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Acute social stress-induced immunomodulation in pigs high and low responders to ACTH

Elodie Bacou [a], Karine Haurogné [a], Grégoire Mignot [a], Marie Allard [a], Laurence De Beaurepaire [a], Jordan Marchand [a], Elena Terenina [b], Yvon Billon [c], Julien Jacques [d], Jean-Marie Bach [a], Pierre Mormède [b], Julie Hervé [a,*,1], Blandine Lieubeau [a,*,1]

[a] IECM, INRA, Oniris, Université de Nantes, LUNAM, La Chantrerie, CS 40706, F-44307 Nantes Cedex 3, France
[b] GenPhySE, Université de Toulouse, INRA, INPT, ENVT, F-31326 Castanet-Tolosan, France
[c] GenESI, INRA, Le Magneraud, F-17700 Saint-Pierre-d'Amilly, France
[d] ERIC, Université de Lyon 2, F-69676 Bron, France

HIGHLIGHTS

- Acute social stress induces leukocyte mobilization in high and low responders to ACTH.
- Acute social stress impairs leukocyte functions (phagocytosis and cytokine secretion).
- One-hour mixing of pig is a relevant model of acute social stress for human research.
- Selection upon HPA axis activity increases resilience of pigs to stress.
- Selection upon HPA axis activity appears as a good tool to increase pig robustness.

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ABSTRACT

Pig husbandry is known as an intensive breeding system, piglets being submitted to multiple stressful events such as early weaning, successive mixing, crowding and shipping. These stressors are thought to impair immune defences and might contribute, at least partly, to the prophylactic use of antibiotics. Robustness was recently defined as the ability of an individual to express a high-production potential in a wide variety of environmental conditions. Increasing robustness thus appears as a valuable option to improve resilience to stressors and could be obtained by selecting piglets upon their adrenocortical activity.

In this study, we aimed at depicting the consequences of an acute social stress on the immune capacity of piglets genetically selected upon divergent hypothalamic-pituitary-adrenocortical (HPA) axis activity. For this purpose, we monitored neuroendocrine and immune parameters, in high- (HPA^{hi}) and low- (HPA^{lo}) responders to ACTH, just before and immediately after a one-hour mixing with unfamiliar conspecifics.

As expected, stressed piglets displayed higher levels of circulating cortisol and norepinephrine. Blood cell count analysis combined to flow cytometry revealed a stress-induced leukocyte mobilization in the bloodstream with a specific recruitment of $CD8\alpha^+$ lymphocytes. Besides, one-hour mixing decreased LPS-induced IL-8 and TNFα secretions in whole-blood assays (WBA) and reduced mononuclear cell phagocytosis. Altogether, our data demonstrate that acute social stress alters immune competence of piglets from both groups, and bring new insights in favour of good farming practices.

While for most parameters high- and low-responders to ACTH behaved similarly, HPA^{hi} piglets displayed higher number of $CD4^+$ $CD8\alpha^-$ T cells, as well as increased cytokine production in WBA (LPS-induced TNFα and PIL-induced IL-8), which could confer them increased resistance to pathogens.

Finally, a principal component analysis including all parameters highlighted that overall stress effects were less pronounced on piglets with a strong HPA axis. Thus, selection upon adrenocortical axis activity seems to reduce the magnitude of response to stress and appears as a good tool to increase piglet robustness.

* Corresponding authors.
E-mail addresses: julie.herve@oniris-nantes.fr (J. Hervé), blandine.lieubeau@oniris-nantes.fr (B. Lieubeau).
[1] These authors contributed equally to this work.

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1. Introduction

Pig husbandry is recognised as one of the most stressful breeding systems, individuals being exposed, from birth to slaughter, to numerous physiological, environmental and social stressors. In particular, the weaning period appears as a critical step for piglets that have to cope with multiple stressors: separation from the dam, handling and transportation, mixing with unfamiliar pigs and establishment of a novel social hierarchy, crowding, feed change and exposure to different pathogens [1]. Thus, at weaning, piglets are prone to develop dysbiosis and/or infectious diseases and the management of this period often relies on the prophylactic use of antibiotics with harmful consequences for public health.

Increasing piglet robustness appears as a valuable option to increase resilience of individuals to stressors and by the way to reduce antibiotic use. The concept of robustness refers to the ability of an individual to combine a high production potential with resilience to stressors, thereby allowing a sustained productivity in a wide variety of environmental conditions [2]. We recently suggested that the hypothalamic-pituitary-adrenocortical (HPA) axis plays a central role in the trade-off between production and robustness traits [3,4]. In this context, we developed a strategy to increase robustness by genetic selection of animals with a more active HPA axis, based on their adrenal response to ACTH, a highly heritable trait [5,6]. This strategy raises the question of the consequences of this selection on immune functions.

The immune system has long been considered autonomous but it is now well established that immunity, as part of homeostasis, is tightly controlled by the central nervous system [7]. Although all neuroendocrine hormones can modulate immune responses, the HPA and the sympatho-adreno-medullar (SAM) axes are predominant in conveying efferent signals from the brain to peripheral tissues. Immune cells express glucocorticoid as well as adrenergic receptors and are thus able to respond to cortisol, epinephrine and norepinephrine. Conversely, immune cells signal back to the brain especially through cytokine release (IL-1β, IL-6, TNFα) (reviewed in [8]).

Both HPA and SAM axes are activated by stressors and help the organism to adapt and cope with threats. Stress is usually classified depending upon its type (physical, environmental and/or psychosocial), intensity and duration (acute *vs* chronic) [9]. Although chronic stressors are widely recognised as detrimental to most immune functions, conflicting data were reported using acute stressors (reviewed in [10]). Notably, it was shown that a brief stress might increase some aspects of immune responses, such as skin delayed-type hypersensitivity [11–13] which has led to the emergence of the concept of acute stress-induced immunoenhancement [14]. Edwards and collaborators even suggested that vaccine efficacy could be maximized using stressors or stress mediators as adjuvants. Yet, one study demonstrated clear benefits of co-administering a β2-adrenergic agonist along with Herpes-simplex virus (HSV) vaccine in mice, thereby conferring prolonged survival and reduced inflammation against HSV infection [15].

