extensively (Merian [8]). However, in addition to different forms of pollution also a wide variety of pathogen induced diseases affect the wellbeing of plants, animals and man. One group of pathogens that threaten the life of other organisms are parasites (protozoans and metazoans) which account for approximately more than 50% of all organisms (Price [11]). Parasites can significantly reduce the fitness of their hosts (Barnard and

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Behnke [1]) often due to interactions with the stress hormone system (e.g. Dunlap and Schall [3]; Sures et al. [18]). For example elevation of glucocorticoids like cortisol have deleterious effects on the long run, including inhibition of the reproductive and immune systems (Sapolsky [13]). On the other hand, decreased cortisol secretion may also be fatal, as organisms are not able to re-establish their physiological homeostasis after acute stress if their cortisol concentrations are too low.

Under natural conditions usually a mixture of different forms of stressors like endogenous factors, pathogens, pollution, social interactions, etc. are the causative for deviations from a physiological homeostasis of organisms. Especially the interrelation between host-parasite associations and the impact of environmental pollution on the health of the parasitized host has become of interest to ecologists and parasitologists (see reviews by MacKenzie et al. [7]; Lafferty [6]; Sures et al. [17]; Sures [15]). In a recent experiment the impact of a single isolated stressor (cadmium) and a simultaneous infection with a parasite (the acanthocephalan *Moniliformis moniliformis*) on the stress hormone levels of a vertebrate, the laboratory rat was studied (Sures et al. [18]). The response to stress was investigated by measuring levels of the stress hormone cortisol by radioimmunoassay (RIA). Although corticosterone is the primary glucocorticoid in rats, there is a general agreement that both glucocorticoids, corticosterone and cortisol, are regulated in the same way and released in parallel (Saito et al. [12]) which allows cortisol to stand as a general stress measure for adrenocortical function (see e.g. Milanes et al. [9]). In this experiment, we found some indications that parasite infection reduces cortisol levels in rats. A similar effect was also observed for the oral application of $CdCl_2$ to rats. Additionally, these effects seem to be synergistic as rats which were simultaneously infected with the parasite and exposed to Cd showed the most clear reduction in cortisol levels. As it appears from this study that each treatment has an effect on the stress response of rats, it is our goal to find statistical models which describe these responses over time. The models are used subsequently to assess the significance of differences in the stress response between treatment groups.

2 Material and methods

The experiment was described in detail in Sures et al. [18]. Briefly, male Wistar rats of the CD-M-strain, were kept in cages at 20°C, fed on commercial rat pellets, and were free of intestinal helminths as proven by spot checks of faecal matter. Rats were randomly divided into four groups (exposed to Cd, infected with *Moniliformis moniliformis*, exposed and infected, and unexposed and uninfected controls) each containing

8 animals. Each individual rat was kept in a separate cage, all cages were placed in the same room, and the treatments were synchronised. For metal contamination rats were orally exposed to cadmium twice per week for a period of five weeks beginning at day 25 post infection (p.i.) using a cadmium concentration of $5\mu g/g$ body weight. The cadmium was administered in a cadmium chloride-solution prepared from solid $CdCl_2$. As the rats were conditioned to drink sugar-solution from Eppendorf pipettes these solutions supplemented with $CdCl_2$ were administered to the exposed animals. Untreated control sugar solutions were at the same time administered to the unexposed rats to treat all animals in the same manner. Infected rats were inoculated with 10 cystacanths of the acanthocephalan *M. moniliformis* dissected from cockroaches (*Periplaneta americana*) which had been infected 2 months previously by feeding a glucose solution which contained eggs collected from a gravid female *M. moniliformis*. Blood was taken twice a week from each rat (same day and time) by puncturing the lateral vein of the tail, using a syringe and drain tubes without anaesthetics. After blood samples were centrifuged cortisol was extracted with ethanol. Afterwards tritium-labelled cortisol and cortisol antiserum according to Kloas et al. [5] were added. To isolate the antibody-hormone complex, a dextran-activated carbon suspension was added, centrifuged and the supernatant containing the antibody-hormone complex was transferred to scintillation vials and filled with scintillation solution. A liquid scintillation counter (Tri Carb 1900 T; Packard, Dreieich, Germany) was used according to Kloas et al. [5]. Concentrations of cortisol were determined as ng/ml serum. Logarithm of cortisol concentrations was used for all statistical analyses.

