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Interaction between cadmium exposure and infection with the intestinal parasite *Moniliformis moniliformis* (Acanthocephala) on the stress hormone levels in rats

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"Capsule": *Parasite infections may influence host's reponse to environmental pollutants.*

Abstract

The impact of an infection with the acanthocephalan *Moniliformis moniliformis* and a simultaneous Cd-exposure on the stress hormone levels of rats was studied. Immediately after the application of cadmium to some rats, cortisol levels in all groups of rats, as quantified by radioimmunoassay (RIA), significantly increased. However, infections with *M. moniliformis* as well as the uptake of Cd reduced significantly the cortisol release compared to untreated controls. While catecholamine concentrations, as determined by high-performance liquid chromatography (HPLC), showed no clear tendency during the experimental period, the ratio of $C_{adrenaline}/C_{noradrenaline}$ in the controls showed the significantly lowest value of all four groups after killing the animals. Thus, the acanthocephalan infection as well as the Cd-exposure and the combination of both treatments affect hormone homeostasis in the rats which probably lead to negative effects on the health of the rat. Therefore parasite infections must be carefully considered in environmental impact studies, as an important factor affecting the host's health.

Keywords: Cadmium; Rats; Stress hormones; Catecholamines; Cortisol; Acanthocephalans; Parasites; *Moniliformis moniliformis*

1. Introduction

Parasites can significantly reduce the fitness of their hosts (Barnard and Behnke, 1990; Price, 1980). For example, a field study found that male fence lizards infected with the malarial parasite *Plasmodium mexicanum* had higher levels of corticosterone and lower levels of testosterone as a response to stress than uninfected conspecifics (Dunlap & Schall, 1995). Although glucocorticoids (including corticosterone and cortisol) are important in responding to various challenges, prolonged or repeated elevation of these hormones have deleterious effects, including inhibition of the reproductive and immune systems (Sapolsky, 1987).

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. 1995; Lafferty, 1997; Sures et al., 1997, 1999). Most investigations have dealt with animals from aquatic biotopes (reviewed in Sures, 2001), except for a few that compared heavy metal concentrations in mammalian parasites and their definitive hosts (Ince, 1976; Greichus and Greichus, 1980; Sures et al., 1998, 2000a, b; Scheef et al., 2000).

One of the most toxic metals released into the environment by production, manufaction and waste disposal is cadmium (Merian and Haerdi, 1991). The uptake and accumulation of cadmium in mammals is well known (Goon and Klaassen, 1989; Elsenhans et al., 1994; Shaikh et al., 1995; Elsenhans et al., 1997) and the toxicological effects of this metal are described from all classes of vertebrates (Schäfer et al. 1994). Cadmium is known to increase levels of cortisol in fish (Gill et al., 1993), but in mammals a reduction in corticosterone production was described (Ng and Liu, 1990). Steroids

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themselves can also have a variety of effects on cadmium toxicity including both mitigation and exacerbation of the harmful effects of this metal (Shimada et al., 1988). Another group of stress hormones, the catecholamines have also been found to both increase and decrease in their concentrations in mammals as a result of cadmium exposure (Shanbaky et al., 1978; Shrivastava and Sathyanesan, 1988).

Vertebrates under natural conditions usually are affected by multiple stressors. The aim of this study was to investigate the impact of a single isolated stressor (cadmium) with a simultaneous infection with a parasite (the acanthocephalan *Moniliformis moniliformis*) on the stress hormone levels of a vertebrate, the laboratory rat. The response to stress was investigated by measuring levels of two catecholamines, adrenaline and noradrenaline, using high performance liquid chromatography (HPLC) and levels of the stress hormone cortisol were quantified 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., 1992) which allows cortisol to stand as a general stress measure for adrenocortical function (see e.g. Milanes et al., 1991) using a well established RIA (Kloas et al., 1994).

2. Material and methods

2.1. Maintenance and infection of rats

Male Wistar rats of the CD-M-strain, weighing on average 285 g, and 60–65 days of age, were obtained from a commercial supplier (Charles River Deutschland GmbH, Sulzbach, Germany). The animals 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. Twice a week they were additionally fed with biscuits to guarantee a sufficient supply of saccharose for the parasitizing *M. moniliformis*. Rats were infected with 10 *M. moniliformis* cystacanths each, 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*. After the rats were trained to drink sugar solution from an Eppendorf pipette the cystacanths were given to them in a sugar solution.

