# Social Rank Influences the Distribution of Blood Leukocyte Subsets in Female Growing Pigs

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***Summary***

The effect of high (DOM) and low (SUB) social rank on blood immune variables was examined in female growing pigs. Pigs were mixed with unfamiliar pigs at 9 weeks of age and kept in stable groups of 4 pigs for 5 weeks. Social rank was determined using a feeding competition test. SUB pigs showed reduced growth as compared to DOM pigs confirming their lower social status. Blood was sampled for immunological assessments immediately before grouping the pigs and again after the 5 weeks of social housing. White Blood Cell (WBC) counts, percentage of CD4 positive cells ($CD4^+$), percentage of CD8 positive cells ($CD8^+$), percentage of swine leukocyte antigen II (SLAII) carrying cells, LPS-stimulated Toll-like Receptor 4 (TLR4) expression, and LPS-stimulated tumor necrosis factor-alpha (TNF-α) responsiveness were determined at both times. IgG and IgM concentrations were measured following the 5 weeks of social housing only.

From the WBC counts it was found that the percentage of neutrophils was higher in SUB pigs and the neutrophil to lymphocyte ratio was higher in DOM pigs. The percentage of $CD4^+$ cells decreased with time in both DOM and SUB pigs, but only significantly in SUB pigs. The percentage of CD8+ cells was higher in SUB pigs than in DOM pigs and decreased with time in both DOM and SUB pigs. In addition, SUB pigs had a higher *ex vivo* TNF-α responsiveness as compared to DOM pigs. Both the percentage of SLAII carrying cells and LPS-stimulated TLR4 expression increased with time, but here no significant effect of social rank was found. In addition, neither IgG nor IgM concentrations showed any relationship with social rank. The findings indicate that social rank influences the distribution of blood leukocyte subsets in female growing pigs, suggesting that the pig would be a good model for investigating the effects of long-term immunomodulation on health.

***Introduction***

Individuals show different vulnerability to disease and stress (*Segerstrom et al., 2001*). Part of this variability can be related to the individual's social environment (*Fano et al., 2001*). So, social stressors have been shown to strongly influence various aspects of the immune function. A range of human, primate and rodent studies have addressed the consequences of both acute and chronic socially stressful conditions on the immune system (e.g. *Cunnick et al., 1991; Boccia et al., 1992; Stefanski, 1998; Bartolomucci et al., 2003; Tamashiro et al., 2005; Langhorst et al., 2007; Miller et al., 2008*), though the majority of studies have used rather acute social stressors.

Considering the fact that pigs are gaining increased use in biomedical research, an investigation into the

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effects of chronic social stress on the immune system in this species may, therefore, be of particular interest. Yet, in pigs, the impacts of social stress on the immune system have mainly been studied during disturbance in existing social hierarchies or immediately following formation of social hierarchies.

The social structure of pigs is based on a dominance hierarchy (*Meese & Ewbank, 1973*) and bringing unacquainted pigs together results in a short period of vigorous fighting that ends once dominance relationships have been settled (*Bolhuis et al., 2005*). Indeed, social rank has been shown to influence immune responses, for instance, *in vitro* Natural Killer cell cytotoxicity and mitogen-induced lymphocyte proliferation response, following exposure to acute stressors such as heat, cold, shipping, and social mixing (*McGlone et al., 1993; Morrow-Tesch et al., 1994; Hicks et al., 1998; Tuchscherer et al., 1998*). However, e.g. duration and timing of exposure to stressors is suggested as a critical factor in the immune outcome (*Morrow-Tesch et al., 1994; Wallgren et al., 1994; De Groot et al., 2001*) and the effects may therefore be more pronounced than in a stable social situation.

Thus, the objective of the present study was to describe some important aspects of blood cellular immunity in high and low ranking female growing pigs housed in a stable social environment. The immunological variables were determined before placing the pigs in experimental groups and again after five weeks of group housing. In contrast to earlier studies (*Hessing et al., 1994; Morrow-Tesch et al., 1994; Tuchscherer et al., 1998*), no antigen challenge was used. The pig's social position was determined using a feeding competition test and only the highest and lowest ranking pig from each group was chosen for analysis.