Figure 1 shows the course of log-cortisol concentrations in rats after day 25, separated by treatment group. There are 8 animals in group 1 (controls), 7 in group 2 (infected rats) and 8 in group 3 (Cd-exposed rats); group 4 comprises 6 infected and Cd-exposed rats. Mean values and standard deviations of the log-cortisol concentrations in the different groups are plotted in Figure 2.

3 Results

In Section 3.1, we introduce a nonlinear model describing cortisol concentrations over time and examine several ramifications. To emphasize the within-subject dependence, we consider a nonlinear mixed-effect model in Section 3.2. The following section treats the construction of asymptotic confidence intervals and simultaneous confidence bands for the group mean values. By comparing our parametric model with a nonparametric smoothing spline model, we examine in Section 3.4 the appropriateness of the parametric

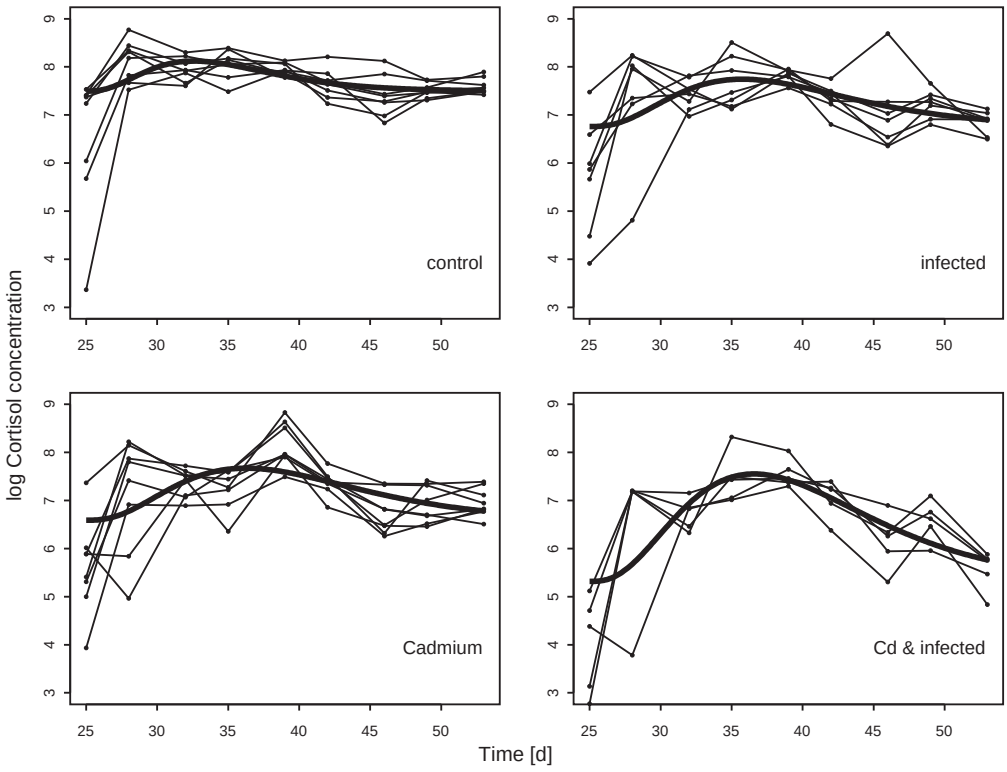

Figure 1: Course of log-cortisol concentrations in rats after day 25 following different treatments with 1-8: controls, 9-15: *Moniliformis moniliformis* infected rats, 16-23: Cd-exposed rats and 24-28: *M. moniliformis* infected and Cd-exposed rats; *M. moniliformis* infection at day 0; Cd exposure started at day 25. The smooth curves are the fits resulting from model (1) with variance function (2) and correlation structure (4) (described in Section 3.1)

