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Different stressors elicit different responses in the salivary biomarkers cortisol, haptoglobin, and chromogranin A in pigs

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- We aimed to find salivary markers for stress other than cortisol in pigs
- Pigs were subjected to mixing and feed deprivation stress
- Salivary cortisol, chromogranin A and Haptoglobin were positively correlated
- Responses of the respective salivary biomarkers differed between stressors
- Including various salivary biomarkers in stress evaluation seems useful

ABSTRACT

Most commonly, salivary cortisol is used in pig stress assessment, alternative salivary biomarkers are scarcely studied. Here, salivary cortisol and two alternative salivary biomarkers, haptoglobin and chromogranin A were measured in a pig stress study. Treatment pigs (n=24) were exposed to mixing and feed deprivation, in two trials, and compared to untreated controls (n=24). Haptoglobin differed for feed deprivation vs control. Other differences were only found within treatment. Treatment pigs had higher salivary cortisol concentrations on the mixing day ($P<0.05$). Chromogranin A concentrations were increased on the day of refeeding ($P<0.05$). Haptoglobin showed a similar pattern to chromogranin A. Overall correlations between the salivary biomarkers were positive. Cortisol and

Chromogranin A were moderately correlated ($r=0.49$, $P<0.0001$), correlations between other markers were weaker. The present results indicate that different types of stressors elicited different physiological stress responses in the pigs, and therefore including various salivary biomarkers in stress evaluation seems useful.

KEY WORDS: Pig, stress, saliva, cortisol, haptoglobin, chromogranin A

INTRODUCTION

Pigs are exposed to many stress factors during their life. Stress can have a negative impact on their welfare and health status and can result in economic losses due to e.g. reduced growth or reproduction (von Borell, 1995; Hyun et al., 1998). To study pig welfare and health status, easy and non-invasive sampling methods such as saliva sampling are preferred (Gutiérrez et al., 2009). The hypothalamic pituitary adrenocortical (HPA) axis and the sympathetic adrenomedullary (SAM) system play a key role in the stress response (Koolhaas et al., 2011). SAM and HPA, stimulate the secretion of salivary biomarkers such as cortisol (Cort), acute phase proteins (APP) and chromogranin A (CgA) (Murata et al., 2004; Obayashi, 2013). The measurement of salivary Cort is a common and validated technique in pigs (Kirschbaum and Hellhammer, 1994). The advantage of salivary Cort over plasma Cort is that only the biological active free cortisol is measured (Mormède et al., 2007). However, Cort has shown to be influenced by several internal and external factors such as age, breed, sex and circadian rhythm (Mormède et al., 2007). Acute phase proteins are released during the acute phase response, the non-specific innate immune response (Gruys et al., 2005). Although the exact linkage between the acute phase response and the stress response is not known, it is hypothesized that catecholamines and glucocorticoids are directly or indirectly (through cytokines) involved in activation of the liver cells to produce and release APP (Bürger et al., 1998; Murata et al., 2004). Haptoglobin (Hp), a type of APP, determined in serum was found to be a valuable indicator for the effects of road transport, mixing and thermal stress in pigs (Piñeiro et al., 2004; 2007a; 2007b; Salamano et al., 2008). Salivary Hp has shown to be present and determinable in pig saliva and had a high correspondence with serum (Gutiérrez et al., 2009; Gómez-Laguna et al., 2010). Chromogranin A is a major protein of secretory vesicles of endocrine, neuroendocrine and neuronal cells (Winkler and Fischer-Colbrie, 1992). Chromogranin A is considered to reflect SAM activity (Nakane et al., 1998; Kanno et al., 1999) and is used as biomarker in human stress studies (Nakane et al., 1998; Toda et

al., 2005; Lee et al., 2006; Gallina et al., 2011). Recently, CgA has been reported as an acute stress indicator in pigs (Escribano et al., 2013).

Salivary CgA and Hp are relatively new in pig stress assessment and have never been measured together with Cort in one study.

