

Effects of environmental enrichment and regrouping on natural autoantibodies-binding danger and neural antigens in healthy pigs with different individual characteristics

L. Luo, R. Geers, I. Reimert, B. Kemp, H. K. Parmentier and J. E. Bolhuis[†]

Adaptation Physiology Group, Department of Animal Sciences, Wageningen University & Research, PO Box 338, 6700 AH, Wageningen, The Netherlands

(Received 28 November 2016; Accepted 7 March 2017; First published online 6 April 2017)

Pigs living in commercial husbandry systems may experience both acute stress due to standard management procedures and chronic stress through limitations in their barren housing environment. This might influence their immune status, including antibody responses to neural and danger autoantigens. Levels of natural autoantibody (NAAb)-binding phosphorylcholine-conjugated bovine serum albumin (PC-BSA) and myelin basic protein (MBP) were measured over time in pigs that were kept in environmental enriched v. barren housing, and that underwent a regrouping test. In total, 480 pigs were housed in 80 pens in either barren or straw-enriched pens from 4 through 23 weeks of age. Blood samples were taken from pigs before (week 8), and 3 days after a 24 h regrouping test (week 9), and at 22 weeks of age. Phosphorylcholine-conjugated bovine serum albumin (PC-BSA) and MBP antibody titres in serum were measured using ELISA. Enriched-housed pigs had higher levels of IgM-binding MBP, and tended to have higher levels of IgG-binding MBP and IgA-binding PC-BSA than barren-housed pigs. Each NAAb measured in this study was affected by gender and litter. These results suggest that enriched housing conditions, as well as acute regrouping stress, have an influence on levels of serum NAAb-binding danger and neural antigens in pigs.

Keywords: environmental enrichment, natural autoantibody, pig, stress, immunity

Implications

Since January 2003, the provision of appropriate environmental enrichment to pigs has been mandatory across the European Union (Directives 2001/88/EC and 2001/93/EC). Pigs may experience stress and develop abnormal behaviour due to barren housing conditions in commercial pig husbandry and standard management procedures. This might affect their immune status and reduce welfare. The present paper suggests that enriched housing influenced the levels of natural autoantibodies (NAAb)-binding danger and neural antigens, which may reflect or affect their immune competence, (mental) health and welfare.

Introduction

Pigs in modern intensive husbandry systems can experience acute stress while being exposed to standard management procedures, such as tail docking, ear tagging and regrouping. The circumstances, under which pigs are kept, moreover, can lead to more sustained stress in these animals. Pigs in

commercial husbandry usually live in barren, stimulus-poor conditions. The absence of suitable substrates for oral manipulation thwarts pigs from performing highly motivated behaviours such as rooting and chewing and may, therefore, induce chronic stress. Indeed, compared with pigs kept under more enriched conditions, barren-housed pigs show changes in hypothalamus–pituitary–adrenal (re)activity (de Jong *et al.*, 2000), a decline in spatial cognitive performance (Grimberg-Henrici *et al.*, 2016), suggesting impaired hippocampal functioning, and an increase in abnormal behaviours such as tail biting (e.g. Camerlink *et al.*, 2014). Moreover, barren housing also negatively affects mood of pigs, as reflected in a more pessimistic view of life (Douglas *et al.*, 2012) and a decrease in play behaviours (Bolhuis *et al.*, 2005). Part of the differences between animals in barren and enriched housing might be explained by cellular and molecular changes in the brain induced by environmental enrichment (Scholz *et al.*, 2015).

There are indications for an effect of environmental enrichment as opposed to barren housing conditions on immune functions in pigs (Bolhuis *et al.*, 2003). Both acute and chronic stress have been shown to result in altered

[†] E-mail: liesbeth.bolhuis@wur.nl

immune functions (Dhabhar, 2014). Moreover, psychosocial factors can affect functioning of the immune system (Dhabhar, 2014), as both a negative and a positive mood may trigger immune alterations (Koh, 1998). Conversely, evidence is accumulating that mood and behaviour are affected by immune reactivity, including autoimmune responses towards brain and neuro-endocrine structures (Maier and Watkins, 1998).

Natural antibodies (NAb) are defined as immunoglobulins derived from self-renewing CD5⁺ B-1 cells (Casali and Notkins, 1989), without exogenous antigenic stimulation, and are found in all animal species tested so far (e.g. chickens: Sun *et al.*, 2011; bovine: Mayasari *et al.*, 2015; and wild boar: Rossi *et al.*, 2013). Natural antibodies are important as first line of defence, and participate in physiological activities (Panda and Ding, 2015). A large part of the NAb repertoire is directed to self-antigens, and these antibodies are referred to as NAAb. Blood levels of autoantibodies-binding neural antigens have been related to acute and chronic stress (Andrejević *et al.*, 1997; Zhou *et al.*, 1999), and could both reflect and affect neuronal functioning (Gold *et al.*, 2012). Myelin basic protein (MBP) is an important protein in the process of myelination of nerves in the nervous system, and it has been found that high levels of autoantibodies to MBP and other neural antigens were associated with neurological damage (Abou-Donia *et al.*, 2013). Other studies reported relations between stress and autoantibodies-binding antigens reflecting cell damage and inflammation, such as phosphorylcholine (PC) (Lutz *et al.*, 2009). Phosphorylcholine is a component of cell membranes which is recognized by IgM autoantibodies after cell damage (Kim *et al.*, 2002).

