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# URINE CORTISOL IN PIGS OF DIFFERENT MH-GENOTYPES *

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## ABSTRACT

Urine cortisol concentration was evaluated in German Landrace barrows of two MH-genotypes (NN, Nn) housed in metabolic cages. Furthermore, cortisol in urine was evaluated as a substitute measure of plasma cortisol values in pigs. Three replicates were done, each including 4 animals. Altogether there were 12 animals included in the experiment. The effect of MH-genotype on urine cortisol concentration was evaluated and correlations between urine and plasma cortisol were calculated. Higher urine cortisol values in NN- in comparison to Nn-pigs indicated stronger response to stressors by stress resistant NN-genotype. The correlation between urine (12-hr urine values) and plasma (the average of 12 collections per day, taken between 8.00 a.m. and 11.00 a.m.) cortisol values was practically negligible in samples taken on the same day ($r = 0.07$, $P = 0.71$). In time-lag samples (urine taken the day after blood collection) correlation was increased ($r = 0.22$), but still not significant ($P = 0.22$). A connection between plasma and urine cortisol values was indicated, but it should be better understood before urine cortisol is used as a substitute of plasma cortisol measuring in pigs.

Key words: pigs / animal physiology / endocrinology / malignant hyperthermia / urine / plasma / cortisol

# KONCENTRACIJA KORTIZOLA V URINU PRI PRAŠIČIH RAZLIČNIH MH-GENOTIPOV †

## IZVLEČEK

Namen raziskave je bil oceniti koncentracijo kortizola v urinu pri prašičih dveh MH-genotipov (NN, Nn). Poleg tega smo ocenili koncentracijo kortizola v urinu kot nadomestilo določanju koncentracije kortizola v plazmi pri prašičih. Prašiči so bili pasme nemška landrace, uhlevljeni v metabolne kletke. Opravljeni so bili trije poskusi (tri ponovitve), v vsakem so bili 4 kastrati. Skupno je bilo v raziskavo vključenih 12 živali. Ovrednotili smo vpliv MH-genotipa na

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koncentracijo kortizola v urinu in izračunali korelacije med vrednostmi kortizola v urinu (12-urne vrednosti) in plazmi (povprečje 12-ih dnevnih odvzemov, odvzetih med 8.00 in 11.00 uro). Večja koncentracija kortizola v urinu pri NN- v primerjavi z Nn-genotipom kaže na močnejši odziv na stresorje pri NN-genotipu, odpornem na stres. Korelacija med koncentracijo kortizola v plazmi in urinu je bila statistično neznačilna pri odvzemu vzorcev na isti dan ($r = 0,07$, $p = 0,71$) in višja ($r = 0,22$), vendar še vedno statistično neznačilna pri odvzemu urina dan po odvzemu krvi ($p = 0,22$). Povezava med koncentracijo kortizola v krvi in urinu je nakazana, vendar jo je potrebno bolje pojasniti pred rutinsko uporabo kortizola v urinu kot nadomestila za določanje kortizola v plazmi pri prašičih.

Ključne besede: prašiči / fiziologija živali / endokrinologija / maligna hipertermija / urin / plazma / kortizol

## INTRODUCTION

Plasma levels of adrenocortical hormone cortisol are often used as a stress indicator. However, collection of blood is invasive and can, in itself, stimulate cortisol release. Furthermore, the insertion of vein catheters is a skilled technique and catheters are often difficult to maintain over longer periods. Therefore, only a very limited number of animals can be included in the experiment. Cortisol is mainly excreted in urine, which can be collected noninvasively when spontaneously voided. It is therefore considered as an alternative to plasma sampling.