2.2. Experimental design

Rats were randomly divided into four groups each containing eight animals (Table 1). Each individual rat was kept in a separate cage, all cages were placed in the same room, and the treatments were synchronised. Rats in groups III and IV were orally exposed to cadmium twice per week for a period of 5 weeks beginning at day 25 post infection (p.i.) using a cadmium concentration of 5 μg/g body weight. The cadmium was administered in a cadmium chloride-solution prepared from solid $CdCl_2$ (Merck, Darmstadt, Germany). 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. Rats in groups II and IV were infected with cystacanths of the acanthocephalan *M. moniliformis* as described earlier (infection intensities, see Table 1). The animals in group I served as uninfected and unexposed controls.

Table 1
Experimental design

Group	Number of rats	Infection (No. of *M. moniliformis*)[a]	Cd-application
I	8	−	−
II	8	+ (2.0±1.5; 1−4)	−
III	8	−	+
IV	8	+ (1.2±1.8; 1−5)	+

[a] Number of worms detected in the intestine (mean±SD; min–max).

2.3. Sampling of blood

Blood was taken twice a week (same day and time). The first blood sample was drawn prior to the infection of rats in groups II and IV at day 0. Prior to blood sampling, the animals were warmed at 37 °C for 15 min in an incubator to ensure adequate blood flow. Blood (1 ml) was collected by puncturing the lateral vein of the tail by using a syringe and drain tubes (Henke Sass Wolf, Tuttlingen, Germany) without anaesthetics. A final blood sample was taken from the posterior vena cava at the end of the investigation after killing the rats at day 53.

2.4. Determination of cortisol

After blood samples were centrifuged for 5 min at $2000\times\boldsymbol{g}$, sera were collected and stored at −70 °C until analysis. Cortisol was extracted by adding 1000 μl of 96% ethanol to each sample of 50 μl serum, centrifuged and the organic phase including cortisol, was transferred in open glass vials. The ethanol was allowed to evaporate overnight. The following day, 1000 μl of 5% ethanol was added to the samples to dissolve the cortisol again. The sample was divided into three aliquots each containing 300 μl of extract, to which 100 μl of tritium-labelled cortisol (3000 cpm) and 500 μl of cortisol antiserum (dilution 1:10,000 in lysocym buffer B) according to Kloas et al. (1994) were added; these were

then held on ice for about 3 h. To isolate the antibody-hormone complex, 100 μl of dextran-activated carbon suspension was added. After centrifugation, the supernatant containing the antibody-hormone complex, was transferred to LSC-tubes and filled with scintillation solution (Ultima Gold; Packard, Dreieich, Germany). A liquid scintillation counter (Tri Carb 1900 T; Packard, Dreieich, Germany) was used according to the methods described by Kloas et al. (1994).

2.5. Determination of the catecholamines

The catecholamines (adrenaline and noradrenaline) were determined in 200 μl of serum. To all sera samples was added 1100 μl of distilled water and 100 μl of dihydroxybenzylamine (1 ng/100μl 1% acetic acid) as an internal standard. After adding 500 μl of 2 M Tris–HCl buffer (pH 8.7) containing 0.065% Na_2EDTA, the catecholamines and the internal standard were adsorbed with 10 mg of activated aluminium oxide (Sigma, Deisenhofen, Germany). Following 15 min shaking and centrifugation the supernatant was removed and the aluminium oxide containing the adsorbed catecholamines and the internal standard was washed three times with 1 ml of distilled water before the catecholamines and the standard were eluted from the aluminium oxide. For the acidic elution 100 μl of 1% acetic acid containing 0.05% $Na_2S_2O_5$ and 0.025% Na_2EDTA was added, and after 15 min shaking the samples were centrifuged. The supernatant was removed and stored at −20 °C until analysis by HPLC. The method of HPLC analysis of adrenaline and noradrenaline closely followed that published by Kloas and Hanke (1992).