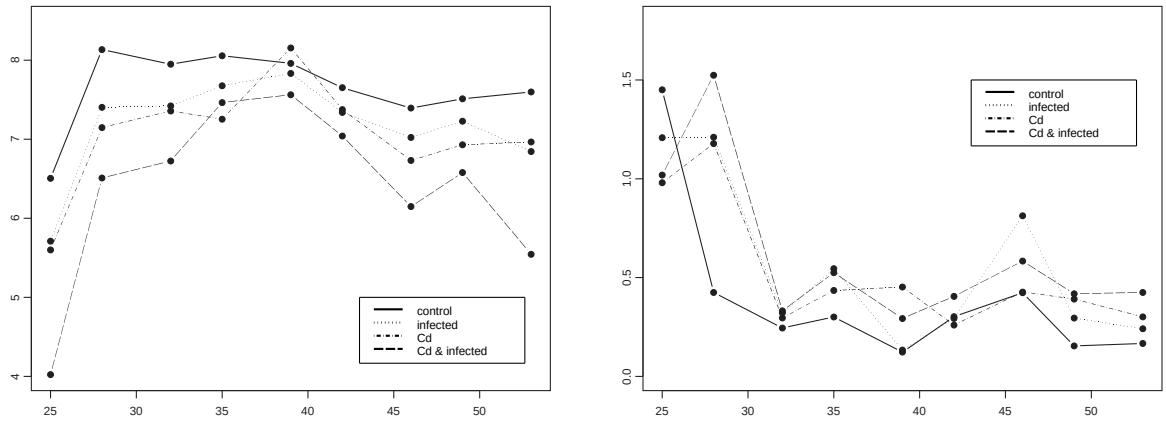

Figure 2: Mean values (left) and standard deviations (right) of the log-cortisol concentrations in the different treatment groups

model.

3.1 A nonlinear model describes cortisol concentrations over time

Our aim is to model the logarithm of the cortisol levels after day 25 and to elaborate differences between the different treatment groups. After the beginning of the metal exposure, the mean curves show a sharp increase and a subsequent decline to the original level (see Figure 2). Hence, we propose the following nonlinear model relating the logarithm of cortisol concentration to time. Note that there are two levels of grouping in the data: the four treatment groups and the subject within group.

$$y_{ijk} = \beta_{1i} + \beta_{2i}\,(\beta_{3i}\,(t_{ijk} - 25))^3\;\; e^{-\beta_{3i}\,(t_{ijk}-25)} + \varepsilon_{ijk} \qquad (t_{ijk} \geq 25) \qquad (1)$$
$$i = 1, \ldots, 4, j = 1, \ldots, M_i, k = 1, \ldots, 9,$$

where $M_1 = 8, M_2 = 7, M_3 = 8, M_4 = 5$, and the error terms ε_{ijk} are assumed to be independently distributed as $\mathcal{N}(0, \sigma^2)$. The value of the parameter vector $\beta_i = (\beta_{1i}, \beta_{2i}, \beta_{3i})$ depends on the treatment group.

Hand and Crowder [4], p. 118-120, used the model $y = \tilde{\beta}_1 + \tilde{\beta}_2 t^3 e^{-\tilde{\beta}_3 t}$ to model blood glucose levels. The two models only differ in the parametrization. Our choice has the advantage that β_3 can be interpreted as time scale.

We fitted the model by the maximum likelihood method, using S-PLUS and the nlme library of Pinheiro and Bates [10]. An inspection of the standardized residuals and of a quantile-quantile plot of the residuals shows strong deviations from the model assumptions.

3.1.1 A heteroscedastic model

As a first step towards a valid model, we dismiss the assumption of equal variance of the error terms, since Figure 2 shows a sharp decline of the variance function over time.

To this end, we introduce the variance function

$$Var(\varepsilon_{ijk}) = \sigma^2 \, (t_{ijk} - 24)^{2\delta_i}, \qquad (t_{ijk} \geq 25). \tag{2}$$

Again, the parameter may depend on the group. Fitting the model yields the values

	control	infected	cadmium	Cd & infected
δ	-0.61	-0.43	-0.42	-0.38

A visual comparison shows that the structure of the empirical standard deviation of the log-cortisol concentrations in the different groups is matched very well by the fitted values of model (2).

The mean curves (estimated again by the maximum likelihood method) are

$$\begin{aligned}
E[y_{1jk}] &= 7.50 + 0.46 \; (-0.39 \, (t_{ijk} - 25))^3 \; \exp(-0.39(t_{ijk} - 25)), \\
E[y_{2jk}] &= 6.76 + 0.73 \; (-0.28 \, (t_{ijk} - 25))^3 \; \exp(-0.28(t_{ijk} - 25)), \\
E[y_{3jk}] &= 6.59 + 0.80 \; (-0.27 \, (t_{ijk} - 25))^3 \; \exp(-0.27(t_{ijk} - 25)), \\
E[y_{4jk}] &= 5.32 + 1.66 \; (-0.26 \, (t_{ijk} - 25))^3 \; \exp(-0.26(t_{ijk} - 25)).
\end{aligned} \tag{3}$$

They are drawn in Figure 1. To facilitate comparison, the four fitted curves are plotted together in Figure 3. Whereas the curves for the infected group and the exposed group are nearly identical, the control group lies above, and the infected and exposed group has the lowest values.