## *Material and Methods*

### *Animals and housing*

The experiment used crossbred Landrace×Yorkshire female growing pigs from the resident herd at the University of Aarhus, Faculty of Agricultural Sciences (DJF), Tjele, and was run in 3 blocks. Each block contained 4 x 4 pigs drawn from four different litters. All pigs were weaned at 4 weeks of age (28 ± 2 days) and moved to weaning pens in intact litters. At 9 weeks of age (62 ± 3 days) the pigs were transferred in intact litters to the experimental building and the pigs chosen for experimentation were grouped with 3 unfamiliar pigs. The whole experiment thus comprised 12 groups of 4 pigs per pen. The experimental pens measured 1.5 m x 3.0 m, ¾ of this area being solid floor. Each pen was equipped with a two-holed food trough fitted in the right-hand corner at the front of the pen and a nipple drinker in the rear end. Thus, the pigs had *ad libitum* access to water and a standard pelleted feed (Farm Management and Research Facilities, Faculty of Agricultural Sciences, Denmark, SV-02 SV-1138). The ventilation system was an equal pressure system (Fristamat, Langeskov, Denmark) and the air exchange was approximately 50 $m^3$ per hour per pig or 2.2 times per hour in the room. Room temperature was maintained at approximately 20 °C (21.4 ± 1.1). In addition to daylight, artificial lights were on from 0730 to 1530 hours. Pens were cleaned and 200 g fresh straw per pig was provided daily between 0800 and 0930 hours. No additional bedding material was used.

The pigs were weighed weekly throughout the experimental period starting 3 days after arrival to the experimental pens. At the first weighing of the pigs, the mean weight of the groups varied from 15.9 to 23.7 kg. The lightest pig weighed 9.2 kg and the heaviest 31.5 kg. At the end of the experiment the average weight gain was calculated. Individual pigs were identified by a plastic earmark in addition to numbering using a pig marker spray (Hatting Mærkespray, Hatting KS, Denmark), which was refreshed twice a week. A daily clinical record was kept, and animals showing any clinical signs of disease (leg problems, treatment-demanding illnesses) were excluded from the experiment.

All procedures involving animals were approved by the Danish Animal Experiments Inspectorate in accordance with the Danish Ministry of Justice Law no. 382 (June 10, 1987) and Acts 333 (May 19,

1990), 726 (September 9, 1993) and 1016 (December 12, 2001).

*Feeding competition test and assessment of rank position*

A feeding competition test was performed at 11 weeks of age (78 ± 4 days), two weeks after grouping the pigs in the experimental pens. By then, the pigs weighed 28.7 ± 7.3 kg (mean ± SE). The test, adapted from *Lawrence et al.* (1991), was conducted between 0900 and 1100 hours and lasted for a total of 15 min (three consecutive series of 5 min periods each). Feeders were allowed to run empty by 1530 hours on the day before testing. Each period was started by pouring 150 g of the pigs' standard feed in the feeder's skid leading to the outer feeding hole (directed towards the pen). Thus, only one pig could feed at a time. To measure the total feeding time of each pig, the test was recorded on video. If a pig had its head inserted in the trough, it was defined as having precedence of the feeder. Social rank was assigned based on the outcome of observed durations of having precedence of the feeder (*Craig, 1986*). Pigs having precedence of the feeder for the longest time were termed dominant (DOM), while those having precedence for the shortest time were termed subordinate (SUB). One DOM pig was found in all 12 pens resulting in 12 DOM pigs. In addition, all pens housed a SUB pig but in three pens there were two pigs, which did not gain access to the feeder during the test and consequently they also were nominated as SUB pigs leading to a total of 15 SUB pigs. Only DOM and SUB pigs were included in further analysis.

*Analysis of blood samples*

The pigs were bled by jugular vein puncture while restrained by snaring. Blood was sampled on two occasions. The first sample (baseline) was collected at 9 weeks of age before mixing the pigs in the experimental groups. The second sample was collected at 14 weeks of age (100 ± 4 days) after 5 weeks in the stable groups (social stress). Blood was collected in both sodium heparin (Na-heparin) stabilised plastic tubes (Becton Dickinson, Franklin Lakes, USA) and in ethylenediaminetetraacetic acid (EDTA) coated plastic tubes (Greiner bio-one GmbH, Kremsmünster, Austria). The earlier sampling was performed between 0930 and 1200 hours and the later sampling was performed between 0930 and 1400 hours.

