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Hepatic expression patterns in psychosocially high-stressed pigs suggest mechanisms following allostatic principles

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2 Hepatic expression patterns in psychosocially high-stressed pigs suggest mechanisms
3 following allostatic principles.

4

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41 Highlights

- 42 • Psychosocial stress altered essential and unessential metabolic pathways
- 43 • High-stressed pigs show decreased expression of catechol-O-methyltransferase
44 (COMT)
- 45 • mRNA alterations could be summarized with reference to the concept of allostasis
46

47 Abstract

48 Psychosocial challenges are known to introduce cellular and humoral adaptations in various
49 tissues and organs, including parts of the sympatho-adrenal-medullary system and
50 hypothalamic–pituitary–adrenal axis as well as other peripheral tissue being responsive to
51 cortisol and catecholamines. The liver is of particular interest given its vital roles in
52 maintaining homeostasis and health as well as regulating nutrient utilization and overall
53 metabolism. We aimed to evaluate whether and how response to psychosocial stress is
54 reflected by physiological molecular pathways in liver tissue. A pig mixing experiment was
55 conducted to induce psychosocial stress culminating in skin lesions which reflect the
56 involvement in aggressive behavior and fighting. At 27 weeks of age, animals prone to
57 psychosocially low- and high-stress were assigned to mixing groups. Skin lesions were
58 counted before mixing and after slaughter on the carcass. Individual liver samples (n=12)
59 were taken. The isolated RNA was hybridized on Affymetrix GeneChip porcine Genome
60 Arrays. Relative changes of mRNA abundances were estimated via variance analyses.
61 Molecular routes related to tRNA charging, urea cycle, acute phase response, galactose
62 utilization, and steroid receptor signaling were found to be increased in psychosocially high-
63 stressed animals, whereas catecholamine degradation and cholesterol biosynthesis were
64 found to be decreased. In particular, psychosocially high-stressed animals show decreased
65 expression of catechol-O-methyltransferase (COMT) which has been linked to molecular
66 mechanisms regulating aggressiveness and stress response. The expression patterns of
67 high-stressed animals revealed metabolic alterations of key genes related to energy-
68 mobilizing processes at the expense of energy consuming processes. Thus, the coping
69 following psychosocial challenges involves transcriptional alterations in liver tissue which
70 may be summarized with reference to the concept of allostasis, a strategy which is critical for
71 survival.

72

73 Keywords: Allostasis: Liver: Microarray: Mixing: Pig: Psychosocial stress

74

75 1. Introduction

76 In pigs, the mixing of unfamiliar animals is considered to cause psychosocial stress [1,2],
77 thereby negatively affecting animal welfare [3,4]. It has been reported, that pigs exposed to
78 unfamiliar conspecifics alter their neuroendocrine system [5,6,7] which may culminate in
79 fighting and aggressive behavior [8,9] resulting in injuries as indicated by the 'skin lesion
80 score' [10,11]. Hence, multiple effects on production traits, endocrine system and metabolism
81 have been stated. We have previously reported that psychosocial stress induced by mixing
82 unfamiliar animals was indicated by a higher number of carcass skin lesions and higher
83 levels of plasma cortisol at slaughter [2]. Furthermore, gene expression patterns of the
84 adrenal stress response in these pigs were analyzed, revealing altered mRNA abundances
85 for transcripts associated with biosynthesis of steroids, cell growth and cell death [12]. It has
86 been suggested that immune alterations following psychosocial stress are part of
87 evolutionary conserved mechanisms to face potential injuries (e.g. skin lesions) [13].
88 Consistently, gene expression of porcine peripheral blood mononuclear cells (PBMC)
89 showed adjusted immunological pathways in psychosocially high-stressed females [14].