To date, consequences of acute stress on immunity have been poorly documented in pigs. In 2009, Tuchscherer and collaborators submitted 7 to 35 day-old piglets to a 4-hour social isolation stress and compared their endocrine and immune responses to control animals [16]. Using whole blood assay (WBA), which measures cytokine release in whole blood in response to LPS stimulation and was proposed to predict individual susceptibility to infectious disease [17], they found lower LPS-induced IL-10 and IL-1β secretions in stressed *versus* control 21-day old piglets, suggesting stress-induced immune alterations. Besides, few studies evidenced stress-induced lymphopenia in pigs [18,19].

The present experiments were designed to study the consequences of divergent genetic selection for HPA axis activity on immune functions in basal conditions and in response to acute stress. For this purpose, we monitored endocrine and immune parameters in piglets belonging to the second generation of divergent selection based on the cortisol response to ACTH, just before and right after a one-hour mixing with unfamiliar conspecifics.

2. Materials and methods

2.1. Animals and experimental design

All experimental procedures involving animals were carried out in accordance with the EU directive 2010/63 for animal experiments and submitted to and approved by the C2EA 84 Poitou-Charentes Region ethic committee (decisions CE2013-1 and APAFIS#2446-201510121422769). Ninety male Large White pigs, born and raised at the INRA experimental unit of Le Magneraud (EU 1206, GenESI), were used in this study. These pigs belonged to the second generation of a divergent genetic selection experiment based on hypothalamic-pituitary-adrenal (HPA) axis activity. For this purpose, breeders were selected upon extreme variability in their cortisol response following ACTH stimulation, allowing generation of low (HPA^{lo}) and high (HPA^{hi}) responders to ACTH [6].

In this study, three experimental batches, each involving 15 HPA^{lo} and 15 HPA^{hi} piglets, were studied. After weaning at 4 weeks, piglets were housed by groups of approximately 20 animals per pen (7.7 m^2), separated by solid walls so that they had no direct contact. Of note, HPA^{lo} and HPA^{hi} piglets were housed in separate pens and were never mixed together. When piglets were 6 week-old, cortisolemia was measured 1 h after IM administration of a single 222 μg dose of synthetic 1-24 ACTH peptide (Pepscan Presto BV, Lelystad, the Netherlands). We confirmed that ACTH-induced cortisolemia was significantly higher in HPA^{hi} as compared to HPA^{lo} piglets (data not shown).

One week later, piglets were submitted to an acute social stress. For this purpose, five piglets were taken from their home pen in the morning (between 8:00 and 9:00 am), placed in an adjacent pen with approximately 20 unfamiliar conspecifics for 1 h. Blood samples were collected from the jugular vein of pigs slightly maintained in a supine position as previously described [20], just before and immediately after 1 h of mixing, by experienced staff, in <30 s per pig. Total volume collected never exceeded 25 ml per piglet.

2.2. White blood cell count

White blood cell (WBC) counts were determined from EDTA blood samples using a clinical-grade MS9-5s analyser calibrated for pigs (Melet Schloesing, Osny, France).

2.3. Flow cytometry

Lymphocyte subpopulations were identified by triple staining using monoclonal antibodies and enumerated. Fifty microliters of EDTA blood samples were stained with FITC conjugated anti-pig CD4, PE conjugated anti-pig CD21 (both from Acrys Antibodies, Herford, Germany), and Alexa Fluor® 647 conjugated anti-pig CD8α (BD Biosciences, Le Pont de Claix, France) antibodies in the dark for 15 min at room temperature. Erythrocytes were then removed using a lysing solution (BD Biosciences). After washing with phosphate-buffered saline, samples were analysed with a FACS Aria flow cytometer (BD Biosciences) and data were computed using FlowJo software (FlowJo, Ashland, Oregon). This combination of three antibodies allowed the identification of five subpopulations in whole blood samples namely $CD21^+$ B lymphocytes, $CD4^+$ $CD8\alpha^-$ and $CD4^+$ $CD8\alpha^+$ helper T lymphocytes, $CD4^-$ $CD8\alpha^{hi}$ cytotoxic lymphocytes and $CD4^-$ $CD8\alpha^{med}$ cells, which includes NK cells [21]. Cell count for each specific subpopulation was then determined as the product of the percentage of this specific subpopulation in a (lymphocyte plus monocyte FSC/SSC) gate by the absolute number of lymphocytes plus monocytes (obtained from the MS9-5s analyser).

2.4. Phagocytosis

Phagocytosis was assessed using the Phagotest™ Kit (BD Biosciences) according to the protocol given by the manufacturer. Briefly, heparinized blood samples were incubated for 10 min at 37 °C with opsonized fluorescein isothiocyanate (FITC)-labelled *E. coli* bacteria. For each analysis, ice-incubated negative controls were included. Samples were finally analysed by flow cytometry using FSC/SSC dot plot to discriminate granulocytes from mononuclear cells.

2.5. Whole blood assay

Heparinized blood samples were five-fold diluted in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin and stimulated in duplicate either with the already described [22] PIL mixture (10 ng/ml phorbol myristate acetate, 1 μg/ml ionomycin and 1 μg/ml *Escherichia coli* O111:B4 LPS, all from Sigma-Aldrich, St-Quentin-Fallavier, France) or with a 10 ng/ml LPS stimulation [17]. All stimulations were tested in duplicate. After an 18-hour incubation at 37 °C, supernatants were collected, centrifuged at 500 g for 5 min at 4 °C and stored at −80 °C before cytokine concentration measurements. Porcine TNFα, IL-10 and IL-8 were quantified by ELISA with respective detection limits of 31, 23 and 125 pg/ml (R&D Systems, Abingdon, UK). Supernatants in duplicate prepared from samples collected before (t0) and after (t1) mixing were analysed in the same plates. For each plate, we evaluated the intra-assay variation to validate differences between t0 and t1 samples. For statistical analysis, we attributed to undetected samples half of the corresponding threshold value.