Previously, barren-housed and enriched-housed pigs were found to differ in levels of NAb binding the model antigen keyhole limpet hemocyanin (KLH) (Reimert *et al.*, 2014a). However, effects of environmental enrichment on NAAb-binding danger and neural antigens in healthy pigs (i.e. without clinical signs of illness) have not studied before. Our objective was to investigate the chronic effects of environmental enrichment (straw-enriched housing) *v.* barren housing on levels of NAAb-binding PC and MBP over time in pigs, and to study the effect of regrouping (inducing acute stress) on these autoantibodies. Individual characteristics of the pigs such as gender and personality, which influenced immune responses in other studies (Bolhuis *et al.*, 2003; Reimert *et al.*, 2014a) were taken into account as well.

Material and methods

The established principles of laboratory animal use and care were followed, as well as the Dutch law on animal experiments. The Animal Care and Use Committee of Wageningen University & Research approved the experiment.

Animals and housing

A total of 480 pigs, equally divided over five batches, were used in this study and were the same pigs as described in Reimert *et al.* (2014b). Briefly, pigs were born at the Topigs Norsvin experimental farm in Beilen, the Netherlands, where

they were housed in conventional farrowing pens with their sow. On the day of weaning, at 28 days of age, pigs were transported to experimental farm 'de Haar' of Wageningen University & Research, the Netherlands. Half of the pigs were housed in straw-enriched pens (~1 m²/pig) with 1.5 kg of straw and 12 kg of wood shavings ($n = 40$ pens). The other half of the pigs were housed in barren pens (~1 m²/pig) with a partially slatted and partially concrete solid floor ($n = 40$ pens). Litter mates were equally distributed over barren and enriched pens. Two hands of wood shavings were given to barren-housed pigs each day from 6 weeks of age onwards. Each pen was cleaned daily and afterwards enriched-housed pigs received 3 kg of fresh wood shavings and fresh straw (250 g at the start of experiment and then gradually increasing to 1.5 kg). A metal chain with a ball was present in each pen and at 8 weeks of age a jute sack was attached to the wall of each pen. The jute sack was replaced when worn.

Each pen contained six non-littermate pigs, three gilts and three barrows. Within each group, at least two pigs were classified as high resister (HR) and two pigs as low resister (LR) using a backtest. Groups diverged in estimated breeding values for indirect genetic effects (IGE) for growth during the finishing period (from ~25 to 110 kg), that is the heritable effect on the growth of their group members. During the finishing period, the growth of a pig is theoretically affected by each of its pen mates, and the genetic part of this effect is referred to as an IGE for growth. All pigs in a pen had either an estimated relatively positive (+IGE) or an estimated relatively negative (–IGE) on the growth of their pen mates (see Camerlink *et al.*, 2013; Reimert *et al.*, 2014a for details).

Behavioural tests

Backtest. Pigs were subjected to a backtest at ~2 weeks of age to assess their personality or coping style (Bolhuis *et al.*, 2003). Briefly, a piglet was manually restrained in supine position for 60 s. Classification of pigs was based on the number of struggles and the number of vocalizations. Piglets which showed two struggles and at least 25 vocalizations, or three struggles or more were classified as HR. The ones that showed zero or one struggle or two struggles with < 25 vocalizations were classified as LR.

Regrouping test. A regrouping test was conducted at 9 weeks of age as an acute stressor. Regrouping usually leads to fighting to establish a new rank order and is known to induce acute stress in pigs (Camerlink *et al.*, 2013). In short, a pair of pigs was regrouped for 24 h in a new pen with two other pairs of unfamiliar pigs. The same housing condition and IGE class were maintained when mixing pairs of pigs, as well as a balanced group composition for gender and coping style. All pigs were returned to their original pen and group after the 24 h test.

Blood collection and analyses

Blood samples were taken before (week 8) and 3 days after the regrouping test (week 9) and at 22 weeks of age. Blood was collected by immobilizing a pig on its back in a crib (weeks 8 and 9) or fixating the pig using a nose sling

(week 22) after which blood was taken by venepuncture from the jugular vein. Housing condition and IGE class were taken into account in the order of blood collection. Blood was collected in serum separating tubes (Greiner bio-one, Alphen aan den Rijn, The Netherlands) kept at room temperature (RT) until incubation for 1 h at 37°C. After that samples were centrifuged at $5251 \times g$ for 12 min at -20°C . Sera were stored at -80°C until analysis.