2.6. Statistical analysis

Mean concentrations ($\bar{x} \pm$ SD) of the catecholamines and of cortisol were determined as ng/ml serum. A detailed analysis of the cortisol concentration of the different treatment groups at a given time were conducted. As a preliminary check, the variances were tested for homogeneity using the Fligner–Killeen test with significant results only on days 14 ($P = 0.01$) and 25 ($P = 0.005$).

To test for differences of means, a one-way ANOVA was used. Normal probability plots (QQ plots) and the Shapiro–Wilk test for normality were used to assess whether the distribution of the residuals is close to normality. The Shapiro–Wilk test was significant for day 0 ($P = 0.02$) and day 39 ($P = 0.03$) and highly significant for day 18 ($P = 0.001$) and day 46 ($P < 0.001$). Hence, the results of parametric tests for day 18 and day 46 may be invalid. If the ANOVA showed a significant result, pairwise *t*-tests were used (this corresponds to Fisher's least significant difference method). To correct for multiple testing, Tukey's studentized range test was applied.

Since there is some doubt whether the parametric assumptions hold for all dates, we repeated the analysis using nonparametric procedures: as overall test, we applied the Kruskal–Wallis test. Pairwise comparisons of different treatments were conducted using the Mann–Whitney *U*-test. As correction for multiple testing, we used Holm's method.

Such corrections notwithstanding, there is still a multiple testing situation since (at least) one test for each day was performed. A further correction taking into account the different tests for different points in time is not appropriate due to the substantial loss of power. Despite the well-known problems of multiple testing for repeated measurements, we think that the results of the tests are still informative.

A possible simple remedy for the multiple testing problem for repeated measurements is the use of summary measures, as advocated by Matthews et al. (1990). Since the time intervals are nearly equally spaced, the arithmetic mean of cortisol concentration over a suitable time period is a sensible per subject summary measure. Additionally, a model that takes into account the development over time, was developed (Section 3.2).

Friedman and Wilcoxon tests were applied to test for significant differences between hormone levels of following days in each group.

3. Results

3.1. Cortisol

Mean cortisol concentrations (± SD) for all groups of rats are shown in Fig. 1. To visualize differences between the groups, individual cortisol concentrations were compensated by subtraction of each value during the study by the initial value and plotted in Fig. 2 according to Sures et al. (2001). Cortisol levels significantly increased in all four groups immediately after the Cd-exposure of the rats of groups III and IV (Wilcoxon-test, $P \leqslant 0.01$ in all cases). Following the first metal exposure on day 25 the infected, the exposed and the infected and exposed rats showed significantly lower cortisol levels on several days compared to the control (Tables 2 and 3). Additionally, the infected and exposed rats had also significantly lower cortisol levels than those rats which were either only Cd treated or only infected. Accordingly, there is a synergistic interaction of *M. moniliformis* and Cd resulting in decreased serum cortisol levels. Prior to the Cd exposure cortisol levels of the infected rats were on several days significantly lower (Tables 2 and 3) than those of the controls or the Cd exposed animals (which were untreated until day 25). Thus, infection with *M. moniliformis* alone has an influence on the hormone status of the host.

