A Combination of Behavioral and Physiological Indicators for Assessing Pig Welfare on the Farm

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The purpose of this research was to identify pig welfare indicators that could help in recognizing stressful practices on farm. The study evaluated behavioral and physiological indicators (cortisol and negative acute phase proteins) in 2 groups of 20 female pigs 4 months old after a 48-hr transport. The first group (A) was transported at the end of May, the second (B) in June. Behavioral observations and blood collection
occurred at arrival (D1) and 28 days later (D28). Compared with within-animal control samples obtained 28 days later, pigs of Group A had increased cortisol levels and decreased albumin concentrations after arrival. As demonstrated by lesion and behavior observations, the effect on cortisol and albumin was higher in Group B pigs after a tail-biting episode occurred. The study has reported no evidence of Retinol Binding Protein (RBP) in pigs. A method developed for swine RBP quantification found RBP strongly reduced in D28 samples of Group B, confirming it to be a negative protein in pigs. The suggested combination of physiological and behavioral indicators could provide useful information on the welfare state of an animal.

Welfare can be defined as the state of a nonhuman animal regarding the animal’s attempts to cope with the environment (Broom, 1996). Welfare therefore varies from good to bad: A good welfare is reached when animals are in harmony with their respective environments and with themselves, both physically and mentally. A poor welfare occurs when animals are exposed to adverse conditions. Animal welfare can be reduced on farm: Many management practices in commercial swine production—handling by humans, transport (Hall & Bradshaw, 1998), and disruption of social contacts—may influence feed intake, growth, behavior, and stress susceptibility of the pigs (Biensen, Von Borell, & Ford, 1996).

Welfare can be assessed in a quantitative manner. Indicators of animal welfare during housing include behavior, productivity, health, and physiology. Standardizing objective measurable welfare indicators could improve the monitoring system of animal welfare at farm level and help in recognizing stressful practices so that preventive and corrective action can be taken within the growing cycle of the animals.

Behavioral changes are the most manifest symptoms of poor welfare: Animals who are lethargic—unwilling to move or to vocalize (Grandin, 1980, 1982)—or who have become aggressive (Dougherty, 1976) are unlikely to be coping with the aversive situation.

It is also well recognized that physiological changes, such as theactivation of the hypothalamic pituitary adrenal axis (HPA) with the release of cortisol and catecholamines, are indicators of poor welfare. A recent work reports increased blood cortisol concentrations—together with an increased level of resting behavior—after mixing of piglets at weaning (Merlot, Meunier-Salaun, & Prunier, 2004); cortisol was also found to be increased in pigs after transport (Geers et al., 1994), after mixing of unfamiliar animals and agonistic interactions (Otten, Puppe, Kanitz, Schon, & Stabenow, 2002).

In addition to the alterations in the HPA axis regulation, animals react to disturbances in their homeostasis with a set of physiological changes known as acute phase response. During this response, there is a change in the rate of synthesis and release of certain proteins such as haptoglobin, C-reactive protein, and serum
amyloid A (Heegard et al., 1998)—collectively called “positive acute phase proteins”—and concurrently a decrease in the rate of production of other plasma proteins—such as albumin and transthyretin—called “negative acute phase proteins” (Eckersall, 2000; Koj, 1985). The quantification of Acute Phase proteins (APP) concentration has been used to monitor pathological processes in farm animals (Gruys, Vanederen, Alsemgeest, Kalsbeek, & Wensing, 1993); recently it has been demonstrated to be useful in the assessment of animal welfare (Eckersall, 2000; Murata, Shimada, & Yoshioka, 2004). APP can help to identify poor hygiene in pig production (Knura, Lipperheide, Petersen, & Wendt, 2000) and to analyze various noninflammatory conditions such as pregnancy (Concannon, Gimpel, Newton, & Castracane, 1996), parturition (Uchida, Katoh, & Takahashi, 1993), metabolic diseases, and stress associated with road transport (Murata & Miyamoto, 1993).

Retinol binding protein (RBP) is the plasmatic vitamin A carrier and is a single polypeptide chain protein with a molecular mass of 21 kDa (Peterson, 1971). Studies on changes in vitamin A metabolism have led to defining the lowering of retinol as a part of the acute phase response to inflammation and infection in humans (Filteau, 1999): During infections, plasma retinol can decrease to as low as about a third of its standard concentration (Arroyave & Calcano, 1979). RBP has been demonstrated to be a negative acute phase protein in cattle (Ingenbleek & Young, 1994); however, no evidence on RBP as negative APP in pigs has yet been reported, and no methods are available for its determination in porcine serum.