90 Experimental designs inducing psychosocial stress indicate that mammals exhibit a broad
91 spectrum of adaptive mechanisms on both the cellular and humoral level. Hence, different
92 tissues and organs have to interact extensively in order to cope with environmental
93 challenges. That can also be described as the concept of allostasis [15] reflecting 'the ability
94 to achieve stability through change', a strategy which is critical for survival [16]. Therefore, to
95 adapt to a stressful challenge, a variety of parameters must be orchestrated in different body
96 components, including transcriptional alterations in metabolically active tissues such as in the
97 liver.

98 To evaluate whether differences in the response to stress introduced by mixing are reflected
99 by physiological molecular pathways that contribute to homeostasis or allostasis we
100 investigated hepatic gene expression in psychosocially high- and low-stressed pigs.

101

102 2. Materials and methods

103 2.1. Animals, balanced mixing, sample collection

104 A pig mixing experiment was performed when high and low levels of psychosocial stress
105 were induced as described by D'Eath *et al.* [2]. In brief, the progeny (n=271) derived from a
106 crossbreed including Landrace, Large White, Duroc (sows) and Pietrain (boars) were mixed
107 at approximately 10 weeks of age (Figure 1: MIXING 1). The pigs were assigned to new
108 weight-balanced single-sex groups of eight or ten animals. Immediately before and at 24 hrs
109 after mixing, skin lesions were counted, dividing the body into front (head, neck, shoulder,
110 and front legs), middle (flanks, and back) and rear (rump, hind legs, and tail) sections [10].
111 The skin lesion score reflects the involvement in aggressive behavior and fighting [10,11] and

112 was used to distinguish the examined animals as either prone to high-aggression (high skin
113 lesion score) or low-aggression (low skin lesion score). For each mixing group the cut-off
114 criteria for the total lesion score was individually calculated. Thereby, the distribution of skin
115 lesion scores in each group was estimated. Half of the pigs were designated as high
116 aggressiveness (those with front lesions above average) and the remaining half was
117 designated as low aggressiveness (those with front lesions below average), respectively.
118 The first mixing revealed an increased skin lesion score (mean \pm SEM) in H animals than in L
119 animals (140.2 ± 69.5 vs. 86.5 ± 30.2 , respectively). Pigs remained in the established rearing
120 groups until they reached slaughter weight at approximately 27 weeks of age. Here, the pigs
121 were assigned to mixing groups based on their aggressiveness (Figure 1: MIXING 2), as
122 they were loaded onto a vehicle for a 270 km transport to the abattoir. In detail, single-sex
123 groups were built by mixing four pigs from one rearing group and four pigs from another
124 rearing group. Thus, H pigs were mixed with H pigs resulting in a HH batch, H pigs were
125 mixed with L pigs resulting in a HL batch, or L pigs were mixed with L pigs resulting in a LL
126 batch. Skin lesions were counted before mixing and after slaughter on the carcass, thereby
127 the skin lesion score was calculated (Table 1). In all these batches animals with high and low
128 lesion scores were observed. Apparently, high and low levels of psychosocial stress were
129 induced independently from the initial mixing group. Accordingly, groups of animals with
130 divergent response were built over all batches and termed HS and LS (for high stress/score
131 and low stress/score), respectively. In detail, the second mixing revealed a skin lesion score
132 (mean \pm SEM) on HS and LS animals originated from the HH mixing of 181.0 ± 55.6 and,
133 46.3 ± 18.4 respectively, and a skin lesion score on HS and LS animals originating from LL
134 mixing of 119.7 ± 47.9 and 32.7 ± 6.6 , respectively. Liver samples were taken and stored at -
135 80°C until analyses. Based on parameters of different stress levels (skin lesion score,
136 creatine kinase, and cortisol as stated in Table 1) those animals were selected for gene
137 expression profiling which represented extremes within mixing groups (Figure 1: ANALYSIS).
138 Each sampling group was represented by 4 castrates and 2 females.