2.6. Biochemical analysis

Heparinized blood samples were centrifuged and plasma collected for hormone content measurements. Plasma catecholamine (epinephrine and norepinephrine) concentrations were determined in duplicate from heparinized blood samples using the CAT Plasma ELISA high sensitive kit (LDN, Nordhorn, Germany). Briefly, epinephrine and norepinephrine were extracted from plasma using a cis-diol-specific affinity gel, acylated and then modified enzymatically before performing competitive ELISA. Plasma cortisol was assayed in the same samples using a Cortisol RIA kit (Demeditec Diagnostics, Kiel-Wellsee, Germany). Post-ACTH plasma cortisol concentrations were measured by direct automated immunoassay (AIA-1800, Tosoh Bioscience, San Francisco, CA).

2.7. Statistical analysis

Statistical analyses were performed using R software (version 3.3.1) while charts were graphed using Graph Pad Prism (version 6.0).

To describe acute social stress consequences upon immune and endocrine systems in high and low responders to ACTH, a linear mixed-effects model was developed using the lme4 package [23]. Data were transformed using natural logarithm and normality was assessed for each parameter using the Shapiro test. The model included as fixed effects batch (Ba: 1, 2, 3), HPA axis activity (HPA: low and high), mixing (Mix: before and after) and all possible interactions and, as random effects, blood sampling rank (BSR) and individual (Id). Residual plots did not reveal any obvious deviations from homoscedasticity for all response variables. We tested significance for the fixed effects influence on each parameter with the Wald Chi-square test, using the anova function from the car package [24]; $p < 0.05$ was considered significant. For all response variables, least square means (LS-means) and standard errors were calculated using the lsmeans package [25] and are graphed on charts. Whenever the Wald Chi-square test demonstrated a significant interaction effect, pair-wise differences between LS-means were subsequently tested using the Tukey-Kramer procedure ($p < 0.05$ was considered significant).

Lastly, to have a whole-scale overview of our data, we performed a Principal Component Analysis (PCA) on log-transformed variables using the FactoMineR package [26].

3. Results

3.1. Data overview

Endocrine and immune parameters were analysed in blood samples from 45 HPA^{lo} and 45 HPA^{hi} piglets, divided into three batches, each piglet being sampled just before and right after one-hour mixing with unfamiliar pigs. Table 1 summarizes biological values of endocrine and immune parameters obtained on HPA^{lo} and HPA^{hi} piglets before and after mixing.

To assess the effects of acute social stress and HPA axis activity upon these parameters, we applied a mixed-effect model, which included mixing, HPA axis activity, batch and all interactions as fixed effects, while individual and blood sampling rank were specified as random factors.

We observed a batch effect on 13 parameters upon 18 (Supplemental Table 1). For 6 of them, there was a batch effect and no interaction between batch and any other fixed effect, highlighting similar mixing- and/or HPA axis activity-related effects in all batches. When the Wald Chi-square test indicated a significant interaction between the batch and a second fixed effect (mixing, HPA axis activity or both), the batch effect was further on analysed and discussed.

As shown in Table 2, acute social stress significantly affected 16 parameters upon 18. Indeed, only 2 parameters were not affected by mixing: PIL-induced IL-10 secretion also unaffected by HPA axis activity and PIL-induced IL-8 secretion, which was different in HPA^{lo} and HPA^{hi} piglets. Three parameters were significantly modulated by both stress and HPA axis activity, namely cortisol level, $CD4^+$ $CD8\alpha^-$ cell number and LPS-induced TNFα secretion. All these results are detailed in the following sections.

3.2. Effects of one-hour mixing on stress hormone levels

For plasma cortisol levels, the Wald Chi-square test indicated significant effects of mixing, HPA axis activity and mixing × HPA interaction (Table 2). As expected, pair-wise comparisons of the LS-means (Tukey-Kramer test) from HPA^{lo} and HPA^{hi} at baseline demonstrated that plasma cortisol levels were significantly higher in HPA^{hi} as compared to HPA^{lo} individuals (Fig. 1A). Mixing increased cortisol levels in both HPA^{lo} and HPA^{hi} piglets. The Wald Chi-square test also evidenced a significant batch × mixing interaction effect (Supplemental Table 1), attributable to a non-significant increase in cortisolemia observed in one batch upon three (Supplemental Fig. 1).

For epinephrine levels, the Wald Chi-square test indicated a significant mixing effect (Table 2) spoiled by a significant batch × mixing × HPA interaction effect (Supplemental Table 1). Pair-wise comparisons of the LS-means before and after mixing in each batch showed that mixing actually had no significant effect on epinephrine levels either in HPA^{lo} or in HPA^{hi} piglets in any batch (data not shown).

In contrast, plasma norepinephrine concentrations were significantly affected by mixing only (Table 2). As shown in Fig. 1B, acute social stress increased norepinephrine levels in all piglets.

Altogether, these results confirmed stress-induced activation of HPA and SAM axes and validated our protocol as a reliable model of acute stress.

3.3. Acute social stress-induced leukocyte mobilization

The Wald Chi-square test indicated significant effects of mixing on every white blood cell population (Table 2). As shown in Fig. 2, mixing significantly increased granulocyte, lymphocyte and monocyte

Table 1
Values of endocrine and immune parameters.
Endocrine and immune parameters values measured before and after acute social stress in HPA^{lo} and HPA^{hi} piglets. For each parameter, the median and range of raw data are reported. For most parameters, measurements were made on 90 piglets, except phagocytic activity ($n = 43$ piglets) and lymphocyte subset determination (2 batches, $n = 60$). nd: not detected.

