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# Early and later life environmental enrichment affect specific antibody responses and blood leukocyte subpopulations in pigs

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ABSTRACT

This study addressed the impact of early and later life environmental enrichment, and their combination, on specific antibody responses and peripheral blood leukocyte subpopulations in pigs. Pigs were kept in either barren (B1) or enriched (E1) housing from birth, and half of the pigs switched to barren or enriched housing on day 47, resulting in four treatment combinations: B1B2, B1E2, E1B2, E1E2). Pigs were immunized with keyhole limpet hemocyanin-conjugated trinitrophenyl (KLH-TNP) on day 74 and 109 to induce primary and secondary antibody responses. Blood samples were taken weekly until day 130, and IgM and IgG antibody responses were measured. Leukocyte subpopulations were measured on day 74 and 130. Time course of the antibody responses was not affected by housing. Early life enrichment increased the IgG response to KLH, particularly the primary one. At day 74 the relative frequency of lymphocytes, DC and SLA-II expression on monocytes were higher in E1 pigs, whereas the percentage of granulocytes tended to be lower in E1 pigs at day 74. Early life enrichment increased the SLA-II expression on monocytes, the granulocyte to lymphocyte ratio, and tended to increase the percentage of granulocytes, but tended to decrease the percentage of monocytes at day 130. Later life enrichment reduced percentages of CD4 + CD8α$^+$ T cells before and after immunization and the SLA-II expression on monocytes at day 74, the percentage of granulocytes and the granulocyte to lymphocyte ratio at day 130. Notably, early and later life housing interacted in their effects on several immune parameters. KLH-IgM responses (both primary and secondary) were affected by the interaction between early and later life housing. IgM titers were higher for B1B2 than for E1E2, with the switched animals (B1E2 and E1B2) moving towards the titers of the animals kept in their later life environment from birth onwards. At day 130 the percentage of gamma delta T cells, CD8α$^+$ cytotoxic T cells and DC were not different between pigs kept in B1B2 and E1E2, but there was a clear impact of the switch in housing conditions, particularly for the pigs that changed from barren to enriched housing. We also found effects of coping style (personality) and sex on some immune parameters. In conclusion, both early life and later life enrichment, and, notably a switch in housing conditions influenced specific antibodies and leukocyte subpopulations in pigs. The current study implies that the early life history of animals and the (mis)match with their current environment could thus be of major importance for their immune system. Further research is needed to investigate potential consequences for the pigs' health.

## 1. Introduction

Pigs in commercial farms may experience chronic stress caused by the barren housing environment in which they are usually kept. Limited living space, and the lack of materials to forage prevent pigs from expressing natural behaviours, leading to damaging behaviours like biting the tails and ears of pen mates [1], cognitive impairment [2, 3], and negative emotions [4, 5]. Stress can result in altered innate [6] and adaptive [7] immune functions, and also positive and negative emotions can trigger immune alterations [8, 9]. Environmental enrichment is defined as an increase of the biological relevance of captive environments by appropriate modifications (such as more space and, in the case of pigs, the provision of rooting substrates) resulting in an improvement of the biological functioning of captive animals [10]. Enriched environments, as opposed to barren housing, have been shown to reduce damaging behaviours, induce a more positive emotional (affective) state [4], and influence the levels of natural (auto) antibodies [11-13] and viral clearance in pigs [14]. We recently found

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that enriched housed pigs had higher levels of natural IgM antibodies binding myelin basic protein (MBP), a neural antigen, and tended to have higher levels of natural IgG binding MBP [12]. Besides, the effect of infections with porcine reproductive and respiratory syndrome virus (PRRSV) and *Actinobacillus pleuropneumoniae* on natural (auto)antibodies was influenced by housing in pigs in either barren or enriched conditions from birth [13].

Apart from the current housing environment of pigs, however, early life conditions may also have profound effects on later performance. It has been shown in humans that stressful early life experiences may lead to increased vulnerability to immune dysregulation later in life [see 15 for review, 16] and, in rats, maternal separation in early life was found to increase plasma corticosterone and the systemic immune response after an *in vitro* lipopolysaccharide challenge [17]. There are indications that also in pigs early life experiences affect immune status. Lewis et al. [18] showed that pigs isolated in early life had more $CD4^+$ and $CD4^+CD25^+$ effector T-cell staining in the intestinal mucosa, and a reduced $CD4^+CD25^+Foxp3^+$ regulatory T-cell population at weaning, compared to pigs reared with sows, resulting in a higher T-reg-to-effector ratio in the latter animals. This suggests that the early life environment may profoundly affect local development of regulatory components of the mucosal immune system. Schokker et al. [19, 20] showed that exposure to stress in early life affected gut microbial colonization and intestinal immune development, although it should be noted that effects of antibiotic administration at day 4 of life were more pronounced. Although, to our knowledge, no data are available on the specific impact of early life enrichment on systemic immune competence of pigs in later life, evidence has been presented for long-term effects of being raised in a spacious, stimulus-rich environment on their social skills [21] and sensitivity to reward loss [78]. It can be hypothesized that the differences induced by early life enrichment may exert long-term effects on the pigs' immune system as well. Moreover, the potential impact of early life enrichment on immunity may depend on the housing conditions of pigs in later life, which may or may not match with the way they were reared. This is of relevance given that commercial pigs usually experience at least one move (but often more) to a different pen, room, and sometimes farm, in their lives. Although a handful of studies indicate that the effects of current housing on behaviour and welfare of pigs partly depend on early life conditions (and vice versa) (e.g., [4, 5, 22]), whether such an interaction also holds for immune parameters, is, to the best of our knowledge, unknown.

We therefore aimed to investigate the effects of early and later life enrichment and the combination of those on 1) specific primary and secondary antibody responses and 2) the relative frequencies of peripheral blood leukocyte subpopulations in pigs to assess their immune status. Treatment groups were balanced for sex and coping style, a personality trait, which affected immune parameters in other studies [11, 12, 23–25].

## 2. Material and methods

Established principles of laboratory animal use and care and the Dutch law on animal experiments were followed. The Animal Care and Use Committee of Wageningen University approved the experiment.

Fig. 1 gives an outline of the experimental set-up. Briefly (for details see below), pigs were raised in either barren or enriched housing conditions from birth. At 47 days of age, they experienced either a switch in housing (from barren to enriched or vice versa), or remained in their original housing type (barren or enriched). Thus, four treatment groups were formed in a 2 × 2 arrangement, with early life and later life (post-switch) housing as factors using $n = 64$ pigs from 32 pens in total. At 74 and 119 days of age, all pigs were immunized with the same antigen to mount a primary and secondary antibody response.

### 2.1. Animals and housing

Piglets (*Tempo* × *Topigs 20*) originated from 27 litters, divided over two batches. Pigs were not castrated, tail docked or teeth clipped. From birth till weaning, half of the piglets were housed with sow in 8.6 $m^2$ barren (B) pens with solid floor, and the other half in 17.1 $m^2$ enriched (E) pens with the same B part and an additional enriched part with 1.7 kg of straw, 300 L of sawdust, and 270 L of peat. Daily, 0.8 kg straw and 40 L of sawdust were added, and weekly 30 L peat. B pens contained two toys, one chain with a ball and one with screws. E pens contained one permanent toy (a chain with a ball) and one toy selected from four different toys that rotated daily. All sows were housed in farrowing crates in the B part. In the first week after birth, heating lamps were provided. Each pen had drinking nipples for pigs and sows. Sows were fed a standard diet twice a day, and piglets received fresh feed from day 5. Temperature was 25 °C, and gradually decreased to 21 °C.

At 13 days of age, piglets were subjected to a backtest to assess their coping style, also referred to as personality [26]. Briefly, in this test, piglets are restrained in supine position for 1 min and the number and latency of escape attempts and vocalizations are recorded (see [27] for details). Pigs were either classified as "high resisters" (HR) or "low-resisters" (LR) as described before [11].

### 2.2. Weaning, regrouping and switch

Pigs were weaned at on average 28 days of age, and their housing treatment (B vs. E) was kept the same as before. In each of 32 pens, six unfamiliar pigs were grouped, balanced for sex (1:1) and coping style (3 HR and 3 LR) to minimize between-pen variation, as these two factors may influence immune parameters. Taking into account these criteria, piglets with body weights close to the mean of their litter were selected. Selection of the two pigs per pen to be immunized followed the same criteria. B pigs were moved to 5.6 $m^2$ B pens, with partly solid and partly slatted floor. E pigs were moved to 11.2 $m^2$ E pens with 2.5 kg straw, 400 L of sawdust, and 360 L of peat. Daily, 1.25 kg straw and 60 L sawdust were added, and weekly 45 L peat. Toys were same as before. From 39 days of age, E pigs received extra enrichment (toys or substrates) weekly. The housing conditions before the switch are labelled with a "1" (i.e., B1 or E1), which means the early life housing experience, in the text referred to as "pre-housing".

Pens had one drinking nipple and pigs received solid food *ad libitum*. One heating lamp was provided for two weeks. Pens were cleaned daily and lights and radio were on from 7:00 until 19:00. Temperature was 25 °C at weaning and gradually decreased to 21 °C.

