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MHS STATUS AND SALIVARY CORTISOL CONCENTRATION IN INDIVIDUALLY HOUSED PIGS *

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ABSTRACT

Salivary cortisol was evaluated as stress measure in pigs of two malignant hyperthermia (MH) genotypes (NN and Nn), housed individually in metabolic cages and in comfortable large pens with straw. Three replicates were done, each including 8 German Landrace barrows, four (2 NN, 2 Nn) housed in pens and four (2 NN, 2 Nn) in metabolic cages. Altogether there were 24 animals included in the experiment. Saliva samples of all animals were collected simultaneously every 15 minutes between 8.00 and 11.00 a.m. on days 8, 22 and 36 of the experiment. Pigs in more stressful conditions (metabolic cages) had higher salivary cortisol values than pigs in pens, indicating that salivary cortisol might be a suitable indicator of stress in pigs. Higher salivary cortisol values in NN- in comparison to Nn- pigs indicated stronger response to stressful conditions in NN-genotype.

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

MHS – STATUS IN KONCENTRACIJA KORTIZOLA V SLINI PRI INDIVIDUALNO UHLEVLJENIH PRAŠIČIH †

IZVLEČEK

Kortizol v slini je bil ovrednoten kot pokazatelj stresa pri prašičih dveh MH – genotipov (NN in Nn) v dveh individualnih uhlevitvah (metabolne kletke in prostorni boksi z nastilom). V vsaki od treh ponovitev je bilo 8 kastratov pasme nemška landrace. Štiri živali (2 NN, 2 Nn) so bile uhlevljene v bokse, štiri (2 NN, 2 Nn) pa v metabolne kletke. Skupno je bilo v raziskavo vključenih 24 živali. Vzorci sline so bili odvzeti istočasno od vseh živali, vsakih 15 minut med 8. in 11. uro, in sicer 8., 22. in 36. dan poskusa. Živali v bolj stresnem okolju (metabolne kletke) so imele višjo koncentracijo kortizola v slini kot živali v boksih, kar nakazuje, da je kortizol v slini primeren pokazatelj stresa pri prašičih. Višje koncentracije kortizola pri živalih NN – genotipa v primerjavi z genotipom Nn kažejo na močnejši odziv na stresne razmere pri genotipu NN.

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

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INTRODUCTION

The adrenocortical hormone cortisol measured in plasma is often used as a stress indicator in pigs. However, the procedures for obtaining blood can, in itself, stimulate cortisol release, especially in case of venipuncture. The use of vein catheters is considered less stressful, but health problems can occur and only a very limited number of animals can be included in the experiment. In addition, insertion of catheters is a skilled technique and catheters are often difficult to maintain over longer periods. Measurement of cortisol in saliva is a noninvasive way of sampling. Saliva samples can be obtained also on farms, without any health risk for the animals. Furthermore, since there is no question of catheter functioning, the experiments can last for a longer period.

Salivary cortisol measurement was found to be a suitable replacement for plasma cortisol measurement in various species. In man, Vining *et al*. (1983) described the correlation between salivary and serum cortisol as excellent. High correlations were found in different situations: in patients with adrenal insufficiency and in healthy people, in tests of adrenal function (dexamethasone suppression, ACTH stimulation) and circadian variation as well as in random samples. The results of Laudat *et al*. (1988) confirmed excellent correlation in normal people and in patients with adrenal disfunction. According to Fell *et al*. (1985) and Fell and Shutt (1986), salivary cortisol was a reliable indicator of stress in sheep (transport and confinement) and calves (transport, castration) as well as in goats during transport (Greenwood and Shutt, 1992). Steinhardt and Thielscher (2000) confirmed good agreement between plasma and salivary cortisol in calves. Patzl *et al*. (1992) tested reliability of salivary cortisol in dogs in non-stressful conditions. Results were in good agreement with plasma cortisol. For horses, van der Kolk *et al.* (2001) found high correlations between total plasma and salivary cortisol, while Elsaesser *et al*. (2001) did not find salivary cortisol a reliable marker of the training status and fitness in horses. The results for pigs are unclear. In prepubertal boars and gilts housed in metabolic cages the response to ACTH stimulation was lower in salivary than in plasma cortisol, indicating that salivary cortisol was less sensitive indicator of adrenal activity than plasma cortisol. However, it was concluded, that salivary cortisol is of use for stress assessment in intensively housed pigs (Parrott *et al*., 1989). Other research confirmed the usefulness of salivary cortisol as stress indicator in pigs: in prepubertal boars, stress (transport, mixing) resulted in salivary cortisol levels similar to those seen after ACTH stimulation (Parrott and Misson, 1989). On the other hand, Blackshaw and Blackshaw (1989) did not find salivary cortisol useful in the assessment of cortisol secretion, because the correlations between salivary and either total or free plasma cortisol in different categories of pigs housed in intensive conditions were low. The research of Cook *et al*. (1996) gave different results. High correlations were found between serum and salivary cortisol values following ACTH stimulation and restraint in female pigs. In the study of Bushong *et al.* (2000), the correlation between total cortisol concentration in plasma and saliva was affected by the duration of ACTH stimulation.

