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RESEARCH

Time course of the response to ACTH in pig: biological and transcriptomic study

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Abstract

Background: HPA axis plays a major role in physiological homeostasis. It is also involved in stress and adaptive response to the environment. In farm animals in general and specifically in pigs, breeding strategies have highly favored production traits such as lean growth rate, feed efficiency and prolificacy at the cost of robustness. On the hypothesis that the HPA axis could contribute to the trade-off between robustness and production traits, we have designed this experiment to explore individual variation in the biological response to the main stress hormone, cortisol, in pigs. We used ACTH injections to trigger production of cortisol in 120 juvenile Large White (LW) pigs from 28 litters and the kinetics of the response was measured with biological variables and whole blood gene expression at 4 time points. A multilevel statistical analysis was used to take into account the longitudinal aspect of the data.

Results: Cortisol level reached its peak 1 h after ACTH injection. White blood cell composition was modified with a decrease of lymphocytes and monocytes and an increase of granulocytes ($FDR < 0.05$). Basal level of cortisol was correlated with birth and weaning weights. Microarray analysis identified 65 unique genes of which expression responded to the injection of ACTH (adjusted $P < 0.05$). These genes were classified into 4 clusters with distinctive kinetics in response to ACTH injection. The first cluster identified genes strongly correlated to cortisol and previously reported as being regulated by glucocorticoids. In particular, *DDIT4*, *DUSP1*, *FKBP5*, *IL7R*, *NFKBIA*, *PER1*, *RGS2* and *RHOB* were shown to be connected to each other by the glucocorticoid receptor NR3C1. Most of the differentially expressed genes that encode transcription factors have not been described yet as being important in transcription networks involved in stress response. Their co-expression may mean co-regulation and they could thus provide new patterns of biomarkers of the individual sensitivity to cortisol.

Conclusions: We identified 65 genes as biological markers of HPA axis activation at the gene expression level. These genes might be candidates for a better understanding of the molecular mechanisms of the stress response.

Keywords: Stress; Hypothalamic-pituitary-adrenal (HPA) axis; Cortisol; Time-course; Systems biology; Microarray; Pig

1

2

3 Background

4 Farm animals have been highly selected for favorable production traits such as lean
5 growth rate, feed efficiency, and prolificacy in pigs. In parallel their robustness has
6 tended to decrease, as shown by the sensitivity to diseases, locomotor weakness
7 or behavioral problems [1]. The hypothalamic-pituitary-adrenocortical (HPA) axis

8 plays a major role in physiological homeostasis including metabolism, brain func-
9 tion and behavior, the immune system and inflammatory processes. Together with
10 the autonomic nervous system, it is also involved in stress and adaptive responses
11 to environmental challenges. On the basis of available literature, we have hypothe-
12 sized that the HPA axis could contribute to the trade-off between production and
13 robustness traits, and that genetic variation in HPA axis activity could be used to
14 select more robust animals [2, 3]. HPA axis activity shows a large variation among
15 individuals and genetic influences are well documented [4]. For example, in pigs,
16 the sensitivity of the adrenal cortex to adrenocorticotrophic hormone (ACTH) and
17 the production of corticosteroid binding globulin (CBG), the carrier of cortisol in
18 blood are the two main mechanisms responsible for genetic differences in circulat-
19 ing cortisol levels [5, 6]. In a previous paper, Hazard *et al.* [7] have studied at the
20 gene expression level the molecular mechanisms of genetic differences in the adrenal
21 gland response to ACTH. However little is known about the individual variation in
22 the biological activity of cortisol, the main glucocorticoid hormone, and the genetic
23 mechanisms involved.

24 Corticosteroid hormones exert their biological actions via two intracellular recep-
25 tors (gluco- and mineralo-receptor) that, upon activation by their ligand, influence
26 the expression of a large number (several hundreds) of genes in a wide range of
27 cell types [8]. In pigs, glucocorticoid receptor polymorphisms have been described
28 with their functional consequences [9–13]. The present experiment was designed to
29 explore in pigs individual variation in the biological response to cortisol in order
30 to identify possible biomarkers of this sensitivity. To that end, juvenile pigs were
31 submitted to an ACTH challenge to release cortisol and the kinetics of the response
32 was measured with biological variables and with gene expression analysis in blood
33 cells. Taken together with biological information, this approach will serve as an im-
34 portant step to understand HPA axis regulation and will identify key genes involved
35 in signaling pathways relevant to stress responses. The final goal of this work is to
36 develop a strategy for further genetic studies in order to overcome the unfavourable
37 consequences of stress in farm animals.

38 **Animals and methods**

39 **Animals, treatment and blood sampling**

40 All animal use was performed under European Union and French legislation (di-
41 rective 201063UE, décret 2013-118). The protocol and procedures were approved
42 by the local (Poitou-Charentes) ethics committee (decision CE2013-1, 21012013).
43 Experimental animals were 120 piglets (63 females and 57 males) randomly selected
44 from 28 litters (4-5 animals per litter) of purebred Large White pigs and produced
45 in 3 successive batches raised 3 weeks apart. They were weaned at 4 weeks and
46 animals from 2-3 litters were mixed at weaning in each post-weaning pen. Experi-
47 mental animals were not isolated from their littermates. At 6 weeks, each animal was
48 injected in the neck muscles with 222 μg of synthetic 1-24 ACTH (Pepscan Presto
49 BV, Lelystad, The Netherlands) diluted in 1 mL of 0.9 % saline. Injections occurred
50 from 10:00 to 11:00 AM to avoid nycthemeral variations. Blood samples were col-
51 lected before the injection ($t = 0$) and 1 ($t = +1$), 4 ($t = +4$) and 24 ($t = +24$)
52 hours later. At each time, animals were slightly restrained on their back in such a

53 way that the effect on their stress level can be regarded as insignificant. Two blood
54 samples were then taken by puncture of a jugular vein in Vacutainer[®] tubes with
55 20 G needles. The whole handling procedure lasted less than 30 seconds. One 10
56 mL tube with lithium heparin was used for chemical biology. After centrifugation
57 (2355 g, 10 min), plasma aliquots were frozen at -80°C until analysis. One 5 mL
58 tube with EDTA (di-potassium salt) was used for blood cell count and an aliquot
59 (400 μ L) was mixed with the same volume of DL buffer (Macherey-Nagel), frozen
60 at -20°C for 4 h and then at -80°C until analysis for gene expression.