The objective of the present study was to investigate the usefulness of relatively new salivary biomarkers, Hp and CgA in stress assessment in grower pigs exposed to mixing and a 24-h feed deprivation.

MATERIALS AND METHODS

Animals and housing

The experiment took place at the pig husbandry site of Biocentrum Agri-vet of Ghent University Merelbeke, Belgium and was approved by the Animal Ethical Committee of Ghent University (EC2012/125). Two trials of 24 pigs each were conducted using crossbred grower pigs (Piétrain Plus x Rattlerow Seghers, Hypor KI). In each trial the same experimental unit with four pens (2.35 m x 1.60 m) was used, with six littermates in each pen. Pens were equipped with a single feeder space and one drink nipple and the pigs had ad libitum access to water and to a commercial grower feed (except for the feed deprivation period). Pens were separated by solid walls (1.5 m) so pigs could hear pigs from adjacent pens, but could not make physical contact. Pigs had a timer controlled twelve hour light period from 07:00h-19:00h. The indoor climate was controlled by a system of Hotraco (Horst, The Netherlands). During the trials the mean barn temperature was $22.8\text{ C} \pm 1.6$ (mean $\pm$ SD).

Before the start of each trial, pigs were kept in groups of eight littermates. At approximately ten weeks of age, three gilts and three barrows with the most homogeneous weights were selected from each litter. In the first trial, one pen consisted of four gilts and two barrows because three equal barrows were unavailable. Start weight (day 0) averaged 21.0 ± 2.2 kg (mean $\pm$ SD) and 31.5 ± 3.5 kg in trial 1 and 2 respectively. Weights were not different between control and treatment groups in both trials.

Experimental design

Each trial lasted 15 days, containing three periods: pre-stressor, post-mixing and post-feed deprivation (Figure 1). Before each trial, pigs received seven days adaptation to their new environment and were habituated to the weighing and saliva sampling procedure. Per trial, two experimental pens were

randomly chosen to receive the stressor treatments, the remaining two pens served as controls. During the pre-stressor period all pens were treated the same. On day 6 (Figure 1), all four pens (control and treatment) were relocated. The relocation of all pens was necessary to compensate for the relocation effect. Control pigs remained in the same group composition, while treatment pigs were mixed during the relocation. Three pigs from one treatment pen were exchanged with three pigs from the other treatment pen; gender and weight of pigs were kept balanced in both pens. On day 11, treatment pigs were feed deprived from 12:00h at day 11 to 12:00h at day 12 (24-hours), after this fasting period they were refed by gaining ad libitum access again. Control pigs remained undisturbed.

Physiological data collection

The physiological data collection always took place between 19:00h and 21:30h. Body weights, saliva samples and lesion scores were collected for each individual pig. Saliva samples were collected twice in every experimental period (Figure 1), the order of pens and pigs within a pen were chosen randomly. Each pig had to chew on a dental cotton role attached to a dental floss wire (Xtradent, Waregem, Belgium) for approximately one minute. Four weighing moments were performed using a mobile metal framed weighing box (Fancor, Panningen, The Netherlands). Average daily gain (ADG) of each pig was calculated per experimental period (pre-stressor, post-mixing and post-deprivation). Lesion scores were recorded once in every experimental period based on the procedure of Turner et al (2006). When different physiological measurements were collected on one day, first the saliva sample was taken so that it was unaffected by weight or lesion recordings. The fixed time of the physiological data collection was to account for the circadian rhythm of the salivary Cort and Hp, showing a peak level in the morning and a lower level during the evening and night period (Ruis et al., 1997; Gutiérrez et al., 2013). To minimize stress during physiological data collection, the week prior to the experimental period, pigs had been habituated to the saliva sampling and weighing procedure.