Despite unclear results, salivary cortisol is frequently used as a stress measure in pigs; for example, in the evaluation of different treatments of pregnant sows (Spoolder *et al*., 1996) and in the investigation of welfare of the pigs during transport (Bradshaw *et al*., 1996; Geverink *et al*., 1998). More recently, salivary cortisol was used as a stress measure in mixing of unfamiliar pigs (de Groot *et al*., 2001), as a physiology measure of coping styles in gilts (Geverink *et al*., 2002; Ruis *et al*., 2002) and as a measure of individual responses to weaning (van Erp-van der Kooij *et al*., 2003; Mason *et al*., 2003). O′Connell *et al*. (2003) used salivary cortisol as a stress measure in sows of different social status.

Salivary cortisol was used also in the evaluation of housing conditions in pigs. Space allowance, social contacts and control of the environment are important factors in adaptation to the environment. Ekkel *et al*. (1995) compared so called Specific-Stress-Free (SSF) housing

system to a conventional housing system and found lower cortisol concentrations in SSF pigs. In the study of Broom *et al*. (1995), pregnant sows in three housing systems did not show clear differences in salivary cortisol responses on different environment, while in the study of de Jong *et al*. (1998) enriched-housed growing pigs had higher salivary cortisol values than poor-housed pigs. The results of de Groot *et al*. (2000) are even more complex. Enriched-housed pigs showed higher concentrations of cortisol in saliva than barren-housed pigs, but only in the light period of the day. In the dark period of the day salivary cortisol concentrations were low in both housing conditions. Klont *et al*. (2001) compared the reactions to transport between poor- and enriched-housed pigs. Pigs from the barren environment had higher increase in salivary cortisol from farm to slaughter. De Leeuw *et al*. (2003) found higher salivary cortisol values in individually housed gilts with no substrate in comparison with those on wood shavings litter spread on the foraging area.

Adrenocortical response to stressful conditions is one important aspect, the other interesting one is the possible connection between malignant hyperthermia syndrome (MHS) and adrenocortical action. MHS is a reaction to acute stressors, like transport, high ambient temperature, mixing with unknown animals. It is inherited by a single recessive gene (n) and characterised by hyperthermia and muscle rigidity. These symptoms are often followed by sudden death within minutes of acute stress. Two malignant hyperthermia (MH) genotypes (NN and Nn) differed in adrenocortical response to stressful conditions. NN animals had higher cortisol concentration in plasma (Siard *et al*., 2003a) and urine (Siard *et al*., 2003b). To our knowledge, no comparisons between different MH genotypes have been made for salivary cortisol concentration in pigs.

The aim of the study was to evaluate two individual housing conditions of fattening pigs, pens with straw (enriched individual housing system) and metabolic cages (very barren environment), with salivary cortisol monitoring. Further aim was to assess the relationship between two MH genotypes (NN and Nn) and salivary cortisol concentration.

MATERIAL AND METHODS

The experiment was done in three replicates, each including eight German Landrace barrows housed individually, four in large pens with litter (1.98 x 1.93 m) and four in metabolic cages with wire mesh floor. Altogether, there were 24 animals included in the experiment. Pens bedded with straw were separated by solid wooden partitions. In the metabolic cages without straw, animals were able to stand up and lie down, but not to turn around.

The MH genotype was determined by a DNA-based test (Fujii *et al*., 1991). Altogether, 95 animals were checked. There were only six animals with nn-genotype and they were unevenly spred over the three replicates. Thus, it was not possible to include nn animals into the experiment. Therefore, half of the pigs in each housing condition were dominant homozygous (NN) and the other half were heterozygous (Nn).

Pigs were housed in the experimental environment 14 days prior to the experiment. The animals had no previous experience with the two housing conditions. The health condition of the animals was followed by daily measurement of body temperature. Dry meal and water were available *ad libitum*. The illumination and ventilation were natural.

Pigs were made familiar with chewing the cotton buds for saliva collection in a 14-day period prior to the experiment. There were three sampling days, in-between each day of collection there was a 14-day interval. Saliva samples were therefore collected on days 8, 22 and 36 of the experiment. On each sampling day the samples were collected simultaneously from all animals between 8.00h and 11.00h in 15 min. intervals. The collection of saliva did not take more than one minute; no restraint was needed. During the sampling period the water was withdrawn. Pigs

were always handled by a familiar person. They chewed the buds until they were thoroughly moistened. The buds were then fitted into glass centrifuge tubes. The saliva was extracted from the buds by centrifugation. All samples were centrifuged immediately at 2500 x g for 15 minutes and stored at –20 °C.