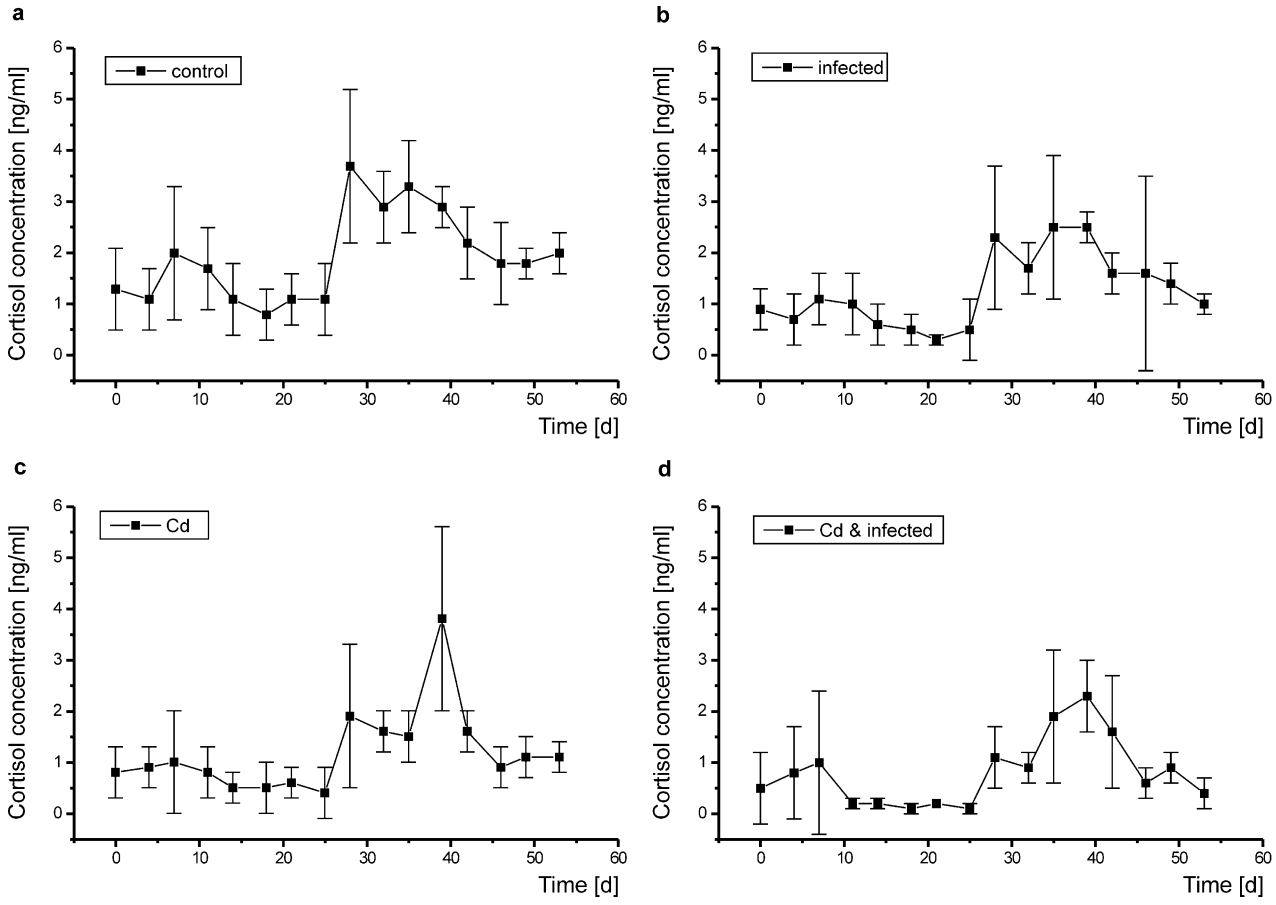

Fig. 1. Course of cortisol concentrations in rats following different treatments with a: controls, b: *Moniliformis moniliformis* infected rats, c: Cd-exposed rats and d: *M. moniliformis* infected and Cd-exposed rats; *M. moniliformis* infection at day 0; Cd exposure started at day 25.

3.2. A nonlinear model describes cortisol concentrations over time

Looking at Figs. 1 and 2, a linear or polynomial model does not seem adequate to describe the relationship between cortisol concentrations and time. To simplify matters, we decided to model only the cortisol levels after day 25. After the beginning of the metal exposure, the mean curves show a sharp increase and a subsequent decline to the original level (Fig. 1). Hence, we propose the following nonlinear model relating the expected cortisol concentration to time.

$$\text{Cortisol} = a + b(\text{day-25})^3 \exp(-c(\text{day-25})) \quad (\text{day} > 25) \tag{1}$$

A similar model was used by Hand and Crowder (1996). Fig. 3 shows the cortisol concentrations for day 25 to day 53 in the four groups along with model (1), fitted by the nonlinear least squares method. The estimated values are

$$\text{Cortisol (control)} = 1940 + 243\ (\text{day-25})^3 \exp(-0.56\ (\text{day-25})),$$

$$\text{Cortisol (infected)} = 1077 + 28\ (\text{day-25})^3 \exp(-0.30\ (\text{day-25})),$$

$$\text{Cortisol (cadmium)} = 738 + 23\ (\text{day-25})^3 \exp(-0.27\ (\text{day-25})),$$

$$\text{Cortisol (Cd and infected)} = 199 + 25\ (\text{day-25})^3 \exp(-0.28\ (\text{day-25})).$$

To facilitate comparison, the four fitted curves are plotted together in Fig. 4. Whereas the curves for the infected and the exposed group are nearly identical, the control group lies above, and the infected and exposed group has the lowest values.