Half of the E and B groups were allocated to switch conditions at 47 days of age, and the other half not, resulting in treatment groups B1B2, E1B2, B1E2, and E1E2. For the switch, each group of pigs was moved to a different pen, including the B1B2 and E1E2 groups, without a change in enrichment conditions. Pigs remained in the same group. Substrates were used and added as before, but only 30 L of sawdust was given daily to E pigs. Conditions after the switch are referred to as "post-housing". Part of the pigs within a pen were exposed to tests of emotional state and all of them were observed to study their behaviour in the home pens. These results are presented elsewhere [28, 29] (Luo et al., submitted).

### 2.3. Immunization and blood sampling

Per pen, two pigs (balanced for sex and coping style within treatment), $n = 16$ per treatment, i.e., in total 64 pigs were primary immunized subcutaneously with 1mg/ml keyhole limpet hemocyanin-conjugated trinitrophenyl (KLH-TNP, LGC Biosearch Technologies, Petaluma, CA, USA) in 1 ml PBS at 74 days of age, and secondary immunized with the same dose of antigen at 109 days of age was done. Blood samples were collected (order balanced for treatment) in heparin

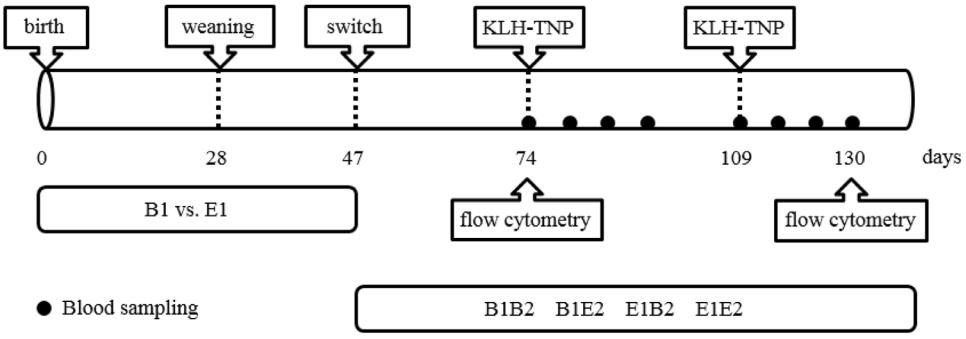


**Fig. 1.** Experimental set-up. Pigs were housed in either barren (B1) or enriched (E1) condition from birth till 47 days of age, when half of pigs experienced a housing switch, creating B1B2, B1E2, E1B2, and E1E2 housing conditions. Two pigs per pen were immunized with KLH-TNP on 74 and 109 days of age, and blood samples were taken to measure antibody and leukocytes.

tubes (Greiner Bio-one, Alphen aan den Rijn, The Netherlands) on 74, 81, 88, 95, and 109 days of age (i.e., 0, 1, 2, 3 and 5 weeks after first immunization), and on 116, 123, and 130 days of age (i.e., 1, 2, and 3 weeks after second immunization). Blood was kept on ice, and centrifuged at 5251 x g for 10 min at 4 °C. Plasma was stored at –20 °C until analysis. In addition, whole blood samples were collected at 74 and 130 days of age (before and after immunization) in the same heparin tubes and kept at RT for flow cytometric analysis of immune cell subsets.

### 2.4. Enzyme-linked immunosorbent assay

To study specific IgM and IgG responses to KLH-TNP, and KLH separately, specific IgM and IgG antibody titers binding KLH-TNP (LGC Biosearch Technologies) and KLH (Sigma-Aldrich, St. Louis, MO, USA) were determined by a two-step indirect enzyme-linked immunosorbent assay (ELISA). Medium-binding microtiter plates (Greiner Bio-One) were coated overnight at 4 °C with 4 μg/ml KLH-TNP or 4 μg/ml KLH in coating buffer (5.3 g/L $Na_2CO_3$ + 4.2 g/L $NaHCO_3$, pH 9.6). Plates were washed with tap water containing 0.05% Tween 20, and tapped to remove the excess of washing buffer. Based on earlier tests plasma was prediluted 1/50 and 1/25 for IgM and IgG binding KLH-TNP and KLH respectively. Samples were 4-step wise serially diluted in PBS in plates and incubated for 1.5 h at RT. After washing, plates were incubated for 1.5 h at RT with a 1:40000 diluted goat-anti-swine-IgM-HRP (GASwIgM/PO, Bethyl Laboratories, Montgomery, TX, USA) to detect binding of IgM, or with a 1:20,000 diluted goat-anti-swine $IgG_{FC}$ HRP (GASwIgG$_{FC}$/PO, Bethy Laboratories) to detect binding of IgG to KLH-TNP and KLH, respectively. After washing, tetramethylbenzidine (TMB) was added as a substrate for 10 min. Reaction was stopped with 1.25M N $H_2SO_4$ and absorbance was measured at 450 nm with a Multiskan Go (Thermo scientific, Breda, the Netherlands). Absorbance was expressed relative to that of a standard positive control sample, and antibody titers 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 sample present on every microtiter plate. All laboratory analyses were performed blind to the treatment (also for flow cytometry).

### 2.5. Flow cytometry

Flow cytometry was performed to estimate the frequencies of leukocyte subpopulations in the blood before and after immunization. Twenty-five microliters of whole blood was transferred to a 96 U bottom Deepwell plate (Thermo Fisher Scientific, Waltham, USA) and stained with several combinations of monoclonal antibodies. The antibody panels are shown in Table 1. T-cell subsets and NK cells were stained with mix 1, a combination of mouse-anti-pig CD4-FITC (clone 74-12-4, IgG2b, Southernbiotech, Birmingham, USA), mouse-anti-pig CD8α-PE (clone 76-2-11; IgG2a, Southernbiotech), mouse-anti-pig CD3ε-PeCy7 (clone BB23-8E6-8C8, IgG2a, BD biosciences, San Jose, USA) and rat-anti-pig γδ -APC (clone MAC320, IgG2a,BD Biosciences). B-cells and antigen presenting cells were stained with mix 2, a combination of mouse-anti-pig CD21-PE (clone BB6-11C9.6, IgG1, Southernbiotech), mouse-anti-pig CD14-FITC (clone MIL2, IgG2b Bio-Rad, Puchheim, Germany), mouse-anti-pig pan-myeloid cell marker SWC3/CD172a-biotin (clone 74-22-15A, IgG1, Southernbiotech) and an unconjugated mouse-anti-pig-SLA Class II DR antibody (clone 2E9/13, IgG2b, Bio-Rad). Cells were stained for 20 min at RT. Next, erythrocytes were lysed using lysis buffer (BD biosciences). After 15 min at RT cells were centrifuged for 5 min at 1300 rpm and the pellet was resuspended in PBS supplemented with 0.5% BSA. Next, a combination of streptavidin-PercP (BD biosciences) and goat-anti-mouse-IgG2b APC (Southernbiotech) was added to the samples stained with mix 2 or to samples without a primary antibody mix as negative control. Cells were stained for 20 min at 4°C, washed in PBS supplemented with 0.5% BSA and flow cytometry was performed using a FACS Canto flow cytometer (BD Biosciences). At least 100,000 lymphocytes were collected. Data were analysed using FlowJo software ((Threestar Inc, San Carlo, USA).

Peripheral blood leukocyte subpopulations were analysed by flow cytometry. A representative example of the gating strategy to identify different subsets of innate and adaptive immune cells is shown in Figs. 2 and 3. Lymphocytes were selected based on scatter (Fig. 2A). T cells ($CD3^+$ cells) were gated and within this population several subsets including gamma delta T cells, $CD4^+$ helper T cells, $CD8\alpha^+$ cytotoxic T cells and $CD4^+CD8\alpha^+$ T cells were analysed. Also NK cells ($CD3^-CD4^-CD\alpha^+$ cells) and B cells ($CD21^+$ cells) were studied. In Fig. 3 the large cells were gated based on forward and side scatter. In Fig. 3, based on SWC3/CD172a expression, two SWC3/$CD172a^{hi}$ subsets and a SWC3/$CD172a^{low}$ subset were defined. CD172a is expressed on dendritic cells (DC) in blood [30]. Granulocytes [31] and monocytes are also known to express CD172a, and CD172a expression on monocytes is higher compared to the expression DC [32]. Based on side scatter, SWC3/$CD172a^{hi}$ cells can be further separated into monocytes and granulocytes [31]. Based on literature and SLA-II expression we defined SWC3/$CD172a^{hi}SSC^{low}$ cells as monocytes in the present study, SWC3/$CD172a^{hi}$ $SSC^{hi}$ cells as granulocytes and SWC3/$CD172a^{low}$ $SSC^{low}$ cells as DC. CD14 staining did not result in a clear population of positive cells in these pigs (data not shown) and was therefore not included in further analysis of innate immune cell subsets. Apart from the relative frequencies of leukocyte subsets identified, granulocyte to lymphocyte ratios were analysed as well.

### 2.6. Statistical analyses

SAS (SAS 9.4, SAS Institute Inc.) was used for all statistical analyses. Normality of error distribution, and homogeneity of variance were examined graphically, and based on this, the percentage of monocytes was log-transformed. Two pigs were euthanized (because of umbilical hernia and lameness).