139

140 2.2 Physiological parameters

141 The measurements of cortisol levels and creatine kinase activity were previously described
142 [2]. In brief, cortisol levels were measured with the automated analyzer Centaur (Siemens
143 Healthcare Diagnostics S.A.S., Saint Denis, France). The creatine kinase activity was
144 measured with a clinical biochemistry automat (COBAS-MIRA Plus, Roche Diagnostics).
145 Plasma urea nitrogen was analysed with a commercial assay using Fuji DriChem 4000i
146 purchased from scil animal care company GmbH, Viernheim, Germany.

147

148 2.3. RNA isolation, target preparation and hybridization

149 Total tissue RNA was isolated from individual liver samples (n = 12; balanced for mixing
150 group) using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany) per manufacturer's
151 directions. Quantification and purification were performed as previously described [14]. All
152 RNA samples were stored at -80°C until downstream analyses was performed. For the
153 microarray experiments, individual biotin-labeled cRNA samples were hybridized on
154 Affymetrix GeneChip porcine Genome Arrays (Affymetrix, Santa Clara, CA, USA).

155

156 2.4. Data analyses

157 The microarray data were analyzed as previously described [14]. In brief, the arrays passed
158 the appropriate quality control criteria as proposed by Kauffmann *et al.* (2009) [17]. The data
159 was GC-RMA normalized (Log2) and filtered by MAS5 (present rate > 50% per experimental
160 group), standard deviation (SD > 0.2) and mean (m > 2.5). Relative changes of mRNA
161 abundances were estimated via variance analyses (SAS Institute, Cary, NC, USA), including
162 effects represented by the stress level, sex, slaughter batch, mixing group, and stress
163 level*sex ($V_{ijkl} = \mu + \text{stress level}_i + \text{sex}_j + \text{slaughter batch}_k + \text{mixing group}_l + (\text{stress}$
164 $\text{level*sex})_{ij} + \text{error}_{ijkl}$). Due to multiple testing, p-values were converted to a set of q-values
165 [18]. The level of significance was set at $p \leq 0.05$. The raw data has been deposited in a
166 MIAME compliant database, the National Center for Biotechnology Information Gene
167 Expression Omnibus (www.ncbi.nlm.nih.gov/geo) (accession number: GSE49290).

168

169 2.5. Pathway analyses

170 The probe-sets were annotated by EnSEMBL Susscrofa 9 [19]. Gene lists obtained from the
171 microarray analyses were evaluated with 'Ingenuity Pathway Analysis' (IPA release winter
172 2012, Ingenuity Systems, Redwood City, CA, USA). The level of significance was set at $p \leq$
173 0.05. The significant top five pathways were considered in the discussion.

174

175 2.6. Quantitative real-time PCR

176 Total transcript levels of selected target (*ALDH1B1*, *ALDH9A1*, *COMT*, *DHCR24*, *DHCR7*,
177 *HMGCR*, *SQLE*, *GALP*, and *IL1R1*) and reference genes (*RPL10*, *RPS11*) were quantified
178 by real-time qPCR (Table 2) as previously described in detail [14]. In brief, 12 individual liver
179 mRNA samples were analyzed in duplicate on a LightCycler 480 system using LightCycler
180 480 SYBR Green I Master (Roche, Mannheim, Germany). Data were factorial normalized,
181 the statistical analysis included effects of stress level, sex, slaughter batch, mixing group,
182 and stress level*sex (SAS Institute, Cary, NC). The level of significance was set at $p \leq 0.05$.

183

184 3. Results

185 We investigated hepatic gene expression in psychosocially high- and low-stressed pigs. The
186 aim of the current study was to investigate whether emotional responses will be
187 accompanied by distinct physiological molecular paths in liver tissue. The samples used in
188 this study were selected from a larger animal experiment [2]. Six animals with high stress
189 levels (HS) and six animals with low stress levels (LS) were used to create two microarray
190 experimental groups to analyze their transcriptional responses in porcine liver tissue. The
191 animals differed significantly regarding physical and physiological parameters (Table 1). The
192 pigs were characterized by lesion scores as well as by the physiological stress parameters
193 creatine kinase activity and plasma cortisol level, which are known to reflect both damage of
194 muscle fibres due to strenuous physical activity and adrenal responses due to psychosocial
195 stress, respectively. Plasma glucose, plasma lactate, and blood urea nitrogen were found in
196 physiological concentrations and were unaffected by stress level. The initial microarray
197 analyses identified 12,887 expressed probe-sets (~53 % present calls). Further analyses
198 filtered 7,914 probe-sets, representing 5,906 genes [19].