Urine cortisol values have been used as a measure of adrenocortical activity in many species. Kiecolt-Glaser *et al*. (1984) used urine cortisol as stress measure in lonely psychiatric inpatients. Bertrand *et al*. (1987) confirmed urine cortisol to be of value in assessing adrenocortical function in man. In horses, plasma and urine cortisol were found to be significantly correlated (Ralston *et al*., 1988). Miller *et al*. (1991) showed that monitoring cortisol concentration in urine was a valid approach for studying adrenocortical activity in bighorn sheep. Carlstead *et al*. (1992) made comparable conclusions for felids. As a stress measure urine cortisol values were also used in cattle (Redbo, 1993). Stress conditions – tethering after a grazing period – lead to increased levels of stereotypies and high urine levels of cortisol.

For pigs, the evaluations of urine cortisol as an alternative measure to plasma cortisol are more recent. Hay *et al*. (2000) found diurnal variations of urinary cortisol comparable to those in plasma. Furthermore, cortisol in urine produced during the night and the early morning was highly correlated with its 24-hr excretion rate in blood. Pol *et al*. (2002) showed in pregnant sows that adrenocortical activity could be monitored by urinary cortisol analysis in routine conditions or after ACTH challenge. It was concluded that urine cortisol could be used as the addition in the assessment of chronic stress and could improve the evaluation of animal welfare if used together with other welfare indicators.

Malignant hyperthermia syndrome is a reaction to acute stressors, inherited by a single recessive gene (n). It is characterised by hyperthermia and muscle rigidity, often followed by sudden death within minutes. The connection between malignant hyperthermia (MH) and adrenal function was investigated by monitoring cortisol concentrations in blood (e.g. Aberle *et al*. 1976; Mitchell and Heffron, 1981; Nyberg *et al*., 1988; von Borell and Ladewig, 1989; Schaefer *et al*., 1990). To our knowledge, no comparisons of urine cortisol concentrations have been made between different malignant hyperthermia (MH) genotypes. Hay and Mormède (1998) evaluated urine cortisol values in two breeds of pigs known to differ in mean plasma cortisol concentrations. The difference was comparable with plasma cortisol results (Mormède *et al*., 1984; Bergeron *et al*., 1996).

The aim of the present study was to evaluate urine cortisol in pigs of two malignant hyperthermia (MH) genotypes. In addition, the experiment allowed also evaluating its substitute value for cortisol concentration in plasma for growing pigs.

## MATERIAL AND METHODS

### Animals and housing

Three replicates were done, each including four German Landrace barrows housed in metabolic cages. Altogether, there were 12 animals included in the experiment. In the metabolic cages without straw, animals were able to stand up and lie down, but not to turn around. Pigs were housed in the experimental environment 14 days before intravenous catheters for blood sampling were inserted. They were always handled by a familiar person in order to reduce additional stress caused by handling. Health condition of the animals was monitored by daily measurement of body temperature. Dry meal and drinking water were available *ad libitum*. The illumination and ventilation were natural.

Two MH-genotypes were included in the experiment. The MH-genotype was determined by a DNA-based test (Fujii *et al*., 1991). Altogether 95 animals were checked. There were only 6.13 % of animals carring nn-genotype. They were unevenly spred over the three replicates. Thus, it was impossible to create groups of recessive homozygotes. Therefore, half of the pigs where dominant homozygous (NN) and nn-animals were replaced with heterozygotes (Nn).

### Collecting blood and urine samples

Catheters for blood collection were inserted at the age of 143 ± 5 days and live weight 63.0 ± 4.0 kg. Blood samples were collected simultaneously from all animals on day 8, 22 and 36 after the insertion of catheters between 8.00 a.m. and 11.00 a.m. in 15 min. intervals. Urine of each animal was collected in plastic containers under the cage between 7.30 a.m. and 7.30 p.m. on the day before, on, and after blood collection. The aliquots (5 ml) for determination of cortisol level were taken from excreted urine and stored at –20 °C. The average of 12 plasma samples collected on the corresponding day was related to urine concentration on and after the day of blood collection.