Enzyme-linked immunosorbent assay

To be able to perform ELISA, IgG, IgM and IgA antibody titres specific for PC were measured using phosphorylcholine conjugated to bovine serum albumin (PC-BSA; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and IgG and IgM antibody titres specific for MBP (Sigma-Aldrich, St. Louis, MO, USA) were determined by a two-step indirect ELISA similar to Bolhuis *et al.* (2003). Preliminary analysis revealed too low-IgA levels binding MBP for further analysis. First, medium binding microtitre plates (Greiner bio-one) were coated overnight at 4°C with $0.5 \mu\text{g/ml}$ PC-BSA or $0.5 \mu\text{g/ml}$ MBP in coating buffer ($0.05 \text{ M Na}_2\text{CO}_3 \times 10 \text{ H}_2\text{O}$, pH 9.6). After washing with tap water containing 0.05% Tween 20, serial dilutions of serum were added and incubated for 1.5 h at RT. After washing, plates were incubated for 1.5 h at RT with a 1 : 20 000 (for MBP) and 1 : 40 000 (for PC-BSA) diluted peroxidase (PO)-conjugated goat antibody directed to swine IgG_{FC} (GASwIgG_{FC}/PO; Bethyl Laboratories, Montgomery, AL, USA) to detect binding of IgG, or with 1 : 20 000 (for MBP) and 1 : 40 000 (for PC-BSA) diluted PO-conjugated goat antibody directed to swine IgM_{FC} (GASwIgM_{FC}/PO; Bethyl Laboratories) to detect binding of IgM, or with 1 : 20 000 diluted PO-conjugated goat antibody directed to swine IgA_{FC} (GASwIgA_{FC}/PO, Bethyl Laboratories) to detect binding of IgA, respectively. After washing, tetramethylbenzidine and 0.05% H_2O_2 was added as a substrate and incubated for 10 min at RT. Reaction was stopped with $2.5 \text{ N H}_2\text{SO}_4$ and absorbance was measured at 450 nm with a Multiskan (Flow, Irvine, UK). Each absorbance was expressed relatively to the absorbance of a standard positive control serum sample, and antibody titres were expressed as \log_2 values of dilutions that gave extinction closest to 50% of E_{max} , where E_{max} represents the highest mean extinction of a standard positive serum present on every microtitre plate.

Statistical analyses

SAS (SAS 9.3; SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. Titres could not be obtained from all 480 pigs at each sampling period due to technical problems, and due to pigs being removed from the experiment for health reasons (see Reimert *et al.*, 2014b for details). From the enriched pigs, at 8, 9 and 22 weeks of age 236, 235 and 223 samples were analyzed and from the barren pigs 237, 234 and 226 samples. Phosphorylcholine-conjugated bovine serum albumin -IgA titres were log transformed to obtain normality of residuals. Antibody titres were analysed using a repeated linear mixed model. The fixed effects housing, IGE class, backtest classification, week, and their

interactions, and gender, and its interaction with week and batch were included in the model, as well as the random effect of pen and litter. Values in time of individual pens, pigs and litter were taken as repeated measurements, that is IGE class, housing and batch effects were tested against the random effect of pen, and backtest classification and gender effects were tested against the random effect of pig.

Preliminary analyses revealed strong week effects for all variables. Therefore, effects of housing, IGE class, backtest classification and gender were also analysed for each week separately. A linear mixed model was used for this, with housing, IGE class, backtest classification, their two-way interactions, gender and batch as fixed effects and pen and litter as random effects. To investigate effects of housing, IGE class, backtest classification and gender on titres after acute stress, the Δ between weeks 9 and 8 was calculated and subsequently analysed with a linear mixed model with the same fixed, except week and random effects as used for the per week analysis. Only significant effects ($P < 0.05$) and tendencies ($P < 0.10$) are reported. Significant interactions were further investigated with *post hoc* pairwise comparisons using the differences of the least square means. Results are presented as means \pm standard error of mean.

Results

Titer of IgM, IgG and IgA antibodies in serum binding PC-BSA and IgM and IgG binding MBP are shown in Figure 1 (housing effects) and in Table 1 (gender effects). The analyses showed (additive, not interactive) effects of particularly housing and gender on titres over time, and therefore these two factors are highlighted separately. The change in antibody titres due to regrouping (Δ week 9 – week 8) for both housing conditions is shown in Figure 2.

IgM, IgG and IgA titres binding phosphorylcholine-conjugated bovine serum albumin

IgM binding phosphorylcholine-conjugated bovine serum albumin. Titers of IgM binding PC-BSA tended to be affected by gender ($P < 0.10$), with overall lower levels for gilts (6.56 ± 0.06) than for barrows (6.60 ± 0.07 , see also Table 1) and were also affected by week ($P < 0.001$). *Post hoc* analysis showed that titres increased from weeks 8 to 9 to 22 (Figure 1a). When analysing the effects per week, barren-housed pigs had higher IgM titres binding PC-BSA than enriched-housed pigs in week 8 ($P < 0.05$), and they tended to do so in week 9 ($P < 0.10$, Figure 1a). The increase in PC-BSA-IgM titres after regrouping (i.e. Δ of week 9 – week 8 as an effect of regrouping) was larger for barrows (1.17 ± 0.08) than for gilts (0.99 ± 0.07 , $P < 0.05$). There was no effect of housing on titre changes after regrouping (Figure 2).

IgG binding phosphorylcholine-conjugated bovine serum albumin. Phosphorylcholine-conjugated bovine serum albumin -IgG titres were affected by week ($P < 0.001$) and decreased from weeks 8 to 9 to 22 (Figure 1b). When analysed for each