The aim of this work was to identify a combination of behavioral and physiological parameters for welfare monitoring in farming pigs. Because animal welfare is particularly compromised under stress conditions (Dwyer & Bornett, 2004), emphasis was put on the swine response to an acute stressor, the transport. Different indicators were evaluated: (a) behavioral parameters, (b) hormone content, and (c) negative acute phase proteins levels. The second aim of the work was to investigate whether RBP is a negative APP in pigs.

**MATERIALS AND METHODS**

**Experimental Design**

Twenty female pigs of the same genotype (Large White-Landrace cross) were purchased from a commercial pig farm in Denmark at the end of May 2004. Pigs were 4 months old; their weight was about 40 kg (Group A).

After purchase, pigs were exported to Italy, enduring a 48-hr transport (about 1700 km). Loading density was about 188 kg/m²; according to the European Union directives for animal transport, different stops were made during the journey for inspection, watering, and feeding the animals.
A second group of pigs of the same sex, age, and genotype were subjected to the same transport 15 days later (Group B).

The pigs were assigned to two separate pens (respectively, Pen A and Pen B). Each pen was 3 x 2.5 m, with woven-wire mesh flooring. Food and water were provided ad libitum by a 6-hole feeder and a nipple waterer.

Behavioral observations and blood samples for physiological analysis were collected in both groups at the beginning of the experiment immediately after transport (D1) and 28 days later (D28) to obtain within-animal control samples. Pigs were restrained for a short time by a wire noose to obtain blood by jugular venapuncture; blood collection always occurred between 09.00 and 10.00 a.m. Each blood sample was centrifuged at 3000 rpm for 20 min after coagulation within the first 3 hr. Serum was separated and stored at –80 °C for subsequent analysis.

Behavioral Observations

Assessments of pig welfare were made by recording their behavior during two 2-hr periods (09.00–11.00 a.m.): immediately after transport (T1) and at the end of the monitoring period (T28). Observations were carried out by a single observer. Based on the method of Day, Spoolder, Burfoot, Chamberlain, & Edwards (2002), at each 10-min interval, the number of pigs performing the behaviors listed in the ethogram (Table 1) was counted. This ethogram details the animal’s posture, the aggressive interaction levels, and pig-directed activity. Results are given as percentages of the total observation number.

Skin lesions, as indicators of aggression (Barnett, Cronin, McCallum, & Newman, 1996), were also assessed by a second observer during the behavioral observations. Skin damages (penetration of the epidermis of > 1 cm length) were counted in each of three body regions (head/shoulders, flank, tail/vulva) and were

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing</td>
<td>The pig’s body is supported by the legs.</td>
</tr>
<tr>
<td>Lying</td>
<td>The pig’s sternum is touching the ground.</td>
</tr>
<tr>
<td>Aggression</td>
<td>Pig is interacting aggressively (bites, nosing, pushes) with another pig.</td>
</tr>
<tr>
<td>Nose penmates</td>
<td>Pig is nosing any part of the body of another pig.</td>
</tr>
<tr>
<td>Nose environment</td>
<td>Pig is nosing the floor, walls, or other pen components.</td>
</tr>
<tr>
<td>Feeding-drinking</td>
<td>Pig’s mouth is in the feeder or in the drinker.</td>
</tr>
<tr>
<td>Other</td>
<td>Pig is not performing any of the above behaviors.</td>
</tr>
</tbody>
</table>
totaled to give an overall damage score. Results are given as percentages of this overall score.

Cortisol

Cortisol levels were measured in duplicate with a competitive solid phase enzyme-linked immunosorbent assay from IBL-Hamburg. According to the manufacturer’s instructions, two standard cortisol from Bio-Rad, (40161 and 40162), respectively, corresponding to a 23.5 and 176 ng/ml concentration, were included in the test. The inter-assay coefficients of variations (cv) for plasma controls at concentrations 254.2, 156.6 and 29.2 ng/ml were 0.061, 0.043, and 0.069. The intra-assay precision was < 0.07 for cortisol concentrations between 0.5 and 150 ng/ml. The lowest detectable level was 2.5 ng/ml.