### Cortisol analysis

The total plasma cortisol concentration was quantitated by means of RIA as described by Ladewig and Smidt (1989). Total urine cortisol concentration was determined with chemiluminescence method, following the instructions of the kit producer – Nichols Institute Diagnostica. We were assured by the producer that the kit for the quantitative determination of cortisol in human serum, plasma and urine was suitable also for the cortisol determination in pigs. The intra-assay variability was up to 10 % for plasma and urine cortisol concentrations.

### Statistics

Cortisol measurements were logarithmically transformed to achieve normality. For urine cortisol, the following fixed effects were included in the model 1: the three replicates ($R_i$), the two MH-genotypes ($G_j$), the individual animals ($A_{ijk}$) and the trial ($T_l$). Within each replicate there were three blood samplings while urine was taken on “the day before, the day after and the day of blood sampling” assigned as trials. Interactions did not show a significant effect and were therefore excluded from the model.

$$y_{ijkl} = \mu + R_i + G_j + A_{ijk} + T_l + e_{ijkl} \quad [1]$$

The data were checked for normality with UNIVARIATE, correlations between plasma and urine cortisol concentration were estimated with CORR, and model evaluation with GLM procedure (SAS/STAT, 1990).

## RESULTS

### Cortisol concentration in urine

Cortisol concentration was variable as shown on Figure 1, where each dot displayed single observation of each animal. The values ranged from 0.6 to 94.3 ng $ml^{-1}$. However, the majority of measurements were gathered under 20 ng $ml^{-1}$. Nine urine samples were collected for each animal within each replicate, except for animal 8, for which one urine sample was lost due to spilling. Three outliers were detected: one on animal 8 and two on animal 16 (Figure 1); they were excluded from further analyses.

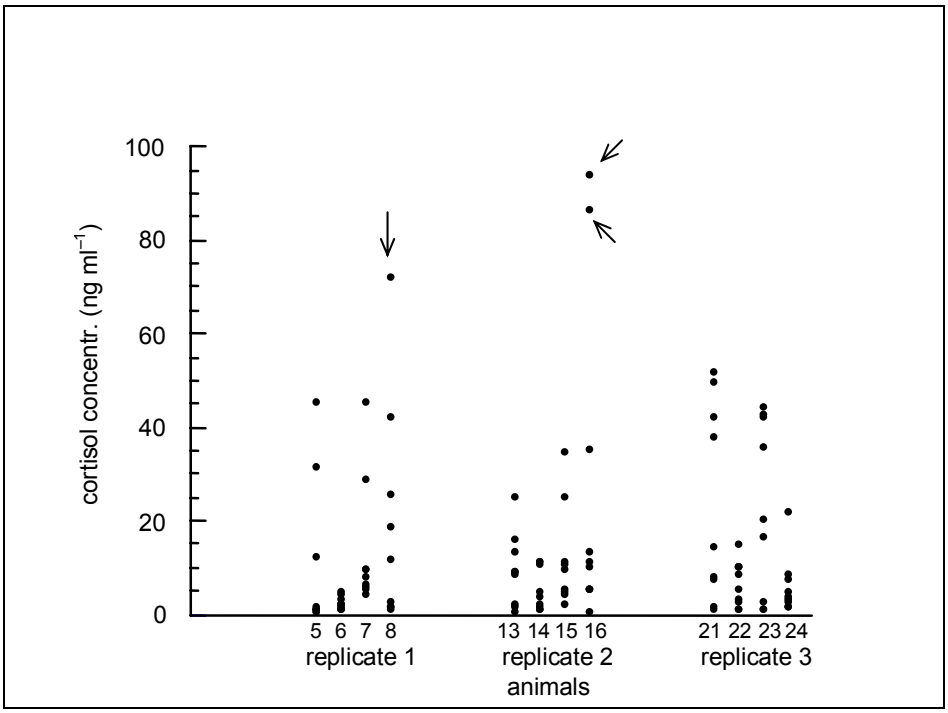


Figure 1. Urine cortisol concentrations (ng $ml^{-1}$) for individual animals in replicates 1, 2 and 3. Every point represents one 12-hr sample. The arrows are pointing to outliers.
Slika 1. Koncentracija kortizola v urinu (ng $ml^{-1}$) pri posamezni živali v ponovitvah 1, 2 in 3. Vsaka točka predstavlja en 12-urni vzorec. Puščice kažejo osamelce.

