Effect of Repeated Confined Single Housing of Young Pigs on Faecal Excretion of Cortisol and IgA

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Summary
During 48 days four young male, castrated Yorkshire x Landrace pigs (growers) were singly housed alternatively in standard pig pens (4 x 6 days) and metabolic cages (4 x 6 days). The faecal excretion of cortisol metabolites and immunoglobulin A (IgA) was quantified by enzyme-linked immunoabsorbent assays (ELISAs). The first stay in the metabolic cage was associated with an increase in faecal cortisol levels, which may be interpreted as a symptom of acute stress. But when the pigs' visits to the metabolic cages were repeated, the faecal cortisol generally reverted to fairly low levels. Concomitantly, faecal IgA excretion decreased steadily during the study period suggesting sustained stress. The body weight gain was lower during periods when the pigs were housed in metabolic cages than when they were in pens. These results suggest that metabolic cages caused a continued stress condition in pigs. One pig showed consistent high IgA excretion and a smaller decline in body weight gain during periods in the metabolic cage than the other pigs. This pig thus appeared to be less stressed than the other pigs, but maintained high cortisol levels throughout the study period when housed in the ordinary pen. This indicates that cortisol may have a protective effect against the stress caused by housing in metabolic cages.

Introduction
Metabolic cages are frequently used in biomedical studies and it is uncertain whether very confined single housing of this nature may induce anxiety and psychological stress. Animals and humans react to confinement with an activation of the hypothalamus-pituitary-adrenal (HPA) axis (*Gauquelin-Koch et al. 1996; Kraft et al., 2003*). The activation of the HPA axis leads to, among other things, an increased synthesis and release into the circulation of glucocorticosteroids (*for* review see *Woodman, 1997*). Quantification of glucocorticosteroids in blood unfortunately requires capture, restraint and blood sampling, which results in a rapid release of corticosteroids into the circulation making hormone blood levels of little use in studies of chronic stress in animals. Consequently, there has been an increase in the efforts during the past decade to develop non-invasive methods for corticosteroid quantification in secreted or excreted material. Stress has been documented to be associated with a decrease in the secretion of mucosal immunoglobulin A (IgA) in humans and different animal species (*Green et al., 1988; Skandakumar et al., 1995; Guhad and Hau, 1996; Deinzer and Schuller, 1998; Carver and Hau; 2000, Royo et al., 2004*).
The aim of the present study was to quantify faecal cortisol, as a measure of acute stress, and IgA, as a measure of chronic stress, in pigs housed alternately in normal pens and in metabolic cages, which is a common procedure in nutrition studies.

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Materials and Methods

Animals and housing conditions

Four 11-week old castrated Specific Pathogen Free male pigs (Yorkshire x Landrace, Vallrums Lantbruks AB, Uppsala, Sweden) at an average weight of 26 kg were allowed to acclimatize for 12 days in their individual pens before the study. The pens were 230 x 145 cm with no bedding but with a rubber mat to lie on. Between each pen there were openings so the pigs could see each other. The metabolic cages were 125 x 70 cm and they were placed close together so the pigs had visual contact with each other. The light regime was a 12/12 h dark/artificial light cycle. The temperature was maintained at 20 ± 1° C and the relative humidity was 50% (± 10%). The cages and pens were cleaned twice a day. Throughout the study the pigs were fed twice a day with a diet based on wheat and barley (Svenska Lantmännen, Malmö, Sweden), equivalent to a daily amount of 4% of the body weight and adjusted for body weight change every 12 days. Water was available *ad libitum*.

Experimental design

The experiment was designed as 4 time periods (period 1-4) each consisting of one cycle of 12 days. Each cycle consisted of two periods, 6 days in a pen (P) followed by 6 days in a metabolic cage (M). During the first period (P1) only one sample was collected, and this value is referred to as the "pre-experimental" value. During the second period, the first day's sample (M1, day-1) was lost. The pigs were weighed each time they were moved from one housing type to another.

During both the pen- and cage-periods, faeces were collected twice a day – in the morning and in the late afternoon. The individual pigs' total production of faeces per day was weighed, blended with an electric mixer and 40g samples taken from each day of the trial and frozen at -20° C until analysis.