199

200 3.1. Transcriptional responses due to different psychosocially induced stress levels

201 The comparison of liver tissue transcripts derived from HS and LS samples revealed 1,274
202 different probe-sets ($p \leq 0.05$; corresponding $q \leq 0.19$) (Table S1). Of these, 694 probe-sets
203 showed increased transcript abundances in HS animals ($H > L$). Analysis of these
204 differences suggested molecular routes related to tRNA charging, urea cycle, acute phase
205 response, estrogen receptor signaling, and glucocorticoid receptor signaling (Table 3).
206 Additionally, two transcripts associated to galactose degradation showed increased
207 abundances in HS animals: *GALE* (UDP-galactose-4-epimerase; $p = <0.05$; Fold change
208 1.75) and *GALK1* (galactokinase 1; $p = <0.05$; Fold change 2.39). Furthermore, 580 probe-
209 sets showed lowered mRNA abundances in HS animals ($HS < LS$), related to epoxysqualene
210 biosynthesis, noradrenaline and adrenalin degradation, methylglyoxal degradation, dopamine
211 degradation, and cholesterol biosynthesis (Table 3).

212

213 3.2. Alterations in mRNA abundances of selected transcripts

214 Both microarray and qRT-PCR data were correlated in order to validate the differences in
215 mRNA abundance between the experimental groups. According to Ingenuity pathway
216 analysis no particular pathway was significantly altered ($p < 0.05$) when transcripts with
217 decreased expression in HS compared to LS samples were investigated (Table 3: mRNA
218 abundance: $HS < LS$). In order to verify this issue, selected transcripts represented mainly
219 such gene products, which were involved in those pathways. In total, nine transcripts
220 encoding genes associated with catecholamine degradation (*ALDH1B1*, *ALDH9A1*, *COMT*),

221 cholesterol biosynthesis (*DHCR24*, *DHCR7*, *HMGCR*, *SQLE*), central nervous system
222 (*GALP*), and acute phase response (*IL1R1*) were analyzed (Table 4). The qRT-PCR data
223 reflected the statistical validity of the microarray analyses. The transcripts *HMGCR* and
224 *GALP* appeared to be false negatives in the microarray dataset and showed significantly
225 lowered mRNA abundances in HS animals when analyzed by qRT-PCR. The mRNA
226 abundance of the transcript encoding *DHCR7* remained unaltered in both MA and qRT-PCR.
227 The differences in mRNA abundances (i.e. Fold change) appeared reproducible in both
228 microarray and qRT-PCR analyses. The correlation coefficients of the data obtained by the
229 different approaches were highly significant and ranged between 0.85 and 0.99, except for
230 *IL1R1* ($\rho = 0.57$; $p = <0.10$).

231

232 4. Discussion

233 In order to gain detailed knowledge about transcriptional responses and mechanisms that
234 might contribute to allostasis we conducted a microarray experiment in porcine liver tissue of
235 animals involved in a mixing experiment [2]. On the transcriptional level we investigated the
236 interrelation between psychosocially induced stress levels and supporting metabolic
237 mechanisms in porcine liver tissue. The differences of cortisol level, CK activity and lesion
238 scores indicate that the experimental groups used different strategies to cope with
239 psychosocial stress induced by aggressive temperament, social dominance, and exogenous
240 stressors associated with the mixing treatment. Hence, the transcriptional alterations found in
241 liver tissue display the basic requirements in terms of allostasis, which enables the organism
242 to respond to psychosocial stress via sophisticated mechanisms covering stress-related,
243 immune and metabolic alterations. In this context, although the liver is seen as subordinated
244 tissue, it has a central metabolic position, thereby processing concomitantly efferent and
245 afferent signals to afford its multiple functions. However, there might be limitations to
246 extrapolate hepatic expression patterns to the organismal level.