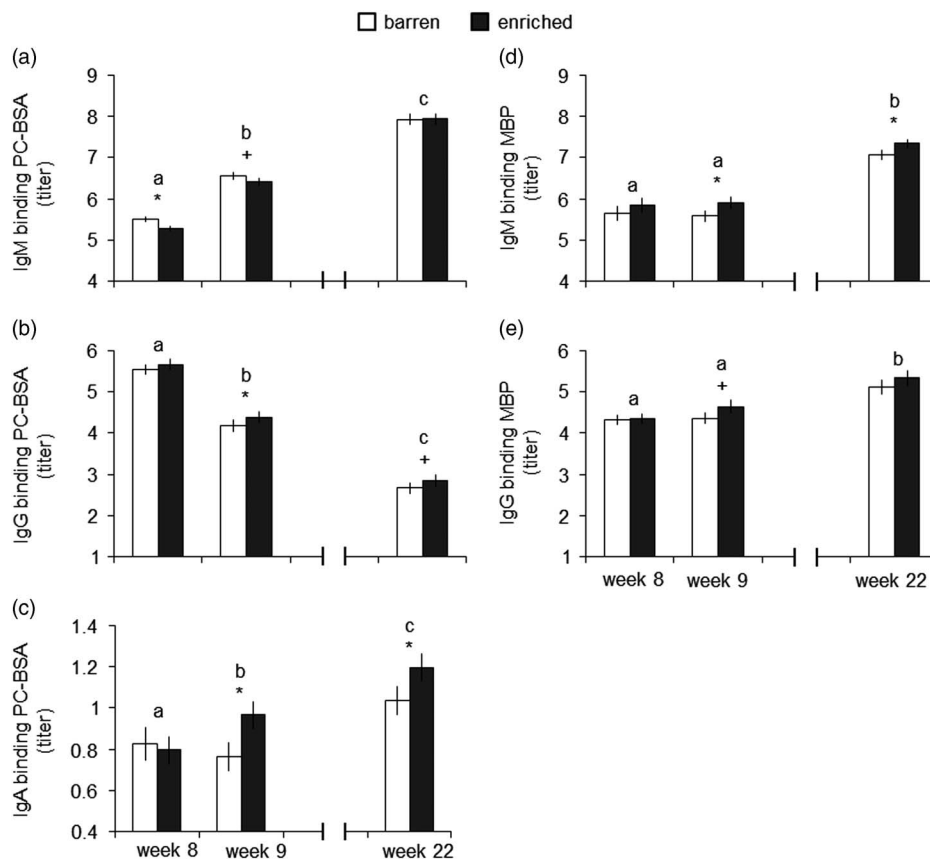


Figure 1 Means and standard error of mean of antibody titres. IgM (a), IgG (b) and IgA (c) titres to phosphorylcholine-bovine serum albumin (PC-BSA) and IgM (d) and IgG (e) titres to myelin basic protein (MBP) of pigs ($n = 80$ pens) in barren and enriched housing conditions measured before a 24 h regrouping test at 8 weeks of age, 3 days after the regrouping test at 9 weeks of age and at 22 weeks of age. Effects of housing over all 3 weeks are reported in the text; significances of housing differences per week are indicated: * $P < 0.05$; + $P < 0.10$. Week differences are indicated by letters; weeks lacking a common letter significantly differ.

Table 1 Means and standard error of mean of antibody titres of barrows and gilts determined before a 24 h regrouping test at 8 weeks of age, 3 days after the regrouping test, at 9 weeks of age and at 22 weeks of age

	Week 8			Week 9			Week 22		
	Barrow	Gilt	<i>P</i> -values	Barrow	Gilt	<i>P</i> -values	Barrow	Gilt	<i>P</i> -values
PC-BSA-IgM	5.37 ± 0.06	5.42 ± 0.06	Ns	6.56 ± 0.08	6.41 ± 0.08	+	7.98 ± 0.13	7.88 ± 0.11	*
PC-BSA-IgG	5.64 ± 0.12	5.57 ± 0.13	Ns	4.23 ± 0.13	4.34 ± 0.13	Ns	2.78 ± 0.13	2.74 ± 0.13	Ns
PC-BSA-IgA	0.86 ± 0.06	0.75 ± 0.06	+	0.81 ± 0.05	0.92 ± 0.07	Ns	1.20 ± 0.05	1.02 ± 0.06	*
MBP-IgM	5.80 ± 0.09	5.69 ± 0.09	Ns	5.74 ± 0.07	5.75 ± 0.07	Ns	7.24 ± 0.06	7.15 ± 0.06	+
MBP-IgG	4.40 ± 0.08	4.28 ± 0.08	Ns	4.63 ± 0.10	4.35 ± 0.09	*	5.25 ± 0.10	5.18 ± 0.10	Ns

PC-BSA = phosphorylcholine-bovine serum albumin; MBP = myelin basic protein.

Effects of gender over all 3 weeks are reported in the text; significances of gender differences per week are indicated: * $P < 0.05$; + $P < 0.10$.

week separately, barren-housed pigs had lower titres than enriched-housed pigs in week 9 ($P < 0.05$) and in week 22 ($P < 0.10$), with no housing effect in week 8 (Figure 1b). Gender did not affect PC-BSA-IgG titres (see Table 1 for means per week). The change in PC-BSA-IgG titres after regrouping (i.e. Δ) was not affected by any of the factors tested (Figure 2).