Extraction of IgA and Cortisol

After being thawed, 5g faeces were diluted with 10 ml distilled water, and the mixture homogenized using a tissue homogenator (Tissue Tearer, Biospec Products, Inc., Bartlesville, OK, USA). For quantification of cortisol, 4.5g homogenate was subjected to extraction with 7 ml dichloromethane in a glass tube, following the procedures described by Pihl and Hau (2003). For IgA extraction, 1g homogenate was diluted with 2 ml PBS, 0.05% Tween 20 (pH 7.4) and the subsequent purification procedure was done as described by Hau et al. (2001). The between-sample variation of these extraction procedures was assessed extracting six samples in duplicate and calculating the coefficient of variation of the results. The coefficient of variation of the extraction replicates was 1.2 % for IgA and 3 % for cortisol.

Quantification of IgA and Cortisol

All samples were run in duplicate. The IgA was quantified with a specific pig-IgA ELISA kit (Bethyl Laboratories Inc., Montgomery, TX, USA). Secretory IgA is produced in the intestinal mucosa and it is uncertain to what extent IgA metabolites from the proteolytic degradation of faecal IgA retained immunoreactivity and thus reacted in the kit.

The cortisol was measured with a pan-specific cortisol ELISA kit (DRG Diagnostics, Marburg, Germany). According to the manufacturer, the Cortisol kit presents the following cross reactivity: 60% with prednisolone, 29.9% with corticosterone, 3% with cortisone, less than 1% with 11-deoxycortisol and 17-OH progesterone, less than 0.1% with prednisone, progesterone, dexamethasone, desoxycorticosterone, dehydroepiandrosterone sulphate, estradiol, estriol, estrone, and testosterone, respectively. It is uncertain to what extent native molecules and immunoreactive metabolites of cortisol were quantified in the kit used. A more correct terminology would have been to use the term "cortisol and immunoreactive cortisol metabolites". However, for clarity this was not done.

Stability of IgA and Cortisol

The stability of immunoreactive cortisol metabolites and IgA in fresh pig faeces was analyzed by quantification of these molecules in pools of faeces which were mixed, aliquoted and frozen after having been stored at room temperature for various time spans: 1, 4, 8, 12 and 24 hours. There were no trends towards decreasing or increasing concentrations of either cortisol or IgA associated with storage in room temperature. The concentration of the respective molecules did not differ significantly between the samples regardless of the time (up to 24 hours) the samples had been left at room temperature prior to freezing.

Data Treatment
The statistical analysis was performed through ANOVA tests. Differences with p-values < 0.05 were considered significant. In the present study we measured the total amounts of corticosterone and IgA excreted in faeces per time unit per kg body weight. This is in contrast to most other studies measuring concentrations in excreted products only. In most endocrinological studies using repeated measures of concentrations in e.g. blood the 'area under the curve' is a frequently used integrative method to attempt to obtain measures of secretion over a specific time period (*Preussner et al, 2003*). An obvious advantage of our approach, collecting all faeces, is that by measuring the total amounts excreted we obtain accurate information. In this context it is important to bear in mind that corticosteroid concentration changes taking place in the animal's circulation will not be apparent in faecal excretions until 6-18 hours later.

Project licence
The regional ethics committee of Tierp, Uppland, Sweden, approved the experimental procedures.

Results and Discussion
The increase of the pigs' body weights is presented in Figure 1. The body weight gain during the pen housing periods was significantly higher than the increase recorded during the periods the pigs were housed in metabolic cages ($p< 0.001$).
The profile of the individual pigs' IgA excretion during each period expressed as micrograms/day kg bw, is presented in Figure 2. For pigs 1, 2 and 3, the values obtained during the periods M1, P2 and M2 was significantly higher than values obtained in M3, P4 and M4 ($p < 0.05$). In contrast to the other pigs, pig-4 presented a significant increase in IgA excretion and the values in P2 were significantly higher than those recorded in the other periods.