61 Biological analyses

62 Cortisol was measured by direct automated immunoassay (AIA-1800, Tosoh Bio-
63 science, San Francisco, CA). Glucose and free fatty acid (FFA), were measured by
64 colorimetry with an ABX Pentra 400 clinical chemistry analyzer from Horiba Med-
65 ical (Grabels, FR). Blood cell counts were measured with a MS-9-5 hematology
66 analyzer from Melet Schloesing Laboratories (Osny, FR), calibrated for pig blood
67 by the manufacturer. Blood cell count variables included: white cells count, pro-
68 portion of lymphocytes, monocytes and granulocytes, red cells count, percentage
69 of hematocrit, concentration of hemoglobin, red cells width and volume, concentra-
70 tion of platelets and platelets width and volume. The biological variables contained
71 thus 15 variables measured on the 120 pigs. In addition, birth and weaning weights
72 were also measured for each pig. Outlying observations were visually identified and
73 treated as missing data. Missing data were imputed using a k -nearest neighbour
74 ($k = 5$) imputation (R package **DMwR** [14]). To ensure normality, cortisol, platelets
75 and white cells count were \log_{10} transformed and FFA was transformed using the
76 square-root. Batch effects were removed by aligning the within-batch medians for
77 all measurements.

78 RNA extraction and whole blood gene expression analysis

79 A total RNA isolation and purification was done according to the manufacturer's
80 instructions using the Nucleospin RNA Blood kit (Macherey-Nagel, France) followed
81 by DNase treatment. The quality of each RNA sample was checked through the
82 Bioanalyser Agilent 2100 (Agilent Technologies, Massy, France) and low-quality
83 RNA preparations were discarded (RIN < 8).

84 *Microarray description*

85 A porcine microarray GPL16524 (Agilent, 8 \times 60K) was used to hybridize the RNA
86 samples as previously described [15]. This microarray contained 61625 spots. Among
87 them, 308 were negative controls and 49 were used for aligning. One probe was
88 duplicated twice on each array. Thus the microarray contained 60305 unique porcine
89 probes. After quality control, quantile normalization and filtering, 35429 transcripts
90 were found to be expressed in blood in our experimental conditions.

91 *Hybridization protocol*

92 Blood samples from 30 female pigs from only 2 batches were used. Each of the 15
93 arrays used contained 8 microarrays which corresponded to the 4 observations of two
94 individuals, each from one batch. This design secured the kinetics of the response

95 for each individual and prevented confounding effects between batch and array.
 96 After quality control and filtering, 35419 probes were kept and \log_2 transformed.
 97 Technical biases were handled by aligning the within-array medians for all genes
 98 and by a quantile normalization within animal (function **normalize.quantiles** in the
 99 R package **preprocessCore** [16]). No missing data were reported. Normalized data
 100 were submitted to GEO/NCBI with the GSE71207 accession number.

101 Statistical analyses

102 All analyses were performed with the R software, version 3.1.0 [17].

103 *Multilevel approach*

104 A multilevel approach was used to investigate the relationships between the re-
 105 peated measurements while taking advantage of multivariate approaches [18]. The
 106 multilevel approach, as described by Liquet *et al.* [18], uses a split-up variation com-
 107 ing from the mixed-model framework. Let $X = (x_{it}^k)_{i=1,\dots,n, t \in \{0,+1,+4,+24\}, k=1,\dots,p}$
 108 be the $(N \times p)$ observation matrix (biological variables or gene expressions) on n
 animals with 4 times of measurements ($N = n \times 4$). X can be split up as:

$$X = \underbrace{X_{..}}_{\text{offset term}} + \underbrace{X_b}_{\text{between-animal deviation}} + \underbrace{X_w}_{\text{within-animal deviation}} \quad (1)$$

109 The matrix $X_{..}$ represents the offset term defined as $1_N x_{..}^T$ where 1_N is a
 110 vector of length N containing only ones and $x_{..}^T = (x_{..}^1, \dots, x_{..}^p)$ (with $x_{..}^k =$
 111 $\frac{1}{N} \sum_{t \in \{0,+1,+4,+24\}} \sum_{i=1}^n x_{it}^k$). X_b is the between-animal matrix of size $(N \times p)$ defined
 112 by concatenating $1_4 x_{bi}^T$ for each animal into X_b with $x_{bi}^T = (x_{i.}^1 - x_{..}^1, \dots, x_{i.}^p - x_{..}^p)$
 113 ($x_{i.}^k = \frac{1}{4} \sum_{t \in \{0,+1,+4,+24\}} x_{it}^k$). $X_w = X - X_{i.}$ is the within-animal deviation matrix of
 114 size $(N \times p)$ with $X_{i.}$ the matrix defined by concatenating the matrices $1_4 x_{i.}^T$ for
 115 every animal into $X_{i.}$, with $x_{i.}^T = (x_{i.}^1, \dots, x_{i.}^p)$.

116 By splitting the different parts of the variation in the data while taking into
 117 account the repeated measurements on the subjects, the multilevel step allows us
 118 to study the effect of different conditions within a subject separately from the
 119 variation between subjects. This method is especially relevant when a high between-
 120 subject variability is observed in repeated data: multivariate approaches (such as
 121 principal components analysis, PCA [19] and partial least square regressions, PLS
 122 [20]) can then be performed on X_w to highlight the most relevant correlations
 123 between variables in the dataset, independently from individual variations.

124 *Statistical analysis of plasma metabolites and hormone*

125 First, all variables were subjected to a one-way ANOVA with repeated measures. P-
 126 values were adjusted using a Benjamini-Hochberg (BH) approach in order to control
 127 the false discovery rate (FDR) [21]. Variables with an adjusted P-value (FDR)
 128 < 0.05 were then subjected to 3 paired t-tests to assess the difference between $t = 0$
 129 and $t = +1$, between $t = 0$ and $t = +4$ and between $t = 0$ and $t = +24$. The full
 130 list of P-values was adjusted using a BH approach.

131 In addition, the influence of sex on the biological variables was tested using a
132 two-way ANOVA with repeated measures including sex as a variable. P-values were
133 adjusted using a BH approach.

134 Then, variability between individuals before the ACTH injection was studied by
135 performing a PCA on the observations at $t = 0$ for variables responding to ACTH
136 and birth and weaning weights. The overall effect of ACTH over time was investi-
137 gated with a multilevel PCA as previously described.