*White Blood Cell counts*

Within 4 hours of sampling, EDTA-stabilised whole blood was analysed to determine the total number of leukocytes and the differential proportions of neutrophils and lymphocytes using a haemocytometer (Cell-Dyn 3500 Coulter counter, ABBOTT laboratories, Wiesbaden, Germany). Samples were counted twice. The haemocytometer was monitored with whole blood reference controls (Cell-Dyn$^{R}$22control) according to the manufacturer's instructions. The neutrophil to lymphocyte ratio (N:L ratio) was calculated as the percentage of neutrophils divided by the percentage of lymphocytes.

The remaining EDTA stabilised blood samples were centrifuged for 20 min at 4 °C and 2000 x *g*, after which the plasma was separated and stored at −80 °C for later assaying of IgG and IgM concentrations.

*Phenotyping of leukocyte subsets*

Phenotyping of subpopulations of peripheral blood leukocytes was done by flow cytometry. Primary antibody staining was performed by incubating 100 µl whole heparinised blood with FITC-conjugated mAb MCA2061F (anti-TLR4), FITC-conjugated MCA1749F (anti-CD4), PE-conjugated MCA1223PE (anti-CD8), or MCA 1335 (anti-SLAII) in the dark for 20 min. The staining with anti-CD4 and anti-CD8 was done using a double staining protocol. After the primary staining, erythrocytes were lysed using Optilyse®C Lysing solution (Immunotech, Beckman Coulter, Marseille, France) in accordance with the manufacturer's guidelines. The remaining cells were washed twice in FACS buffer (0.2% BSA, 0.2% sodium azid, 0.05% horse serum in PBS pH 7.4). Secondary

antibody staining of anti-SLAII was performed in a total of 100 µl FACS buffer containing FITC-conjugated Goat anti-mouse IgG (1:50). All antibodies used were purchased from Serotec (Serotec Ltd, 22 Bankside, Kidlington, Oxford, UK). The blood for anti-TLR4 staining was first incubated for 2 hours at 39 °C with 25 µg/ml lipopolysaccharide (LPS) (Sigma, 3050 Spruce Street, St Louis; Missouri, USA). The remains of the LPS-stimulated blood sample were centrifuged for 20 min at 4 °C and 2000 x *g* after which the plasma was separated and stored at −80 °C until tumor necrosis factor-alpha (TNF-α) analysis.

All steps in the flow cytometry analysis were carried out at room temperature. Centrifugation was performed between each step for 5 min at 296 x *g*. The cells were resuspended in 1 ml FACS buffer, before being analysed on an EPICS$^{R}$ XL-MCL flow cytometer, equipped with an argon laser providing incident light at 488 nm (Coulter Immunotech, Miami, FL, USA). Alignment was performed with Flow-Check fluorospheres and day-to-day standardisation was performed with Flow-Set COULTER® Fluorospheres (Coulter Immunotech, Miami, FL, USA). For each pig, individual gates for granulocytes and lymphocytes including monocytes were set using a dot plot of cell size versus cell granularity (FSC×SSC), excluding cellular debris. Monocytes were led into the lymphocyte gate, as monocyte and lymphocyte populations are overlapping in pigs (*Wang et al., 1987*). Marker bars were set relative to negative controls. Negative controls were samples with no reagents, with irrelevant isotype-matched mAbs or with secondary mAb only. Mean Fluorescence intensity (MFI) was measured. From each sample, 10000-20000 events were counted.

*Concentration of IgG and IgM in serum*

A commercial enzyme-linked immunosorbent assay (ELISA) was used for quantification of IgG and IgM (pig IgG [E100-104] and pig IgM [E100-100], Bethyl Laboratories, Montgomery, TX, USA). Freshly thawed plasma samples were analysed in duplicate according to the manufacturer's guidelines, together with a high and a low positive control. The IgG assay detection limit was 7.8 ng/ml. On IgG measures, the intra-assay coefficient of variation (CV) was 11.8-15.8 % in high controls and 7.7-13.5 % in low controls. The inter-assay CV was 12.4% in high controls and 8.9 % in low controls. The IgM assay detection limit was 16 ng/ml. On IgM measures, the intra-assay CV was 3.0-3.4 % in high controls and 3.4-4.0 % in low controls. The inter-assay CV was 5.6 % in high controls and 3.4 % in low controls.