247

248

249 4.1. Transcriptional alterations related to stress mediators

250 Fighting behavior and resulting aggression were shown to impact meat quality parameters
251 [2,20]. In particular, considering all treated animals within our experiment, an increased meat
252 pH at 24 hours appeared when aggressive animals were mixed with unfamiliar mates [2].
253 Interestingly, due to pre-slaughter stress an increased meat pH is positively correlated with
254 urinary adrenaline and noradrenaline levels at slaughter [21]. Because in our study HS
255 animals showed decreased mRNA abundances of catecholamine degrading enzymes a
256 delay of catecholamine degradation might have occurred which would lead to a prolonged
257 catecholamine appearance. Catecholamines are known as important stress mediators to

258 conduct signals to tissues and body compartments. In this context, the transcript encoding
259 catechol-O-methyltransferase (COMT) is of particular interest. The mammalian gene *COMT*
260 encodes a key enzyme which contributes to eliminate the catecholamine neurotransmitters,
261 in particular dopamine. Interestingly, research on the homologous mouse gene *COMT1* has
262 shown its association with aggressiveness and stress response [22,23]. Furthermore, a study
263 investigating the molecular equivalents of different aggressive phenotypes revealed lowered
264 mRNA abundances of *COMT* in aggressive male mice [24]. Accordingly, in our study both
265 microarray and qRT-PCR data revealed a decreased mRNA abundance of *COMT* in HS
266 animals. This observation supports data which link *COMT* to molecular mechanisms
267 regulating aggressiveness leading to psychosocial stress [22,25].
268 Furthermore, HS samples showed increased mRNA abundance of transcripts associated
269 with estrogen receptor signaling and glucocorticoid receptor signaling. However, the analysis
270 revealed unaltered mRNA abundances of the particular receptors, *NR3A1* and *NR3C1*,
271 respectively. Because cortisol levels were found to be numerically increased in HS animals,
272 a transcriptional response of glucocorticoid receptor associated transcripts was expected.
273 The large overlap regarding the assigned transcripts in estrogen receptor signaling and
274 glucocorticoid receptor signaling rather indicate an adjusted crosslink between stress-
275 response and metabolism than an impact of a particular sexual hormone.

276

277 4.2. Transcriptional alterations related to the immune system

278 As reviewed elsewhere, the adjustment of the immune system is a major concern to cope
279 with environmental challenges [26]. Regarding this context, we recently reported that female
280 HS animals alter the expression profiles in peripheral blood mononuclear cells (PBMC),
281 possibly to retain a prepared immune system [14]. In face of psychosocial stress, our
282 microarray experiment revealed that porcine liver tissue focused on immunological
283 components. In particular, transcripts associated with the non-specific acute phase response
284 showed increased mRNA abundances in HS animals. These alterations may contribute to
285 establish immune barriers antagonizing potential microorganisms intruding via skin lesions.
286 Under physiological conditions, the acute phase proteins remain elevated for a minimum of
287 24 hours after an initial stimulus [27]. Consequently, the revealed hepatic expression pattern
288 in our study fit to the sampling time point.

289

290 4.3. Transcriptional alterations related to metabolism

291 The microarray experiment revealed increased mRNA abundances of key genes associated
292 with galactose utilization, possibly to mobilize alternative energy metabolites. Furthermore,
293 the increased mRNA abundances of transcripts associated with urea cycle may indicate a
294 higher deamination of amino acids, possibly to gain energy-rich amino acid carbons. In such

295 energy-mobilizing processes the increased cortisol levels [2] may be involved, highlighting
296 the potential of cortisol as catabolic hormone [28]. However, these transcriptional alterations
297 did not appear to be transduced to the metabolite level, at least urea nitrogen in plasma was
298 unaffected and both blood glucose and lactose maintained in physiological concentrations. In
299 contrast, hyperglycaemia was observed after mixing unfamiliar pigs [5] and due to
300 aggressive behavior in male rats [29].