IgA binding phosphorylcholine-conjugated bovine serum albumin. Phosphorylcholine-conjugated bovine serum albumin-IgA titres tended to be affected by housing ($P < 0.10$), with

lower levels for barren-housed pigs than for enriched-housed pigs (overall: 0.99 ± 0.04 v. 0.87 ± 0.04 , Figure 1c), particularly in weeks 9 and 22 (per week analysis, both $P < 0.001$). In addition, effects of gender ($P < 0.05$), week ($P < 0.001$) and the interaction between gender and week ($P < 0.05$) were found. *Post hoc* analysis showed that overall titres in weeks 8 and 9 were not significantly different, but differed from those in week 22. In gilts, however, PC-BSA-IgA titres in week 8 differed from those in week 9 ($P < 0.05$, see Table 1 for means). Barrows had higher titres than gilts in week 22 (Table 1). The change in

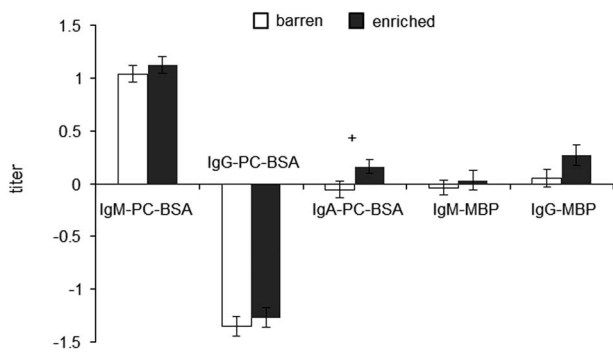


Figure 2 Means and standard error of mean of the variation in antibody titres between week 9, 3 days after a regrouping test, and week 8, before this test. IgM, IgG and IgA titres to phosphorylcholine-bovine serum albumin (PC-BSA) and IgM and IgG titres to myelin basic protein (MBP) of pigs ($n = 80$ pens) in barren and enriched housing conditions for Δ (week 9–week 8). Significances of housing differences are indicated: $^+P < 0.10$.

titres from weeks 8 to 9 (i.e. Δ) tended to be affected by housing (see Figure 2, $P < 0.10$) and was affected by gender (gilts: 0.17 ± 0.08 ; barrows: -0.06 ± 0.07 , $P < 0.05$).

IgM and IgG titres binding myelin basic protein

IgM binding myelin basic protein. Myelin basic protein-IgM titres were affected by housing ($P < 0.001$) (Figure 1d), with overall lower titres for barren-housed pigs (6.10 ± 0.10) than enriched-housed pigs (6.36 ± 0.10). Separate analyses per week revealed that MBP-IgM titres were particularly affected by housing in weeks 9 ($P < 0.05$) and 22 ($P < 0.05$) (Figure 1d). Moreover, a tendency for a gender effect was found ($P < 0.10$), with overall lower titres for gilts (6.19 ± 0.05) than for barrows (6.24 ± 0.05). Myelin basic protein-IgM titres were higher in week 22 (7.20 ± 0.04) compared with week 8 (5.75 ± 0.05) and week 9 (5.75 ± 0.05 , week effect, $P < 0.05$). Even though titres in weeks 8 and 9 were overall not significantly different, gender ($P < 0.10$) tended to affect the Δ in MBP-IgM titres (gilts: 0.10 ± 0.08 ; barrows: -0.10 ± 0.08).

IgG binding myelin basic protein. Myelin basic protein-IgG titres tended to be affected by housing ($P < 0.10$) (Figure 1e), and were affected by gender ($P < 0.05$) and week ($P < 0.001$, Figure 1e). Barren-housed pigs (4.60 ± 0.09) tended to have lower titres than enriched-housed pigs (4.78 ± 0.10). Analysis of the effects for each week separately showed that MBP-IgG titres particularly tended to be affected by housing ($P < 0.10$) in week 9. Gilts (4.60 ± 0.05) had lower titres than barrows (4.75 ± 0.06). *Post hoc* analysis showed those titres in week 22 were higher than those in weeks 8 and 9. The change in MBP-IgG titres from weeks 8 to 9 tended to be influenced by gender ($P < 0.10$; Δ gilts: 0.05 ± 0.08 ; barrows: 0.28 ± 0.10).

Effect of litter and individual characteristics

All autoantibody titres were affected by litter ($P < 0.001$). Litter did not affect the titre changes from weeks 8 to 9 (after the regrouping test), with the exception of changes in

PC-BSA-IgG titres ($P < 0.05$). Backtest classification or IGE class did not affect antibody titres, except that PC-BSA-IgM tended ($P < 0.10$) to be higher for – IGE pigs (6.59 ± 0.08) than for + IGE pigs (6.38 ± 0.08) in week 9.

Discussion

In this study, we investigated the chronic effects of a straw-enriched v. a barren housing environment, and the acute effects of regrouping on levels of NAAb-binding PC-BSA and MBP in pigs differing in gender, coping style and genetic background. Our study shows that these antibodies can be detected in blood of healthy pigs, and that their levels are related to housing conditions, regrouping stress and gender.

Housing effects

In the present study, serum levels of autoantibodies binding MBP were higher in pigs kept in an enriched environment from weaning at 4 weeks onward as compared with barren-housed pigs. As we distributed pigs from the same litters in a balanced manner over the two different post-weaning housing conditions and studied 40 pens per housing condition (80 pens in total), it is highly unlikely that these housing differences found in week 8 (and 9 and 22) are an artefact of coincidental differences that might have already existed in week 4. The housing effect on MBP titres was strongest for the IgM isotype. In man, NAAb (including MBP antibodies) in cerebrospinal fluid and blood have frequently been associated with neurologic diseases (Gold *et al.*, 2012). The sera of healthy humans, however, also contain MBP autoantibodies (Hedegaard *et al.*, 2009). Autoantibodies-binding neural antigens may both have a pathological role in neural tissue and a protective role by regulating immune homeostasis (Gold *et al.*, 2012). The latter may hold for our healthy pigs as antibody titres to MBP were higher in enriched-housed pigs. Other studies have shown an impact of enrichment on other immune characteristics of pigs (Bolhuis *et al.*, 2003; Reimert *et al.*, 2014a), including reduced haptoglobin levels (Reimert *et al.*, 2014b), and provided evidence that pigs reared in an enriched environment were less susceptible to a porcine reproductive and respiratory virus combined with *Actinobacillus pleuropneumoniae* challenge (van Dixhoorn *et al.*, 2016).