The volumes of saliva samples were not larger than 300 µl. Due to low volumes, salivary cortisol was determined by chemiluminescence method (kit provided by Nichols Institute Diagnostica). The intra-assay variability was up to 10%.

Salivary cortisol data had to be logarithmically transformed ($\mathbf{y_{ijklmn}}$) to achieve normality. The following fixed effects were included in the model: the three replicates ($\mathbf{R_i}$), the two housing conditions ($\mathbf{H_j}$), the two MH genotypes ($\mathbf{G_k}$), the individual animals ($\mathbf{A_{ijkl}}$) and the sampling day ($\mathbf{D_{im}}$). Possible changes of cortisol concentration within sampling day (trial) were described by the linear regression ($\mathbf{b_{im}}$). The model also included two and three level interactions, which showed the significant effects in the preliminary analysis:

$$y_{ijklmn} = \mu + R_i + H_j + G_k + A_{ijkl} + D_{im} + b_{im}(x_{imn} - 5.5) + RH_{ij} + RG_{ik} + RHG_{ijk} + e_{ijklmn}$$

RESULTS

Salivary cortisol values for individual pig from the three replicates are presented in Figure 1. Considerable variability between and within the animals was observed in all replicates (Figure 1). The average values per animal were ranging from 3.3 to 36.0 ng ml^{-1}. Standard deviation within animal was high. However, the average cortisol concentration in saliva was the smallest in replicate three (in the first replicate the average ± SD was 17.3 ± 11.7 ng ml^{-1}, in the second 16.8 ± 7.9 ng ml^{-1} and in the third replicate 8.9 ± 6.3 ng ml^{-1}). Transformed data from the model included also the extreme values from Figure 1, which presents untransformed data.

Table 1. Statistically significant effects on salivary cortisol concentration
Preglednica 1. Statistična značilnost vplivov na koncentracijo kortizola v slini

Effects Vplivi	Degrees of freedom Stopinje prostosti	P-value p-vrednost
Replicate Ponovitev	2	0.0001
Animal Žival	12	0.0001
Housing condition Uhlevitev	1	0.0001
MH genotype MH - genotip	1	0.01
Sampling day Dan odvzema vzorca	6	0.0001
Trial – linear regression Odvzem – linearna regresija	9	0.0001
Replicate × housing Ponovitev × uhlevitev	2	0.002

High variability of salivary cortisol values between and within the animals (Figure 1) was reflected in the high significance of animal and replicate. The effects of housing condition and MH genotype were significant (Table 1). Pigs in metabolic cages had higher cortisol values than

pigs in individual pens. In addition, NN animals had higher cortisol values than Nn animals (Table 2). The effect of sampling day within the replicate was highly significant. There was no trend up or down in the 4-week period within the replicates. The effect of trial (3-hr period of sampling within the sampling day) was also highly significant. The possible reason for the significant effect of interaction between replicate and housing (Table 1) is again high variability in cortisol values between and within the animals.