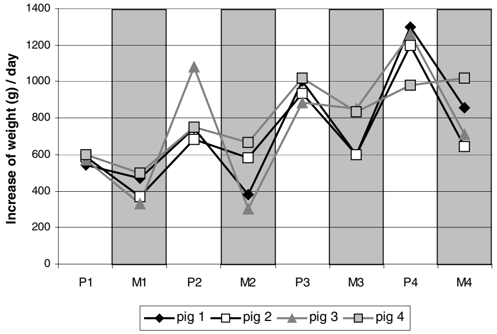

Figure 1: Body weight gain during each period (P for pens, M for metabolic cages) expressed in g of bw per day. White bars indicate days in pens, and grey bars days in metabolic cages.

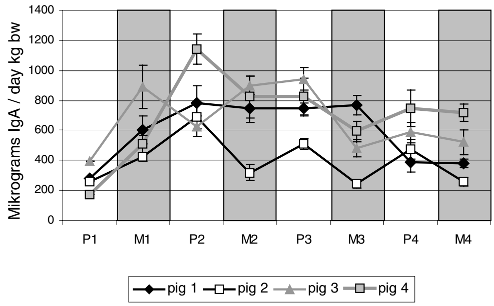

Figure 2: Mean of fecal IgA excretion per period (P for pens, M for metabolic cages). Bar errors represent ±SEM (n=6). White bars indicate days in pens, and grey bars days in metabolic cages.

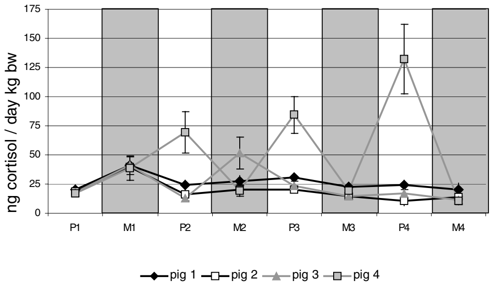

Figure 3: Mean of fecal cortisol excretion per period (P for pens, M for metabolic cages). Bar errors represent ±SEM (n=6). White bars indicate days in pens, and grey bars, days in metabolic cages.

In all pigs the amounts of cortisol excreted during M1 were higher than excretions in the preceding time period, and for pigs 1, 2 and 3, the values in M1 were higher than any of those recorded in pens (Figure 3). For all pigs, the values obtained in M1 and M2 were higher than in M3 and M4 ($p< 0.01$). There was a significant correlation between the faecal (micrograms/g faeces) concentration of IgA ($r = 0.67$, $p < 0.001$, $n = 167$) and the faecal concentration of cortisol (ng/ g faeces) ($r = 0.92$, $p < 0.001$, $n = 167$) and the total excreted amount of these substances per day and kg body weight, respectively. A similar relationship was also observed for the rat (*Pihl and Hau, 2003*). Taken together, the results indicate that pigs generally react with an activation of the HPA axis and an increase of excreted cortisol when first transferred to a metabolism cage. This is in agreement with what has been reported in other species (*Gomez-Sanchez and Gomez-Sanchez, 1991; Heidbreder et al. 2000; Eriksson et al, 2004*). When the pigs were housed repeatedly in metabolic cages, a gradual reduction of HPA activation was observed, and the last housing period in a metabolism cage did not result in any increase in faecal cortisol excretion. This is in agreement with previous observations demonstrating that a cortisol response cannot be maintained during long term stress (*Craigh et al., 1986*). A sustained stress response throughout the study period was indicated by a reduced weight gain during the periods the pigs were housed in metabolic cages. This prolonged stress was confirmed by recordings of a steady reduction in IgA excretion during the last periods in metabolic cages when compared with the first ones. These results are in complete agreement with our findings in rats (*Eriksson et al, 2004*).

Judging from the IgA and weight gain results, pig-4 seemed to be less stressed than the other pigs during the entire study. Interestingly, pig-4 was the only one, which showed an increase of faecal cortisol excretion during the later stages in the pen, and this excretion was increasing cycle after cycle. Consequently, it can be speculated that there may exist a protective effect of cortisol against the long-term stress of the present study - a coping mechanism. Supporting this suggestion, there are numerous publications on cortisol as a protective agent after traumatic events (*Yehuda et al., 1990; Delahanty et al., 2000; Anisman et al., 2001*).