301 Additionally, the diminished mRNA abundances for cholesterol biosynthesis in HS samples
302 indicate metabolic alterations at the expense of energy consuming endogenous steroid
303 biosynthesis. Indeed, the transcriptional clues for a lowered cholesterol biosynthesis are in
304 line with the known phenomenon that psychosocial stress lead to a suppression of
305 unessential anabolism, including growth, digestion, and reproduction [30]. Thus, these
306 mRNA profiles indicate a catabolic state, probably driven by superior mechanisms
307 cumulating in increased energy demand and metabolic costs of psychosocially high-stressed
308 animals.

309 The HS animals showed clues for an increased translational activity as indicated by the
310 pathway tRNA charging. Interestingly, due to the cellular nutritional status the tRNAs
311 themselves differ in their nucleocytoplasmic distribution while their transcription rate is
312 independently from metabolic state [31]. In contrast, transcripts encoding tRNA-synthases,
313 which gene product act as one catalytic principle of the translation machinery, showed
314 increased mRNA abundances in HS animals, specifically for cysteine, glutamine, glutamic
315 acid, valine, phenylalanine, asparagine, aspartic acid, tryptophane, threonine, serine, and
316 proline. Such tRNA-synthases evolved early in evolution and are highly conserved. These
317 enzymes both interpret the RNA code and catalyze the attachment of specific amino acids to
318 the tRNAs containing the cognate trinucleotide anticodons. Of course, in a microarray
319 approach it appears to be difficult to characterize the metabolic relevance. However, the
320 character of tRNA charging contributes to anabolic processes in protein metabolisms (e.g.
321 immunoglobulins, enzymes).

322 Encoding a neuropeptide, galanin-like peptide (*GALP*) is mainly expressed in neurons of the
323 arcuate nucleus of the hypothalamus (ARC) [32]. To date, there is only poor knowledge
324 about *GALP* expression in peripheral tissues and its biological function. However, it has been
325 suggested that *GALP* is an important mediator at the crossroad between nutritional status,
326 reproduction and energy balance [32]. Interestingly, in adrenal gland the transcript encoding
327 *GALP* showed an increased mRNA abundance in HS animals [12], while in liver tissue a
328 diminished transcript yield was observed. Thus, the expression of *GALP* appears to be
329 tissue-specific in psychosocially high-stressed animals.

330

331 5. Conclusions

332 In order to maintain physiological functions, coping following psychosocial challenges involve
333 a variety of body compartments, including tissues acting in metabolism (liver), endocrine
334 system (adrenal gland [12]), immune system (lymphocytes / PBMC [14]), and neural system
335 (hippocampus [33,34]). These alterations reflect that complex organisms are able to respond
336 to varying environmental conditions in a multidimensional manner, although there are
337 evidences that mixing of animals differing in temperament is likely affecting animal welfare in
338 an undesired way [3,4]. The broad behavioral, phenotypic, and transcriptional alterations
339 observed in our experiment [2,12,14] could be summarized with reference to the concept of
340 allostasis [15,16]. In this context, the brain is seen as key organ which perceives
341 psychosocial stress and produces both behavioral and physiological responses via e.g.
342 hypothalamic-pituitary-adrenal (HPA) axis hormones, catecholamines, and cytokines [35].
343 Upon corresponding receptors, adaptive effects are produced in various tissues and organs.
344 According to the mRNA pattern in our study, one may speculate that macronutrients (e.g.
345 galactose, amino acids) are redistributed via liver tissue towards body components
346 responsible for e.g. physical exercise and immune function. Further, expression levels of
347 *COMT* seem to be associated with aggressiveness and stress level and may impact related
348 molecular mechanisms. Notably, these findings reflect the need to distinguish between acute
349 and chronic stressors and other factors like age, social status and genetics. These results
350 complement the findings, that psychosocial stress activates an array of adrenocortical,
351 immunological and neurobiological adaptations [30]. In order to cope with an exogenous
352 stressor, a strong activation of allostatic responses is required, evidently as part of
353 evolutionary inherited mechanisms to face potential injuries during fighting [13]. In this
354 context, liver tissue appeared to be capable to ensure survival by adjusting essential and
355 unessential metabolic paths, possibly orchestrated by superior mechanisms (e.g. in brain
356 and adrenal gland).