3.3. Catecholamines

There were no significant changes (Friedman, $P > 0.05$) between the catecholamine concentrations in

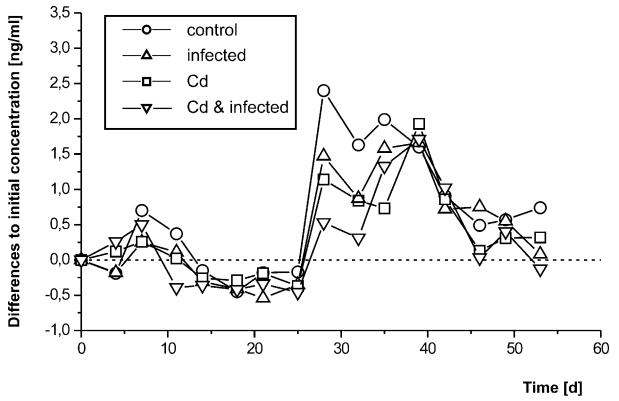

Fig. 2. Differences to basal cortisol levels in rats following different treatments; *Moniliformis moniliformis* infection at day 0; Cd exposure started at day 25.

Table 2
Results of parametric tests[a]

Day	*P*-value of ANOVA	I–II[b]	I–III	I–IV	II–III	II–IV	III–IV
11	0.002	>	(≫)[c]	≫	(>)		
14	0.009	>	(>)	≫			
18	0.06[d]						
21	<0.001	≫	(≫)	≫		>	
25	0.02		>	≫			
28	0.01		>	≫			
32	<0.001	≫	≫	≫	>	>	
35	0.02		≫	>			
39	0.03					≫	>
42	0.02	>	>	≫			
46	0.17[d]						
49	<0.001	>	≫	≫	≫		
53	< 0.001	≫	≫	≫	≫	≫	
0–21	0.02	>	>	≫			
25–53	<0.001	>	≫	≫		>	

[a] ANOVA not significant for days 0 ($P=0.22$), 4 ($P=0.61$) and 7 ($P=0.33$).

[b] >, Significant difference following Fisher's least significant difference method; ≫, correction for multiple testing with Tukey's studentized range test.

[c] The parenthesis indicate that the differences are not significant at the 0.01 level.

[d] ANOVA results questionable for day 18 and day 46 since Shapiro–Wilk test for normality of residuals is highly significant ($P<=0.001$ in both cases).

each of the four groups during the study period, nor did the ratio of $C_{adrenaline}/C_{noradrenaline}$ (see Gfell et al., 1997) vary for the different treatments. The concentrations of the catecholamines and the resulting ratio of $C_{adrenaline}/C_{noradrenaline}$ after killing the animals are shown in Fig. 5. Comparison of the adrenaline and noradrenaline concentrations between treatments revealed no significant differences (Kruskal–Wallis test, $P>0.05$). In contrast, different values were found for the ratio of $C_{adrenaline}/C_{noradrenaline}$ (Kruskal–Wallis test, $P\leqslant 0.05$). The controls were found to show the significantly lowest value of all four groups (Mann–Whitney U, $P\leqslant 0.05$, in all cases), whereas no difference was found among the remaining

Table 3
Results of nonparametric tests[a]

Day	p-value of Kruskal–Wallis test	I–II[b]	I–III	I–IV	II–III	II–IV	III–IV
11	0.003		>	≫	≫	≫	
14	0.02			≫		>	
18	0.02		>	>			
21	<0.001	≫	(>)[c]	≫		≫	>
25	0.02			>	>	>	
28	0.01		>	≫	>		
32	<0.001	≫	≫	≫	≫	≫	
35	0.03		≫				
39	0.03			>		>	
42	0.03			>			
46	0.006		>	≫	>		
49	0.001	>	≫	≫	≫		
53	<0.001	≫	≫	≫	≫	≫	
0–21	0.049	>		>			
25–53	0.003	>	>	≫	>		

[a] Kruskal–Wallis test not significant for days 0 ($P=0.22$), 4 ($P=0.61$) and 7 ($P=0.33$).