Neither a plot of the standardized residuals nor a quantile-quantile plot of the residuals of model (1) with variance structure (2) show noticeable deviations from the model assumptions.

3.1.2 Modelling the correlation structure

As it has to be expected, the sample autocorrelation function (acf) of the residuals of the heteroscedastic model indicates some dependence between the residuals of the same subject (Figure 4, left). Hence, it is worth considering models with correlated errors.

As the first and simplest correlation structure, we use the model

$$\mathrm{cor}(\varepsilon_{ijk}, \varepsilon_{ijk'}) = \rho, \tag{4}$$

which assumes equal correlation among all errors pertaining to the same subject. The estimated value of ρ is 0.24. Figure 4 (right) shows the sample acf of the heteroscedastic

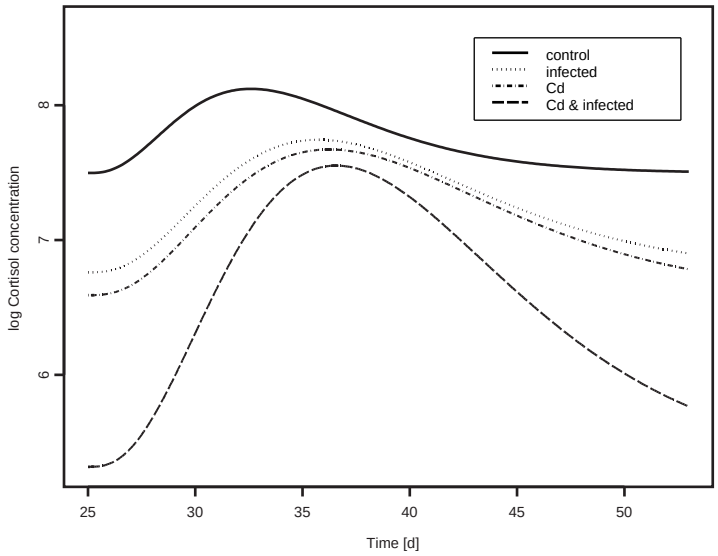

Figure 3: Cortisol concentrations

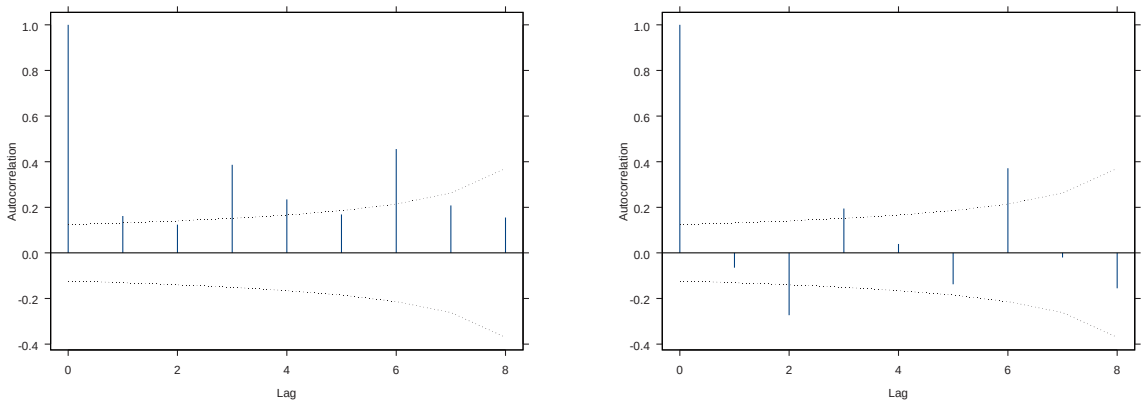

Figure 4: Left: Sample acf of heteroscedastic model with uncorrelated errors. Right: Sample acf of heteroscedastic model with constant correlation structure

Table 1: Parameter values in different groups compared to control group for the constant error model.