Sample analysis

Directly after the collection of each saliva sample, the cotton role was stored in a tube and cooled on ice. Within three hours after the collection, the saliva samples were processed by centrifuging them for twelve minutes (3000 rpm, 4°C). Each saliva sample was aliquoted in duplo and stored at -80°C until the sample analysis. Salivary Cort was analysed using an automated chemiluminescent immunoassay (Immulite 1000 cortisol, Siemens Medical Solutions Diagnostics) validated for pigs (Escribano et al.,

2012). The intra- and inter-assay coefficients of variations (CVs) were lower than 16% and the detection limit was 0.016 µg/dL. The concentration of salivary Hp, and CgA were determined by time-resolved immunofluorometry assays (TR-IFMA), as previously described (Gutiérrez et al., 2009; Escribano et al., 2013). The assays showed intra- and inter-assay CV lower than 10% and 15%, respectively, and the detection limit was 4.27 ng/mL for CgA and 0.52 ng/mL for Hp.

Statistical analysis

Mean baseline levels and SD for Cort, Hp and CgA were calculated based on the sampling data of all pigs on the two sampling days in the pre-stressor period. This to compare the salivary concentrations with previous research.

To test the effect of mixing and feed deprivation on the concentration of the biomarkers in the saliva of both trials, two datasets were used. One dataset included the days around mixing (days 0, 5, 6 and 10) and one dataset the days around the feed deprivation (days 10, 12 and 15). Both datasets were tested using a general linear mixed model PROC MIXED in SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). The model included the main effects of treatment (2 levels), day (4 or 3 levels, depending on the number of sampling days of each dataset) and the interaction effect of treatment x day. Litter (8 litters) nested into trial (1 and 2) was taken as a random effect and the sampling data of each individual pig were included as repeated measures. Start weight (day 0) and sex were first included as covariates, but both had no significant effect ($P>0.1$) on the concentration of the salivary biomarkers and were therefore removed from the model. The effect of mixing and feed deprivation on ADG and total number of body lesions were tested in a separate general linear mixed model in SAS. The model included the main effects of treatment (2 levels) and experimental period (pre-stressor, post-mixing and post-deprivation) and the interaction between these effects. Start weight was included as a covariate because it showed a significant influence ($P<0.1$), both on body lesions and ADG. Sex had no significant influence ($P>0.1$) and was removed from the model. Litter nested into trial was taken as a random factor and measures on each individual pig were repeated over period.

For both models, an interaction term with $P < 0.2$ was kept in the model. Least squares means were compared pairwise, correcting for multiple comparisons by using a Tukey test. In order to obtain normally distributed residuals, Cort and Hp were log transformed, CgA and body injuries square rooted and average daily gain needed a power 1.5 transformation. For the data presentation LSMEANS were back-transformed.

To get an indication about the interrelationships between the biomarkers Cort, Hp and CgA, Pearson's correlations were calculated on pooled data of all pigs and sampling days.

RESULTS

In total five pigs were excluded from data-analysis. Two pigs could not be habituated to the saliva sampling and showed high Cort levels (ranging from 3.6 to 6.6 $\mu\text{g/dL}$) on days before and after stressor exposure. One pig got lame after the mixing and showed high levels of Hp (3.6 and 7.7 $\mu\text{g/mL}$ on day 10 and day 12 respectively). Two pigs in one control pen were excluded from the analysis because they lost body weight.

Baseline levels of salivary biomarkers

The mean $\pm$ SD baseline levels ($n=95$) were 0.29 ± 0.55 $\mu\text{g/dL}$ for Cort, 0.46 ± 0.36 $\mu\text{g/mL}$ for Hp and 0.72 ± 0.48 $\mu\text{g/mL}$ for CgA.

Mixing and feed deprivation effect on salivary cortisol, Hp and CgA

On most of the sampling days, a large variability in biomarker concentration between individual pigs was measured, as is shown by the 95% confidence limits (Figure 2).

Mixing (Figure 2). An interaction effect (treatment $\times$ day) was found for Cort. Within the treatment group, mixing resulted in higher Cort levels ($P < 0.05$) on the day of mixing (day 6) compared to the first sampling day pre-stressor (day 0). Haptoglobin or CgA levels did not significantly change after mixing. No significant differences ($P > 0.05$) in biomarker concentrations between the control and treatment group were found on any of the sampling days around mixing.