[b] >, Significant difference following Mann–Whitney U-test; ≫, correction for multiple testing with Holm's method.

[c] The parenthesis indicate that the differences are not significant at the 0.01 level.

three groups (Mann–Whitney U test, $P>0.05$ in all cases). Each single stressor as well as the combination of Cd-exposure and infection causes higher adrenaline than noradrenaline concentrations which is in contrast to the controls (Fig. 5 a).

4. Discussion

A significant increase of cortisol levels was evident for all rats correlating with the beginning of Cd-application of the respective exposed animals. This phenomenon can be explained by communication between individual rats of the different groups as all rats were faced with the same physical stress of administering the respective solutions. All rats were kept together in one room, allowing acoustic, olfactory and visual contact between the animals. After each cadmium dose the rats scrape on the floor of their cages. This behaviour seems to be an alarming signal which may be responsible for a stress reaction in the unexposed animals. Interestingly, the control rats showed the significantly highest cortisol release following Cd exposure. Infection with the acanthocephalan *M. moniliformis* as well as cadmium contamination significantly reduce cortisol release. Additionally, the lowest values of cortisol following Cd exposure were obtained for those rats which were infected and exposed. One may expect an even more pronounced effect if higher infection intensities in the rats were obtained (see e.g. Sures et al., 2000b). However our data reveal that a parasitic infection acts as a