We can only speculate about the origin of the effect of enrichment on levels of MBP binding autoantibodies of the IgM isotype. First, the higher antibody levels may be related to the effects of enrichment on brain and behaviour of pigs, including their mental state. In humans, it has been suggested that emotional states, for instance anxiety and depression, could trigger immune alterations (Postal and Appenzeller, 2015), which may, in turn, affect (neural) cell injury, and as a consequence the release of cell constituents such as MBP (Abou-Donia *et al.*, 2013). Second, a difference in hygienic conditions between the enriched and barren environment may have played a role, even though all pigs were housed in the same room. In this study, enrichment was provided in the form of straw bedding which is beneficial for

pigs' behaviour and welfare (Camerlink *et al.*, 2015), but can also be a suitable environment for microbes (Tuytens, 2005) and in this manner affect immune competence. Alternatively, lower MBP binding antibody titres in barren-housed pigs may reflect a continuous usage of the antibodies due to enhanced formation of immune complexes with MBP. Further research is needed to disentangle these potential effects of enrichment on the immune state of pigs, including their autoantibody levels.

Enrichment as compared with barren housing led to lower IgM and higher IgG antibodies binding PC-BSA at some time points when we analyzed levels of antibodies per week, and tended to increase overall titres of IgA binding PC-BSA. The response of PC-BSA to regrouping was generally not affected by housing, except for a tendency of enriched-housed pigs to show a higher increase in IgA-PC-BSA antibodies after regrouping. Apart from the potential influencing factors as discussed above for MBP, a housing difference in aggressive behaviour of these pigs over time, causing body lesions and thus cell damage (Camerlink *et al.*, 2013) may explain the effects of enrichment on PC-BSA autoantibody levels. The exact consequences of these subtle differences in autoantibody levels in our study are unknown, but previous studies have found (even modest differences in) NAb to be linked with survival (Sun *et al.*, 2011) and fitness (Rossi *et al.*, 2013).

In brief, effects of housing on MBP autoantibody (mainly IgM) titres and to a lesser extent on PC antibody titres tentatively suggest that environmental conditions affect levels of autoantibodies binding (neural) antigens as a homeostatic response, which could have consequences for both pig health and welfare.

Regrouping effects

Remarkably, IgM and IgG binding PC-BSA, titres were significantly different between weeks 8 and 9, 3 days after the regrouping test. A similar effect was found for the titres of IgA binding PC-BSA, but in gilts only. An age effect seems unlikely here, given the short time span between both sampling days, suggesting an effect of acute stress. A previous study on the same pigs revealed that temporary regrouping activated the classical complement pathway and increased serum haptoglobin levels, suggesting acute regrouping stress (Reimert *et al.*, 2014a). Moreover, others reported a rise in serum autoantibodies after acute (restraint) stress (Andrejević *et al.*, 1997). Regrouping leads to mutual ranking fights, which can be quite vigorous. It is known to be stressful for pigs and the aggressive behaviour results in body lesions and scratches, in this regrouping test on average >70 per animal (Camerlink *et al.*, 2013). The cell damage associated with stress and/or body lesions induced release of PC could explain why merely NAb binding PC-BSA, but not those binding MBP, was affected by regrouping.

Week effects

All antibody titres measured were affected by week, and were generally highest at 22 weeks of age, except PC-BSA-IgG. The increase in antibody titres with time is in line with

other studies reporting a rise of natural antibody titres with age (Reimert *et al.*, 2014a). In contrast, the level of IgG binding PC-BSA was lower in week 22 compared with weeks 8 and 9. A Spearman correlation test showed that PC-BSA-IgM and PC-BSA-IgG titres were significantly negatively correlated. Kim *et al.* (2002) tested the idea that IgM is responsible for complement activation on apoptotic cells via binding of antibodies targeting lysophospholipids, including PC. Under normal circumstances, lysophospholipids are recognized by IgM which activates the classical complement pathway preventing inflammation. In this study, pigs were all healthy and environmental enrichment or barren conditions may not have led to a shortage of IgM antibodies. When enough IgM is available, the animals can handle the clearance of PC that has been formed as a waste product due to stress. If, however, there is not enough IgM, IgG may be formed which might explain why there was a negative correlation between levels of PC-BSA-IgM and levels of PC-BSA-IgG.

Gender effects

Natural autoantibody can also be affected by gender (Nagele *et al.*, 2013). Except for IgG titres binding PC-BSA, all antibody titres measured in this study were affected or tended to be affected by gender. In general, barrows showed higher antibody titres binding PC-BSA and MBP compared with gilts. Nagele *et al.* (2013) also found higher NAAb levels in human males which would be in line with our study, although it should be noted that the male pigs were castrated. In general, the immune system of males and females differs significantly (Bupp, 2015).