Negative Acute Phase Proteins

The concentrations of negative APP (albumin and RBP) and total proteins were determined in the serum samples. APP concentration (mg/ml) was calculated as a percentage of each sample’s total protein content. Total protein concentrations were measured with a commercial assay kit (BCA™ Protein Assay Reagent Kit, Pierce, Rockford) based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total proteins.

Albumin (Alb) concentration was determined by the Paragon Serum Electrophoresis Kit (Paragon SPE Kit, Beckman Coulter). The assay is based on the electrophoretic separation of proteins in a buffered agarose gel. RBP was determined by means of a homemade monoclonal antibody (mAb) and a recombinant RBP. First, RBP coding mRNA was isolated from pig liver with a polyA mRNA isolation system. RBP gene sequence was then amplified by RT-PCR and ligated into the pGEM-T easy vector for expression. The recombinant plasmid was incorporated into E. Coli DH-5a bacteria by electroporation and colonies of recombinant clones were amplified on selective plates. Transformants were picked and grown at 37 °C in LB medium. After inducing protein expression, cells were collected by centrifugation, lysed, and the recombinant protein purified. The sequence of the protein was analyzed and showed a high homology with pigRBP M68860 (Internet, GenBank).

The mAb were produced in two Balb/c mice. Eighty µg of the synthesized RBP, diluted in 100 µl Specol adjuvant, was injected in the two mice. Three and 6 weeks later a booster was injected. Spleen cells were isolated and fused with a plasmacytoma cell line (Sp2/0) to generate the Ab-producing hybridomas. Hybridomas were selected by an immunoenzimatic assay where micro titer plates were coated.
with the purified antigen or normal pig serum (Dako), 5 µg/well, and incubated with HRP-goat anti mouse IgG (H+L) (Zymed, 62-6520).

RBP was determined in serum samples by reducing SDS-page in a Mini Protean II Electrophoresis apparatus (BioRad, CA, USA), using low molecular weight BioRad markers. Equal amounts of total proteins were loaded into the slots of the gel for every sample (7.5 µg/ml). Separated proteins were electroblotted to nitrocellulose membranes (Hybond-C Super, Amersham, UK) at 100 V for 1 hr using the mini Trans-blot Cell (Bio-Rad, CA). After blotting, the membranes were incubated with the mouse anti-pig RBP mAb (1 µg/ml) 100X diluted in 0.1% Tween20 PBS (TPBS) containing 0.5% nonfat dried milk. After washing with TPBS, the membranes were incubated for 1 hr in HRP-conjugated mouse EnVision™ + reagent (Dako Cytomation) 200X diluted in TPBS/0.5% nonfat milk.

The proteins were detected by the ECL Western blotting detection reagents (Amersham Pharmacia) and the blots developed with Hyperfilm™ ECL (Amersham Biosciences).

Developed films were read with the Bio-Rad imaging densitometer (model GS 700) and the densities of defined bands were calculated using the program Molecular Analyst–Software for Bio-Rad (Version 4.1). Determination of the relative RBP band concentrations was carried out by comparing the bands with the band formed by the purified RBP (4 mg/ml).

Statistical Analysis

Statistical analyses were performed by the software SPSS 13.0, statistic for Windows. After describing observed data by parameters of position and variability (mean, median and SD), normality of data distribution was checked by the Shapiro-Wilkes test.

For normal distributed data, significance of the specific comparisons was analyzed by the paired T test; when data were not normally distributed a nonparametric test was applied (Wilcoxon test). Data were considered significant where \( p < .05 \).

RESULTS

Effect of Transportation on Behavior

In Group A pigs, there was a significant \( (p < .05) \) increased number of pigs observed nosing the environment by D1 than by D28. No other significant effect of time on any of the listed behaviors was detected (Table 2).

Total level of skin damage was greater immediately after transport (D1) than by D28 (Table 3). Significant differences were observed for lesions in the flank area.
In Group B pigs, behaviors were mostly directed toward penmates; a significantly higher percentage of pigs developed aggressive behaviors on D28 as compared with D1. The level of skin damage in the tail area was significantly greater than that in other body regions in Group B pigs (Table 3). Total level of skin damage was higher by D28 than by D28.

**Cortisol**

Results obtained for cortisol concentrations are presented in Table 4 as mean and median values (ng/ml) with the standard deviation (SD).