Antibody titers were analysed using a repeated linear mixed model. Fixed effects of pre-housing (housing before the switch, B1 or E1), post-housing (housing after the switch, B2 or E2), sampling day, their interactions, batch, sex, and coping style (HR vs. LR) were included in the model. Values in time of individual animals were taken as repeated

**Table 1**
Monoclonal antibodies used for flow cytometry.

| Antibody name | Panel | Isotype | Clone | Target |
|---|---|---|---|---|
| Mouse-anti-pig CD4 FITC | Mix 1 | IgG2b | 74-12-4 | CD4+ T helper cells |
| Mouse-anti-pig-CD8α PE | Mix 1 | IgG2a | 76-2-11 | CD8+ cytotoxic T cells |
| Mouse-anti-pig CD3ε PeCy7 | Mix 1 | IgG2a | BB23-8E6-8C8 | T cells |
| Rat-anti-pig γδ APC | Mix 1 | IgG2a | Clone MAC320 | Gamma delta T cells |
| Mouse-anti-pig CD21 PE | Mix 2 | IgG1 | BB6-11C9.6 | B cells |
| Mouse-anti-pig CD14 FITC | Mix 2 | IgG2b | MIL2 | Myeloid cells |
| Mouse-anti-pig SWC3/CD172a BIOTIN | Mix 2 | IgG1 | clone 74-22-15A | Myeloid cells |
| Mouse-anti-pig-SLA Class II DR UNL | Mix 2 | IgG2b | 2E9/13 | SLA-II |
| Streptavidin PercP | | – | – | Biotin |
| goat-anti-mouse-IgG2b APC | | – | – | IgG2a |

measurements. A linear model with pre-housing, post-housing, their interactions, batch, sex and coping style as fixed effects was used to analyse their influence on antibody titres before immunization (at 74 days of age) and on percentages of leukocyte subpopulations at 74 and 130 days of age.

Significant interactions ($p < 0.05$) were further investigated with post hoc pairwise comparisons using the difference of the least square means. If significant sampling day effects were found, pairwise comparisons between days were adjusted using Tukey corrections. Results are presented as means ± SEM.

## 3. Results

### 3.1. Effects of housing conditions on antibody responses

Specific IgM and IgG antibody responses to KLH and KLH-TNP were similar, indicating that the KLH-TNP antibody responses mainly reflected antibodies binding KLH (data not shown). Therefore, only responses to KLH are reported.

#### 3.1.1. KLH-IgM

*3.1.1.1. Before immunization.* KLH-IgM titers before immunization at day 74 were affected by post-housing ($F_{(1,57)} = 6.47$, $p = 0.014$); B2

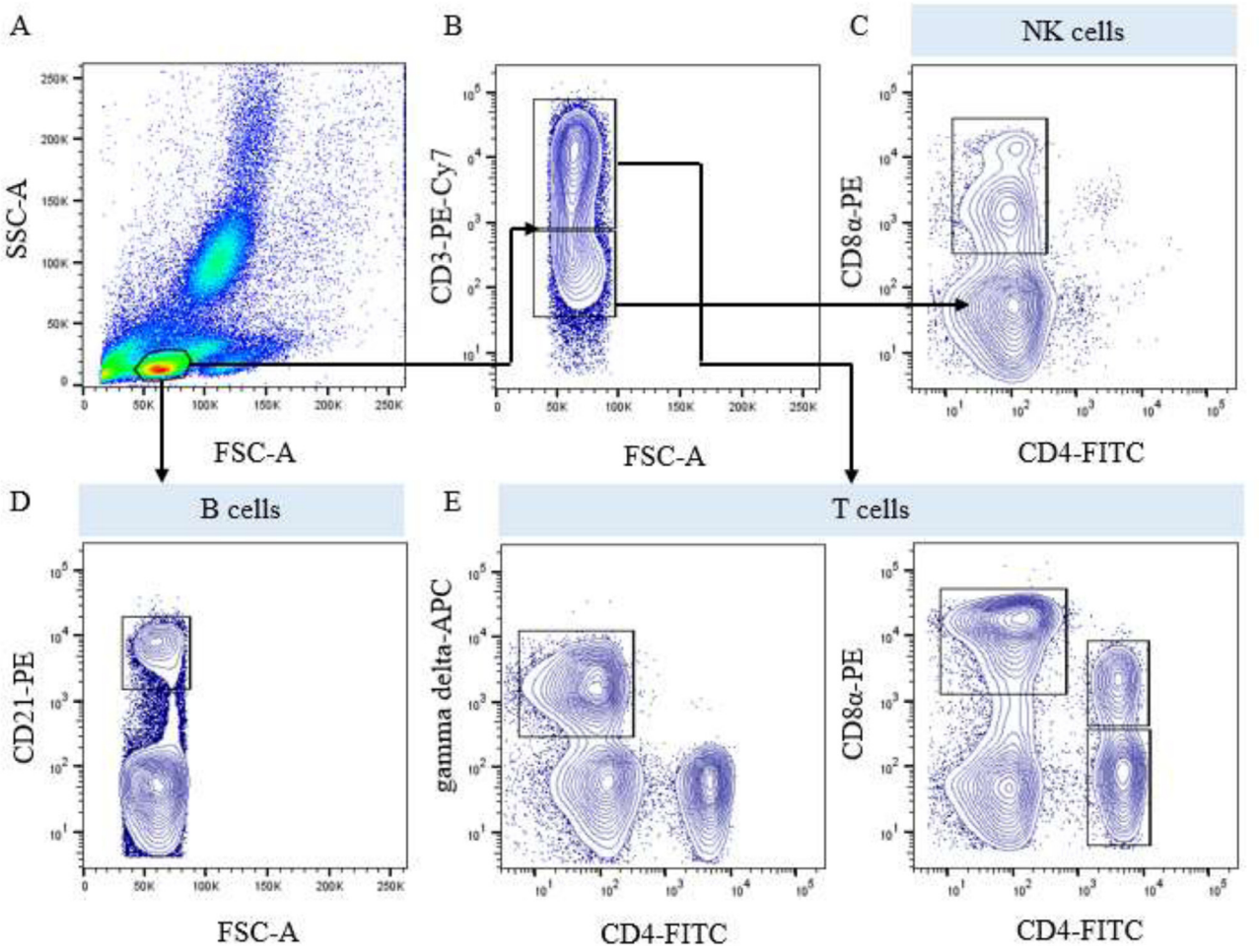


**Fig. 2.** Gating strategy to identify lymphocyte subsets. (A) Lymphocytes were gated based on forward and side scatter. (B) Lymphocytes were divided into $CD3^-$ non T cells and $CD3^+$ T cells. (C) Within the $CD3^-$ non T cell population, NK cells were defined as $CD4^-CD8\alpha^+$ cells. (D) B cells were identified as $CD21^+$ cells within the lymphocyte population. (E) $CD3^+$ T cells were further separated into gamma delta T cells, $CD8\alpha^+$ T cells, $CD4^+$ T cells and $CD4^+CD8\alpha^+$ T cells.