	Coefficient	Value	Std.Error	p-value
control	β_{11}	7.51	0.06	< 0.001
infected	$\beta_{12} - \beta_{11}$	-0.71	0.18	< 0.001
cadmium	$\beta_{13} - \beta_{11}$	-0.87	0.19	< 0.001
Cd&infected	$\beta_{14} - \beta_{11}$	-2.16	0.27	< 0.001
control	β_{21}	0.48	0.09	< 0.001
infected	$\beta_{22} - \beta_{21}$	0.26	0.17	0.13
cadmium	$\beta_{23} - \beta_{21}$	0.32	0.17	0.055
Cd&infected	$\beta_{24} - \beta_{21}$	1.18	0.23	< 0.001
control	β_{31}	0.40	0.04	< 0.001
infected	$\beta_{32} - \beta_{31}$	-0.11	0.05	0.033
cadmium	$\beta_{33} - \beta_{31}$	-0.13	0.05	0.013
Cd&infected	$\beta_{34} - \beta_{31}$	-0.14	0.05	0.004

model with constant correlation structure. There, the sample acf does not show a systematic pattern, but exceeds sometimes the confidence bounds. One should note, however, that these bounds are based on pointwise 95% confidence intervals.

Table 1 shows the (difference of) parameter values in the different groups for the constant error model. The p-value in the last columns indicates if the parameter value or the difference of parameter values significantly differs from zero.

Since the time points are nearly equidistant, we can also use time-series models for the correlation structure of the errors. An autoregressive-moving average model of order (p, q) ($ARMA(p, q)$-model) is defined by

$$\varepsilon_t = \phi_1 \varepsilon_{t-1} + \ldots + \phi_p \varepsilon_{t-p} + \theta_1 a_{t-1} + \ldots + \theta_q a_{t-q} + a_t,$$

where $\phi = (\phi_1, \ldots, \phi_p)$ and $\theta = (\theta_1, \ldots, \theta_q)$ are (unknown) parameters, a_t is a centered homoscedastic noise term (independent of the previous observations), and the time points t are assumed to be equidistant. We examined several models; an autoregressive model of order 3 ($AR(3)$-model), which corresponds to an $ARMA(3, 0)$-model, clearly yields the best fit. Hence, we choose the model

$$\varepsilon_{ijk} = \phi_1 \varepsilon_{ij(k-1)} + \phi_2 \varepsilon_{ij(k-2)} + \phi_3 \varepsilon_{ij(k-3)} + a_{ijk}, \tag{5}$$

where $a_{ijk} \sim N(0, \sigma^2)$. The estimated values of the coefficients are

$$\phi_1 = 0.113, \quad \phi_2 = 0.016, \quad \phi_3 = 0.468.$$

Looking at the sample acf of the heteroscedastic model with AR(3)-correlation structure, all values are now within the confidence bounds. This seems to be a clear improvement over the previous model with constant errors; however, the small values of ϕ_1 and ϕ_2, and the quite large value for ϕ_3, are conspicuous, and not easy to explain.

The fitted values of β_i, σ^2 and δ_i for both models with correlated errors are nearly identical with the values in Section 3.1.1. Hence, Figures 1 and 3 remain unchanged when using correlation structure (4) or (5).

3.2 A nonlinear mixed-effects model

As an alternative for modelling the in-subject dependence, we use the following nonlinear mixed-effects model.

$$y_{ijk} = (\beta_{1i} + b_{1ij}) + (\beta_{2i} + b_{2ij})\ ((\beta_{3i} + b_{3ij})(t_{ijk} - 25))^3\ \ e^{-(\beta_{3i}+b_{3ij})\,(t_{ijk}-25)} + \varepsilon_{ijk}$$
$$t_{ijk} \geq 25,\ i = 1, \ldots, 4,\ j = 1, \ldots, M_i,\ k = 1, \ldots, 9,$$

where the random effects $b_{ij} = (b_{1ij}, b_{2ij}, b_{3ij})$ are assumed to be independently distributed as $\mathcal{N}(0, \Psi)$. The statistical analysis showed that a random effect should only be applied to the first coefficient. Hence, we have the model

$$y_{ijk} = (\beta_{1i} + b_{1ij}) + \beta_{2i}\ (\beta_{3i}(t_{ijk} - 25))^3\ \ e^{-\beta_{3i}\,(t_{ijk}-25)} + \varepsilon_{ijk} \qquad (t_{ijk} \geq 25), \quad (6)$$

where $b_{1ij} \sim \mathcal{N}(0, \tau^2)$. Fitting the model yields the value $\tau = 0.204$ which is rather small compared to the residual error with value $\sigma = 1.55$.

For the error terms, we used again the variance function (2) and uncorrelated errors, since the the sample acf of the residuals of model (6) does not show positive dependence.

A dotplot of the estimated random effect shows no particular structure (as, for example, between group differences). Since further diagnostic plots does not indicate violations of the model assumptions, the mixed-effects model seems to be well justified. However, the actual difference of the individual mean curves and the group mean curves is quite small, as can be seen by a visual inspection of the group predictions versus the within subject predictions. So one may question if the additional complexity of model (6) compared to the models of Section 3.1 is justified.