In Group A pigs, cortisol levels were found to be significantly higher in D1 serum samples than by D28 samples (Wilcoxon p < .05). No significant differences were found between D1 and D28 cortisol concentrations in Group B pigs.
Results obtained for the negative APP Alb are presented in Table 5. Albumin concentrations in blood samples from Group A pigs were found to be slightly lower in D1 samples, following transport, compared with D28 samples. A significant decrease (Wilcoxon test: \( p < .01 \)) was instead observed for Alb concentration in D28 samples of Group B pigs, after the tail-biting episode, when compared with D1 samples.

**Table 4**

Cortisol Mean and Median Values (ng/ml) With SD

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Group A</th>
<th></th>
<th></th>
<th>Group B</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D28</td>
<td>( p )</td>
<td>D1</td>
<td>D28</td>
<td>( p )</td>
</tr>
<tr>
<td>Cortisol mean</td>
<td>34.3</td>
<td>22.3</td>
<td></td>
<td>37.6</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>36.8</td>
<td>24.2</td>
<td>( a )</td>
<td>38.1</td>
<td>37.3</td>
<td>ns</td>
</tr>
<tr>
<td>SD</td>
<td>10.8</td>
<td>6.1</td>
<td></td>
<td>11.1</td>
<td>12.4</td>
<td></td>
</tr>
</tbody>
</table>

Note. D1 = day 1 after transportation; D28 = day 28 after transportation. \( p \) value: \( a = p < .01; \) ns = not significant.

**Table 5**

Negative APP Values Expressed as Percentage of Total Protein Contents

<table>
<thead>
<tr>
<th>Protein</th>
<th>Group A</th>
<th></th>
<th></th>
<th>Group B</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D28</td>
<td>( p )</td>
<td>D1</td>
<td>D28</td>
<td>( p )</td>
</tr>
<tr>
<td>Albumin mean</td>
<td>53.8</td>
<td>58.4</td>
<td>ns</td>
<td>49.4</td>
<td>34.8</td>
<td>( a )</td>
</tr>
<tr>
<td>median</td>
<td>53.1</td>
<td>56.2</td>
<td></td>
<td>47.3</td>
<td>32.9</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>5.4</td>
<td>7.1</td>
<td></td>
<td>6.7</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>RBP mean</td>
<td>0.45</td>
<td>0.44</td>
<td>ns</td>
<td>0.50</td>
<td>0.27</td>
<td>( a )</td>
</tr>
<tr>
<td>median</td>
<td>0.39</td>
<td>0.51</td>
<td></td>
<td>0.42</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.14</td>
<td>0.11</td>
<td></td>
<td>0.19</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>

Note. APP = Acute Phase Proteins; RBP = Retinol Binding Protein; D1 = day 1 after transportation; D28 = day 28 after transportation. \( p \) value: \( a = p < .01; \) ns = not significant.

**Negative Acute Phase Proteins**

Albumin/ Results obtained for the negative APP Alb are presented in Table 5. Albumin concentrations in blood samples from Group A pigs were found to be slightly lower in D1 samples, following transport, compared with D28 samples. A significant decrease (Wilcoxon test: \( p < .01 \)) was instead observed for Alb concentration in D28 samples of Group B pigs, after the tail-biting episode, when compared with D1 samples.

**Retinol binding protein.** Serum RBP of blood samples reacted avidly with the homemade mouse anti-pig RBP monoclonal antibody, demonstrating the antibody’s efficacy in RBP recognition. The protein was detected as a band in the 21 kDa region. In Figure 1a and 1b, western blots for five samples from Group A and five samples from Group B are shown. Standard RBP is presented on the left while on the right D1 and D28 patterns for each sample are shown. D1 and D28 samples...
from Group A did not differ substantially in concentration. On the other hand, a significant reduction in RBP contents is shown in D28 samples from Group B.

In Table 5, RBP concentrations are presented as percentage of total protein contents: the trend confirms the pattern seen by blotting. The Wilcoxon test showed a significant difference ($p < .01$) between D1 and D28 samples of Group B.

### DISCUSSION

There is recently a growing societal concern about animal welfare; thus, there is a growing need for developing a welfare assessment method. The assessment of animal welfare commonly involves behavioral and physiological indicators. Although single indicators can show that welfare is poor, a wide range of indicators must be used for an adequate assessment of welfare (SCAHAW, 2002).