*Ex vivo TNF-α responsiveness of cells in plasma*

A commercial enzyme-linked immunosorbent assay (ELISA) was used for quantification of TNF-α (Quantikine® Porcine TNF-α/TNFSF2 Immunoassay, R & D systems, Minneapolis, MN, USA) in LPS simulated heparinised plasma. Freshly thawed LPS-stimulated plasma samples were analysed in duplicate according to the manufacturer's guidelines, together with dilutions of recombinant porcine TNF-α standards and controls. The detection range was from 3.7 to 237.0 pg/mL. The intra-assay variation coefficient (CV) on controls ranged from 2.8 to 10.2 %, while the inter-assay CV on controls was 8.7 %.

*Statistical analyses*

The MIXED procedure of SAS (*Littell et al., 1996*) was used for analysis of variance using the Restricted Maximum Likelihood method with multiple error terms. The effects of rank (DOM, SUB), time of sampling (9 weeks of age, 14 weeks of age), and their interactions were used as fixed effects in the model. Pen, block, and the interaction between sow and block were considered random effects. Variables measured twice were fitted with time as a repeated measure specifying the individual pig as subject. A heterogeneous compound symmetry variance structure was used to allow for different residual variance for each of the two observation times. Weight and its interactions with the fixed effects were included as covariates if $P<0.10$. Due to unbalanced data as a result of lacking observations (i.e. clotting of blood

samples), Satterthwaite's approximation was used to find the denominator degrees of freedom in all F-tests. The univariate procedure of SAS was used to determine the normality of distribution of each set of data. When necessary, $\log_{10}$ transformations were performed to normalize data distribution. In addition, residuals were inspected to check that the assumptions of normal distribution and variance homogeneity were satisfactory. All analyses were run as two tailed tests.

Results are reported as Least Square Means ± standard error (*Littell et al., 1996*) by social status only. Significance is reported for $P<0.05$ and for tendencies at $0.05<P<0.10$. The corresponding F-value and the degrees of freedom are indicated by $F(df_{investigated\ effect}, df_{error})$. Significant effects that included weight are described with their regression coefficients, ß, and t-test values.

## *Results*

*Behaviour in feeding competition test and growth*

The duration of time having precedence of the feeder classified 12 pigs as dominant (DOM) and 15 pigs as subordinate (SUB). In 3 of the pens there were 2 pigs that did not gain access to the feeder at all during the feeding competition test. Consequently, each of these 3 pens had 2 pigs classified as SUB pigs.

Weight increased significantly with time [F(1,31)=189.7; P<0.0001] (Table 1) and tended to be higher in DOM pigs than in SUB pigs by 14 weeks of age as indicated by the interaction between social rank and time [F(1,31)=3.5; P<0.10]. Initially, by 9 weeks of age, the weight of DOM and SUB pigs did not differ (P>0.10), but growth during the 5-week experimental period was significantly higher in DOM pigs as compared to SUB pigs ([F(1,6)=6.1; P<0.05].

*Rank effects on White Blood Cells*

Mean values for White Blood Cell (WBC) counts, by social rank only, are shown in Table 2. The total number of leukocytes tended to be higher in DOM pigs than in SUB pigs [F(1,27)=3.2; P<0.10]. Overall the percentage of neutrophils was higher in SUB pigs than in DOM pigs [F(1,26)=619.8; P<0.0001], and higher at 14 weeks of age than at 9 weeks of age [F(1,26)=16.7; P<0.001]. The percentage of neutrophils was negatively related to weight [F(1,26)=87675.3; P<0.001] (ß=-0.51; P<0.0001). The percentage of lymphocytes was influenced by time and weight as shown by a significant effect of the interaction between the two factors [F(1,25)=4.8; P<0.05]. The percentage of lymphocytes was higher at 9 weeks of age than at 14 weeks of age. There was a positive relationship between the percentage of lymphocytes and weight at 9 weeks of age (ß=1.07; P<0.01), whereas no relationship was evident at 14 weeks of age. The percentage of lymphocytes was not affected by social