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References

Anisman, H., J. Griffiths, K. Matheson, A. V. Ravindran, and Z. Merali, 2001. Posttraumatic stress symptoms and salivary cortisol levels. Am. J. Psychiatry, 2001, *158*, 1509-1511.

Carver, J. F., J. Hau, 2000. Development of a saliva sampling method and a rocket immunoelectrophoretic assay for quantification of salivary IgA in the squirrel monkey (Saimiri sciureus). In Vivo. *14*, 735-739.

Craig, J. V., J. A. Craig, J. Vargas, 1986. Corticosteroids and other indicators of hens' well-being in four laying-house environments. Poult. Sci. *65*, 856-863.

Deinzer, R., N. Schuller, 1998. Dynamics of stress-related decrease of salivary immunoglobulin A (sIgA): relationship to symptoms of the common cold and studying behavior. Behav. Med. Winter. *23*, 161-169.

Delahanty, D. L., A. J. Raimonde, E. Spoonster, 2000. Initial posttraumatic urinary cortisol levels predict subsequent PTSD symptoms in motor vehicle accident victims. Biol. Psychiatry. *48*, 940-947.

Eriksson, E., F. Royo, K. Lyberg, HE. Carlsson, J. Hau, 2004. Effect of metabolic cage housing on Immunoglobulin A and corticosterone excretion in faeces and urine of young male rats. Exp. Physiol. *89*, 427-433.

Gauquelin-Koch, G., J. P. Blanquie, M. Viso, G. Florence, C. Milhaud, C. Gharib, 1996. Hormonal response to restraint in rhesus monkeys. J. Med. Primatol. *25*: 387-396.

Gomez-Sanchez, E. P., CE. Gomez-Sanchez, 1991. The effect of gonadectomy and aromatase inhibition on the excretion of 19-nordeoxycorticosterone in rats. J Steroid Biochem Mol Biol. *39*, 185-188.

Green, M. L., R. G. Green, W. Santoro, 1988. Daily relaxation modifies serum and salivary immunoglobulins and psychophysiologic symptom severity. Biofeedback Self. Regul. *13,* 187-199.

Guhad, F. A., J. Hau, 1996. Salivary IgA as a marker of social stress in rats. Neurosci. Lett. *216*, 137-140.

Hau, J., E. Andersson, H-E. Carlsson, 2001. Development and validation of a sensitive ELISA for quantification of secretory IgA in rat saliva and feces. Lab. Anim. *35*, 301-306.

Heidbreder, C. A., I. C. Weiss, A. M. Domeney, C. Pryce, J. Homberg, G. Hedou, J. Feldon, M. C. Moran, P. Nelson, 2000. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience. *100*, 749-68.

Kraft, N. O., T. J. Lyons, H. Binder, 2003. Group dynamics and catecholamines during long-duration confinement in an isolated environment. Aviat Space Environ Med. *74*, 266-272.

Pihl, L., J. Hau, 2003. Faecal corticosterone and immunoglobulin A in young adult rats. Lab. Anim. *37*, 166-171.

Preussner, J. C., K. Kirchbaum, G. Meinschmid, D. K. Hellhammer, 2003. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology *28*, 916–931.

Royo, F., N. Björk, H-E. Carlsson, S. Mayo, J. Hau, 2004. Impact of chronic catheterization and automated blood sampling (Accusampler®) on serum corticosterone and fecal corticosterone and immunoglobulin A in male rats. J. Endocrinol. *80*, 145-153.

Skandakumar, S., G. Stodulski, J. Hau, 1995. Salivary IgA: a possible stress marker in dogs. Anim. Welf. *4*, 339-350.

Woodman, D. D., 1997. The adrenal glands. In: J. Whiley (Ed.), Laboratory animal endocrinology. Hormonal action, control mechanisms and interactions with drug. Whiley & Sons Ltd. Chichester, England, pp 253-286.

Yehuda, R., S. M. Southwick, G. Nussbaum, V. Wahby, E. L . Jr. Giller, J. W. Mason, 1990. Low urinary cortisol excretion in patients with post-traumatic stress disorder. J. Nerv. Ment. Dis. *178*, 366-369.