The differences among animals were expressed also in mean values (Table 1) varying from 2.4 to 23.8 ng $ml^{-1}$. Standard deviations within animals were high and depended on mean suggesting need for data transformation. Furthermore, the test for normality proved that the data were skewed toward low values as already expressed in Figure 1. A logarithmic transformation confirmed to be appropriate to achieve normality.

Table 1. Means and standard deviations (SD) for urine cortisol values (ng $ml^{-1}$) in individual animals

Preglednica 1. Povprečja in standardne deviacije (SD) za koncentracijo kortizola v urinu (ng $ml^{-1}$) pri posamezni živali

| | REPLICATE 1 | | REPLICATE 2 | | REPLICATE 3 | |
|---|---|---|---|---|---|---|
| genotype | animal | $\bar{x} \pm$ SD | animal | $\bar{x} \pm$ SD | animal | $\bar{x} \pm$ SD |
| Nn | 5 | 10.6 ± 16.7 | 14 | 4.2 ± 4.0 | 22 | 6.4 ± 4.8 |
| Nn | 6 | 2.4 ± 1.4 | 15 | 12.1 ± 10.9 | 23 | 23.0 ± 18.7 |
| NN | 7 | 13.7 ± 14.0 | 13 | 9.6 ± 7.9 | 21 | 23.8 ± 21.3 |
| NN | 8 | 14.9 ± 15.3 | 16 | 11.6 ± 11.3 | 24 | 6.3 ± 6.3 |
| total | | 10.4 ± 5.6 | | 9.4 ± 3.6 | | 14.9 ± 9.8 |

n = 9 for all animals, except for animal 8 (one sample was lost due to spilling, therefore n = 8)

## Correlations between cortisol concentration in plasma and urine

Correlations were calculated between daily cortisol level in plasma and urine. The correlation between samples taken on the same day (Figure 2a) was practically negligible (r = 0.07) and not significant (P = 0.71). The results revealed that half-day sample of urine did not reflect oscillations of the average cortisol level in blood samples taken between 8 and 11 a.m. In time-lag samples (Figure 2b), when urine was taken the day after blood collection, the correlation increased (r = 0.22), but it was still not significant (P = 0.22). The relationship was positive implying that higher cortisol level in plasma caused increase in urine excreted the following day.

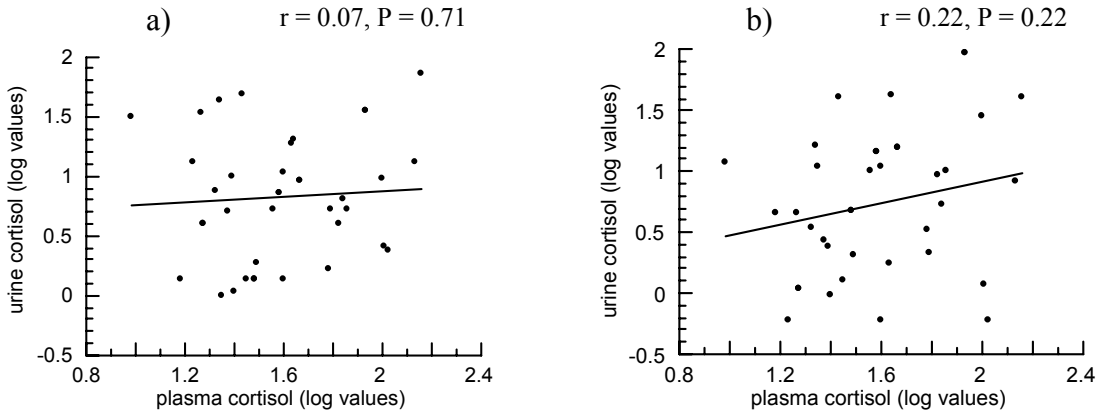


Figure 2. Relationship between plasma and urine cortisol values in samples a) taken on the same day and b) taken the day after blood collection.