138 Cortisol levels at $t = +1$ is the most relevant measure to assess the sensitivity
139 of the adrenals to ACTH. Hence, correlations between biological variables at $t \in$
140 $\{0, +1, +4, +24\}$ and the level of cortisol at $t = +1$ were investigated using paired
141 t-tests. P-values were adjusted using a BH approach.

142 *Statistical analysis of the transcriptome*

143 All transcripts were subjected to 3 paired t-tests to assess the difference in expres-
144 sion between $t = 0$ and $t = +1$, between $t = 0$ and $t = +4$ and between $t = 0$ and
145 $t = +24$. The full list of P-values was adjusted using a Bonferroni approach. As
146 the Bonferroni approach exerts a more stringent control than the BH approach, it
147 was used to obtain a narrowed list of the most significantly differentially expressed
148 (DE) transcripts. Transcripts with at least one adjusted P-value < 0.05 among
149 the three tests were kept. Correlations between the expression levels of different
150 transcripts of the same gene were investigated to highlight genes for which at least
151 $3/4$ of the duplicates were significantly DE and had a correlation of at least 0.65
152 between them. The most significant transcripts per annotated gene were kept and
153 the multilevel approach was used to extract the within-subject deviation matrix for
154 further analysis.

155 A hierarchical ascending classification (HAC) was performed using the Ward
156 method with an Euclidean distance matrix based on the correlations between genes.
157 This allowed for the characterization of clusters of genes having the same evolution
158 over time. Significance of the difference between time measurements was assessed
159 using 3 paired t-tests at the average gene level between $t = 0$ and $t = +1$, between
160 $t = 0$ and $t = +4$ and between $t = 0$ and $t = +24$. P-values were adjusted within
161 the clusters using a BH approach.

162 *Integration*

163 Relations between the main stress variable, the cortisol, the biological variables and
164 the gene expressions were studied using different approaches.

As for biological data, correlations between gene expression at $t \in$
 $\{0, +1, +4, +24\}$ and the level of cortisol at $t = +1$ were investigated. More pre-
cisely, the within-subject components of the transcriptomic data and of the cortisol
level was extracted using the method described in [18] and Section [Multilevel ap-
proach](#). Then, a paired t-test was performed between the within-subject expression
at $t \in \{0, +1, +4, +24\}$ and the within-subject level of cortisol at $t = +1$. The
full list of P-values was globally adjusted using a BH approach. In order to assess
the significativity of change in expression after removing the contribution of cell
population changes, a linear mixed model was fitted for every DEG

$$x_{it} = \beta_0 + \beta_{1,t} + \beta_2(L/G)_{it} + U_i + \epsilon_{it}$$

165 where x_{it} is the expression of the DEG for the animal number i ($i = 1, \dots, 120$)
166 and the time step t ($t \in \{0, +1, +4, +24\}$), $(L/G)_{it}$ is the lymphocytes/granulocytes
167 ratio for the same experiment and U_i is the individual random effect. Both time step
168 (as a factor) and $(L/G)_{it}$ were supposed to have fixed effects on gene expression.
169 Significance of the time effect in this model was checked by testing $\beta_{1,t} = \beta_{1,0}$ for
170 $t \in \{+1, +4, +24\}$ and correcting p-values by time point for multiple tests with a
171 BH approach ($FDR < 0.05$). The effect of changes in white cell populations was
172 also assessed by testing $\beta_2 = 0$ and a correction for multiple tests was applied using
173 a BH approach ($FDR < 0.05$).

174 A multilevel PLS, *i.e.*, a PLS performed on the within-subject components of
175 the biological and transcriptomic data, as described in [18] and Section [Multilevel](#)
176 [approach](#), was used to investigate the overall relationships between biological and
177 transcriptomic data. A sparse version of the PLS (L^1 penalty) as described in [20]
178 was used to select a small subset of variables to explain every axis.

179 Sequence annotation

180 Each cDNA sequence was compared to Refseq_rna mammalian database using the
181 NCBI blastn program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Resulting
182 hits were sorted out according to their closeness to the pig genome, their coverage
183 and sequence identity. The selected cDNA sequences were submitted to the HUGO
184 (Human Genome Organization) gene nomenclature committee, using their RefSeq
185 IDs (<http://genenames.org>). Then, HUGO gene symbols or official gene symbol
186 were used as gene names.

187 Functional enrichment and pathway analysis

188 Functional enrichment was performed on each list of clustered genes identified by
189 HAC and on the list of genes of which expression is significantly explained by
190 cell population changes in blood (according to the mixed model described in Sec-
191 tion [“Integration”](#)). Functional annotation of genes was provided by the BioMart
192 database [22]. To set the statistical enrichment of a particular biological function, a
193 Fisher’s exact test was performed, using the list of genes expressed on the microar-
194 ray as the reference list of genes. Resulting P-values were adjusted for multiple tests
195 using a BH approach. A minimum of 3 genes per gene ontology and a $FDR < 0.05$
196 were necessary to consider a biological function to be enriched.

197 A pathway is an interconnected arrangement of processes, representing the func-
198 tional roles of genes in the genome. Functional integration of gene expression, *i.e.*,
199 identification of gene networks, was performed using the ‘Gene Ontology’ database
200 AmiGO (<http://amigo.geneontology.org/cgi-bin/amigo/go.cgi>). The signif-
201 icantly up- or down-regulated genes could be assembled into networks using ‘Inge-
202 nuity Pathway Analysis’ (<http://www.ingenuity.com>) under licence. This applica-
203 tion provides computational algorithms to identify the enriched biological pathways,
204 functions and biological mechanisms of selected genes and proposes also enriched
205 regulators as transcription factors.

206 A regulatory network could be constructed with the information provided by the
207 option ‘Upstream Regulator’. This option proposes a list of regulators known to
208 have a significant effect on some of the targeted genes in the input list. Ingenuity

209 also provides computational algorithms to identify and to dynamically generate sig-
210 nificant biological networks. Networks are ranked by a score that takes into account
211 the number of focus genes and the size of the networks. This score ($-\log_{10}(\text{p-value})$)
212 indicates the probability for genes to be associated in the same network by chance.
213 A higher score means a smaller probability for genes to be observed in the same net-
214 work by chance. We chose networks displaying direct relationships between genes.
215 Path Designer (an Ingenuity tool) was used to improve the readability of the net-
216 works. Nodes added by Ingenuity were discarded when they were not necessary
217 to connect our genes of interest and the resulting network was merged with the
218 regulatory network.