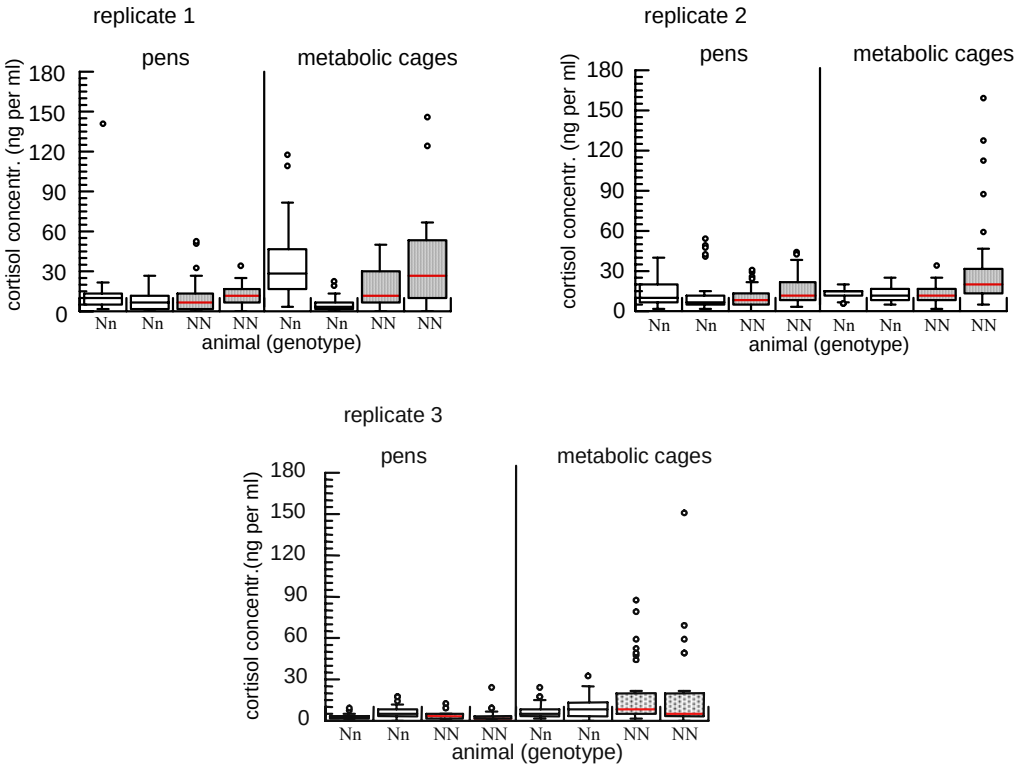

Figure 1. Salivary cortisol values for each pig respectively in the three replicates. Boxes represent the second and the third quartile and bars represent the first and the fourth quartile. The lines in the boxes are medians, the points are extreme values (by default the values larger than 3 SD).

Slika 1. Koncentracija kortizola v slini pri posameznih živalih v treh ponovitvah. Boksi predstavljajo drugi in tretji kvartil, navpične črte pa prvi in četrti kvartil. Prečne črte v boksih so mediane, točke pa ekstremne vrednosti (večje od treh standardnih odklonov).

Table 2. Least square means (LSM) and standard errors (s.e.) for salivary cortisol in two housing conditions and in two MH genotypes

Tabela 2. Vrednosti LSM in s.e. za koncentracijo kortizola v slini pri različnih uhlevitvah in MH – genotipih

Effects Vplivi	LSM ± S.E.	Difference
Housing / Uhlevitev		
Individual pens / Individualni boksi	0.78 ± 0.021	–0.244 ± 0.029
Metabolic cages / Metabolne kletke	1.03 ± 0.020	
MH genotype / MH-genotip		
NN	0.94 ± 0.020	0.069 ± 0.028
Nn	0.87 ± 0.020	

DISCUSSION

High variability in salivary cortisol values between and within the animals was found. That was observed also in plasma (Siard *et al*., 2003a) and urine (Siard *et al*., 2003b). High variability is reflected in the significant effects of replicate and housing, and presumably also in the effect of interaction between replicate and housing (Table 1).

The animals in metabolic cages had higher salivary cortisol values than pigs in pens (Table 2). This is in line with the results for plasma cortisol (Siard *et al*., 2003a) and with the results of de Leeuw *et al*. (2003), who found higher salivary cortisol values in individually housed gilts with no substrate in comparison with those on substrate. However, considering other studies in which they evaluated different housing conditions with salivary cortisol, the picture is not clear. In the study of de Jong *et al.* (1998), pigs in enriched, presumably less stressful environment, had higher salivary cortisol values than pigs in poor environment. They found these results surprising and they tried to explain them with the possible underlying physiological mechanisms. One of the possible explanations was flattened circadian rhythm in stressed animals. De Groot *et al.* (2000) indeed found blunted circadian rhythm of salivary cortisol secretion in more stressful conditions (small pens with no straw in comparison to large pens with bedding). However, in their study the samples were taken every two hours and that is quite a long interval. Considering our results, where the linear regression within 3-hr period was significant (Table 1), the 2-hr interval might have not reflected adequately the circadian rhythm of cortisol secretion. The effect of sampling day was also significant (Table 1), however, no trend was observed in a 4-week period.

NN animals had higher cortisol values than Nn animals. This is in line with plasma (Siard *et al.,* 2003a) and urine (Siard *et al.,* 2003b) cortisol results. To our knowledge, no studies were done on the relationship between salivary cortisol concentration and MH genotypes. However, results from this study and from the previous ones on plasma and urine cortisol with relation to MH status, show higher adrenocortical activity in stress resistant NN-animals. These results are comparable with the results of Mitchell and Heffron (1981). They found higher plasma cortisol values in stress resistant pigs (the animals were classified into resistant and susceptible groups by the halothane test). They suggested that reduced cortisol secretion contributed to the activation of MHS. Higher ability of adrenal cortex reactivity is perhaps good for better acute stress coping in NN genotype.

CONCLUSIONS

The animals in metabolic cages (more stressful environment) had higher salivary cortisol values than pigs in large individual pens with straw. This indicates that saliva cortisol might be, like plasma cortisol, a suitable indicator of stress in pigs. This is important, because saliva sampling can be done in practice, while blood samples can only be taken in research conditions. Furthermore, contrary to blood sampling, saliva collection is not an invasive method. Higher cortisol concentration in saliva in NN- in comparison to Nn- pigs indicates stronger response to stressful conditions in stress resistant NN-genotype. Presumably that is in favour for better acute stress coping in this genotype.

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