Parameters	HPA^{lo}		HPA^{hi}	
Stress hormones				
Cortisol (ng/ml)	Before	25.2 [nd–65]	Before	36.4 [nd–98.2]
	After	46.5 [nd–128.1]	After	57.7 [nd–174.3]
Epinephrine (pg/ml)	Before	607 [269–1,643]	Before	556 [223–2,246]
	After	715 [167–1,464]	After	616 [110–2,217]
Norepinephrine (pg/ml)	Before	1,311 [695–2,951]	Before	1,279 [507–3,465]
	After	1,725 [860–6,656]	After	1,467 [680–12,800]
White blood cell count ($\times 10^3$/μl)				
Granulocytes	Before	6.0 [2.5–20.5]	Before	5.4 [2.8–10.1]
	After	6.3 [2.4–18]	After	6.2 [3.3–13.2]
Lymphocytes	Before	11.4 [5.9–15.5]	Before	10.7 [7–15.7]
	After	11.9 [5.3–21.6]	After	11.5 [7–16.9]
Monocytes	Before	1.3 [0.6–2.1]	Before	1.2 [0.6–1.9]
	After	1.4 [0.6–2.9]	After	1.3 [0.6–2.2]
Lymphocyte subsets ($\times 10^3$/μl)				
$CD8\alpha^{hi}$ $CD4^-$	Before	1.37 [0.67–3.95]	Before	1.54 [0.7–3.49]
	After	1.72 [0.79–6.27]	After	1.76 [0.72–3.53]
$CD8\alpha^{med}$ $CD4^-$	Before	0.90 [0.33–2.14]	Before	0.91 [0.27–2.58]
	After	1.58 [0.52–4.69]	After	1.47 [0.27–3.31]
$CD4^+$ $CD8\alpha^+$	Before	0.80 [0.35–3.11]	Before	0.82 [0.44–1.79]
	After	1.04 [0.37–5.47]	After	1.01 [0.4–2.79]
$CD4^+$ $CD8\alpha^-$	Before	1.73 [0.88–2.38]	Before	1.74 [1.09–3.33]
	After	1.45 [0.86–2.74]	After	1.71 [0.97–3.2]
$CD21^+$	Before	1.62 [0.62–3.35]	Before	1.86 [0.72–3.12]
	After	1.38 [0.43–2.63]	After	1.39 [0.57–2.54]
Phagocytic capacity (%)				
Mononuclear cells	Before	22.5 [15.1–28.2]	Before	21.0 [13.7–32.5]
	After	18.4 [12.4–31.1]	After	18.2 [14–30.5]
Polynuclear cells	Before	90.2 [79.8–95.5]	Before	87.9 [34.2–94.2]
	After	90.3 [65.9–95.7]	After	89.0 [33.0–97.0]
Whole blood assay (pg/ml)				
LPS-induced IL-8 secretion	Before	268 [nd–3,747]	Before	341 [nd–3,040]
	After	189 [nd–1,867]	After	241 [nd–918]
LPS-induced TNFα secretion	Before	191 [97–595]	Before	253 [90–919]
	After	169 [82–739]	After	218 [90–1,145]
LPS-induced IL-10 secretion	Before	70.0 [nd–166]	Before	88.0 [nd–254]
	After	65.5 [nd–188]	After	71.5 [29–272]
PIL-induced IL-8 secretion	Before	18,446 [5,789–42,386]	Before	23,305 [7,963–50,816]
	After	18,091 [5,789–32,367]	After	21,063 [7,963–69,473]
PIL-induced TNFα secretion	Before	14,884 [8,263–20,000]	Before	14,467.5 [11,007–>20,000]
	After	13,649 [9,520–18,644]	After	13,630.5 [9,571–18,901]
PIL-induced IL-10 secretion	Before	6,362 [3,163–12,755]	Before	6,346 [3,303–13,981]
	After	6,123 [3,163–11,990]	After	6,408 [2,965–13,981]

numbers. For monocyte numbers only, the Wald Chi-square test also revealed a significant batch × mixing interaction effect (Supplemental Table 1). Detailed analysis of the data highlighted a non-significant increase in monocyte numbers following mixing in the batch with the lower monocyte numbers (Supplemental Fig. 2), likely due to the poor resolution of the hemocytometer for smaller cell numbers.

We next explored acute stress-induced lymphocytosis using flow cytometry. For this purpose, we enumerated $CD8\alpha^{hi}$ $CD4^-$ cytotoxic T lymphocytes, $CD8\alpha^{med}$ $CD4^-$ cells, $CD4^+$ $CD8\alpha^+$ double positive cells (which are activated helper T lymphocytes [27], well-represented in pigs compared to humans or mice), $CD4^+$ $CD8\alpha^-$ T cell and $CD21^+$ B lymphocytes (Table 1). The Wald Chi-square test revealed significant effects of mixing on $CD8\alpha^{hi}$ $CD4^-$ and $CD8\alpha^{med}$ $CD4^-$ cell subpopulations

Table 2
Results of the statistical analysis for main effects (p-values).
For each parameter, in the linear mixed-effects model, the effect of mixing, HPA activity and interaction between both were tested using the Wald-Chi square test. A p-value < 0.05 was considered significant.

Parameters	Mixing	HPA activity	Mixing × HPA
Cortisol	<0.001	<0.001	<0.05
Epinephrine	<0.05	0.62	0.24
Norepinephrine	<0.001	0.41	0.39
Granulocytes	<0.001	0.34	0.89
Monocytes	<0.001	0.96	0.15
Lymphocytes	<0.001	0.91	0.13
$CD8\alpha^{hi}$ $CD4^-$ lymphocytes	<0.001	0.17	0.81
$CD8\alpha^{med}$ $CD4^-$ lymphocytes	<0.001	0.95	0.89
$CD4^+$ $CD8\alpha^+$ lymphocytes	<0.001	0.65	0.54
$CD4^+$ $CD8\alpha^-$ lymphocytes	<0.01	<0.05	0.78
$CD21^+$ lymphocytes	<0.001	0.51	0.53
% phagocytes in mononuclear cells	<0.001	0.64	0.51
LPS-induced IL-8 secretion	<0.001	0.09	0.78
LPS-induced TNFα secretion	<0.01	<0.05	0.91
LPS-induced IL-10 secretion	<0.001	0.13	0.63
PIL-induced IL-8 secretion	0.77	<0.05	0.58
PIL-induced TNFα secretion	<0.001	0.45	0.08
PIL-induced IL-10 secretion	0.34	0.67	0.95

(Table 2) with a significant effect of the batch × mixing interaction for the $CD8\alpha^{med}$ $CD4^-$ cells (Supplemental Table 1). For this latter population, pair-wise comparisons of the LS-means before and after mixing in each batch showed significant increased $CD8\alpha^{med}$ $CD4^-$ cell numbers after mixing in each batch (data not shown). We concluded that mixing significantly enhanced both $CD8\alpha^{hi}$ $CD4^-$ and $CD8\alpha^{med}$ $CD4^-$ cell numbers (Fig. 3A).