Feed deprivation (Figure 2). Interaction effects (treatment $\times$ day) were found for Hp and CgA. Within the treatment groups, CgA levels were higher ($P < 0.05$) on the day of refeeding (day 12)

compared to the day before (day 10) and several days after the refeeding (day 10 and day 15 respectively). Haptoglobin levels followed a similar pattern on days 12 and 15, but day 12 was not significantly different from day 10. Furthermore, Hp levels were significantly higher between the control and treatment group on the day of refeeding (day 12). Cortisol levels were not affected by the feed deprivation.

Stressor effects on ADG and body lesions and correlations between the salivary biomarkers

Average daily gain was not affected ($P>0.05$) by mixing and feed deprivation. Significant interaction effects (treatment x period) were found for the total number of body lesions. Within treatment group, pigs had a higher number of body lesions in the post-mixing ($P<0.05$) and post feed deprivation period compared to the pre-stress period. Also the number of body lesions were higher ($P<0.05$) in the post-mixing period compared to the post-deprivation period. The treatment group had a higher number of body lesions ($P<0.05$) compared to the control in the post-mixing period (Figure 3).

Cortisol was positively correlated with Hp ($r = 0.34$, $P<0.0001$) and CgA ($r = 0.49$, $P<0.0001$).

Haptoglobin and CgA showed a positive correlation as well ($r = 0.34$, $P<0.0001$).

DISCUSSION

The present research investigated the usefulness of three salivary biomarkers for pig stress evaluation. The mean baseline levels of salivary Cort and Hp were comparable to earlier research that used similar analysis techniques (Escribano et al., 2012; Gutiérrez et al., 2013). Chromogranin A levels were similar to the values reported for the cold season by Escribano et al. (2014). The latter also reported higher CgA values in the hot season and hypothesized that the ambient temperature can affect base line salivary CgA levels.

We exposed pigs to stressors that have been described to cause significant physiological changes. Mixing of unfamiliar pigs is a common management procedure affecting both the social and physical status of pigs, demonstrated by increased stress metabolites, suppression of the immune function, growth retardation and skin lesions (Fernandez et al., 1995; Spoolder et al., 2000; Ruis et al., 2001; Coutellier et al., 2007). Fasting and refeeding has also shown to cause significant (metabolic) stress (Toscano et al., 2007; Lallès and David, 2011).

All three salivary biomarkers are considered to be stimulated after activation of the physiological stress response. Cortisol is released after the activation of the HPA-axis (Mormède et al., 2007), and CgA is thought to be stimulated through the SAM pathway (Kanno et al., 1999). It has been hypothesized that APP can be induced by metabolites released through the SAM and/or HPA pathway (Murata et al., 2004; Gruys et al., 2005; Aninat et al., 2008). It seems that the physiological stress response consists of complex interactions, and hence the interrelationships between metabolites of the acute phase response, SAM and HPA-activity are not exactly known. What is known is that Cort and CgA can both be released during neuroendocrine activity (Black, 2002) and both can be detected early in blood and saliva, from several minutes up to a few hours in acute stress situations (Merlot et al., 2004; Prunier et al., 2005; Merlot et al., 2011; Escribano et al., 2013). Haptoglobin is released during the acute phase response (non-specific innate immune response) and can be triggered by the neuroendocrine pathways (Black, 2002). Haptoglobin has a much slower response time and is measurable in blood after several hours to days after stressor exposure (Saco et al., 2003; Gruys et al., 2005; Salamano et al., 2008). Furthermore, Hp and CgA are supposedly produced mainly locally (Saruta et al., 2005; Soler et al., 2013), while Cort is transferred to the saliva by passive diffusion, reflecting the free fraction from the blood stream (Kirschbaum and Hellhammer, 1994).