219 Results and discussion

220 Plasma cortisol, metabolites and hematology

221 Table 1 shows baseline values of the biological variables, birth weight and weaning
222 weight. Mean evolution over time of the biological variables is shown in Figure 1. A
223 global effect of time was observed for 14 out of the 15 biological variables ($FDR <$
224 0.05). However, when considered at each time point individually, only 9 variables
225 presented a level significantly different from their basal level for at least one time
226 point ($t = +1, +4$ or $+24$).

227 As expected [23], ACTH induced a strong cortisol response peaking 1 h after
228 injection ($FDR = 1.39e - 10$). Plasma cortisol levels at $t = +1$ were 2.7-fold higher
229 than and highly correlated with basal levels ($r^2 = 0.63$, $FDR = 1.95e - 14$). They
230 were significantly lower than baseline values at $t = +4$ ($FDR < 2.2e - 16$) and at
231 $t = +24$ ($FDR = 1.39e - 10$), as can be expected from the feedback of cortisol
232 on its own secretion. Plasma glucose levels showed a slight increase at $t = +4$
233 after ACTH injection, but FFA levels showed a large increase at $t = +1$ ($\times 3.21$,
234 $FDR < 2.2e - 16$), with a strong variation across animals, and were almost back
235 to basal levels at $t = +4$. The values measured at $t = +1$ were correlated with
236 basal values ($r^2 = 0.42$, $FDR = 2.30e - 06$). Cortisol induces a weak increase
237 in circulating glucose and also potentiates the effect of other counter-regulatory
238 hormones [24, 25] and increases FFA levels via acute lipolysis [26].

239 The data obtained here for clinical hematology measures are in the range of pub-
240 lished values in pigs. These values show large variations with age and breed among
241 other sources [27–29]. Although the total number of leucocytes was only marginally
242 influenced by ACTH, large changes in leucocyte subpopulations were observed with
243 an increase of the proportion of granulocytes and a decrease of the proportion of
244 lymphocytes and monocytes at $t = +1$ and $t = +4$. These effects are consistent
245 with the literature [30, 31] and result from the redistribution of leucocytes between
246 blood and tissues [32]. Red blood cell related variables (red cells count, hematocrit
247 and hemoglobin levels) were decreased after injection of ACTH and remained low
248 at $t = +24$. Platelets were not influenced by injection of ACTH.

249 Sex did not influence any of the variables ($FDR > 0.05$).

250 Between-subject variability at $t = 0$

251 Results of the PCA at $t = 0$ on variables responding to ACTH and birth and
252 weaning weights are shown in Figure 2. As red cells count (RC), hematocrit (Hct)

253 and hemoglobin (Hgb) are redundant variables, decision was made to keep only RC
254 for the PCA. No outliers are identified on the projection of the individuals on the
255 two first dimensions of the PCA. On this PCA, sex was not found to be related to
256 the main variability (*i.e.*, to the first axes of the PCA) in the dataset.

257 The first dimension shows an opposition between the proportion of granulocytes
258 (positively correlated with this axis) and the proportion of lymphocytes (negatively
259 correlated with this axis). The second axis shows an opposition between cortisol
260 (positively correlated with this axis) and birth and (to a lesser extent) weaning
261 weights (negatively correlated with this axis) with a strong opposition between
262 these variables on the whole plan formed by the first and second axis. The other
263 variables were not correlated with either of the first two dimensions of the PCA.

264 Overall effect of the injection of exogenous ACTH on clinical biology variables

265 Extraction of the within-subject data matrix prior to the application of a PCA
266 analysis allows for the separation of the observations according to their time of
267 measurement (see Figure 3). The first component of the multi-level PCA opposes
268 the observations at $t = 0$ and $t = +24$ (positive coordinates on this axis) to the
269 observations at $t = +1$ (negative coordinates on this axis), this time step corre-
270 sponding to the peak of cortisol. The second component opposes the observations
271 at $t = +4$ (positive coordinates on this axis) to the observations at $t = +1$ (negative
272 coordinates on this axis). The representation of the variables shows that the first
273 axis is mainly driven by an opposition between the proportion of granulocytes, FFA,
274 cortisol and red cell count (high measures at $t = +1$), on one side, and lympho-
275 cytes and monocytes (high measures at $t = 0/+24$), on the other side. The second
276 axis shows an opposition between glucose (positively correlated with this axis, high
277 measures at $t = +4$) and cortisol, FFA and red count (negatively correlated with
278 this axis, high measures at $t = +1$).

279 Specific links to the level of cortisol at $t = +1$

280 Correlations between cortisol at $t = +1$ and other variables at $t = 0$ allows for the
281 identification of variables which baseline levels may be directly or indirectly linked
282 to the intensity of the cortisol level in response to ACTH, a measure of individual
283 variation in HPA axis activity. Correlations are shown in Table 2. Only glucose and
284 FFA levels at $t = 0$ were significantly positively correlated with the level of cortisol
285 at $t = +1$ ($FDR < 0.05$). Correlations between cortisol at $t = +1$ and other
286 variables at $t = +1$, $t = +4$ or $t = +24$ allows for the identification of variables
287 which are directly linked to the level of cortisol when it reaches its peak during the
288 stress response. FFA at $t = +1$ were positively correlated with cortisol at $t = +1$
289 and glucose at $t = +1$, $t = +4$ and $t = +24$ was negatively correlated with cortisol
290 at $t = +1$ ($FDR < 0.05$). No other variable was found to be significantly linked to
291 the intensity of the cortisol level in response to ACTH.