Changes in antibody titres from weeks 8 to 9 after regrouping were also affected by gender, with the most marked effect on IgA titres which clearly increased in gilts, but decreased in barrows. In week 9, IgA titres binding PC-BSA in gilts were higher than in barrows, which may show a difference in how gilts and barrows deal immunologically with stress after regrouping. Whether sex steroids could underlie the immunological differences found between gilts and barrows remains to be studied. However, gilts and barrows, including the animals in this study, have been found to behave very differently in various novelty tests (e.g. Reimert *et al.*, 2014b) and in regrouping tests (Camerlink *et al.*, 2013). Differences in dealing both behaviourally and immunologically with stress may partly explain the different autoantibody responses to regrouping in gilts and barrows.

Coping style and indirect genetic effects

Previous studies have shown that pigs diverging in coping style, that is the way they deal with stress as part of their personality, differ in immune characteristics, including NAb to KLH, and/or modulate the effects of environmental factors on immune responses (Schrama *et al.*, 1997; Bolhuis *et al.*, 2003; Oster *et al.*, 2014; Reimert *et al.*, 2014a). No effects on levels of autoantibodies binding MBP and PC were, however, found in this study. Classification of pigs based on their IGE on the growth of their pen mates, which is known to

influence fear levels (Reimert *et al.*, 2014b), aggression (Camerlink *et al.*, 2013), occurrence of abnormal behaviour (Camerlink *et al.*, 2015) and some (other) immune characteristics (Reimert *et al.*, 2014a) generally did not affect titres of autoantibodies binding MBP and PC either, except that PC-BSA-IgM tended to be higher for – IGE pigs than for + IGE pigs in week 9. The latter could be related to the higher level of aggression found in – IGE pigs when reunited with their own pen mates after the regrouping test (Camerlink *et al.*, 2013).

Litter effects

Litter strongly affected titres of NAAb binding MBP and PC, in spite of the fact that at weaning, litters were split up, with siblings being equally distributed to barren *v.* enriched pens. The effect of litter on these antibodies could partly be related to the shared prenatal and early postnatal environment (until weaning at 4 weeks of age) of the sibling piglets, but could also reflect genetic effects. A genetic basis for NAb has been demonstrated in other species, such as cattle (Wijga *et al.*, 2013) and poultry (Bao *et al.*, 2016). To the best of our knowledge, it is unknown whether NAAb levels and their response to acute challenges are heritable in pigs.

Conclusion

In conclusion, environment, gender, litter and possibly regrouping stress affected blood levels of autoantibodies related with neural antigen (MBP) and cell damage (PC). In this study, enriched-housed healthy pigs were found to have higher levels of IgM and IgG binding MBP, and IgA binding PC-BSA in serum than barren-housed pigs which may reflect or affect their immune competence, (mental) health and welfare. More research is warranted to investigate the mechanisms by which environmental enrichment affects NAAb.

Acknowledgements

The authors would like to thank Mike Nieuwland, Ger de Vries Reilingh, Monique Ooms, Fleur Bartels, Irene Camerlink, Merel Verhoeven, Nanda Ursinus and Bjorge Laurensen for skilful assistance in conducting the experiment and analyses. We are also grateful to the animal caretakers and students involved. Luo is financed by the China Scholarship Council.

References

Abou-Donia MB, Abou-Donia MM, ElMasry EM, Monro JA and Mulder MF 2013. Autoantibodies to nervous system-specific proteins are elevated in sera of flight crew members: biomarkers for nervous system injury. *Journal of Toxicology and Environmental Health, Part A* 76, 363–380.

Andrejević S, Bukilica M, Dimitrijević M, Laban O, Radulovic J, Kovacevic-jovanovic V, Stanojevic S, Vasiljevic T and Marković BM 1997. Stress-induced rise in serum anti-brain autoantibody levels in the rat. *International Journal of Neuroscience* 89, 153–164.

Bao M, Bovenhuis H, Nieuwland M, Parmentier H and van der Poel J 2016. Genetic parameters of IgM and IgG antibodies binding autoantigens in healthy chickens. *Poultry Science* 95, 458–465.

Bolhuis JE, Parmentier HK, Schouten WG, Schrama JW and Wiegant VM 2003. Effects of housing and individual coping characteristics on immune responses of pigs. *Physiology & Behavior* 79, 289–296.

Bolhuis JE, Schouten WG, Schrama JW and Wiegant VM 2005. Behavioural development of pigs with different coping characteristics in barren and substrate-enriched housing conditions. *Applied Animal Behaviour Science* 93, 213–228.

Bupp MRG 2015. Sex, the aging immune system, and chronic disease. *Cellular Immunology* 294, 102–110.

Camerlink I, Bolhuis J, Duijvesteijn N, Van Arendonk J and Bijma P 2014. Growth performance and carcass traits in pigs selected for indirect genetic effects on growth rate in two environments. *Journal of Animal Science* 92, 2612–2619.

Camerlink I, Turner SP, Bijma P and Bolhuis JE 2013. Indirect genetic effects and housing conditions in relation to aggressive behaviour in pigs. *PLoS ONE* 8, e65136.

Camerlink I, Ursinus WW, Bijma P, Kemp B and Bolhuis JE 2015. Indirect genetic effects for growth rate in domestic pigs alter aggressive and manipulative biting behaviour. *Behavior Genetics* 45, 117–126.