In the present study, overall correlations (using pooled data of all pigs) between the concentrations of salivary Cort, Hp and CgA were weak, but were all positive. A similar positive correlation between salivary Cort and CgA have been reported earlier by Escribano et al. (2013). This is in accordance with the assumption that all three salivary stress markers are stimulated during physiological stress. Significant different stress reactions were found only within treatment group. Cortisol was elevated after mixing while Hp and CgA were significantly increased after feed deprivation. The fact that no differences were found between control and treatment can possibly be attributed to the relatively low power and the large variability between individual pigs. Furthermore, it can not be excluded that due to the different kinetics of the biomarkers, peak levels could have been missed at the sampling times used. Concerning Cort, it was significantly elevated after about 11 hours post-mixing. Previous studies on mixing have shown increases in salivary only in the first hours (de Groot et al., 2001; Merlot et al., 2004; Coutellier et al., 2007). Possibly, our pigs were stressed for a longer period post-mixing.

Chromogranin A was expected to react in a similar way as Cort, based on the similar acute reaction in both human (Nakane et al., 2002) and pig stress studies (Escribano et al., 2013). In this study CgA was not influenced by the mixing but did show a significant elevation after the feed deprivation period. Chromogranin A has been related to psychological stress in human (Saruta et al., 2005) and has not been investigated in relation to metabolic stressors. The current results indicate a possible influence of metabolic stress on CgA.

We found no significant elevation in salivary Hp after mixing. To the authors knowledge only one study measured salivary Hp in relation to an acute stressor (Soler et al., 2013). The latter found no significant increase in Hp one hour after stressor exposure. Therefore, it is recommended to measure Hp over a longer time span in future research. Because our sampling took place at approximately 11 hours after mixing, it was expected to find increases in salivary Hp. Other pig stress studies measured Hp in blood and found significant increases after various types of stressors: (Piñeiro et al., 2004, 2007a, 2007b; Salamano et al., 2008). However, in the latter, the sampling points after the stressor varied from hours to days. After feed deprivation, Hp was increased at approximately 8 hours. In the literature, acute phase proteins have been associated with anorexia and other changes in metabolism (Gruys et al., 2005). It has also been shown that fasting and refeeding can be pro-inflammatory and has other detrimental effects on the gastrointestinal tract (Ferraris and Carey, 2000; Lallès and David, 2011). Therefore, it is likely that the intestinal stress caused by the fasting and refeeding was the main trigger for the Hp response.

Limitations of the present research are the significant differences in biomarkers responses mainly found within the treatment group and, except for Hp, not between control and treatment groups. In addition, it cannot be excluded that applying two consecutive stressors did affect our treatment pigs. In the literature, repeated triggering to a stressor has been reported to cause a higher response APP (Salamano et al., 2008). Nevertheless, it should be noted that despite the relatively low power and the large variability between individual pigs, significant differences were found.

It is concluded that the results obtained are promising for further applications in pig stress assessment. Saliva sampling is far more practical than blood sampling, and can be applied more frequently. The most important result is that different salivary biomarkers appear to react differently to different types of stressors and therefore the use of various salivary stress markers seems essential in stress research.

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FIGURE CAPTIONS

Figure 1. Experimental timeline showing pre-stressor; post-mixing; post-feed deprivation periods. The red arrows indicate the application of mixing and relocation and feed deprivation; the black arrows indicate the days on which physiological measurements took place: saliva sampling (S), body lesions scoring (L) and weighing (W).

Figure 2. Least squares (LS) means (backtransformed) of salivary cortisol (Cort), haptoglobin (Hp) and chromogranin A (CgA) for the control (n=22) and treatment group (n=21) per sampling day. Data of both datasets; days around mixing (day 0, 5, 6 and 10) and days around feed deprivation (day 10, 12 and 15) are presented. For clarity, only the upper or lower 95% confidence limit is given for each LS mean. Within group and between group effects ($P < 0.05$) are indicated by a different letter, a,b and x,y, respectively.

Figure 3. Least squares (LS) means (backtransformed) of total number of body lesions for the control (n=22) and treatment group (n=21) per sampling period. For clarity, only the upper or lower 95% confidence limit is given for each LS mean. Within group and between group effects ($P < 0.05$) are indicated by a different letter, a,b,c and x,y, respectively.