292 Differentially expressed genes

293 We used a comprehensive gene expression profiling by means of microarray analy-
294 sis to identify clusters of genes differentially expressed in peripheral blood cells,
295 taking into consideration the kinetics of the response with 4 time points ($t \in$

296 {0, +1, +4, +24}). Differential analysis revealed 158 DE transcripts (adjusted $P <$
297 0.05) matching 65 unique genes. Among them, 23 genes were differentially expressed
298 at $t = +1$ (5 down regulated / 18 up-regulated), 25 were differentially expressed at
299 $t = +4$ (8 down-regulated / 17 up-regulated) and 17 were differentially expressed
300 at $t = +24$ (all down-regulated). The only gene DE at both $t = +1$ and $t = +4$ was
301 *SUCNR1* (Table 3). The adjusted P-values were smaller for tests between $t = 0$ and
302 $t = +1$ and between $t = 0$ and $t = +4$ than between $t = 0$ and $t = +24$ (see [Addi-](#)
303 [tional file 2 — Distribution of the rank of the significant adjusted P-values in the](#)
304 [tests for DE transcripts between \$t = 0\$ and \$t = +1\$, \$t = 0\$ and \$t = +4\$ and \$t = 0\$](#)
305 [and \$t = +24\$ ‘.pdf’ file](#)). This shows that the transcripts were more differentially
306 expressed between $t = 0$ and $t = +1$ and between $t = 0$ and $t = +4$ than between
307 $t = 0$ and $t = +24$. Main effects of cortisol released by ACTH injection on gene
308 expressions are thus observed at $t = +1$ and $t = +4$ with a return to baseline levels
309 at $t = +24$.

310 HAC performed on the within-subject deviation matrix with the list of DE genes
311 identified 4 groups of genes. Figure 4 shows that the 65 unique DE genes allow
312 for an almost perfect classification of the observations with respect to their time of
313 measurement. For every cluster, Figure 5 shows the average evolution of each gene
314 (gray) and the average evolution over the genes in the cluster (red).

315 Each cluster was then subjected to a functional analysis (results shown in [Addi-](#)
316 [tional file 3 — Complete list of enriched GO \(Biological process \(BP\), Molecular](#)
317 [function \(MF\) and Cellular Component \(CC\) for each of the cluster identified with](#)
318 [the hierarchical ascending clustering ‘.xls’ file](#)). In each cluster, genes were DE
319 ($FDR < 0.01$) at each time point except for $t = +24$ in cluster 3 ($FDR = 0.57$).

320 The first cluster (17 genes) was characterized by genes increasing with a peak of
321 expression at $t = 1$ and stable between $t = +4$ and $t = +24$. The DE genes of
322 this cluster could be assembled into a functional network principally involved in
323 neuroimmune functions. The present analysis reveals novel effects of ACTH on at
324 least five genes related to immunoregulation (*FKBP5*, *IL7R*, *CEBPD*, *CEBPB* and
325 *NFKB1A* in cluster 4). *FKBP5* (FK506 binding protein 51) is a decisive factor for
326 the physiological stress response [33] and has an important role in stress-related phe-
327 notypes [34]. It modifies steroid hormone receptor sensitivity [35]. *CEBPB*, *DUSP1*,
328 *FKBP5* and *NFKB1A* genes from this cluster are also involved in glucocorticoid
329 receptor signaling. Glucocorticoids exert their classic anti-inflammatory role by act-
330 ing on nearly all cell types of the immune system. The CCAAT/enhancer binding
331 proteins (C/EBPs) are key regulators of cell differentiation and are also involved
332 in the expression and production of inflammatory cytokines [36]. The increase of
333 Period 1 gene (*PER1*) expression in peripheral blood cells by glucocorticoids was
334 previously reported in humans [37]. Physical and inflammatory stressors induce the
335 release of the adrenal glucocorticoid hormone that rapidly alter the expression of
336 *PER1* in peripheral tissues through a GRE enhancer present in the gene promo-
337 tor [38–40]. This gene is involved in the circadian rhythm, in which the glucocorticoid
338 mechanism plays a predominant role [41]. Another DE gene *DDIT4* (regulated in
339 development and DNA damage response 1) was described as a surrogate biomarker
340 of the efficiency of glucocorticoid receptor blockade in skeletal muscle [42]. Britto
341 and collaborators showed that *DDIT4* expression was low under basal conditions

342 but was highly increased in response to several catabolic stressors, like hypoxia and
343 glucocorticoids [43]. Glucocorticoids were shown to up-regulate *DUSP1* in periph-
344 eral tissues [44] but constrain the increase of *DUSP1* gene expression in the central
345 components of the HPA axis [45]. *In vitro* studies have shown that glucocorticoid
346 suppression of some MAP-kinase dependent cellular processes depends on glucocor-
347 ticoid mediated up-regulation of *DUSP1* gene expression [46].

348 The second cluster (17 genes) was characterized by genes with an increase between
349 $t = 0$ and $t = +4$ and a decrease between $t = +4$ and $t = +24$. This cluster with
350 genes up-regulated at $t = +4$ is largely related to biological processes such as in-
351 flammatory and immune response and genes of which products are located in the
352 plasma membrane. Among these genes, two are particularly interesting. *CD14* gene
353 is a component of the innate immune system and has been shown to be sensitive
354 to stress in pigs [47]. *MEGF9* gene was shown to be induced by cortisol in human
355 fetal cells *in vitro* [48].

356 The third cluster (8 genes) includes the genes decreasing between $t = 0$ and $t = +4$
357 and returning to a basal level between $t = +4$ and $t = +24$. No ontology was
358 significantly enriched by genes of this cluster. It is interesting to underline here
359 the *ALOX15* gene (arachidonate 15-lipoxygenase) which is a member of the ALOX
360 family and related to cancer and immune responses. This gene was also reported
361 as a dexamethasone-responsive gene with nearby glucocorticoid receptor-binding
362 sites [49].

363 The genes related to the fourth cluster (22 genes) decrease between $t = 0$ and
364 $t = +1$, increase between $t = +1$ and $t = +4$ and decrease between $t = +4$ and
365 $t = +24$. The fourth cluster corresponds to genes with an overall expression decreas-
366 ing between $t = 0$ and $t = +24$. They are significantly linked to biological processes
367 such as protein phosphorylation and kinase activity. Among the genes involved in
368 this cluster *ARHGAP31* and *ARHGAP* family genes were found to be differentially
369 expressed in macrophages treated with dexamethasone [50, 51].