Being welfare affected by stress conditions, the pigs of this study were exposed to an acute stressor, the transport, which involves both exposure to social stress (e.g., mixing with unfamiliar pigs) and physical stress (loading and unloading, stocking density). Blood collection and behavioral observations were carried out from all the animals immediately upon arrival and 28 days later. In accordance
with the work of Piñeiro et al. (2007), samples obtained 28 days later were used as within-animal controls, assuming that 28 days is a long enough period for the animals to recover from stress of transport and to establish a normal physiological state.

First, pig behavior has been used as an indicator of swine welfare: Pigs of Group A were found to be more interested in nosing the environment on D1 than on D28, indicating their curiosity in exploring a new pen on D1 and suggesting that the animals had become acclimated to their new environment by D28. No other important effect of the transport on behavior of Group A pigs was observed. On the other hand, pigs of Group B developed aggressive behaviors and tail biting on D28, as demonstrated by aggressive interactions being the most observed behaviors and by lesion scores in the tail area being greater by D28 than by D1. Transport stress may involve a number of factors such as fasting, water deprivation, social mixing, forced physical exercise, handling, noise, vibration (SCAHAW, 2002), and—finally—exposure to a new environment (Hemsworth, 1993). In this study, the lack of transport and housing differences between Group A and Group B and the fact that Group B pigs were monitored 15 days later than Group A pigs suggest that these behavioral variations could be more related to the environmental conditions than to the transport. Tail biting has previously been related to heat stress (Haske-Cornelius, Von Bogner, & Pescheke, 1979; Penny, Walters, & Tredget, 1981); however, it has more recently been confirmed to have a multifactorial origin and to be an unpredictable event on farms (Schroder-Petersen & Simonsen, 2001), hampering the possibility to relate it to a specific factor. Concerning the potential value of behavioral changes as welfare indicators, Mason and Latham (2004) suggested that variations in behavior are not a sign of poor welfare but—only if associated to other specific symptoms—may be a warning sign of potential suffering.

Physiological parameters were also used to assess pig welfare: Plasma cortisol levels were significantly higher by D1 than by D28 in Group A pigs, suggesting that they could have been affected by the transport and the related exposure to a novel environment. Previous works have confirmed this (Breinekovà et al., 2007; Dalin, Magnusson, Haggendal, & Nyberg, 1993).

The trend was inversed in Group B pigs; D28 cortisol was significantly higher. This probably resulted from the effect of the aggressive interactions, related efforts, and physical injuries—all of which are known to increase hormone concentration (Deguchi & Akuzawa, 1998).

To assess the potential of negative APP as welfare indicators, we chose albumin, which has been recognized as a negative APP in pigs (Lampreave et al., 1994) and RBP, which has so far been included in the group of negative APP only in humans (Baeten et al., 2004) and cattle (Ingenbleek & Young, 1994). The concentration of albumin was found decreased after transport and exposure to novelty, although this effect was not as significant as the effect on cortisol levels. These re-
results agreed with those of Brown, Knowles, Edwards, & Warriss (1999), who reported some changes in albumin concentrations in pigs after long transport. Albumin contents were lower in D28 samples of Group B animals, compared with D1 samples, probably for the effect of the aggressive interactions (excessive exercise and injuries).

The efficacy of RBP in swine welfare monitoring was also assessed. A western blot methodology for its detection was developed and permitted to show swine RBP as a band at 21 kDa (Figure 1), as previously reported in studies on humans (Peterson, 1971).

Important changes in its concentration following the aggressive episodes by D28 in Group B pigs were observed, confirming that RBP is a major negative acute phase protein also in swine. No difference in RBP contents was observed among D1 and D28 samples of Group A pigs, highlighting the importance of further studies on the protein.

The research discussed in this article demonstrates that many welfare issues are multifactorial; so, although it is possible to demonstrate a correlation between environmental factors and welfare outcomes, it is difficult to clearly determine actual cause and effect. The results suggest that the joint quantification of cortisol, albumin, and RBP, especially if associated with specific behavioral observations, could be helpful in representing the diverse and dynamic aspects of the welfare experienced by an animal and also confirm that a wide range of animal-based parameters should be taken into account for an accurate analysis of welfare. More research is needed to identify the best complementing range of indicators for on-farm studies: This parameter combination may be used to certify the level of welfare on specific farms; compare the welfare in different production systems; or serve as an advisory tool that allows farmers to identify, prevent, or rectify welfare problems on the farm.

REFERENCES