**Table 1.** Results from the feeding competition test, weights and total growth (Least Squares Means ± standard error) across the 5-week experimental period

| Rank | Precedence of feeder, in seconds at feeder | Weight, kg (9 weeks of age) | Weight, kg (14 weeks of age) | Total growth, kg |
|---|---|---|---|---|
| **Dominant** | 594 ± 149 | 21.2 ± 1.5 | 47.7 ± 3.5[c***] | 26.3 ± 3.2 [a] |
| **Subordinate** | 11 ± 28 | 19.8 ± 1.3 | 39.7 ± 3.2[d***] | 19.5 ± 3.1 [b] |

Within columns, significance levels are indicated by different letters: a,b (P<0.05); c,d (P<0.10). Within rows, differences from 9 to 14 weeks are indicated by *** (P<0.0001).

**Table 2**. Leukocyte subpopulations in female growing pigs of high (dominant) and low (subordinate) social rank (Least Squares Means ± standard error)

| Cell type | Dominant | | Subordinate | |
|---|---|---|---|---|
| | 9 weeks | 14 weeks | 9 weeks | 14 weeks |
| **Cell Count** | | | | |
| White Blood Cells ($10^9$/L) | 19.6 ± 1.2 | 22.5 ± 1.7 | 18.3 ± 1.1 | 18.6 ± 1.7 |
| % Neutrophils | 34.8 ± 2.9 | 45.6 ± 2.9 | 36.9 ± 2.8 | 47.3 ± 2.8 |
| % Lymphocytes | 62.0 ± 4.5 | 47.0 ± 4.0 | 64.7 ± 5.0 | 46.5 ± 3.6 |
| N:L ratio | 0.82 ± 0.01 | 1.06 ± 0.00 | 0.79 ± 0.01 | 1.02 ± 0.00 |
| **Leukocyte subsets (%)** | | | | |
| $CD4^+$ cells | 9.0 ± 2.0 | 5.5 ± 0.9 | 14.3 ± 2.2 | 5.8 ± 0.7 |
| CD8+ cells | 28.9 ± 2.2 | 13.2 ± 2.3 | 33.9 ± 2.1 | 15.6 ± 1.9 |
| CD4:CD8 ratio | 0.58 ± 0.07 | 0.64 ± 0.04 | 0.75 ± 0.04 | 0.65 ± 0.03 |
| SLAII positive cells | 47.1 ± 2.5 | 53.4 ± 2.4 | 46.6 ± 2.4 | 55.1 ± 2.2 |

rank (P>0.10). The neutrophil to lymphocyte (N:L) ratio was in general higher [F(1,26)=6.5; P<0.05] in DOM pig as compared to SUB pigs, and increased in both DOM and SUB pigs from 9 to 14 weeks of age [F(1,26)=20.6; P<0.0001]. A significant influence of weight [F(1,26)=105.2; P<0.0001] revealed a negative relationship between N:L ratio and weight (ß=-0.01; P<0.0001).