Slika 2. Povezava med koncentracijo kortizola v plazmi in urinu a) pri vzorcih, odvzetih na isti dan in b) vzorcih z zamikom.

## Effect of MH-genotype on cortisol concentration in urine

The experiment was carried out in three replicates under the same conditions. Each replicate consisted of three trials with one blood and three urine sampling days. To eliminate any uncontrolled disturbance, replicates and trials were considered as possible effects. As expected,

they did not show a significant effect, which also indicated that collection of blood was not stressful for the animals.

The basal cortisol levels as well as response to stressors differ among animals, therefore differences among animals were expected. The effect of animal was expressed clearly in raw data (Table 1) and confirmed by statistical test (Table 2).

Table 2. Statistical significance of the effects on urine cortisol concentrations
Preglednica 2. Statistična značilnost vplivov na koncentracijo kortizola v urinu

| Effects | Degrees of freedom | P-value |
|---|---|---|
| Replicate | 2 | 0.26 |
| MH-genotype | 1 | 0.03 |
| Animal | 8 | 0.05 |
| Trial | 2 | 0.16 |

Urine cortisol measurements may be used as indicator of stress. Therefore, the differences in urine cortisol concentrations were expected between homozygotes (NN) and heterozygotes (Nn) carrying recessive allele for MH-deficiency. The effect of MH-genotype was indeed significant (Table 2). Animals NN excreted urine with higher cortisol level than animals Nn. The amount on untransformed scale for homozygotes was approximately 1.4 times the amount of heterozygotes (Figure 3a). The results from the model were coherent (Figure 3b). In enriched environment, the differences might not be expected in such extent. However, the pigs were kept in metabolic cages with confinement conditions causing chronic stress. The higher value might be reflected in stronger response to stressors by more stress resistant NN-genotype.

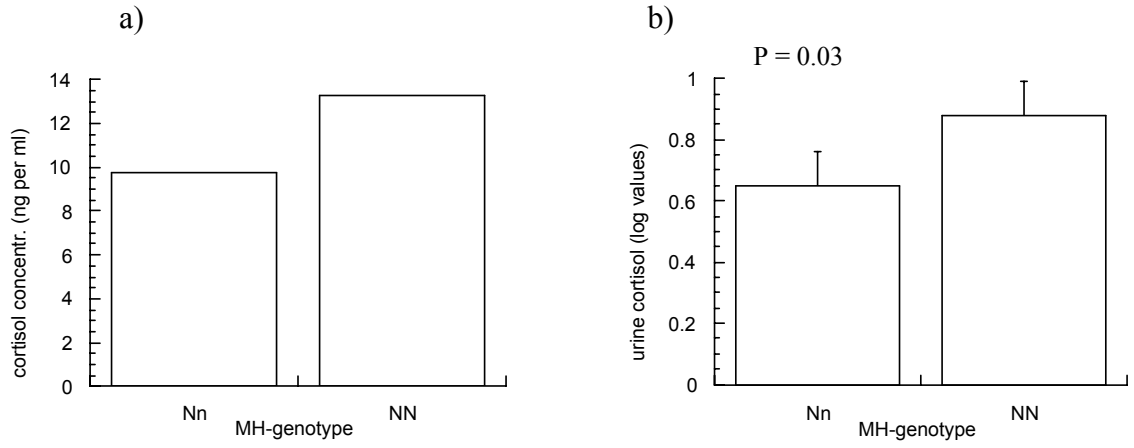


Figure 3. Average urine cortisol values in ng $ml^{-1}$ (a) and least square means (LSM) with standard errors (SE) (b) for the two MH-genotypes.
Slika 3. Povprečni vrednosti za koncentracijo kortizola v urinu v ng $ml^{-1}$ (a) in vrednosti LSM s standardno napako (b) pri dveh MH-genotipih.