357

358 Additional material

359 Supplemental Table S1: Transcripts with higher and lower expression between H and L
360 samples.

361

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375

376 Declaration of interest

377 The authors have declared that no competing interests exist.

378

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503 **Table 1: Phenotype data of the analyzed animals.**

504

Parameter	HS pigs #	LS pigs #	p-value *
Skin lesions §			
Front	72.7 ± 15.7	15.5 ± 4.7	<0.01
Mid	59.3 ± 17.9	18.3 ± 7.5	<0.10
Rear	18.3 ± 11.9	5.7 ± 1.0	>0.10
Total	150.3 ± 37.8	39.5 ± 9.3	<0.05
Creatine kinase (U/l)	10,635.0 ± 1,703.9	2,126.5 ± 212.6	<0.001
Cortisol (ng/ml)	72.3 ± 4.3	57.9 ± 5.1	<0.10
Glucose (nmmol/l)	11.0 ± 1.0	12.9 ± 1.9	>0.10
Lactate (mmol/l)	8.7 ± 2.2	7.6 ± 1.0	>0.10
Blood urea nitrogen (mmol/l)	4.6 ± 0.7	4.4 ± 0.7	>0.10

505 HS – High stressed; LS – Low stressed; # mean ± SEM; n = 6; balanced for mixing groups; * p-value (2 tailed) of a two sample t-test reflecting differences
 506 between animals with high stress level and low stress level; § skin lesions in body sections: front (head, neck, shoulders, and front legs), middle (flanks and
 507 back), and rear (rump, hind legs, and tail);

508 **Table 2: Primer used to verify microarray experiments by qPCR.**

509

Gene symbol	Sequence 5' - 3' For	Sequence 5' - 3' Rev	size (bp)
ALDH1B1	AACGGGATTAGGGCACATTA	CTGTCCACTCCTTCCCTTGA	185
ALDH9A1	TTGGAACTTGGAGGCAAATC	AGGGGACCCATCCTTGTATC	233
COMT	CAACAGAGGTTGGGGTCCTA	CCCACAGGCATTCTCATTCT	164
DHCR24	TCCTTAATGATGGGGAGCAC	TACAGAAGCAGCAGCCACAC	126
DHCR7	GCATGACACTGACTTCTTCTC	CCCACCTCCACTTTATTC	136
HMGCR	CTGCACCATGCCATCCATAG	CTTTGCACGCTCCTTGAACC	104
SQLE	TGTGAATGTCCTTGCTCAGG	GGCATAGACTGCAACAGCAA	196
GALP	CGGACTGTGCCAGGTTTCAC	GCAGGAGTATTTCCCGATTCC	127
IL1R1	TATGACGCTGCTCTGATTGC	GGGAGAACATGGGAAAAGGT	106
RPL10 *	CTGTGTTCTGCTTTTCTTCC	TCATCCACTTTTGCCTTCT	199
RPS11 *	GAAACTGGCAAGGAGAAG	TTCGGATGTAGTGGAGGTAG	214

510 ALDH1B1 - aldehyde dehydrogenase 1 family, member B1; ALDH9A1 - aldehyde dehydrogenase 9 family, member A1; COMT - catechol O-methyltransferase;
 511 DHCR24 - 24-dehydrocholesterol reductase; DHCR7 - 7-dehydrocholesterol reductase; HMGCR - HMG-CoA-reductase; SQLE - squalene epoxidase; GALP -
 512 galanin-like peptide; IL1R1 - interleukin 1 receptor, type I; RPL10 - ribosomal protein L10; RPS11 - ribosomal protein S11; * reference genes;

513
514

Table 3: Top 5 Ingenuity pathways of transcripts with higher and lower expression between HS and LS samples.