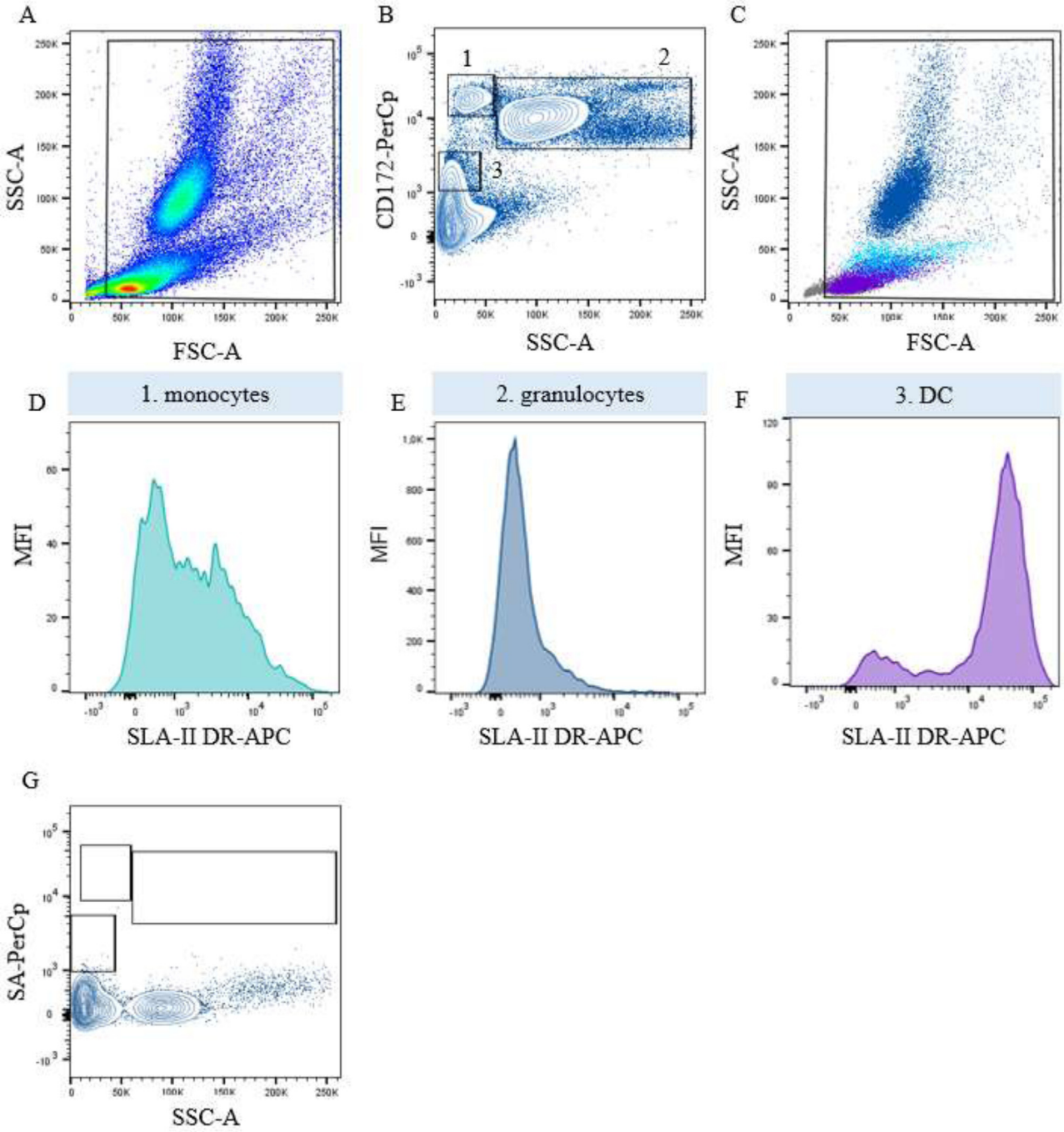


**Fig. 3.** Gating strategy to identify subsets of monocytes, granulocytes and dendritic cells (DC). (A) Large cells were gated based on forward and side scatter. (B) Based on SWC3/CD172a expression and SSC this population was further separated into SWC3/CD172a$^{hi}$ SSC$^{low}$ monocytes (population 1), SWC3/CD172a$^{hi}$ SSC$^{hi}$ granulocytes (population 2) and SWC3/CD172a$^{low}$ SSC$^{low}$ DC (population 3). (C) Backgating of monocytes (light blue), granulocytes (dark blue) and DC (purple). Expression of SLA-II DR on monocytes (D), granulocytes (E) and DC (F) was determined. (G) The conjugate control shows cells stained with streptavidin-PercP only.

pigs (2.46 ± 0.10) had higher titers than E2 pigs (2.09 ± 0.10). No effects of pre-housing ($F_{(1,57)}$ = 0.93, p = 0.338) or the pre-housing × post-housing interaction ($F_{(1,57)}$ = 0.07, $p$ = 0.793) were found.

*3.1.1.2.Overall. antibody response*. Fig. 4A shows changes in specific KLH-IgM responses over time. KLH-IgM titers were affected by sampling day ($F_{(7,413)}$ = 30.57, $p$ < 0.001), showing an increase one week after the first and second immunizations. The change in IgM antibody titers over time did not depend on housing (i.e., no housing and sampling day interaction), which indicates there was no difference in time course (kinetics) of the IgM response to KLH.

Fig. 5A shows the effect of the combination of pre- and post-housing conditions on KLH-IgM titers following primary and secondary immunization. Overall, KLH-IgM titers were affected by post-housing ($F_{(1,57)}$ = 49.46, $p$ <0.001) and tended to be affected by pre-housing ($F_{(1,57)}$ = 3.04, $p$ = 0.086). Importantly, the effect of post-housing depended on its combination with the early life conditions of the pigs (pre-housing × post-housing interaction, $F_{(1,57)}$ = 7.03, p = 0.010). Post hoc analysis showed that B1B2 and E1B2 pigs had the highest titers, followed by B1E2 and then by E1E2 (Fig. 5A, $p$ < 0.05 for all). Thus, the switch from barren to enriched housing lowered antibody

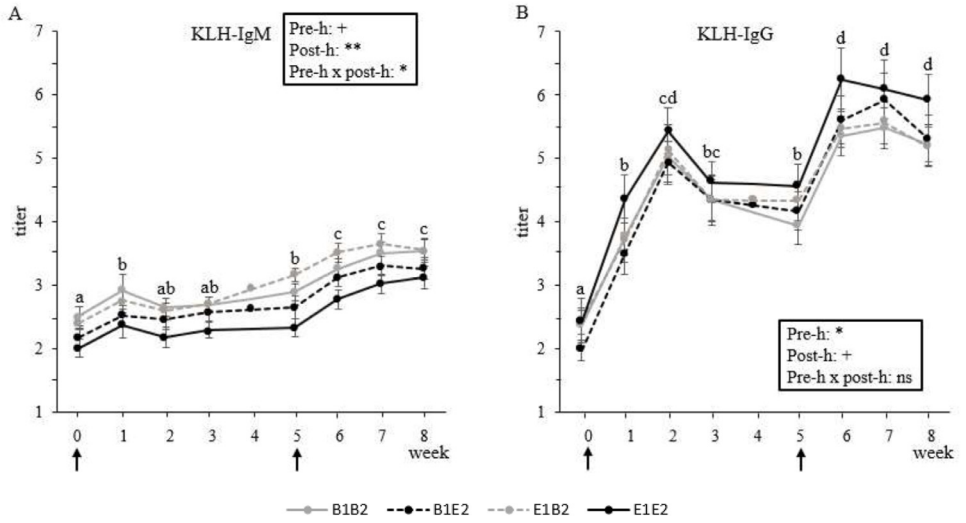


**Fig. 4.** Means and SEM of specific KLH-IgM (A) and KLH-IgG (B) antibody titers over 8 weeks in pigs ($n$ = 62) exposed to different housing situations (B1B2 and E1E2: housed in barren respectively enriched pens throughout the experiment; B1BE2 and E1B2: experiencing a change in environment from barren to enriched respectively vice versa from 47 days of age) in response to primary (at day 74, week = 0) and secondary (at day 109, week = 5) immunization with KLH-TNP antigen. [abc]Sampling day effects are indicated in the figure: days lacking a common letter differ significantly. Housing effects are presented in the text and in Fig. 2. Arrows show the immunization moments. Pre-h and post-h indicate early life housing and later life housing, respectively. Significances of differences are indicated: ** $p < 0.001$, * $p < 0.05$, $0.05 < {}^{+} p < 0.10$, and ns indicates non-significance (exact $p$-values can be found in the text).

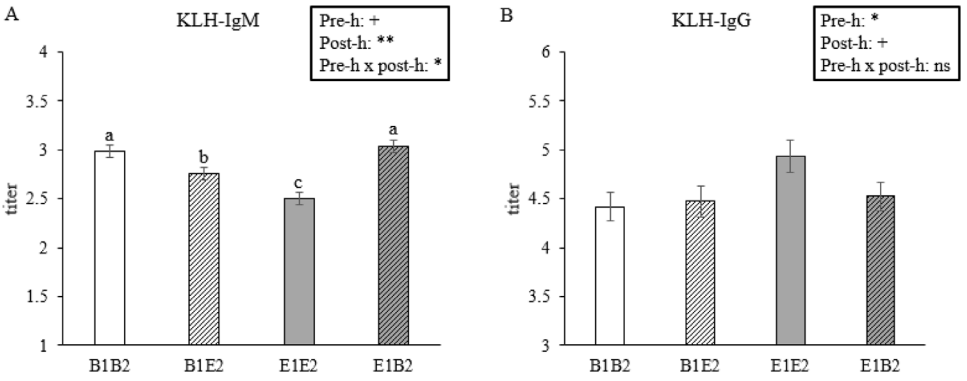


**Fig. 5.** Means and SEM of specific KLH-IgM (A) and KLH-IgG (B) titers over 8 weeks in pigs ($n$ = 62) exposed to different housing situations (B1B2 and E1E2: housed in barren respectively enriched pens throughout the experiment; B1BE2 and E1B2: experiencing a change in environment from barren to enriched respectively vice versa from 47 days of age) in response to primary and secondary immunization with KLH-TNP antigen. [abc]Groups lacking a common letter significantly differ. Pre-h and post-h indicate early life housing and later life housing, respectively. Significances of differences are indicated: ** $p < 0.001$, * $p < 0.05$, $0.05 < {}^{+} p < 0.10$, and ns indicates non-significance (exact $p$-values can be found in the text).

titers in B1 pigs (B1E2 < B1B2), whereas the switch from enriched to barren housing increased titers in E1 pigs (E1B2 > E1E2). In B2 pigs, there was no difference in antibody titers between pigs originating from early life enriched or barren pens (E1B2 = B1B2), whereas in E2 pigs, pigs coming from barren pens had higher titres than those from enriched early-life pens (B1E2 > E1E2, Fig. 5A).

*3.1.1.3.Primary. antibody response.* KLH-IgM titers in the 5-week period after first immunization were affected by post-housing (B2: 2.72 ± 0.05, E2: 2.35 ± 0.05, $F_{(1,57)} = 31.20$, $p < 0.001$), and tended to be affected by the pre-housing ($F_{(1,57)} = 3.30$, $p = 0.074$) and the pre-housing × post-housing interaction ($F_{(1,57)} = 2.90$, $p = 0.094$), similar as described for the overall (primary plus secondary) response.