*Rank effects on lymphocyte subsets*

Mean values for lymphocyte subsets, by social rank only, are also shown in Table 2. The percentage of $CD4^+$ cells in the lymphocyte gate, which included T-helper lymphocytes, regulatory T-lymphocytes and monocytes, was influenced by social rank, time and weight as shown by a significant effect of the interaction between the three factors [F(1,25)=6.0; P<0.05]. In DOM pigs a significant negative relationship existed between $CD4^+$ cells and weight at 9 weeks of age (ß=-0.48; P<0.01), whereas there tended to be a positive relationship at 14 weeks of age (ß=0.09; P<0.10). In SUB pigs a significant positive relationship was seen between $CD4^+$ cells and weight at 14 weeks of age (ß=0.12; P<0.05) only. The percentage of $CD4^+$ cells decreased from 9 to 14 weeks of age in both DOM and SUB pigs, but the decrease was only significant in SUB pigs (P<0.0001). In addition, SUB pigs tended to have a higher percentage of $CD4^+$ cells than DOM pigs (P<0.10) at 9 weeks of age. The percentage of CD8+ cells, i.e. cytotoxic T-lymphocytes and natural killer (NK) cells, was in general higher in SUB pigs than in DOM pigs [F(1,28)=5.0; P<0.05] and overall the percentage of CD8+ cells was higher by 9 weeks of age than by 14 weeks of age [F(1,28)=33.4; P<0.0001]. A significant effect of weight [F(1,28)=10.7; P<0.01] showed that the percentage of CD8+ cells increased with increasing weight (ß=0.30; P<0.01). The CD4:CD8 ratio was influenced by social rank, time and weight as shown by a significant effect of the interaction between the three factors [F(2,25)=7.2; P<0.05]. The effect of weight consisted of a negative relationship between the CD4:CD8 ratio and weight in DOM pigs at 9 weeks of age (ß=-0.03; P<0.05). Although non-significant, the CD4:CD8 ratio increased in DOM

pigs from 9 to 14 weeks, while the CD4:CD8 ratio decreased in SUB pigs. The percentage of SLAII positive cells was in general higher at 14 weeks of age as compared to 9 weeks of age [F(1,28)=7.0; P<0.05] and was significantly influenced by weight [F(1,28)=10.2; P<0.01] represented as a negative relationship between the percentage of SLAII positive cells and weight (ß=-0.31; P<0.01). There were no effects of social rank (P>0.10).

*Rank effects on LPS stimulated leukocytes*

The TLR4 expression on granulocytes after LPS stimulation was higher by 14 weeks of age than by 9 weeks of age [F(1,28)=6.5; P<0.05]. Mean fluorescence intensity (MFI) of the TLR4 expression was 2.6 ± 0.02 at 9 weeks of age and 3.0 ± 0.07 at 14 weeks of age (data not shown). There was no effect of social rank (P>0.10). The *ex vivo* TNF-α responsiveness after LPS stimulation is shown in Figure 1. TNF-α responsiveness was higher in SUB pigs than in DOM pigs [F(1,27)=4.4; P<0.05]. A significant effect of weight [F(1,26)=5.7; P<0.05] showed an increase in TNF-α responsiveness with increasing weight (ß=0.01; P<0.05).

*Rank effects on humoral immune status*

There were no effects of social rank on either plasma IgG (P>0.10) or plasma IgM (P>0.10). At 14 weeks IgG and IgM concentrations were 10.4 ± 1.66 mg/mL and 0.84± 0.04 mg/mL in DOM pigs, and 13.6 ± 1.4 mg/mL and 0.88 ±1 mg/mL in SUB pigs, respectively (data not shown).

## *Discussion*

In the present study, DOM and SUB pigs differed in growth rate as well as in innate and adaptive immune measures. All pigs were housed under identical conditions and in the same building throughout the experimental period. Therefore, the most obvious factor in contributing to the observed immuno-