In addition, mixing also affected $CD4^+$ $CD8\alpha^+$ cell numbers with a significant batch × mixing interaction effect. Pair-wise comparisons of the LS-means before and after mixing, batch per batch, indicated an overall increase of double positive cells even if not significant in one batch ($p = 0.06$) (data not shown).

As far as $CD4^+$ $CD8\alpha^-$ T cells are concerned, the Wald Chi-square test demonstrated that mixing and HPA axis activity influenced their number independently (Table 2). Mixing decreased circulating $CD4^+$ $CD8\alpha^-$ T cell number in a similar manner in piglets from both groups (Fig. 3B). A strong HPA axis activity was associated to higher numbers of $CD4^+$ $CD8\alpha^-$ T cells at any time (Fig. 4A).

Finally, the Wald Chi-square test indicated a significant effect of mixing on $CD21^+$ B cell numbers with a significant batch × mixing interaction effect. Pair-wise comparisons of the LS-means showed significantly reduced cell numbers after mixing in all batches (data not shown). Thus, we concluded that acute social stress reduced circulating B cell numbers in all piglets (Fig. 3C).

3.4. Social stress effects on immune functions in vitro

To document stress-induced modulation of immune capacity, we analysed TNFα, IL-8 and IL-10 secretions by blood cells stimulated *in vitro* using whole blood assay (WBA). As expected, whole blood cells secreted higher amounts of cytokines after PIL stimulation than after stimulation with LPS (Table 1). LPS-induced IL-8 secretion was significantly reduced after mixing with no influence of HPA axis activity (Fig. 5 and Table 2). Regarding LPS-induced TNFα secretion, the Wald Chi-square test highlighted that both mixing and HPA axis activity independently and significantly affected this parameter, while there was also a significant batch × mixing interaction effect (Table 2 and Supplemental Table 1). Mixing induced an overall decrease of LPS-induced TNFα secretion (Fig. 5), although the batch-per-batch analysis on LS-means revealed that this was not observed in one batch out of three (Supplemental Fig. 3). Interestingly, blood cells derived from HPA^{hi} piglets secreted higher levels of TNFα in response to LPS than HPA^{lo} piglets at any time (Fig. 4B). LPS-induced IL-10 secretion was significantly affected

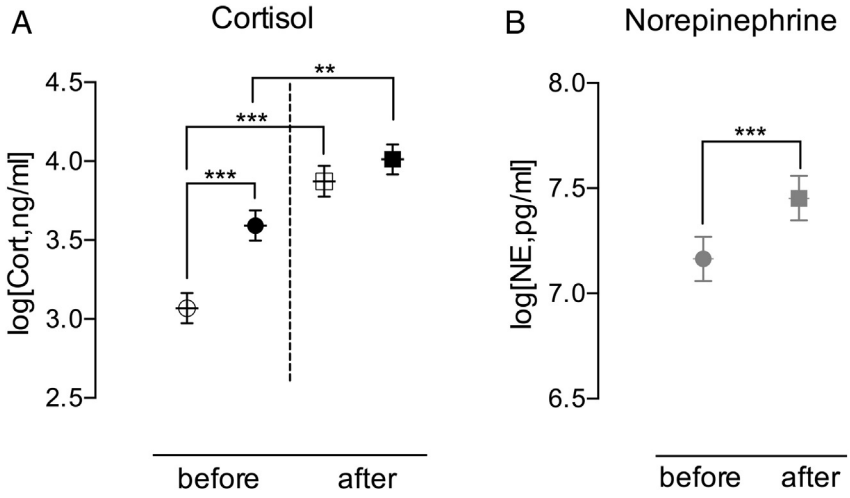

Fig. 1. Stress-related hormone levels. (A) Cortisol levels in plasma of HPA^{lo} (empty symbols) and HPA^{hi} (filled symbols) piglets, just before (circles) and right after (squares) one-hour mixing. Data are expressed as LS-means ± SE. *p*-values were calculated using the Tukey-Kramer procedure. (B) Norepinephrine levels, before (circles) and after (squares) one-hour mixing. Data are expressed as LS-means ± SE with stars denoting statistical significance between before and after mixing (as shown in Table 2).

by mixing (Table 2). However, it was also affected by a significant batch × mixing interaction (Supplemental Table 1), and the comparison of LS-means before and after mixing batch per batch did not give consistent results (data not shown). Regarding PIL-induced cytokine secretions, the Wald Chi-square test indicated that mixing significantly influenced TNFα but neither IL-10 nor IL-8 levels (Table 2). For PIL-induced TNFα secretion, there was a significant batch × mixing × HPA interaction effect. Pair-wise comparisons of LS-means before and after mixing in HPA^{lo} and HPA^{hi} piglets batch-per-batch showed that mixing significantly reduced TNFα secretion in HPA^{lo} piglets in two batches upon three while the mixing effect was never significant in HPA^{hi} piglets (Supplemental Fig. 3). Finally, independently of mixing, we found higher levels of IL-8 after PIL-stimulation of blood cells originating from HPA^{hi} as compared to HPA^{lo} piglets (Table 2 and Fig. 4C).

Since phagocytosis represents an essential mechanism of host defence against bacterial and fungal infections, we also analysed acute social stress consequences on the ability of blood cells to phagocyte FITC-labelled bacteria. Phagocytosis is performed by granulocytes and mononuclear cells. As shown on Fig. 6 and Table 2, mononuclear cell phagocytic activity was significantly impaired by mixing.

3.5. Principal component analysis

At last, we performed a PCA on the whole log-transformed data; the first two components explained 28.3 and 13.4% of total inertia respectively. The circle of correlations showed three bundles corresponding to cytokine secretion in whole blood assays, immune cell numbers and stress hormone levels (Supplemental Fig. 4). Variables' cos^2 analysis highlighted that the first principal component summarised all blood cell counts, while the second one was associated positively to cytokine secretion and negatively to stress-related hormone release (Supplemental Table 2 and Supplemental Fig. 4).

The plot of individuals on the (PC1, PC2) plane is shown in Fig. 7. Confidence ellipses for HPA^{hi} and HPA^{lo} individuals overlapped both before and after acute social stress. Interestingly, 95% confidence ellipses before and after mixing were closer for HPA^{hi} piglets as compared to HPA^{lo} suggesting that stress effects were less pronounced on piglets exhibiting a strong HPA axis activity.