370 Integration of biological and gene expression data

371 All DE genes were found significantly differentially expressed over time in the mixed
372 model described in Section “[Integration](#)” ($FDR < 0.05$). These genes are thus
373 differentially expressed over time even when adjusting for changes in white cells
374 populations. Among them, 34 genes had their expression significantly negatively
375 influenced by L/G ratio (see [Additional file 1 — List of 65 unique genes differen-](#)
376 [tially expressed in response to ACTH in pigs \(\$n = 30\$ \). ‘.xls’ file](#)), meaning that
377 these genes are over-expressed when L/G ratio decreases. Genes with a significant
378 effect of L/G ratio were mainly identified as genes of cluster 2, over-expressed at
379 $t = +4$ (17/17) and cluster 1, over-expressed at $t = +1$ (12/18) and to a lesser
380 extent as genes of cluster 4, under-expressed at $t = +24$ (5/22). No gene of clus-
381 ter 3 was significantly explained by L/G ratio. Results of the functional analysis
382 of this list of 34 genes are shown in [Additional file 4 — Complete list of enriched](#)
383 [GO \(Biological process \(BP\), Molecular function \(MF\) and Cellular Component](#)
384 [\(CC\) for 34 genes for which L/G ratio had a significant effect. ‘.xls’ file](#). Biological
385 functions significantly enriched include regulation of apoptotic process, response to
386 lipopolysaccharide, inflammatory and innate immune response, defense response to
387 bacterium and positive regulation of NF-kappaB transcription factor activity.

388 The 65 DE genes and the biological variables were then subjected to a multilevel
389 PLS. Figure 6 shows that the first axis of the multilevel PLS opposes observations
390 at $t = +1$ after injection to all others, while the second axis opposes observations at
391 $t = +4$ *vs.* all others, similarly as what was already established in multi-level PCA
392 of the biological variables in Section “Overall effect of the injection of exogenous
393 ACTH on clinical biology variables”.

394 On the first axis, cortisol and FFA levels are strongly positively correlated with
395 the expressions of *CEBPB*, *RGS2*, *RHOB*, *PER1*, *FKBP5*, *CEBPD*, *DDIT4*, *CPT1A*
396 and *DUSP1*. All these genes belong to the first cluster identified earlier and are
397 linked to molecular functions such as protein binding and transcription regulation.

398 The second axis of the multilevel PLS is characterized by the opposition between
399 the proportion of lymphocytes and monocytes *vs.* the proportion of granulocytes.
400 This axis is positively correlated with *SUCNR1*, *SLCO2B1*, *FBP1* and *LOC396700*.
401 These genes belong to the third cluster and are related to glycolysis and glycoge-
402 nesis. *SUCNR1* (succinate receptor 1) is decreased at $t = +1$ and increased at
403 $t = +4$. Succinate has a wide range of metabolic actions and regulates the functions
404 of macrophages [52]. The axis is negatively correlated with *CD14*, *CLC4D*, *CHIT1*,
405 *MEGF9* and *C2H19orf59*. These genes belong to the second cluster which is linked
406 to molecular functions such as inflammatory response, but their relationships with
407 cortisol or stress are not yet clearly established.

408 Eight genes (*DDIT4*, *DUSP1*, *FKBP5*, *IL7R*, *NFKBIA*, *PER1*, *RGS2*, *RHOB*, Fig-
409 ure 7) are functionally connected to each other by NR3C1. The NR3C1 (nuclear
410 receptor subfamily 3, group C, member 1) is the glucocorticoid receptor, which
411 can function both as a transcription factor that binds to glucocorticoid response
412 elements in the promoters of glucocorticoid responsive genes, and as a regulator of
413 other transcription factors. Functional consequences of glucocorticoid receptor poly-
414 morphisms were reported in pigs [9–13]. Mutations in *NR3C1* have been previously
415 demonstrated to be associated with generalized glucocorticoid resistance [53]. It is
416 interesting to highlight the DE genes that encode transcription factors. They play a
417 crucial role in regulating gene expression and are fit to regulate diverse cellular pro-
418 cesses by interacting with other proteins. Most of them have not yet been described
419 as important in transcription networks involved in stress responses. If the genes are
420 co-expressed it is highly probable that they are co-regulated. This knowledge can
421 provide new patterns of biomarkers of the individual sensitivity to cortisol that is
422 our field of interest in this study.

423 Our results are in accordance with several studies on the effects of glucocorticoid
424 hormones on peripheral blood cells. Numerous genes related to Cluster 1 and shown
425 as ACTH responsive were found differentially expressed in stress-related investiga-
426 tions. Five genes found in our study (*CXCR4*, *DUSP1*, *FKBP5*, *IL7R*, *TXNIP*)
427 were proposed as markers of differential glucocorticoid sensitivity [54, 55]. *NFK-*
428 *BIA*, *DUSP1*, *CEBPD*, *FKBP5* genes were also found to be associated with up-
429 and down-regulated clusters in response to continuous 24 h cortisol infusion [56].
430 Ponsuksili and collaborators [57] describe *NFKBIA*, *CEBPB* and *CEBPD* as genes
431 of which hepatic expression levels are correlated with plasma cortisol concentrations.
432 Up-regulation of *PER1* gene upon GR activation was confirmed by genome-wide
433 study of glucocorticoid receptor binding sites in neuronal PC12 cells [58]. However,

434 *DDIT4* was shown to be down-regulated by GR activation rather than up-regulated
435 in this analysis.

436 While looking for genes of which expressions at $t = 0$, $t = +1$, $t = +4$ or $t = +24$
437 were significantly correlated with the level of cortisol at $t = +1$, only two genes
438 were identified: *TRMT2A* ($FDR = 0.04$), a gene involved in the methylation of
439 tRNA, and *LOC100626276* ($FDR = 0.04$), a gene of which function has not been
440 identified yet. There is a negative relationship between the expression of cortisol
441 at $t = +1$ and the expression of these two genes at $t = 0$ (-0.45 and -0.63 for
442 *TRMT2A* and *LOC100626276*, respectively). This implies that when their baseline
443 expression is higher, the intensity of the cortisol response to ACTH decreases.

444 The present work shows the interest of transcriptomic data analysis at multiple
445 levels. In other studies, genetic markers found through an analysis of transcription
446 factor binding sites of differentially expressed genes in peripheral blood cells have
447 been proposed in humans to identify the chronic stress related to psychopatholog-
448 ical conditions [59, 60]. In farm animals, this approach was used in horses [61].
449 These studies show chronic stress-related changes in the balance between the ex-
450 pression of stress-related genes regulated by glucocorticoids and those regulated by
451 inflammation-related factors. Furthermore, recent data in humans show that the im-
452 mune system function can also be assessed through blood transcriptomics in health
453 and disease [62].