## DISCUSSION

The experiment was performed to investigate whether presence or absence of recessive allele for malignant hyperthermia syndrome affected urine cortisol levels in pigs. Furthermore, cortisol concentration in urine was evaluated as a substitute measure of plasma cortisol concentration.

The study was carried out in metabolic cages where it was possible to collect urine without disturbing the animals. The urine for that purpose was collected on the day of blood sampling and the day after, while urine samples taken the day before were used as a control.

Pigs exposed different levels of stress hormone cortisol in urine during diurnal excretion (Table 1). The effect of animal proved to be significant (Table 2). The ratio between the lowest and the highest average level was 10-fold. The observations within animal showed high variability as well (Figure 1). The variability between and within animals was higher compared to the proportions in plasma (Siard, 1998). The differences in the averages between animals were a consequence of different ability for cortisol production and release in unstressful and stressful conditions while the deviations within animal were caused by random animal response to poor environment.

The difference between the two MH-genotypes was significant; NN-animals had higher urine cortisol values than Nn-animals (Table 2, Figure 3). Obviously, the adrenocortical activity was higher in resistant NN-animals. The results were in line with outcomes of Mitchell and Heffron (1981) for plasma cortisol that classified animals into resistant and susceptible groups by halothane test. They showed that stress resistant animals excreted more cortisol and reacted faster to halothane exposure. Furthermore, they suggested that reduced cortisol secrection contributed to the triggering of malignant hyperthermia syndrome. A significantly higher cortisol concentration was observed also in plasma of NN-pigs in comparison to Nn-pigs (Siard, 1998).

Differences among genotypes were presented in the study by Hay and Mormède (1998) who monitored urine cortisol concentrations of Meishan, Large white and F1-crossbred pigs. Meishan lactating sows secreted about five times as much cortisol as Large white, while F1 sows were found to produce intermediate level. They claimed that urine cortisol could be used for the assessment of chronic stress. The conclusions were in agreement with plasma cortisol (Mormède *et al*., 1984; Bergeron *et al*., 1996). Meishan pigs having higher cortisol level coped better with a new situation. In behavioural studies, they demonstrated relatively indifferent response to the novel environment (Mormède *et al*., 1984).

The correlation between urine and plasma cortisol in samples taken on the same day (Figure 2a) was slightly positive ($r = 0.07$). The correlation for time-lag samples, where urine was taken the day after blood collection (Figure 2b), was increased to 0.22. However, both correlations did not show significant deviation from zero. In more recent study, Hay *et al*. (2000) compared diurnal variations of urine cortisol with those in plasma in pregnant sows. Average plasma cortisol values, measured in 24-hr samples (blood collected between 0 and 24 hrs) were correlated with cortisol values in urine, produced during the night and early morning of the following day (between 8.00 p.m. and 8.00 a.m.) The correlation was high (0.83) in contrast to our results. The difference between their and our experiment was in blood sampling intervals and in the time-lag used. In our study, urine samples were collected between 7.30 a.m. and 7.30 p.m. on the day of blood collection and the day after blood collection. Furthermore, in our study plasma cortisol values presented 3-hr concentrations of plasma cortisol.

The exact delay of cortisol secretion into urine can be determined with the use of ACTH or corticotropin-releasing hormone (CRH), which stimulate cortisol release. A time-lag in peak responses in plasma and urine is then monitored. Miller *et al*. (1991) reported a 2–6 hr delay in bighorn sheep and Carlstead *et al*. (1992) reported a 2-hr delay in felids. For pigs, a delay was 1,5 hr (Hay *et al*., 2000), while Pol *et al*. (2002) detected the increase in urine cortisol concentration 2–5 hrs after the ACTH injection and return to basal concentrations 24-hrs after ACTH injection. Without stimulation, diurnal oscilation in plasma cortisol was reflected in urine approximately with a 2-hr delay (Hay *et al*., 2000).