*3.1.1.4.Secondary. immunization*. Also after the second immunization KLH-IgM titers in the 3-week period were affected by post-housing ($F_{(1,56)} = 32.39$, $p < 0.001$), and the interaction between pre-housing and post-housing, $F_{(1,56)} = 8.60$, $p = 0.005$). Post hoc analysis showed that E1E2 pigs (2.81 ± 0.08) had lower titers than E1B2 pigs (3.46 ± 0.08, $p < 0.05$), with levels of B1B2 (3.29 ± 0.09) and B1E2 (3.08 ± 0.08) in between.

*3.1.2. KLH-IgG*

*3.1.2.1. Before immunization*. Housing did not affect KLH-IgG titers before immunization (pre-housing: $F_{(1,57)} = 1.54$, $p = 0.220$, post-housing: $F_{(1,57)} = 0.97$, $p$ 0.329, pre-housing × post-housing: $F_{(1,57)} = 0.82$, $p$ 0.370).

*3.1.2.2. Overall antibody response*. KLH-IgG titers were affected by sampling day ($F_{(7,413)} = 48.47$, $p < 0.001$), showing an increase both after the first and second immunization (Fig. 4B). The primary antibody response showed a peak after two weeks, after which titers declined. The secondary immune response peaked after one week, and high titers were retained during the two weeks thereafter (Fig. 4B). The change in IgG antibody titers over time did not depend on housing (i.e., no housing × sampling day interactions).

The effect of the combination of pre- and post-housing conditions on overall KLH titers is shown in Fig. 5B. KLH-IgG titers were affected by pre-housing ($F_{(1,57)} = 5.09$, $p$ 0.028), and tended to be affected by post-housing ($F_{(1,57)} = 3.24$, $p$ 0.077) in the same direction, which showed that B pigs had or tended to have lower titers than E pigs (B1: 4.44 ± 0.11 vs. E1: 4.73 ± 0.11, B2: 4.47 ± 0.10 vs. E2: 4.70 ± 0.12).

*3.1.2.3. Primary immunization*. In the 5-week period after first immunization KLH-IgG titers tended to be affected by pre-housing ($F_{(1,57)} = 3.85$, $p$ 0.055), with higher levels for pre-housing E1 pigs (4.13 ± 0.12) than for B1 pigs (3.84 ± 0.12).

*3.1.2.4. Secondary immunization*. KLH-IgG titers in the 3-week period after second immunization were affected by post-housing ($F_{(1,56)} = 5.05$, $p$ 0.029), as B2 pigs (5.06 ± 0.12) had lower titers than E2 pigs (5.47 ± 0.15).

### 3.2. The effect of housing conditions on peripheral blood leukocyte subpopulations

Pre- and/or post-housing did not affect relative frequencies of $CD3^+$ T cells, $CD4^+$ helper T cells, NK cells, and B cells both before and after immunization (for all means and p-values, see Supplementary Tables 1 and 2 for day 74 and 130, respectively).

#### 3.2.1. Before immunization (day 74)

At day 74, the percentage of lymphocytes ($F_{(1,55)} = 8.16$, $p$ 0.006), granulocytes ($F_{(1,54)} = 3.70$, $p$ 0.060), DC ($F_{(1,54)} = 4.30$, $p$ 0.043), and the SLA-II expression on monocytes ($F_{(1,54)} = 6.05$, $p$ 0.003) tended to be affected or were affected by pre-housing. E1 pigs had a higher percentage of lymphocytes (B1: 41.2 ± 1.6, E1: 47.2 ± 1.5 %) and DC (B1: 4.7 ± 0.5, E1: 6.1 ± 0.7 %) compared to B1 pigs, and showed a higher SLA-II expression on monocytes (B1: 524.1 ± 14.8, E1: 611.7 ± 23.9 MFI), while the percentage of granulocytes tended to be lower for E1 (B1: 34.8 ± 1.7, E1: 30.6 ± 1.5 %). SLA-II expression on monocytes was also affected by post-housing ($F_{(1,54)} = 5.75$, $p = 0.020$), and the expression was higher for B2 pigs than E2 pigs (B2: 1704.2 ± 110.4, E2: 1415.9 ± 49.7). Pre-housing and post-housing tended to interact in their effect on the percentage of monocytes ($F_{(1,54)} = 3.34$, $p$ 0.073, Fig. 6D1) and SLA-II expression on DC ($F_{(1,54)} = 2.83$, $p$ 0.098, Fig. 6G1). When analysing possible effects of housing conditions on adaptive immune cells, only an effect of post-housing on the percentage of $CD4^+CD8\alpha^+$ T cells was observed, with higher percentages for B2 pigs (12.2 ± 0.9 %) than for E2 pigs (9.4 ± 0.5 %, $F_{(1,55)} = 9.49$, $p$ 0.003). No other effects of pre- or post-housing were found on the cells mentioned above, and neither an effect on the granulocyte to lymphocyte ratio was found at day 74.

#### 3.2.2. After immunization (day 130)

At day 130, the percentage of granulocytes ($F_{(1,50)} = 3.23$, $p$ 0.079), monocytes ($F_{(1,50)} = 3.67$, $p$ 0.061), and the SLA-II expression on monocytes ($F_{(1,50)} = 4.49$, $p$ 0.039) tended to be affected or was affected by pre-housing. E1 pigs tended to have a higher percentage of granulocytes (B1: 34.7 ± 1.6, E1: 38.3 ± 1.7 %) and had higher SLA-II expression on monocytes (B1: 1691.0 ± 102.9, E1: 1942.4 ± 88.4 MFI), but tended to have lower percentage of monocytes (B1: 7.0 ± 1.1, E1: 5.4 ± 0.4 %) than B1 pigs. The percentage of lymphocytes ($F_{(1,55)} = 5.19$, $p$ 0.027), granulocytes ($F_{(1,50)} = 13.49$, $p < 0.001$), and $CD4^+CD8\alpha^+$ T cells ($F_{(1,55)} = 4.66$, $p$ 0.035) were affected by post-housing. E2 pigs had a higher percentage of lymphocytes (B2: 47.5 ± 1.7, E2: 50.8 ± 1.7 %), but a lower percentage of