454 In the present study, we identified 65 genes differentially expressed in periph-
455 eral blood cells of pigs in response to ACTH at different times after injection. It
456 therefore supplies biological markers of HPA axis activation at the gene expression
457 level, and the knowledge on functional gene clusters will help to elucidate the bio-
458 logical processes involved. Moreover, these genes might be candidates for a better
459 understanding of the molecular mechanisms related to stress responses. Thus, blood
460 transcriptome analysis appears as a promising avenue to develop multidimensional
461 biological markers related to robustness. These markers should be used in the study
462 of the genetic mechanisms of adaptation in farm animals that will help to deliver
463 genetic strategies to animal breeders in order to balance production objectives and
464 robustness of animals as well as their welfare [2].

465 Availability of supporting data

466 The data sets supporting the results of this article are available in Gene Expression Omnibus (GEO repository,
467 <http://www.ncbi.nlm.nih.gov/geo/>) through the accession number GSE71207).

468 Abbreviations

469 ACTH: Adrenocorticotrophic hormone; BH: Benjamini-Hochberg; BW: Birth weight; CBG: Corticosteroid binding
470 globulin; DE: Differentially expressed; FDR: False discovery rate; GR: Glucocorticoid receptor; GRE: Glucocorticoid
471 response element; HAC: Hierarchical ascendant classification; Hct: Hematocrit; Hgb: Hemoglobin; HPA:
472 hypothalamic-pituitary-adrenocortical; L/ G: lymphocytes/ granulocytes; PCA: Principal component analysis; PLS:
473 Partial least square regression; RC: Red cells; SEM: Standar error of the mean; WW: Weaning weight;

474 Competing interests

475 The authors declare that they have no competing interests.

476 Author's contributions

477 VS and NVV developed and performed all the statistics. PM and ET developed and undertook the experimental
478 design and ensured the biological interpretation. LG, ET and LL ensured the experimental transcriptomic analysis.
479 YL provided the transcriptomic data set VS, ET and LL performed the transcriptomic related biology interpretation.
480 LG performed the sampling and the data management. CL performed the genetics and the choice of animals. YB
481 was responsible of the animal management. VS, NVV, ET, PM and LL wrote the paper. PM is the project manager.

482 All authors read and approved the final manuscript.

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648 **Figures**

649 Tables

Table 1 Reference values (at $t = 0$) for the biological variables, birth weight and weaning weight ($n = 120$). F value and FDR are for the test of the global time effect on each variable. NR: non relevant since the measure is the same at all time steps.

	units	min	max	mean	sem	F value	FDR
Cortisol	log10(ng/mL)	1.04	1.94	1.55	0.04	445.27	< 0.01
Free fatty acids	(mmol/L) ²	0.03	0.39	0.16	0.01	327.69	< 0.01
Glucose	mmol/L	5.62	9.84	7.67	0.17	24.36	< 0.01
White cells	log10(G/L)	0.96	1.50	1.23	0.02	2.90	0.04
Lymphocytes	%	38.95	69.53	56.63	1.12	50.59	< 0.01
Monocytes	%	5.70	11.15	7.82	0.19	15.44	< 0.01
Granulocytes	%	18.40	52.70	34.20	1.10	51.96	< 0.01
Red cells	T/L	3.88	6.60	5.48	0.08	139.26	< 0.01
Mean corpuscular volume	fL	43.10	65.22	53.67	0.77	29.66	< 0.01
Hematocrit	%	20.12	36.96	28.74	0.48	165.87	< 0.01
Hemoglobin	g/dL	7.70	12.10	9.75	0.14	155.15	< 0.01
Red blood cell distribution width	fL	30.25	35.05	32.56	0.15	17.89	< 0.01
Platelets	log10(G/L)	1.85	3.13	2.61	0.03	14.07	< 0.01
Mean platelet volume	fL	8.00	14.20	10.05	0.23	0.68	0.57
Platelet distribution width	%	9.90	11.90	10.76	0.09	4.08	< 0.01
Birth weight	Kg	0.40	2.68	1.50	0.07	NR	NR
Weaning weight	Kg	5.46	16.56	9.4	0.35	NR	NR

Table 2 Correlation coefficients between the biological variables at $t = 0$, $t = +1$, $t = +4$ and $t = +24$ and cortisol at $t = +1$ ($n = 120$). (SE): standard error of the correlation coefficient; **in bold**: significantly $\neq 0$ ($FDR < 0.05$)

variables	$t = 0$ (SE)	$t = +1$ (SE)	$t = +4$ (SE)	$t = +24$ (SE)
Free fatty acids	0.35 (<0.07)	0.45 (<0.07)	-0.13 (0.09)	0.04 (0.09)
Glucose	0.30 (0.08)	-0.25 (0.08)	-0.27 (0.08)	-0.27 (0.08)
Lymphocytes	-0.09 (0.09)	-0.10 (0.09)	-0.13 (0.09)	-0.07 (0.09)
Monocytes	-0.05 (0.09)	-0.02 (0.09)	-0.05 (0.09)	-0.04 (0.09)
Granulocytes	0.12 (0.09)	0.14 (0.09)	0.15 (0.09)	0.09 (0.09)
Red cells	0.06 (0.09)	0.07 (0.09)	-0.11 (0.09)	0.06 (0.09)
Hematocrit	0.06 (0.09)	0.13 (0.09)	-0.08 (0.09)	0.12 (0.09)
Hemoglobin	0.13 (0.09)	0.18 (0.08)	-0.15 (0.09)	0.03 (0.09)

Table 3 List of 65 unique genes differentially expressed in response to ACTH in pigs ($n = 30$). Genes are divided into clusters corresponding to the kinetics of the response to ACTH. Full description of the genes including their probe name and localisation is displayed in [Additional file 1 — List of 65 unique genes differentially expressed in response to ACTH in pigs \(\$n = 30\$ \). 'xls' file.](#)