Due to the small sample size we could not calculate individual correlations between plasma and urine cortisol values. However, it would be interesting to see what happens at the individual level since Carlstead *et al.* (1992) reported differences in individual correlations in felids. The

importance of taking into account individual differences was emphasized also by Hay and Mormède (1998).

Our results indicate a connection between plasma and urine cortisol concentrations in pigs. For more precise information the exact time-lag of cortisol secretion into urine should be considered, as showed by Hay *et al*. (2000), and the possible individual differences should be clarified.

## CONCLUSIONS

Higher cortisol concentration in NN- in comparison to Nn-pigs indicates stronger response to stressful conditions in stress resistant NN-genotype. A connection between plasma and urine cortisol concentration is indicated, but it should be better understood before urine cortisol concentrations are used as a substitute of plasma cortisol measuring in pigs.

## POVZETEK

V poskusu smo ovrednotili vpliv MH-genotipa na koncentracijo kortizola v urinu in poskušali oceniti koncentracijo kortizola v krvni plazmi prašičev pitancev z meritvami koncentracije kortizola v urinu. Vzorce urina, ki jih je izločila vsaka žival med 7.30 in 19.30 uro, smo odvzeli dan pred, na dan in dan po odvzemu vzorcev krvi. Razpon vrednosti je segal od 0,6 do 94,3 ng $ml^{-1}$. Za izračun korelacije med koncentracijo kortizola v plazmi in urinu smo izračunali povprečno koncentracija kortizola v plazmi za posamezno žival na določen dan (povprečje dvanajstih odvzemov na 15 minut) in primerjali s koncentracijo v urinu na isti dan in dan kasneje. Korelacijo med koncentracijo kortizola v plazmi in koncentracijo kortizola v urinu en dan kasneje smo izračunali, ker so pri različnih vrstah navajali nekaj – do večurni zamik izločanja kortizola v urin (Miller in sod., 1991; Carlstead in sod., 1992). Pol in sod. (2002) so pri prašičih ugotovili povečano koncentracijo kortizola v urinu 2–5 ur po aplikaciji ACTH in padec na osnovno vrednost 24 ur po aplikaciji ACTH. Primerjava dnevnih ritmov izločanja kortizola v kri in urin je pokazala 2-urni zamik (Hay in sod., 2000). V naši raziskavi je bila korelacija med kortizolom v plazmi in urinu na isti dan (slika 2a) komaj pozitivna (r = 0,07). Pri upoštevanju zamika izločanja kortizola v urin (Slika 2b) je bila korelacija višja (r = 0,22), vendar še vedno statistično neznačilna (p = 0,22). Hay in sod. (2000) pa so ugotovili visoko korelacijo med kortizolom v urinu, odvzetem v nočnem in zgodnjem jutranjem času, in 24-urno koncentracijo kortizola v plazmi (r = 0.83). Za razliko od našega poskusa je bil čas jemanja vzorcev krvi in urina v njihovem poskusu dugačen, poleg tega so v njihovem poskusu koncentracijo kortizola v plazmi predstavljali 24-urni vzorci. Živali genotipa NN so imele višjo koncentracijo kortizola v urinu kot živali genotipa Nn, kar kaže na močnejši odziv na stresne razmere pri MH-odpornem genotipu NN. Razlika med genotipoma je primerljiva z razliko v koncentraciji kortizola v plazmi (Siard, 1998). Z ugotavljanjem koncentracije kortizola v urinu nismo uspeli oceniti koncentracije kortizola v urinu, vendar lahko sklepamo, da je tudi kortizol v urinu možen pokazatelj stresa pri živalih, če poznamo njegove osnovne vrednosti v primernem okolju. Pri različnih MH-genotipih pa moramo upoštevati, da se lahko pri dominantnih homozigotih skorja nadledvične žleze odzove na stresne razmere močneje kot pri heterozigotih.

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