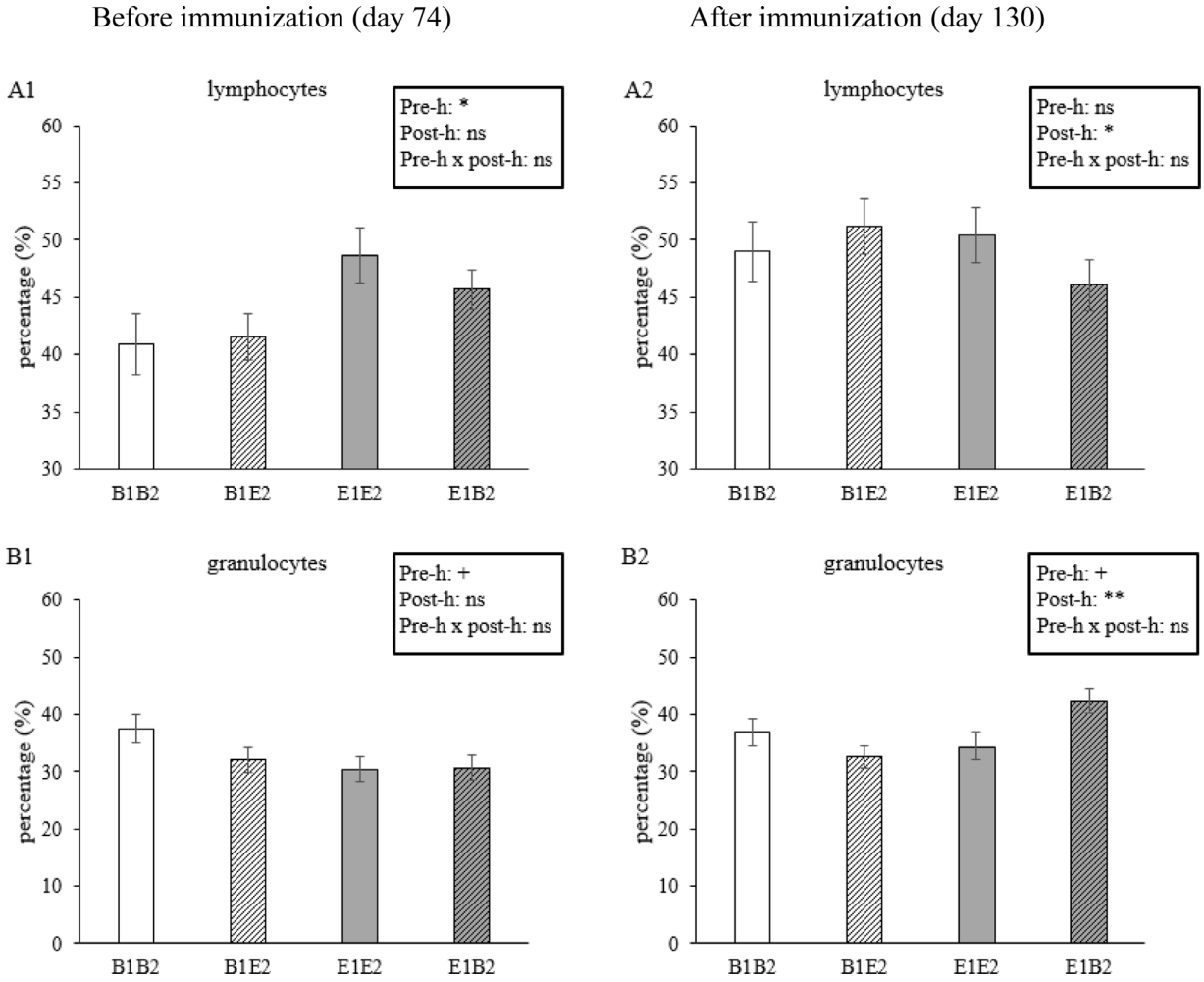


**Fig. 6.** Means and SEM of the percentage of lymphocytes, granulocytes, monocytes, dendritic cells (DC), the granulocyte/lymphocyte (G:L) ratio, the SLA-II expression on monocytes, DC, the percentage of gamma delta T cells, $CD4^+CD8\alpha^+$ T cells, and $CD8\alpha^+$ cytotoxic T cells before immunization, on day 74 (on the left side), and after immunization, on day 130 (on the right side), in pigs ($n = 62$) exposed to four different housing conditions (B1B2 and E1E2: housed in barren respectively enriched pens throughout the experiment; B1BE2 and E1B2: experiencing a change in environment from barren to enriched respectively vice versa from 47 days of age) through primary and secondary immunization with KLH-TNP antigen on day 74 and 109. Significant differences between four housing groups are indicated by letter a and b ($p < 0.05$). Pre-h and post-h indicate early life and later life housing, respectively. Significances of differences are indicated: $^{**}p < 0.001$, $^{*}p < 0.05$, $0.05 < {}^{+}p < 0.10$, and ns indicates non-significance.