4. Discussion

Although the HPA axis impacts many physiological functions (reproduction, cardiovascular system, metabolism…), intense selection for production traits has driven down its activity [28]. Currently, a major goal in farm animal breeding is to increase robustness of individuals *i.e.* to improve their resilience to stressors while not affecting their productivity. From available evidence, we suggest that this could be done by selecting piglets with a strong HPA axis [4,5]. In this study, we sought to evaluate basic functioning of the immune system and the consequences of an acute social stress in piglets genetically selected as high and low responders to ACTH stimulation.

Mixing is a common practice in pig farming. In our study, mixing induced a significant rise in cortisol and norepinephrine levels as previously demonstrated [29]. In contrast, epinephrine level was not

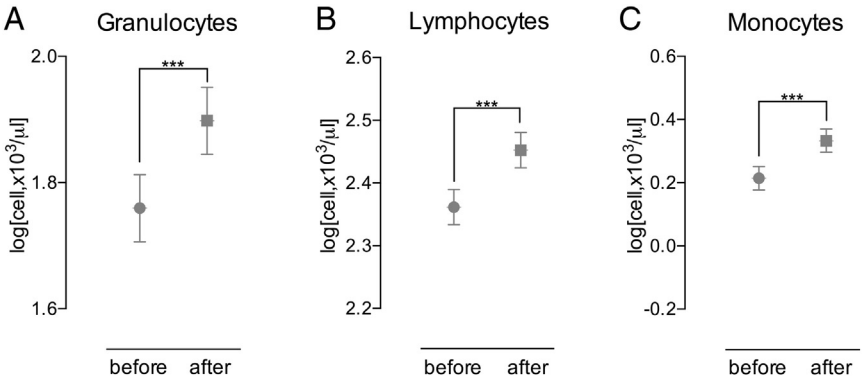

Fig. 2. Stress-induced variations in WBC counts. Granulocytes (A), lymphocytes (B) and monocytes (C) counts from HPA^{lo} and HPA^{hi} piglets before and after mixing. Data are expressed as LS-means ± SE with stars denoting statistical significance (as shown in Table 2).

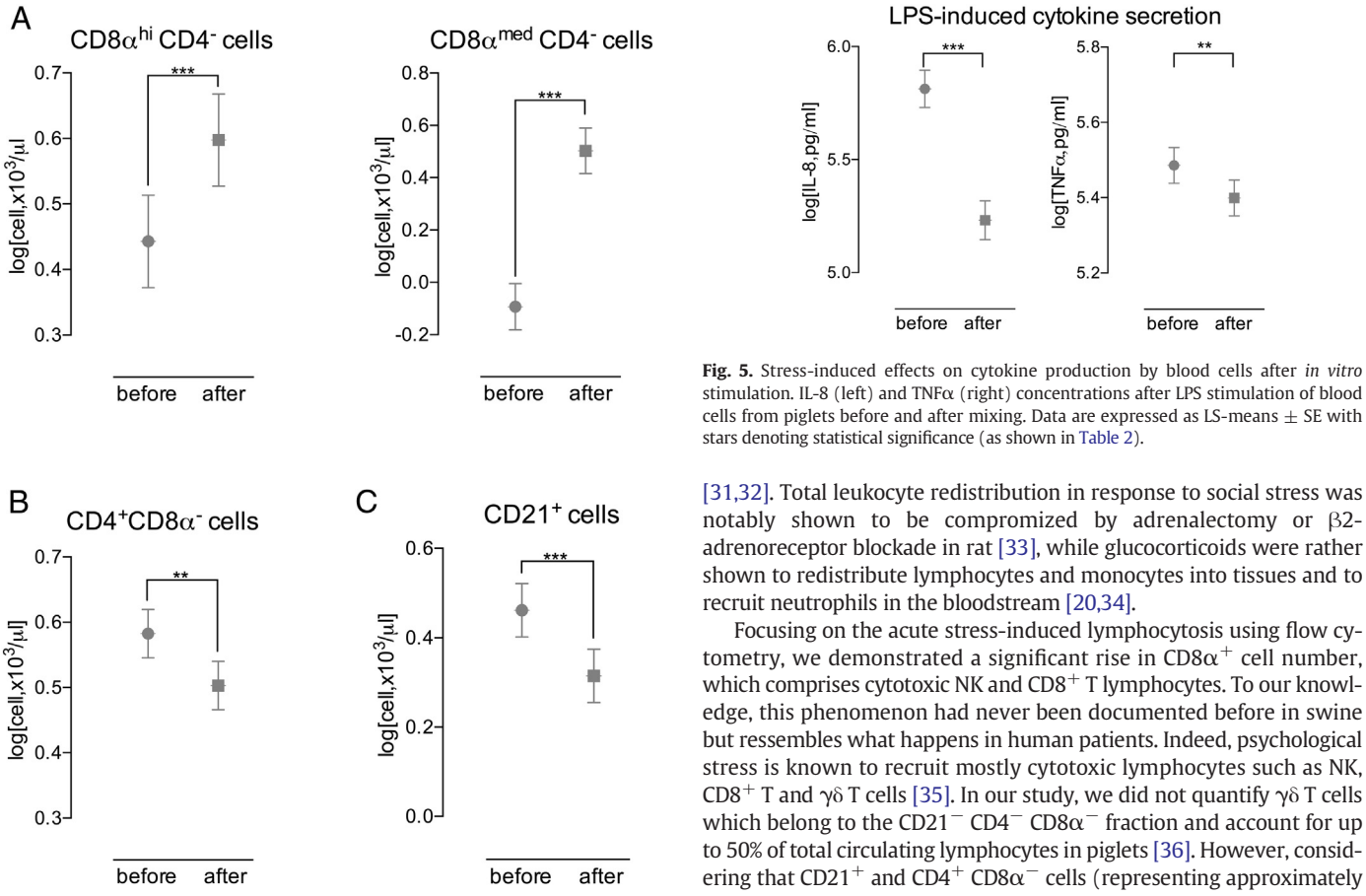

Fig. 5. Stress-induced effects on cytokine production by blood cells after *in vitro* stimulation. IL-8 (left) and TNFα (right) concentrations after LPS stimulation of blood cells from piglets before and after mixing. Data are expressed as LS-means ± SE with stars denoting statistical significance (as shown in Table 2).