Table 2 shows a comparison of the different heteroscedastic models. Both models with correlated errors and the model (6) with random intercept yield statistically significant improvements over model (1).

The nonlinear mixed-effect model and the model with constant correlation structure have the same number of parameters (np) and comparable AIC and BIC values. Due to its simplicity, we tend to favor the latter.

Table 2: Comparison of the different heteroscedastic models.

Model	np	AIC	BIC	logLik	Test	p-value
(1)+(2)	17	425.9	485.9	-195.9		
(1)+(2)+(4)	18	398.0	461.5	-181.0	1 vs 2	< 0.0001
(1)+(2)+(5)	20	391.6	462.2	-175.8	1 vs 3	< 0.0001
(6)	18	399.9	463.4	-182.0	1 vs 4	< 0.0001

The model with AR(3)-error structure has the lowest AIC value and a comparable BIC value. However, in spite of the results of Table 2, we prefer the two other models, since, as mentioned in Section 3.1.2, the estimated values of the autoregressive parameters look rather artificial.

When discussing these model selection criteria, it should be noted again that the fitted values of β_i, σ^2 and δ_i and the corresponding standard errors are nearly identical for the three models. Hence, the statistical findings in this and the following section do not depend on the choice of the correlation structure.

3.3 Asymptotic confidence intervals and bands

Using the usual t-tests, it turns out that there are significant differences between the parameter values of the different groups (Table 1). Whereas such differences are often easy to interpret in linear models, it is more difficult to obtain knowledge about the variability of the mean curve of nonlinear models that way. For this reason, we construct asymptotic confidence intervals and simultaneous confidence bands for the group mean curves of the heteroscedastic model with constant error structure.

Since we use maximum likelihood estimation, the estimator $\hat{\beta}_i = (\hat{\beta}_{1i}, \hat{\beta}_{2i}, \hat{\beta}_{3i})'$ has an approximate normal distribution with mean $\beta_i = (\beta_{1i}, \beta_{2i}, \beta_{3i})'$ and covariance matrix Σ_i (see, for example, Pinheiro and Bates [10], Section 7.5). Σ_i can be consistently estimated by some estimate $\hat{\Sigma}_i$ (which, in case of maximum likelihood estimation, is the inverse of the information matrix). A Taylor expansion of f around β_i then yields the large sample variance $\nabla_i f'(t)\hat{\Sigma}_i \nabla_i f(t)$ of $f(t)$, where $\nabla_i f = (\partial f/\partial \beta_{ji})_{1 \le j \le 3}$. Hence, approximate pointwise confidence intervals for the i-th group are given by

$$\left(f(t, \hat{\beta}_i) - t_{\alpha/2} \sqrt{\nabla_i f'(t) \hat{\Sigma}_i \nabla_i f(t)}, \qquad f(t, \hat{\beta}_i) + t_{\alpha/2} \sqrt{\nabla_i f'(t) \hat{\Sigma}_i \nabla_i f(t)} \right),$$

where $t_{\alpha/2}$ is the $(1 - \alpha/2)$-quantile of the normal distribution.

Approximate simultaneous confidence bands can be constructed in a similar way,

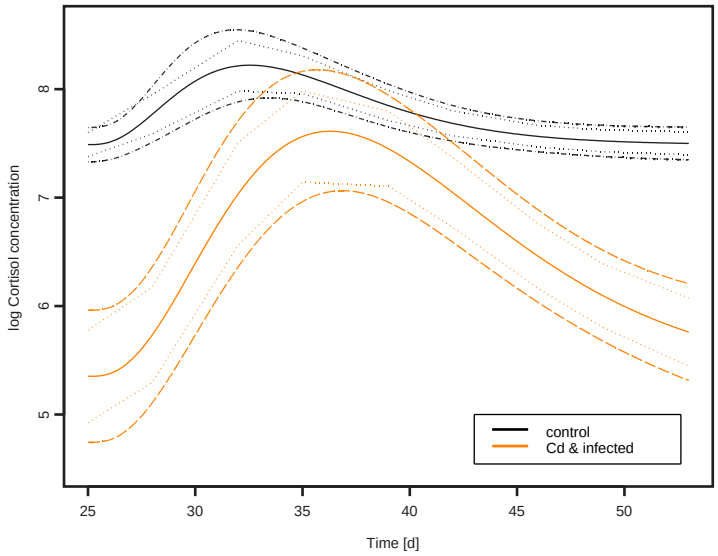

Figure 5: Simultaneous 95% confidence bands (dashed) compared to pointwise 95% CI's (dotted) for groups 1 and 4