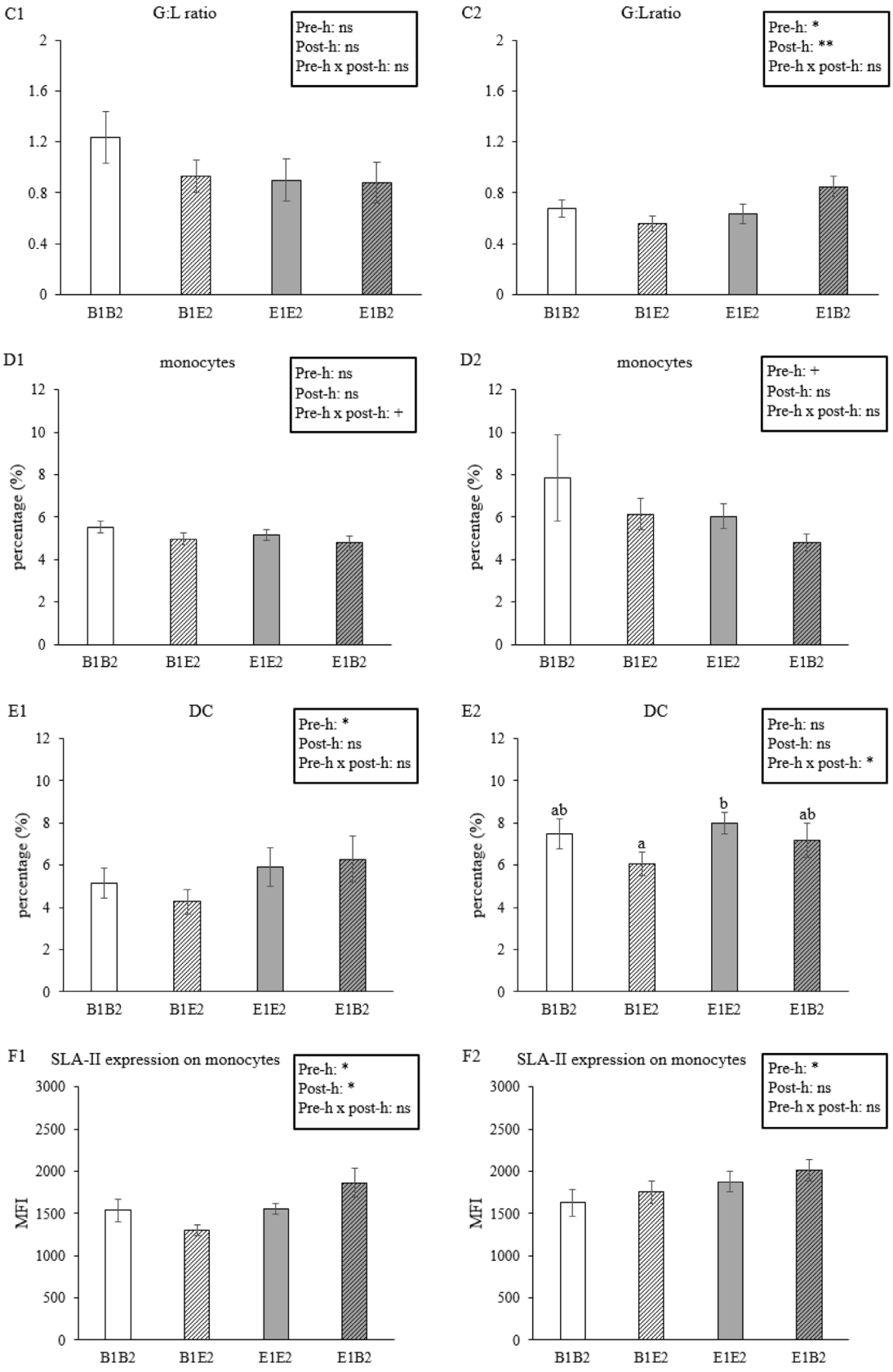


**Fig. 6.** (*continued*)

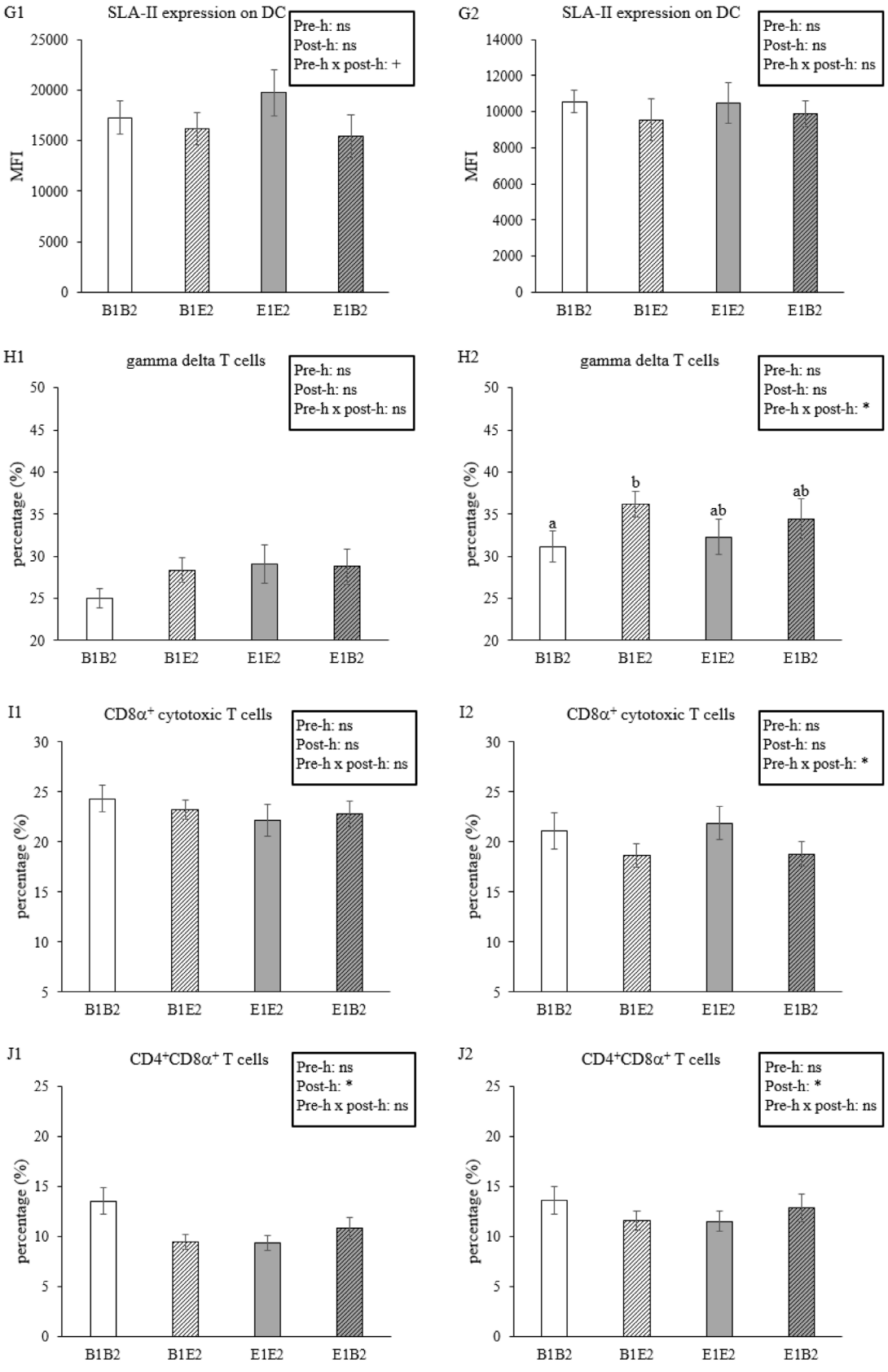


**Fig. 6.** (*continued*)

granulocytes (B2: 39.6 ± 1.6, E2: 33.6 ± 1.6 %) and $CD4^+CD8\alpha^+$ T cells (B2: 13.3 ± 1.0, E2: 11.57 ± 0.7 %) than B2 pigs. The ratio of granulocytes to lymphocytes was affected by both pre- ($F_{(1,50)} = 4.57$, $p$ 0.038) and post-housing ($F_{(1,50)} = 14.16$, $p < 0.001$), but in different directions (B1: 0.62 ± 0.04, E1: 0.74 ± 0.06, B2: 0.76 ± 0.05, E2: 0.60 ± 0.05). Pre- and post-housing interacted in their effect on the percentage of DC ($F_{(1,50)} = 4.64$, $p$ 0.036), gamma-delta T cells ($F_{(}1,55_{)} = 5.60$, $p$ 0.022), and $CD8\alpha^+$ cytotoxic T cells ($F_{(}1,55_{)} = 4.93$, $p$ 0.031). Post hoc analysis showed that the switch from barren to enriched housing reduced the percentage of DC as compared with enriched housing throughout life (B1E2 < E1E2, $p < 0.05$, Fig. 6E2), whereas B1B2 pigs and E1B2 pigs had levels in between. Conversely, B1E2 pigs had a higher relative frequency of gamma delta T cells than B1B2 pigs ($p < 0.05$, Fig. 6H2), with E1E2 and E1B2 in between. The percentage $CD8\alpha^+$ cytotoxic T cells was also affected by the pre-housing × post-housing interaction, but no significant pairwise differences were found, even though both switched groups (E1B2 and B1E2) seemed to have reduced levels (Fig. 6I2). No other effects of pre- or post-housing were found on the cells mentioned above, and neither an effect on the SLA-II expression on DC was found at day 130.

### 3.3. Coping style effects

Coping style did not affect IgM titers before immunization ($F_{(1,57)} = 0.93$, $p$ 0.543), but after immunization, HR pigs had overall lower titers of KLH-IgM (2.73 ± 0.05) than LR pigs (2.91 ± 0.05, $F_{(1,57)} = 11.15$, $p$ 0.002), which held for the primary immune response (HR: 2.43 ± 0.05, LR: 2.65 ± 0.05, $F_{(1,57)} = 11.06$, $p$ 0.002) and tended to hold for the secondary immune response (HR: 3.10 ± 0.06; LR: 3.23 ± 0.06, $F_{(1,56)} = 2.99$, $p$ 0.090). Coping style did not affect IgG responses. Before immunization, coping style affected or tended to affect the percentage of monocytes (HR: 5.4 ± 0.2, LR: 4.8 ± 0.2, $F_{(1,54)} = 5.30$, $p$ 0.025) and the SLA-II expression on monocytes (HR: 1674.4 ± 111.0, LR: 1454.1 ± 59.6, $F_{(1,54)} = 3.60$, $p$ 0.063). On day 130, coping style tended to affect NK cell percentages (HR: 7.8 ± 0.6, LR: 9.5 ± 0.8 %, $F_{(1,55)} = 3.54$, $p$ 0.065). Coping style also affected and tended to affect the percentage of $CD8\alpha^+$ cytotoxic T cells before (HR: 21.7 ± 0.7, LR: 24.6 ± 1.0 %, $F_{(1,55)} = 5.25$, $p$ 0.026), and after immunization (HR: 18.7 ± 0.89, LR: 21.4 ± 1.1 %, $F_{(}1,55_{)} = 3.83$, $p$ 0.055).

### 3.4. Sex effects

Sex did not affect IgM responses, but sex effects were found on KLH-IgG and some leukocyte subsets. Sex did not affect IgG titers before immunization ($F_{(1,57)} = 0.64$, $p$ 0.432), while following immunization, females (4.39 ± 0.11) had overall lower KLH-IgG titers than males (4.76 ± 0.10) ($F_{(1,57)} = 8.39$, $p$ 0.005), which was found for both the primary (F: 3.82 ± 0.14, M: 4.14 ± 0.12, $F_{(1,57)} = 4.16$, $p$ 0.046) and secondary immune response (F: 5.05 ± 0.14, M: 5.46 ± 0.13, $F_{(1,56)} = 6.42$, $p$ 0.014). Sex also affected the percentage of $CD4^+CD8\alpha^+$ T cells (F: 12.1 ± 1.0, M: 9.6 ± 0.5 %, $F_{(1,55)} = 6.59$, $p$ 0.013), and tended to affect NK cells (F: 20.8 ± 2.0, M: 16.8 ± 1.5 %, $F_{(1,55)} = 2.83$, $p$ 0.098) and the SLA-II expression on DC (F: 15462.0 ± 1438.9, M: 18514.4 ± 1217.3 MFI, $F_{(1,54)} = 2.92$, $p$ 0.094) on day 74. No sex effects were found on day 130.

## 4. Discussion

In this study, we investigated the effects of early and later life environmental enrichment in pigs on specific IgM and IgG antibody responses to the T-cell-dependent antigen KLH-TNP as a parameter of specific immunity and memory. In addition, the effects of early life and later life housing experience on the frequencies of cells in the blood related to innate and adaptive immunity were measured. We chose to use the model antigen KLH-TNP to induce mild primary and secondary immune responses and refrained from using adjuvant as this may have overruled potential housing effects. The IgG and low IgM responses likely reflected T-cell-dependent antibody responses [33]. We found long-lasting effects of both housing before the switch of environment (early life environment) and later life housing on several immune parameters, and, moreover, the effects of early and later life conditions often interacted, thus a clear effect of the switch was found. This study reveals that housing conditions and early life housing history can affect specific antibody responses and the frequencies of immune cells, reflecting immune status, in the blood, which suggests differences in immune competence. Both coping style and sex affected some of the immune parameters and will be discussed separately.

### 4.1. Early life housing

We found that early life conditions (from birth until 47 days of age) exerted an effect on humoral immune responses (KLH-IgG) and immune cells (mostly on innate cells) in later life, for some up to 130 days. For distribution of several leukocyte subsets and KLH-IgM titers, however, the impact of early life housing depended on its combination with current housing, as shown by interaction effects (see below for a discussion on those). Pigs from barren housing conditions in early life showed an overall lower IgG response to KLH. Separate analysis of the primary and secondary immune responses revealed that early life housing mainly affected the former, whereas the early life effect seemed to be overruled by the current housing effect on the secondary IgG response to KLH. The (early life) housing effects on IgG titers are in contrast with other studies in which no effects of enrichment throughout life, either with straw and extra space [34] or with sawdust and extra outdoor space [35] on IgG responses to KLH were found [34]. The discrepancy between our and these other studies might be caused by a difference in age at (primary) immunization, in timing of sampling as related to immunization and/or in substrates used as enrichment (in our study, apart from straw bedding or sawdust also peat and extra toys).

Early life enrichment, as compared with barren housing before the switch, not only increased the IgG response to KLH. It also increased percentages of lymphocytes (day 74), DC (day 74), granulocytes (day 130), the SLA-II expression on monocytes (day 74 and 130), while it tended to decrease the percentage of granulocytes at day 74 and the percentage of monocytes at day 130, revealing a (mild) effect of early life enrichment on the subsets of immune cells in peripheral blood.

It is known that environmental enrichment can positively influence behaviour and welfare of pigs, but how early life environmental enrichment exactly influences the immune system is not clear yet. Different effects of enrichment may have played a role here. Firstly, the early oral sampling of rooting substrates and thus enlarged antigenic exposure facilitated by environmental enrichment may have had an impact on intestinal maturation and early microbial colonization, and, consequently, on regulation of innate and adaptive immunity [19, 36]. It has been shown that environmental factors during the early postnatal period that affect microbial exposure, such as natural vs. isolator-rearing [36], outdoor vs. indoor rearing [36], diet [37, 38] or neonatal antibiotic treatment [19], can influence the establishment of gut microbiota in pigs. The gut microbiota play a role in the communication between the gut and brain as well, which is established via a range of chemical, hormonal, immunological and neural signals (reviewed in [39]). Evidence is accumulating that this 'gut-brain axis' can influence stress responses, behaviour and immunity in animals (reviewed in [39, 40]).