Fig. 3. Effects of acute social stress on circulating lymphocyte subpopulations. CD8α^{hi} CD4^{-}, CD8α^{med} CD4^{-} cells (A), CD4^{+}CD8α^{-} (B) and CD21^{+} (C) lymphocyte subset counts before and after mixing. Data are expressed as LS-means ± SE with stars denoting statistical significance (as shown in Table 2).

significantly affected by acute stress in agreement with previous data [30]. We next demonstrated that one-hour mixing with unfamiliar conspecifics induced significant increase in circulating lymphocyte, monocyte and granulocyte (mainly polymorphonuclear neutrophil) numbers in both HPA^{hi} and HPA^{lo} piglets. This leukocytosis had been poorly described in pigs but already documented in humans and rodents and is thought to be mainly related to the catecholamine burst [31,32]. Total leukocyte redistribution in response to social stress was notably shown to be compromized by adrenalectomy or β2-adrenoreceptor blockade in rat [33], while glucocorticoids were rather shown to redistribute lymphocytes and monocytes into tissues and to recruit neutrophils in the bloodstream [20,34].

Focusing on the acute stress-induced lymphocytosis using flow cytometry, we demonstrated a significant rise in CD8α^{+} cell number, which comprises cytotoxic NK and CD8^{+} T lymphocytes. To our knowledge, this phenomenon had never been documented before in swine but ressembles what happens in human patients. Indeed, psychological stress is known to recruit mostly cytotoxic lymphocytes such as NK, CD8^{+} T and γδ T cells [35]. In our study, we did not quantify γδ T cells which belong to the CD21^{-} CD4^{-} CD8α^{-} fraction and account for up to 50% of total circulating lymphocytes in piglets [36]. However, considering that CD21^{+} and CD4^{+} CD8α^{-} cells (representing approximately 30% of total lymphocytes) decreased after stress, we can speculate that γδ T cells were mobilized as CD4^{-} CD8α^{hi} cytotoxic lymphocytes and CD4^{-} CD8α^{med} cells were. In our study, activated CD4^{+} T lymphocytes also tended to increase after acute social stress exposure. A selective redistribution of lymphocytes depending on their activation status was demonstrated in humans following acute stress [37–39]. Altogether we observed a cell-specific demargination after social stress in piglets. Similarly, in humans, β-agonist infusion induced both an increase in NK, CD8α^{+} and γδ T cells and a decrease in circulating B and CD4^{+} T cells [31]. This mobilization of cytotoxic cells is thought to be part of the "fight or flight" response to stress mediated by catecholamines and may provide protection in situations in which exposure to antigen could occur [32,40,41].

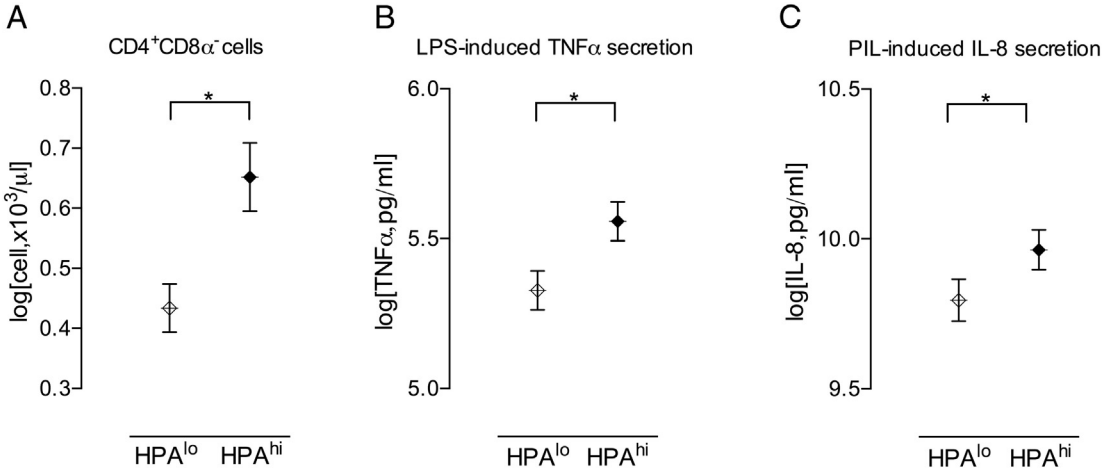

Fig. 4. Immune parameters modulated by HPA axis activity. CD4^{+} CD8α^{-} cell number (A), LPS-induced TNFα secretion (B) and PIL-induced IL-8 secretion (C) in HPA^{lo} as compared to HPA^{hi} piglets. Data are expressed as LS-means ± SE with stars denoting statistical significance (as shown in Table 2).

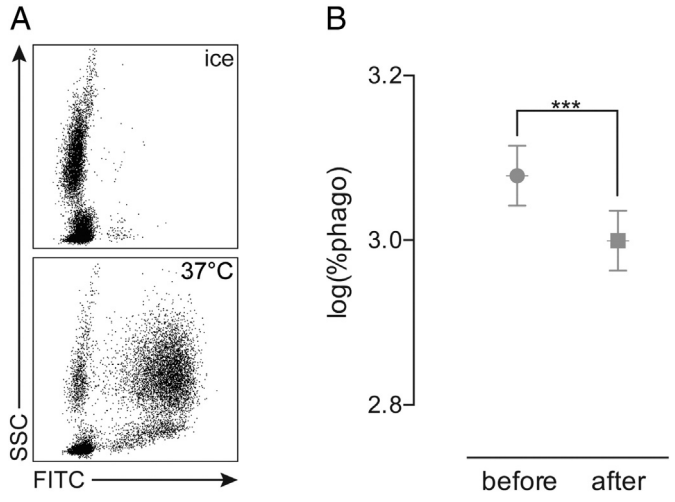

Fig. 6. Acute stress modulation of circulating mononuclear cell phagocytic activity. Phagocytosis was measured by flow cytometry. (A) A representative sample is shown for ice-incubated control cells (top) and 37 °C-incubated cells (bottom). (B) Percentage of FITC-labelled bacteria phagocytosis among mononuclear cells from piglets before and after mixing. Data are expressed as LS-means ± SE with stars denoting statistical significance (as shown in Table 2).