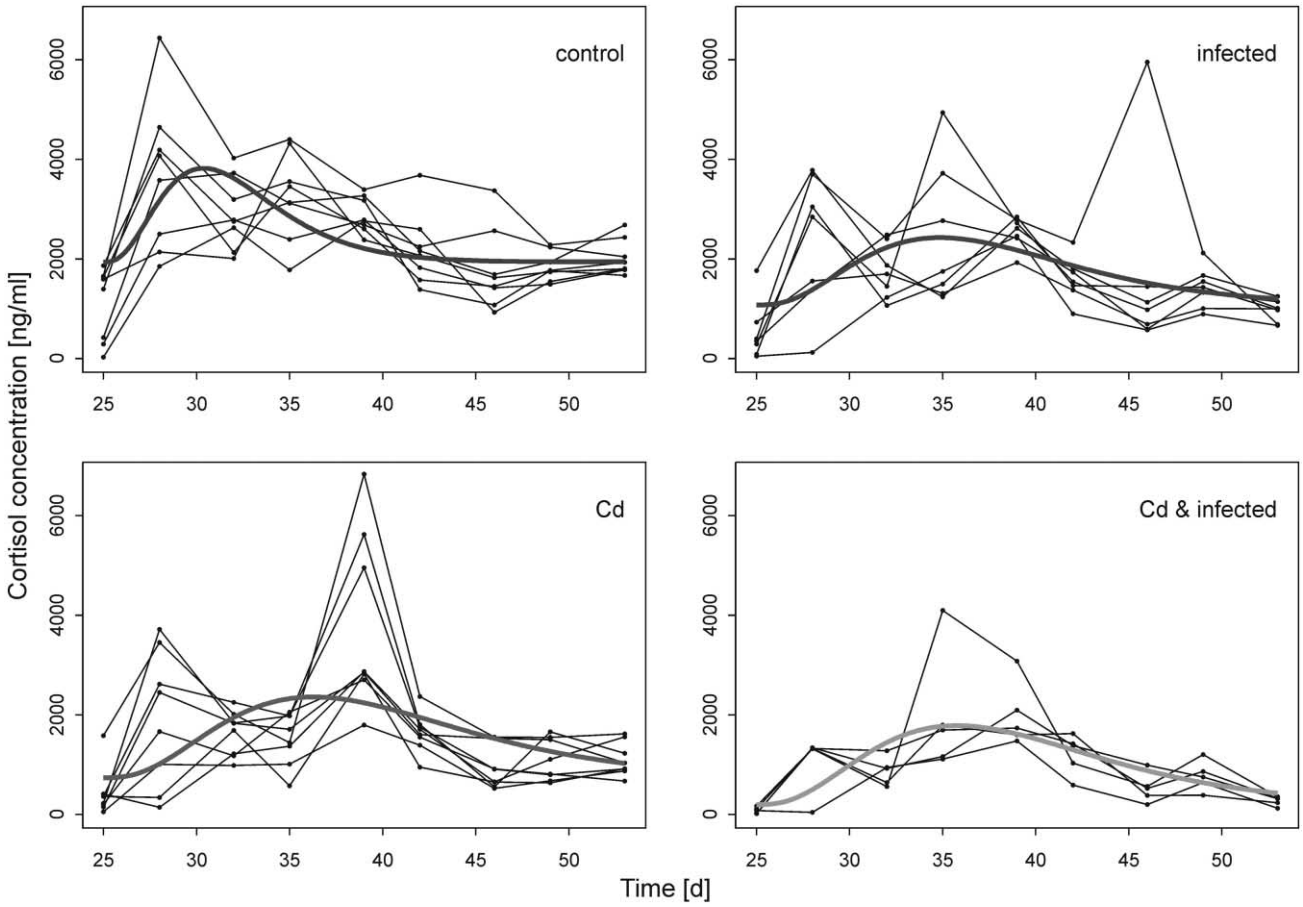

Fig. 3. Cortisol concentrations for day 25 to day 53 in the four groups along with the nonlinear model based on Eq. (1).

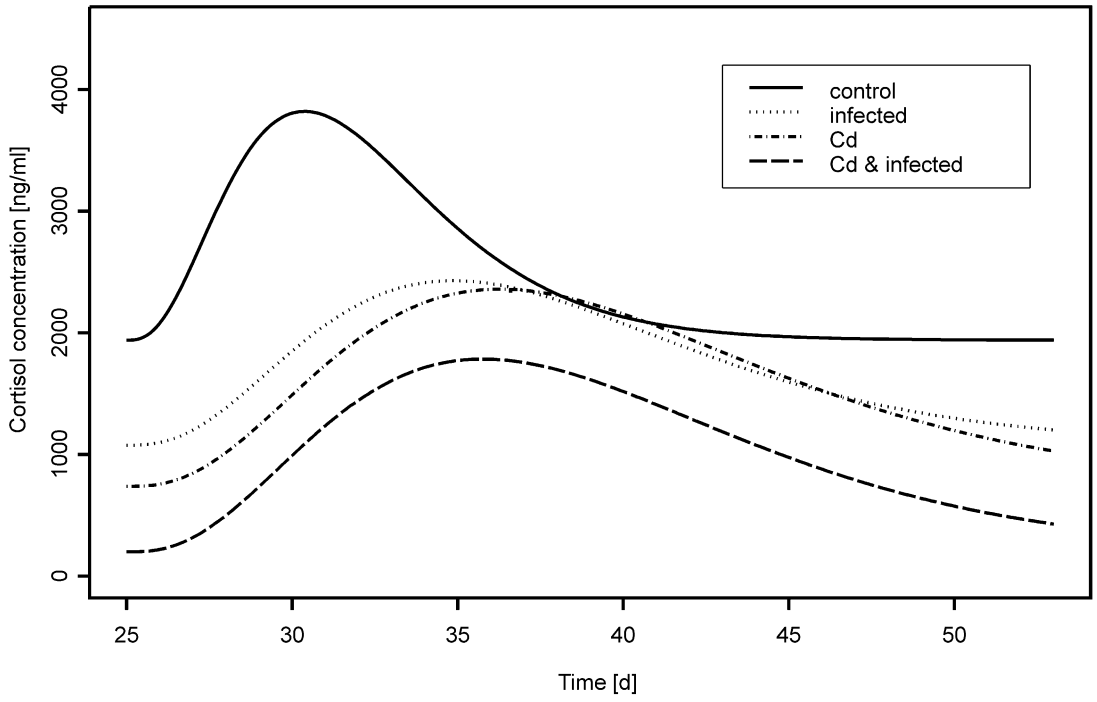

Fig. 4. Comparison of the four fitted curves calculated according to Eq. (1).