	Gene name	adjusted P	Time point	Expression	Cluster
1	ADCY2	1.87E-03	1	UP-regulated	1
2	CEBPB	3.72E-09	1	UP-regulated	1
3	CEBPD	6.65E-07	1	UP-regulated	1
4	CPT1A	2.28E-06	1	UP-regulated	1
5	CXCR4	9.47E-06	1	UP-regulated	1
6	DDIT4	3.96E-07	1	UP-regulated	1
7	DUSP1	7.53E-03	1	UP-regulated	1
8	FKBP5	4.39E-06	1	UP-regulated	1
9	G30866	8.88E-05	1	UP-regulated	1
10	G39878	9.56E-04	1	UP-regulated	1
11	IL7R	6.63E-05	1	UP-regulated	1
12	MXD1	1.71E-03	1	UP-regulated	1
13	NFKBIA	2.72E-03	1	UP-regulated	1
14	PER1	9.37E-05	1	UP-regulated	1
15	PIK3IP1	2.89E-04	1	UP-regulated	1
16	RGS2	8.52E-08	1	UP-regulated	1
17	RHOB	4.08E-02	1	UP-regulated	1
18	TXNIP	1.78E-03	1	UP-regulated	1
19	ALOX5AP	1.20E-03	4	UP-regulated	2
20	ANG1	1.92E-02	4	UP-regulated	2
21	BASP1	4.11E-02	4	UP-regulated	2
22	C2H19orf59	1.06E-02	4	UP-regulated	2
23	CD14	3.99E-04	4	UP-regulated	2
24	CD24	1.82E-04	4	UP-regulated	2
25	CHI3L1	1.73E-02	4	UP-regulated	2
26	CHIT1	2.16E-02	4	UP-regulated	2
27	CLC4D	2.00E-03	4	UP-regulated	2
28	CRLD2	4.40E-02	4	UP-regulated	2
29	G42218	6.47E-03	4	UP-regulated	2
30	MEGF9	1.92E-04	4	UP-regulated	2
31	PDPN	2.08E-02	4	UP-regulated	2
32	RAB31	2.74E-02	4	UP-regulated	2
33	S100A12	5.47E-03	4	UP-regulated	2
34	S100A8	5.26E-03	4	UP-regulated	2
35	S100A9	2.92E-03	4	UP-regulated	2
36	CCL8	1.36E-04	1	DOWN-regulated	3
37	ALOX15	2.03E-07	4	DOWN-regulated	3
38	CAMK1	2.84E-09	4	DOWN-regulated	3
39	CSTA	9.20E-09	4	DOWN-regulated	3
40	FBP1	1.03E-04	4	DOWN-regulated	3
41	G36094	6.98E-10	4	DOWN-regulated	3
42	SLCO2B1	5.40E-13	4	DOWN-regulated	3
43	SUCNR1	2.58E-08	1 & 4	DOWN-regulated	3
44	CD79B	7.92E-04	1	DOWN-regulated	4
45	HHEX	3.32E-02	1	DOWN-regulated	4
46	MZB1	5.04E-03	1	DOWN-regulated	4
47	ST14	2.51E-02	1	DOWN-regulated	4
48	LOC396700	3.78E-06	4	DOWN-regulated	4
49	AKAP13	1.89E-02	24	DOWN-regulated	4
50	ARHGAP31	2.37E-03	24	DOWN-regulated	4
51	CLK1	2.52E-02	24	DOWN-regulated	4
52	DCAF15	1.56E-02	24	DOWN-regulated	4
53	FGF	6.95E-03	24	DOWN-regulated	4
54	G48605	8.08E-04	24	DOWN-regulated	4
55	HOPX	1.54E-02	24	DOWN-regulated	4
56	IGLV_7	3.07E-02	24	DOWN-regulated	4
57	LAS1L	4.12E-02	24	DOWN-regulated	4
58	LOC100626276	1.25E-03	24	DOWN-regulated	4
59	LOC396781	3.36E-02	24	DOWN-regulated	4
60	MAPK6	1.49E-03	24	DOWN-regulated	4
61	ORAI1	1.83E-03	24	DOWN-regulated	4
62	S100A1	5.43E-04	24	DOWN-regulated	4
63	TPST2	9.40E-04	24	DOWN-regulated	4
64	TRMT2A	3.61E-02	24	DOWN-regulated	4
65	XCL1	3.01E-02	24	DOWN-regulated	4

650 **Additional Files**

651 Additional file 1 — List of 65 unique genes differentially expressed in response to ACTH in pigs ($n = 30$). '.xls' file.
652 Genes are divided into clusters corresponding to the kinetics of the response to ACTH. Probe names are those used
653 on the microarray Agilent GPL16524.

- 654 • Gene name: Name of the gene
- 655 • Probe Name, Agilent GPL16524: Probe names used on the microarray Agilent GPL16524
- 656 • L/G coefficient: L/G ratio coefficient estimate
- 657 • Adjusted pval (LG): adjusted P-value associated with the L/G ratio coefficient
- 658 • adj.pval: adjusted P-value of the test at the time measurement where the most significant duplicate of the
659 gene is DE
- 660 • Time point: time measurement where the gene is DE
- 661 • Expression: whether the DEG is up or down-regulated
- 662 • Cluster: cluster in which the gene is classified by HAC
- 663 • Gene description: informations on the gene's molecular function
- 664 • Location: chromosomal location of the gene

665 Additional file 2 — Distribution of the rank of the significant adjusted P-values in the tests for DE transcripts
666 between $t = 0$ and $t = +1$, $t = 0$ and $t = +4$ and $t = 0$ and $t = +24$ '.pdf' file
667 P-values are smaller at $t = +1$ and $t = +4$ than at $t = +24$ implying that the transcripts were overall more
668 differentially expressed between $t = 0$ and $t = +1$ and between $t = 0$ and $t = +4$ than between $t = 0$ and
669 $t = +24$.

670 Additional file 3 — Complete list of enriched GO (Biological process (BP), Molecular function (MF) and Cellular
671 Component (CC) for each of the cluster identified with the hierarchical ascending clustering '.xls' file
672 Features the GO items, the corresponding functions, the class of ontology, the number of genes in the input list
673 (enriching a GO and total number) and in the reference list (enriching a GO and total number), the raw and the
674 adjusted Fisher's exact test P-value and the list of genes.

675 Additional file 4 — Complete list of enriched GO (Biological process (BP), Molecular function (MF) and Cellular
676 Component (CC) for 34 genes for which L/G ratio had a significant effect. '.xls' file
677 Features the GO items, the corresponding functions, the class of ontology, the number of genes in the input list
678 (enriching a GO and total number) and in the reference list (enriching a GO and total number), the raw and the
679 adjusted Fisher's exact test P-value and the list of genes.