Using functional tests, we observed significant stress-induced decreased phagocytic activity by mononuclear cells and reduced cytokine secretions (IL-8 and TNFα) by LPS-stimulated leukocytes; also, PIL-induced TNFα secretion tended to decrease after mixing. These results are all the more interesting since leukocyte number increased after stress. How social stress triggers a reduced responsiveness of leukocytes to the LPS challenge needs to be addressed. Among leukocytes, LPS treatment mainly stimulates monocytes and granulocytes. Few data are available for granulocytes; however, monocytes/macrophages are well known to respond to cortisol and catecholamines (for review see [42]). Even if poorly investigated in swine, acute stress-induced impairment of cytokine production by blood cells was already reported in humans. Notably, a free-speech stress exposure resulted in significant decrease in LPS-induced cytokine (TNFα and IL-6) production in WBA [43]. Also, when exposed to catecholamines, human whole blood cells displayed a reduced ability to secrete TNFα and IL-6 in response to a low dose of LPS [43].

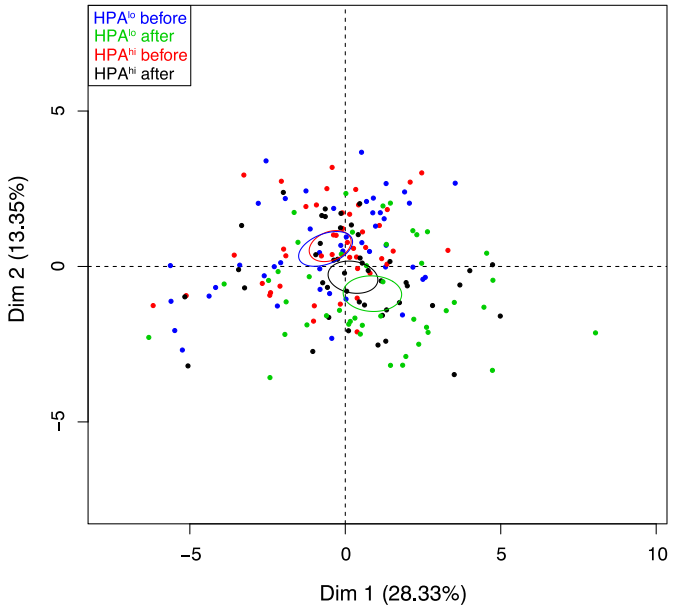

Fig. 7. Results of PCA performed on log-transformed data. Individuals' factor map in the (PC1, PC2) plane is shown. 95% confidence ellipses of the four groups' barycentres are graphed.

Altogether, our data indicate that acute social stress induced a significant rise in circulating leukocyte numbers associated to an impairment in immune functions in both HPA^{hi} and HPA^{lo} piglets. This is in apparent contradiction with the acute stress-induced immunoenhancement hypothesis [14]. Indeed, acute stressors were previously propose to enhance some adaptative immune functions such as vaccination responses. In our study, we focused on innate immune functions that we found impaired by mixing.

Stress effects on immune system were previously shown to depend on social status of pigs. Among stressed pigs, dominant pigs display greater NK cell cytotoxicity [18] and higher lymphoproliferative response [29,44] than subordinate pigs do. In our study, we did not consider social status of pigs since we aimed to depict the immediate consequences of an acute social stress on the immune capacity of divergently selected piglets independently of their social rank. One could argue that the decreased immune reactivity we observed after mixing could result from a biased selection towards subordinate pigs. Although we cannot exclude it, it is unlikely considering sample size and random selection of the piglets.

For most parameters, HPA^{hi} and HPA^{lo} piglets behaved similarly which is not surprising since porcine immune function was already shown to be relatively unaffected by cortisol level [45]. Nevertheless, we evidenced differences in some biological parameters between HPA^{lo} and HPA^{hi} piglets. It is worth noting that selection upon HPA axis activity changed the permanent neuroendocrine environment of the whole body including immune cells. Interestingly, despite well-described immunosuppressive effects of administrated glucocorticoids [46], piglets with higher cortisol levels (at steady-state and after social stress) did not display lower immune cell counts or decreased immune capacity. Three immune parameters were even higher in HPA^{hi} piglets: $CD4^+$ $CD8\alpha^-$ T lymphocyte number, PIL-induced IL-8 secretion and LPS-induced TNFα secretion in WBA. These results are promising since the ability of blood cells to produce key cytokines for host defence was proposed as a relevant indicator of host immunity. Indeed, reduced *ex-vivo* LPS-induced TNFα production was shown to be associated to presence of secondary bacterial infections and increased mortality in critically ill children with influenza [47]. Whether these parameters might confer an increased resistance to pathogens to HPA^{hi} piglets will have to be addressed.

Finally, by performing a principal component analysis on the whole data, we evidenced less pronounced overall effects of acute social stress in piglets genetically selected for a strong HPA axis. Thus selection upon adrenocortical axis activity seems to increase resilience of piglets to acute social stress. We still need to decipher if this results from decreased glucocorticoid sensitivity as already described in stressed mammals (reviewed in [48]).

5. Conclusion

We demonstrated for the first time that one-hour mixing induced leukocyte mobilization in piglets as observed in human patients following psychological stress. Acute social stress in piglets may thus represent a valuable tool to assess functional consequences of stress on immunity in controlled conditions.

Strikingly, stress-induced leukocyte mobilization was associated with impaired immune function. New experiments are mandatory to better characterize this “immune impairment”. Nevertheless, our study already demonstrates that avoiding stressful situations in pig farming could preserve immune capacity of piglets.

Finally, selection upon HPA axis activity seems to reduce the magnitude of the response to stress. Thus it appears as an interesting tool to increase piglet robustness in order to reduce prophylactic use of antibiotics. This is urgently needed since extensive use of antibiotics

participates to the emergence of resistant bacteria and dissemination of resistance genes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.physbeh.2016.11.012.

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