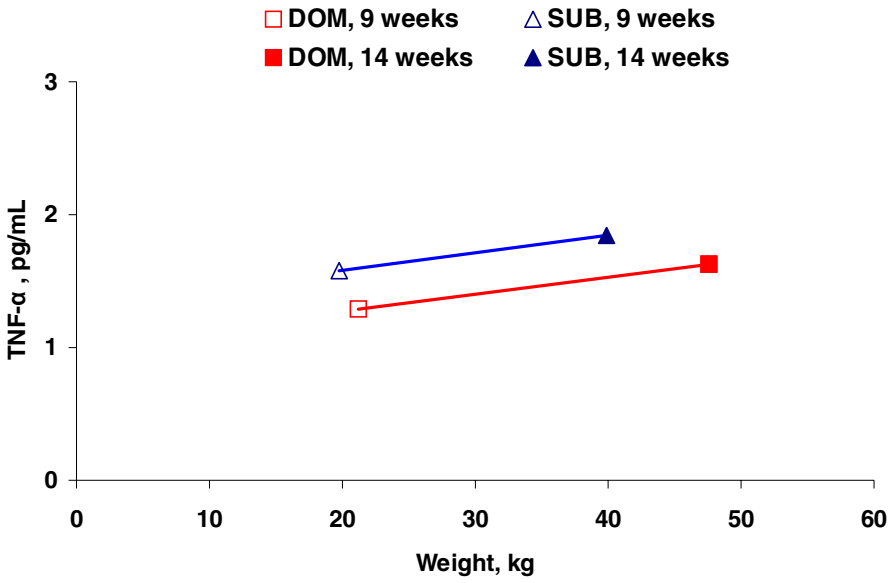


**Figure 1.** Influence of social rank on the *ex vivo* TNF-α responsiveness in dominant (DOM) and subordinate (SUB) female growing pigs. Data are presented as (Least Squares Means ± standard error) TNF-α responsiveness against mean weight per 9 and 14 weeks of age. Significant differences are described in the text.

logical differences between DOM and SUB pigs was their social environment. Thus, the present results suggest that social rank has a modulating effect on the experimental unchallenged immune system in female growing pigs housed in small stable groups. Social rank reflects, in part, an individual's ability to gain priority of access to particular resources (*Craig, 1986*). Therefore, time having precedence of the feeder during a feeding competition test was used in the present study to make an uncomplicated discrimination of the social rank of the pigs. DOM and SUB pigs were initially of equal average weight (Table 1), the experimental groups were rather small, and the feed was provided *ad libitum*. Therefore, daily access to feed was not considered to be limited for any pig in the pens, and accordingly, the lower growth leading to a tendency for a lower body weight in SUB pigs (Table 1) validate the estimation of dominance relationships by using a feeding competition test (*Morrow-Tesch et al., 1994; Hicks et al., 1998; O'Connell & Beattie, 1999; Andersen et al., 2000; Giroux et al., 2000*). Low body weight is considered a characteristic of low social status in rodent social stress models (*Tamasiro et al., 2005*) and is generally considered to indicate that a subordinate position is more stressful than a dominant position. This is in accordance with reports where lower body weight and lower weight gain have been observed in pigs subjected to chronic stress (mixing, heat, and crowding) as compared to unstressed controls (e.g. *Sutherland et al., 2006*).

The rank-related differences in the distribution of leukocytes imply that low and high social rank are not just a persistent stressful situation. The higher percentage of neutrophils in SUB pigs (Table 2) may indicate that SUB pigs occupy a more stressful position (*Widowski et al., 1989*), whereas the higher N:L ratio in DOM pigs (Table 2) may indicate that DOM pigs are occupying a more stressful situation (*Puppe et al., 1997; Sutherland et al., 2006*). However, as evident from the development in growth, the higher N:L ratio did not cause growth impairment in DOM pigs. The apparent contradictions of the responses strengthen the notion that the interpretation of WBC counts in terms of measuring stress should be done with caution (*Leek et al., 2004*). Changes in leukocyte counts causing changes in the N:L ratio have been considered a simple, useful physiological measure of stress (e.g. *Puppe et al., 1997; Gardner et al., 2001*). In rodent models of social stress, stress and stress hormones are known to induce changes in the distribution of leukocytes (*Dhabhar & McEwen, 2001*). Rising levels of cortisol decrease the number (or percentage) of lymphocytes while increasing the number (or percentage) of neutrophils, and, as a consequence, increase the N:L ratio (*Wallgren et al., 1994; Salak-Johnson et al., 1997; Bilandžic et al., 2006*). Taking the negative relationships between weight and number of neutrophils and between weight and N:L ratio into account, the present results indicate that irrespective of social rank, a relatively low weight acts as a stressor. Psychological stress arises from factors such as environmental predictability and control, which varies with rank (*Sapolsky, 2004*). Behavioural control is defined as the expression of a behavioural response that can prevent, reduce, or terminate a stressful situation (*Dantzer, 2001*). A high level of environmental controllability is considered intrinsic to high social rank. Hence, not having the obvious strength to prevent or end agonistic interactions between group members, or to claim rights, could thus make a low weight DOM pig lack some of the controllability and predictability of the environment otherwise assigned to its rank.