following the Scheffé method (Cox and Ma [2]). The resulting bands are

$$\left(f(\cdot, \hat{\beta}_i) - \sqrt{\chi^2_{p,\alpha} \nabla_i f' \hat{\Sigma}_i \nabla_i f}, \qquad f(\cdot, \hat{\beta}_i) + \sqrt{\chi^2_{p,\alpha} \nabla_i f' \hat{\Sigma}_i \nabla_i f} \right), \tag{7}$$

where p is the number of unknown parameters, i.e. in our case we have $p = 3$. The simultaneous bands and the pointwise confidence intervals are compared for groups 1 and 4 in Figure 5.

Note that a Bonferroni correction for the 9 time points would lead to the same width of the confidence intervals at the different times as the simultaneous bands in Figure 5 (since, for $\alpha = 0.05$, $(\chi^2_{3,\alpha})^{1/2} \approx t_{\alpha/(2 \cdot 9)} \approx 2.8$). However, the confidence bands in (7) are simultaneously valid for each t.

Since we are interested in the difference of the cortisol levels between groups, we also constructed (simultaneous) confidence bands for the difference between two treatment models $f(t, \hat{\beta}_{i_1}) - f(t, \hat{\beta}_{i_2})$. Since the estimates $\hat{\beta}_{i_1}$ and $\hat{\beta}_{i_2}$ are asymptotically independent, the large sample variance for the difference is $\left(\nabla_{i_1} f' \hat{\Sigma}_{i_1} \nabla_{i_1} f \right)^2 + \left(\nabla_{i_2} f' \hat{\Sigma}_{i_2} \nabla_{i_2} f \right)^2$. Whereas the lower confidence bound for the difference between control group and the Cd & infected group is nearly always positive, this is no longer the case when comparing the control group with the other treatment groups.

3.4 A smoothing spline model

The main aim of this section is to assess the validity of our parametric model. To this end, we fit a (nonparametric) smoothing spline model to the data, and compare it to our parametric nonlinear model.

As general smoothing spline model we use

$$y_{ijk} = g(t_{ijk}) + \varepsilon_{ijk} \qquad (t_{ijk} \geq 25),$$

$i = 1, \ldots, 4, j = 1, \ldots, M_i, k = 1, \ldots, 9$. Here, we assume that g has a square integrable second derivative and

$$\varepsilon_{ij} = (\varepsilon_{ij1}, \ldots, \varepsilon_{ij9})' \sim N(0, \sigma^2 W_i^{-1}),$$

where $\sigma^2 W_i^{-1}$ denotes the covariance matrix resulting from variance function (2) together with an AR(1)-correlation structure. The nonparametric estimate $\hat{g}_\lambda$ is the solution of

$$\min_g \left\{ \frac{1}{n}(y-g)'W(y-g) + \lambda \int_{t_1}^{t_2} g''(t)\, dt \right\}. \tag{8}$$

The second term in (8) is a penalty to the roughness of g.

We fitted the smoothing spline model using the ASSIST-package of Wang and Ke [20]. A crucial step in the fitting procedure is the choice of the smoothing parameter λ (which may also depend on the treatment group). In ASSIST, several data-adaptive methods are available. We used the generalized maximum likelihood method (Wahba [19]) to estimate the unknown parameters (δ, λ). Figure 6 shows the smoothing spline models of the 4 groups with the observed mean values. The estimated curves follows the mean values very closely. The dashed lines in Figure 6 are Bayesian confidence intervals which also have good frequentist properties (Wang and Wahba [21]).

Instead of assessing the goodness of fit of the parametric model with a formal test, Figure 7 visualizes the parametric mean curve together with the nonparametric fits for groups 1 and 4. Apparently, parametric and nonparametric curves are quite similar, and the parametric curve lies within the confidence bands, indicating the suitability of the parametric model. However, for all four groups, the parametric curves are smoother than the nonparametric models.