Secondly, environmental enrichment can influence the stress level and mental state of pigs as it partly satisfies their behavioural need to explore [4, 41]. It could therefore influence their immune status and responses [9, 42], as the brain and immune system form a bidirectional communication network, amongst others via the hypothalamic-

pituitary-adrenal (HPA) axis (review in [43]). Several studies point to a difference in HPA axis functioning between pigs from barren and enriched housing [1, 41, 44], some of which seem to be established in early life. For instance, absence of enrichment materials up till 4 weeks of age, led to a blunted circadian cortisol rhythm, possibly reflecting chronic stress, in later life, at 21 weeks of age [45]. In addition, we found that pigs housed in the barren environment in early life were more sensitive to reward loss [29], supporting a long-term effect on their adaptive capacity and mental state. It has been found that psychosocial factors can affect functioning of the immune system [43, 46]. For instance, in humans, adverse experiences in early life are associated with long-term effects on both the innate and adaptive immune system, even though it does not appear to affect all elements of the immune system to the same extent [review in 16]. In pigs, 'cognitive enrichment', allowing pigs to successfully cope with a demanding task rewarded by small portions of their food [47], has been reported to result in an increased IgG concentration and in vitro T-cell proliferation to ConA, reduced LPS-induced proliferation of B cells and accelerated wound healing [48]. Given that the cognitive enrichment used did not alter antigenic exposure, this suggests that, also in pigs, one way in which enrichment influences their immune system could be via its effect on their mental state.

### 4.2. Actual housing conditions and their interaction with early life enrichment

Our study revealed an impact of the actual housing conditions on both innate and specific immunity of the pigs as well. KLH-IgM titers before immunization were affected by actual housing, with higher titers for B2 pigs than for E2 pigs. These anti-KLH antibodies circulating in absence of antigenic stimulation are also referred to as natural antibodies [49]. Natural antibodies are important as first line of defence against microbial infection [50], and participate in physiological activities [51]. Also in other studies barren and enriched housed pigs were found to differ in natural (auto)antibody levels binding several antigens including KLH [11–13], although differences were not always in the same direction as in our study. Our results on particularly IgM binding KLH are in contrast with other studies, which showed higher levels of natural KLH-IgM antibodies in enriched housed pigs [11], or no housing effect on KLH-IgM antibodies [13]. The reasons could be a difference in the age at which the enrichment started (before or after weaning), and/or in the exact type of enrichment applied. Other studies used enrichment with straw only, whereas we also provided peat and sawdust to the pigs, as well as extra space. Also the barren conditions applied vary between studies, as we still provided two simple toys that are similar to those used on many commercial pig farms.

Actual housing conditions also affected the frequencies of T cells before and after immunization, as B2 pigs had a higher percentage of $CD4^+CD8\alpha^+$ T cells than E2 pigs. In another study, however, enriched and barren housed pigs did not differ in the percentage of $CD4^+CD8\alpha^+$ T cells [34], possibly, as indicated above, due to differences in experimental design, sample size (larger in our study) and type of enrichment. On day 130, E2 pigs had a higher percentage of lymphocytes than B2 pigs, which is in line with the early life enrichment effect on lymphocyte percentage at day 74. We speculate that after immunization, similar to the KLH-IgG response, the effect of current housing on percentage of lymphocytes overruled the effect of early life at day 130, as there was no early life effect anymore. Actual housing also affected SLA-II expression on monocytes on day 74, with higher levels for barren-housed pigs.

Pigs in barren actual housing also had higher percentages of granulocytes than those in enriched pens on day 130. The granulocyte/lymphocyte ratio on this was affected by both early life and current housing conditions, but in different directions. Adding up the early life and current housing effects revealed the highest ratio in pigs that switched from enriched to barren conditions, and, conversely, the lowest ratio in pigs exposed to the opposite switch. The granulocyte to lymphocyte ratio is a stress marker, and increases for instance following surgical stress in humans [52], restraint stress in rodents [53] and transport stress in pigs [54]. Merlot et al. [35] reported higher granulocyte to lymphocyte ratios in Large White pigs kept in conventional, rather barren, pens, as compared to those provided with sawdust bedding and an outdoor area, suggesting also a long-term stress effect on this marker. The combined impacts of early life and current housing on the granulocyte to lymphocyte ratio in the current study might thus point to increased stress levels in pigs that switched from enriched to barren housing.

Notably, the current study revealed that early life and later life housing often interacted in their effects on both specific and innate immunity variables. Thus, the effects of actual housing often depended on the early life history of the pigs, demonstrating an impact of the switch in housing conditions. Both the switch from barren to enriched and from enriched to barren housing changed KLH-IgM titers. In non-switched animals, IgM titers were higher for B1B2 than for E1E2, and switched animals moved towards the titers of the non-switched animals kept in their later life environment from birth onwards. Still, however, pigs in enriched conditions throughout life (E1E2) differed from enriched-housed pigs originating from early life barren housing (B1E2) in KLH-IgM titers, illustrating that the later life conditions did not completely overrule the effects of housing before the switch. The switch in housing also affected the percentage of DC, $CD8\alpha^+$ cytotoxic T cells (but no pairwise difference here) and gamma delta T cells at day 130. The switch effect on the percentage of DC and gamma delta T cells, however, was only found in the pigs that changed from barren to enriched housing conditions, but not in the pigs that switched from enriched to barren housing. Notably, pigs kept in stable enriched vs. barren conditions from birth onwards did not differ in these leukocyte subpopulations mentioned before, but it was the switch that resulted in a change. Previous studies have shown that pigs that switched from enriched to barren housing conditions show as much behavioural signs of stress [55] and pessimistic mood [4] as barren non-switched pigs or even more [22] (Luo et al., submitted), suggesting that loss of enrichment may be more detrimental than barren housing throughout life [56]. Less is known about the impact of switching from barren to enriched housing, although some studies demonstrated that pigs originating from barren pens showed more enrichment-directed behaviour than pigs reared in enriched pens [57, 58] and a lower occurrence of gastric lesions [55]. Studies in other species found that switching from group housing to individual housing or vice versa had an impact on some indicators of immune function (NK cells) [59].

To our knowledge, this is the first study to show that both early and later life enrichment, and, notably a switch in housing conditions can influence specific antibodies (as a parameter of immune responsiveness) and leukocyte subpopulations in pigs. The underlying mechanism is not known yet. Both the difference in exposure to antigens in the current housing conditions related to the absence or presence of rooting material [36, 60], and the changes in behaviour [5, 47], stress level [41, 55, 61] and mental state [4, 28, 62] may have influenced immune response, memory and status (reviewed in [63, 64, 65]). Based on the latter, i.e., a potential influence of stress and mental state, one might expect the switch from barren to enriched housing to give opposite results on immune function compared with the switch from an enriched to a barren environment, which did not hold for all immune parameters in this study, so other influences of enrichment likely played a role as well. Either switch induced alterations in specific IgM antibody responses and the switch from barren to enriched changed levels of some of immune cells, suggesting that the immune system not only responds to early life and actual housing conditions, but also to a change in environment. Notably, this was not due to relocation to a new pen per se as also the pigs remaining in barren or enriched pens throughout life were relocated to new pens at the time of the switch. This impact of early life conditions and of a change in housing conditions means that

these need to be taken into account when assessing immune competence in pigs. Further studies are needed to investigate how changes in housing affect innate and adaptive immunity, and other components of the immune system that were not included in the present study, and to study their potential impact on disease resistance, vaccine responses and immune competence. A recent study provided evidence that enriched housed pigs more effectively battle an infection with porcine reproductive and respiratory syndrome virus (PRRSV) and *Actinobacillus pleuropneumoniae*, as demonstrated by a faster viral clearance and less lung damage, but did not address the impact of early life conditions [14].

### 4.3. Coping style

Although individual characteristics of pigs were not the main topic of our study, we balanced treatments for coping style, by splitting our population of pigs into low-resisters (LR) and high-resisters (HR) based on their backtest response, without selecting extremes, simply to account for potential effects of this personality trait on the immune parameters measured. The response of pigs in the backtest has been shown to relate to a range of behavioural and (neuro)physiological variables (e.g., [25, 26, 66]) and may therefore indicate a pig's coping style. LR pigs had higher specific KLH-IgM antibody titers than HR pigs in this study, which is alike the previous studies demonstrating a higher specific humoral immune response in LR pigs [67, 68], although others found no difference in adult pigs [69] or found the effect of coping style to be housing-dependent [23]. Previously, also other immune differences were reported, suggesting LR pigs to have a lower cellular immunity [23, 67], a lower complement activity via the alternative pathway [11] and a different gene expression signature for processes acting on cell communication, vasculogenesis and blood coagulation in blood mononuclear cells [70]. It should be noted that in most of these studies the LR and HR selected were more at the extremes of the population. In this study, coping style did not affect leukocyte distribution to a large extent, as only subtle differences were found. HR pigs had a lower percentage of $CD8\alpha^+$ cytotoxic T cells than LR pigs, and higher percentage of monocytes and the SLA-II expression on monocytes before immunization. This further confirms immune differences between the two types of pig, which may be related to their differences in several (neuro)physiological systems [26, 71, 72] that communicate with the immune system [73].

### 4.4. Sex effects

Differences between females and males were found for a number of immune parameters, in line with other studies reporting some influence of sex on immunity [11, 12, 74]. This could be due to the fundamental hormonal and genetic differences between females and males, and also relate to how females and males deal with stress [44, 75–77].

## 5. Conclusions

In conclusion, both early life and later life enrichment, and, notably a switch in housing conditions influenced specific antibodies and leukocyte subpopulations in pigs. The current study implies that the early life history of animals and the (mis)match with their current environment could thus be of major importance for functioning of their immune system, which may have consequences for their responses to vaccination and disease resistance. Further research is needed to investigate potential consequences for the pigs' health.

## Declaration of Competing Interest

The authors declare no competing interests.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2020.112799.

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