Casali P and Notkins AL 1989. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5⁺B lymphocytes. *Annual Review of Immunology* 7, 513–535.

de Jong IC, Prella IT, van de Burgwal JA, Lambouij E, Korte SM, Blokhuis HJ and Koolhaas JM 2000. Effects of environmental enrichment on behavioral responses to novelty, learning, and memory, and the circadian rhythm in cortisol in growing pigs. *Physiology & Behavior* 68, 571–578.

Dhabhar FS 2014. Effects of stress on immune function: the good, the bad, and the beautiful. *Immunologic Research* 58, 193–210.

Douglas C, Bateson M, Walsh C, Bédoué A and Edwards SA 2012. Environmental enrichment induces optimistic cognitive biases in pigs. *Applied Animal Behaviour Science* 139, 65–73.

Gold M, Pul R, Bach JP, Stangel M and Dodel R 2012. Pathogenic and physiological autoantibodies in the central nervous system. *Immunological Reviews* 248, 68–86.

Grimberg-Henrici CG, Vermaak P, Bolhuis JE, Nordquist RE and van der Staay FJ 2016. Effects of environmental enrichment on cognitive performance of pigs in a spatial holeboard discrimination task. *Animal Cognition* 19, 271–283.

Hedegaard CJ, Chen N, Sellebjerg F, Sørensen PS, Leslie RGQ, Bendtzen K and Nielsen CH 2009. Autoantibodies to myelin basic protein (MBP) in healthy individuals and in patients with multiple sclerosis: a role in regulating cytokine responses to MBP. *Immunology* 128, e451–e461.

Kim SJ, Gershov D, Ma X, Brot N and Elkon KB 2002. I-PLA2 activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. *The Journal of Experimental Medicine* 196, 655–665.

Koh KB 1998. Emotion and immunity. *Journal of Psychosomatic Research* 45, 107–115.

Lutz HU, Binder CJ and Kaveri S 2009. Naturally occurring auto-antibodies in homeostasis and disease. *Trends in Immunology* 30, 43–51.

Maier SF and Watkins LR 1998. Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychological Review* 105, 83.

Mayasari N, de Vries Reilingh G, Nieuwland M, Rummelink G, Parmentier H, Kemp B and van Knegsel A 2015. Effect of maternal dry period length on colostrum immunoglobulin content and on natural and specific antibody titers in calves. *Journal of Dairy Science* 98, 3969–3979.

Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC and Nagele RG 2013. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS ONE* 8, e60726.

Oster M, Muráni E, Ponsuksili S, Richard B, Turner SP, Evans G, Thölking L, Kurt E, Klont R and Foury A 2014. Transcriptional responses of PBMC in psychosocially stressed animals indicate an alerting of the immune system in female but not in castrated male pigs. *BMC Genomics* 15, 967.

Panda S and Ding JL 2015. Natural antibodies bridge innate and adaptive immunity. *The Journal of Immunology* 194, 13–20.

Postal M and Appenzeller S 2015. The importance of cytokines and auto-antibodies in depression. *Autoimmunity Reviews* 14, 30–35.

Reimert I, Rodenburg TB, Ursinus WW, Kemp B and Bolhuis JE 2014a. Selection based on indirect genetic effects for growth, environmental enrichment and coping style affect the immune status of pigs. *PLoS ONE* 9, e108700.

Reimert I, Rodenburg TB, Ursinus WW, Kemp B and Bolhuis JE 2014b. Responses to novel situations of female and castrated male pigs with divergent social

- breeding values and different backtest classifications in barren and straw-enriched housing. *Applied Animal Behaviour Science* 151, 24–35.
- Rossi S, Doucelin A, Le Potier M-F, Eraud C and Gilot-Fromont E 2013. Innate immunity correlates with host fitness in wild boar (*Sus scrofa*) exposed to classical swine fever. *PLoS ONE* 8, e79706.
- Scholz J, Allemang-Grand R, Dazai J and Lerch JP 2015. Environmental enrichment is associated with rapid volumetric brain changes in adult mice. *NeuroImage* 109, 190–198.
- Schrama J, Schouten J, Swinkels J, Gentry J, de Vries Reilingh G and Parmentier H 1997. Effect of hemoglobin status on humoral immune response of weanling pigs differing in coping styles. *Journal of Animal Science* 75, 2588–2596.
- Sun Y, Parmentier H, Frankena K and Van der Poel J 2011. Natural antibody isotypes as predictors of survival in laying hens. *Poultry Science* 90, 2263–2274.
- Tuytens FAM 2005. The importance of straw for pig and cattle welfare: a review. *Applied Animal Behaviour Science* 92, 261–282.
- van Dixhoorn ID, Reimert I, Middelkoop J, Bolhuis JE, Wisselink HJ, Koerkamp PWG, Kemp B and Stockhofe-Zurwieden N 2016. Enriched housing reduces disease susceptibility to co-infection with porcine reproductive and respiratory virus (PRRSV) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) in young pigs. *PLoS ONE* 11, e0161832.
- Wijga S, Bovenhuis H, Bastiaansen J, Arendonk J, Ploegaert T, Tijhaar E and Poel J 2013. Genetic parameters for natural antibody isotype titers in milk of Dutch Holstein-Friesians. *Animal Genetics* 44, 485–492.
- Zhou Y, Cheshire A, Howell LA, Ryan DH and Harris RB 1999. Neuroautoantibody immunoreactivity in relation to aging and stress in apolipoprotein E-deficient mice. *Brain Research Bulletin* 49, 173–179.