4 Discussion

In this article, we have introduced a nonlinear heteroscedastic model which is able to describe well the temporal evolution of cortisol concentrations in groups of rats treated by

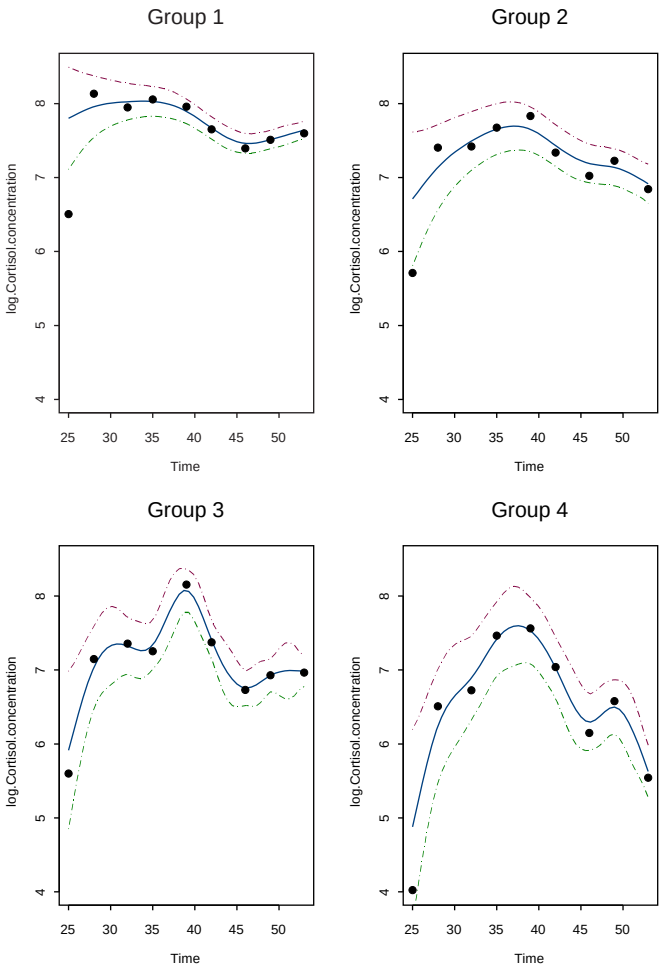

Figure 6: Smoothing spline models of the 4 groups with 95% confidence intervals and observed mean values (group 1: control, group 2: infected, group 3: cadmium, group 4: Cd & infected)

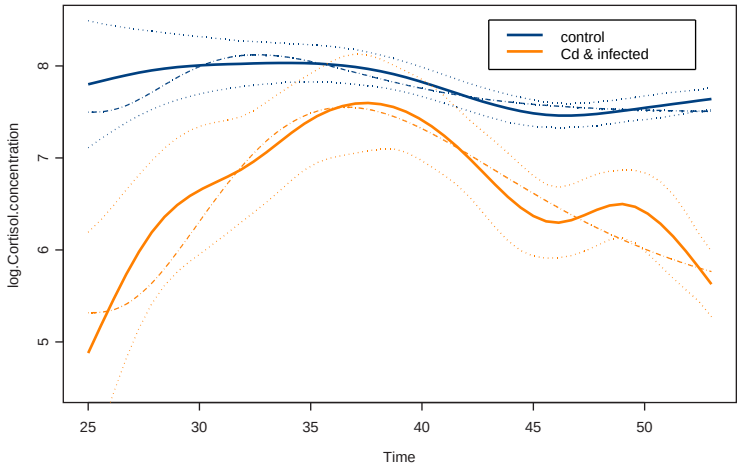

Figure 7: Smoothing spline models of groups 1 and 4 (solid lines) together with 95% confidence intervals (dotted) and mean curve of parametric model (dashed)

cadmium exposure and/or parasite infection. The model reproduces the sharp increase with the beginning of cadmium application and the subsequent decline in all groups.

The fitted model separates the mean curves of the control group and the infected and exposed group from the curves of the other two groups. The latter have nearly identical mean curves. The control group lies significantly higher than the infected and exposed group; the significance bands in Section 5 show nearly no overlap. The other two groups lie in-between, giving strong evidence that the effect of parasite infection and of cadmium exposure affects the stress hormone level of rats in an additive manner.

The similarity of the impact of exposure and infection are of great importance in terms of ecotoxicological research. Especially in studies aiming at the question whether exposure to certain chemicals affect the physiological homeostasis of the test organism, it would be important to use also infected organisms since a vast majority of free living animals is infected with parasites. Accordingly, it would be inappropriate to ignore possible infections in organisms used for ecotoxicological and toxicological tests, as the extrapolation of results obtained from healthy uninfected animals in the laboratory is insufficient to determine the effects on infected hosts under natural conditions.

As a consequence, the host's response to environmental pollution should be evaluated in relation to knowledge about parasite infections.

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