With regard to the immunological measures, SUB pigs differed from DOM pigs in several parameters. Compared to DOM pigs, SUB pigs had a higher percentage of CD8+ cells. Furthermore, the percentage of CD4+ cells decreased significantly during the experimental period in SUB pigs but not in DOM pigs (Table 2). Also, SUB pigs had a higher *ex vivo* TNF-α release after LPS stimulation than DOM pigs (Figure 1). In rodent models, chronic stress induces a decrease in circulating numbers of CD4+ and CD8+ T-cells, and B cells (*Stefanski & Engler, 1999; Engler et al., 2004*) and has been linked with an increase in TNF-α responsiveness

(*Kusnecov and Rossi-George, 2002*). Likewise, a reduction in CD8+ T-cells has been reported in pigs following treatment with the synthetic agent glucocorticoid dexamethasone for three weeks (*Lo et al., 2005*). In sheep, however, isolation stress has been shown to alter the ratio of T-cell phenotypes by increasing CD4+ T-cells and decreasing CD8+ T-cells (*Degabriele & Fell, 2001*). But that study comprised a 2 to 3-week period and a rather radical social change, and the response was consequently described as a recovery from a stressful change of circumstances (*Degabriele & Fell, 2001*). Thus, taking into account that the present measure of CD8+ cells includes CD8+ T-cells as well as NK cells, the present results indicate that SUB pigs occupied the most stressful social position, which is in agreement with the classic view of social rank. In addition, the positive relationships between weight and CD4+ cells and CD8+ cells further support the above mentioned interpretation that low weight acts as a stressor.

Changes in lymphocyte subset frequencies may reflect changes in immunological regulatory environments (*Appleyard et al., 2002*). Thus, the opposite, but non-significant, changes in CD4:CD8 ratio observed across the experimental period in SUB and DOM pigs may indicate that such immunological regulation have taken place. But the present study did not find any overall effect of rank on humoral immune status. This is in agreement with findings on social stress associated with social mixing of pigs (*Sutherland et al., 2006*), though others have found that the specific, humoral immune reactivity of dominant and subordinate pigs and rats is differently affected (*Fleshner et al., 1989; Hicks et al., 1998; Tuchscherer et al., 1998; Hessing et al., 1994*). The different results from studies on immunoglobulins, however, may relate to experimental differences regarding both the type of stressors used (physical, psychological, antigenic etc.), stressor duration and intensity (acute vs. chronic), as well as to previous infection pressures and the current microbial environment.

At present it is not possible to conclude on the consequences on health. The immune profile in the SUB pigs may, however, be indicative of an increased susceptibility to some diseases. The significant decrease in percentage of CD4+ cells in SUB pigs as opposed to DOM pigs may reflect a suppression of the adaptive cellular immunity in SUB pigs, which may reduce disease resistance to opportunistic infections (*Luo & Li, 2008*). In addition, an exaggerated inflammatory response, including an increased TNF-α response, may be associated with an increased disease susceptibility to *Streptococcus suis* in the mouse (*Domínguez-Punaro et al., 2008*). Furthermore, high systemic TNF-α is an inherent factor in the stress-related disease, metabolic syndrome in humans, which is coupled with obesity, insulin resistance and an increased risk of developing type 2 diabetes mellitus and cardiovascular diseases (e.g. *Black, 2006; Xeyda & Stulnig, 2007*). However, a reduced *ex vivo* responsiveness in TNF-α in piglets after weaning has been suggested to be a key factor in the increased susceptibility to post-weaning diarrhoea (*Carstensen et al., 2005*).

In conclusion, the present study demonstrates an influence of social rank on the immunological status of female growing pigs. Despite the fact that the N:L ratio indicated that high social rank acts as a typical chronic stressor, the more specific immunological measures indicate that low social rank reflects chronic stress characterised by reduced competence of the adaptive immune system and an increased activity or reactivity of the innate immune system. This partly implies an interpretation related to weight which, in addition, indicates that low weight is stressful irrespective of social rank. Consequently, social housing of female growing pigs in small stable groups may offer a useful platform for future studies of the consequences of social stressors on immune function and health. The relationship between social rank and specificity and efficiency of functional immune responses, however, needs further investigation as does the establishment of a causal relationship between neuroendocrine state and social rank.

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