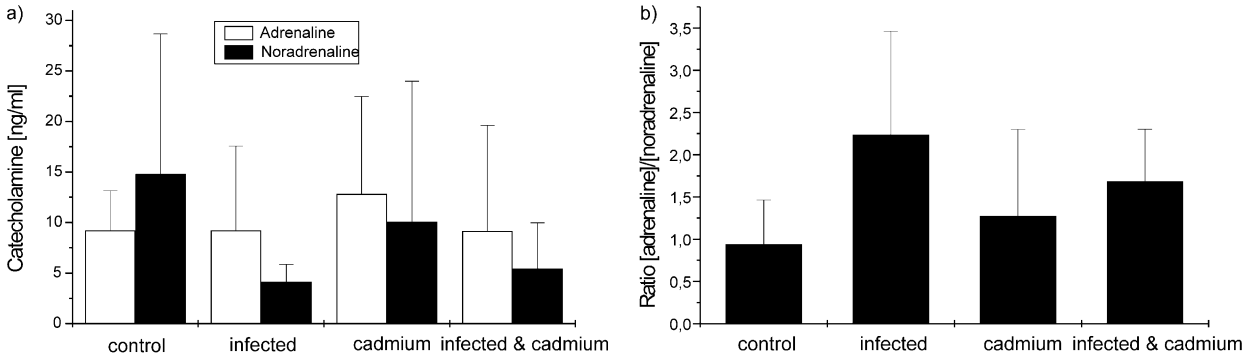

Fig. 5. Catecholamine levels after killing the rats with a: adrenaline and noradrenaline and b: ratio [adrenaline]/[noradrenaline].

synergistic factor in addition to a cadmium exposure resulting in decreased cortisol values. It is already known from literature that Cd may influence corticosteroid synthesis in different ways. Whilst it is known to increase levels of cortisol in fish (Gill et al., 1993), in mammals a reduction in corticosterone production was described (Ng and Liu, 1990). Although the primary glucocorticoid in rats is corticosterone, cortisol and corticosterone are regulated in the same way and are released in parallel (Saito et al., 1992). Due to these similarities decreased cortisol values following the cadmium exposure could be expected.

Reports published on the impact of a Cd-exposure on catecholamine synthesis have also contrasting results. Both Shanbaky et al. (1978) and Hart and Borowitz (1974) described a Cd-induced stimulation of the catecholamine synthesis in the adrenal medulla. In contrast, Shrivastava and Sathyanesan (1988) found a significant decrease in noradrenaline levels following Cd-exposure. In organisms which are not stressed, noradrenaline levels in the serum are usually higher than the adrenaline levels, whereas in stressful situations this relation will be reversed due to the activity of the adrenal medulla, which synthesizes 80% adrenaline and 20% noradrenaline (Chrousos and Gold, 1992). Considering these findings, it is obvious that the acanthocephalan infection as well as the Cd-exposure and the combination of both treatments causes stress to the rats. Thus, in addition to Cd, also the infection with *M. moniliformis* has a stimulating effect on the catecholamine synthesis.

It is commonly accepted that a considerable number of free living animals are infected with large numbers of different protozoan and metazoan parasites (Esch et al., 1990). The results of our study indicate that parasite infections must be carefully considered in environmental impact studies, as another important factor affecting the host's physiological homeostasis. Infection with *M. moniliformis* as well as Cd exposure causes a stimulation of the sympathetic nervous system via the adrenal medulla resulting in higher serum adrenaline/noradrenaline ratios. The hypothalamus-pituitary-adrenal cortex axis seems also to be affected by exposure and infection in a similar way: both parameters reduce the cortisol release in a stressful situation compared to untreated controls. Thus, it would be inappropriate to ignore possible infections with parasites in organisms used for ecotoxicological and toxicological tests, as the extrapolation of results obtained from healthy uninfected animals is insufficient to determine the effects on infected hosts.

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