Canonical pathway	mRNA abundance	p-Value	Number of involved genes	Metadata of involved genes																	
tRNA charging	HS > LS	<0.001	10	Gene symbol	CARS	DARS	EARS2	EPRS	FARSA	NARS	SARS	TARS	VARS	WARS							
				p-value	<0.05	<0.05	<0.05	<0.05	<0.001	<0.05	<0.01	<0.05	<0.01	<0.001							
				FC	1.56	1.22	1.34	1.42	1.73	1.30	1.29	1.22	1.53	1.61							
urea cycle	HS > LS	<0.05	3	Gene symbol	ARG1	ARG2	CPS1														
				p-value	<0.01	<0.05	<0.01														
				FC	1.52	1.82	1.61														
acute phase response signaling	HS > LS	<0.05	14	Gene symbol	APCS	CRP	FGG	HRAS	IL1R1	LBP	NOLC1	OSMR	PTPN11	RBP5	SERPINA3	SHC1	SOD2	STAT3			
				p-value	<0.01	<0.01	<0.05	<0.001	<0.01	<0.001	<0.05	<0.01	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05		
				FC	1.39	3.87	2.16	1.17	1.92	3.00	1.51	2.57	1.24	1.30	1.77	1.18	1.51	1.72			
estrogen receptor	HS > LS	<0.05	11	Gene symbol	DDX5	GTF2H3	H3F3A/H3F3B	HRAS	MED30	POLR2H	POLR2I	SHC1	TAF12	TAF13	TAF5L						
				p-value	<0.05	<0.05	<0.05	<0.001	<0.05	<0.01	<0.05	<0.05	<0.01	<0.05	<0.05						
				FC	1.30	1.76	1.33	1.17	1.31	1.31	1.18	1.18	1.32	1.33	1.26						
glucocorticoid receptor signaling	HS > LS	<0.10	16	Gene symbol	CD163	CREBZF	FGG	GTF2H3	HRAS	HSP90B1	HSPA5	POLR2H	POLR2I	PRKAB2	SGK1	SHC1	STAT3	TAF12	TAF13	TAF5L	
				p-value	<0.05	<0.05	<0.05	<0.05	<0.001	<0.05	<0.01	<0.01	<0.05	<0.01	<0.05	<0.05	<0.05	<0.05	<0.01	<0.05	<0.05
				FC	1.56	1.50	2.16	1.76	1.17	1.33	1.42	1.31	1.18	1.84	1.35	1.18	1.72	1.32	1.33	1.26	
epoxysqualene biosynthesis	HS < LS	>0.10	2	Gene symbol	FDFT1	SQLE															
				p-value	<0.05	<0.05															
				FC	-2.20	-2.01															
noradrenaline and adrenaline degradation	HS < LS	>0.10	5	Gene symbol	ADH4	ALDH1B1	ALDH9A1	COMT	IL4I1												
				p-value	<0.01	<0.001	<0.05	<0.05	<0.05												
				FC	-2.70	-3.01	-1.25	-2.03	-1.33												
methylglyoxal degradation	HS < LS	>0.10	2	Gene symbol	GLO1	HAGH															
				p-value	<0.05	<0.05															
				FC	-1.18	-1.30															
dopamine degradation	HS < LS	>0.10	4	Gene symbol	ALDH1B1	ALDH9A1	COMT	IL4I1													
				p-value	<0.001	<0.05	<0.05	<0.05													
				FC	-3.01	-1.25	-2.03	-1.33													
cholesterol biosynthesis	HS < LS	>0.10	3	Gene symbol	DHCR24	FDFT1	SQLE														
				p-value	<0.01	<0.05	<0.05														
				FC	-1.40	-2.20	-2.01														

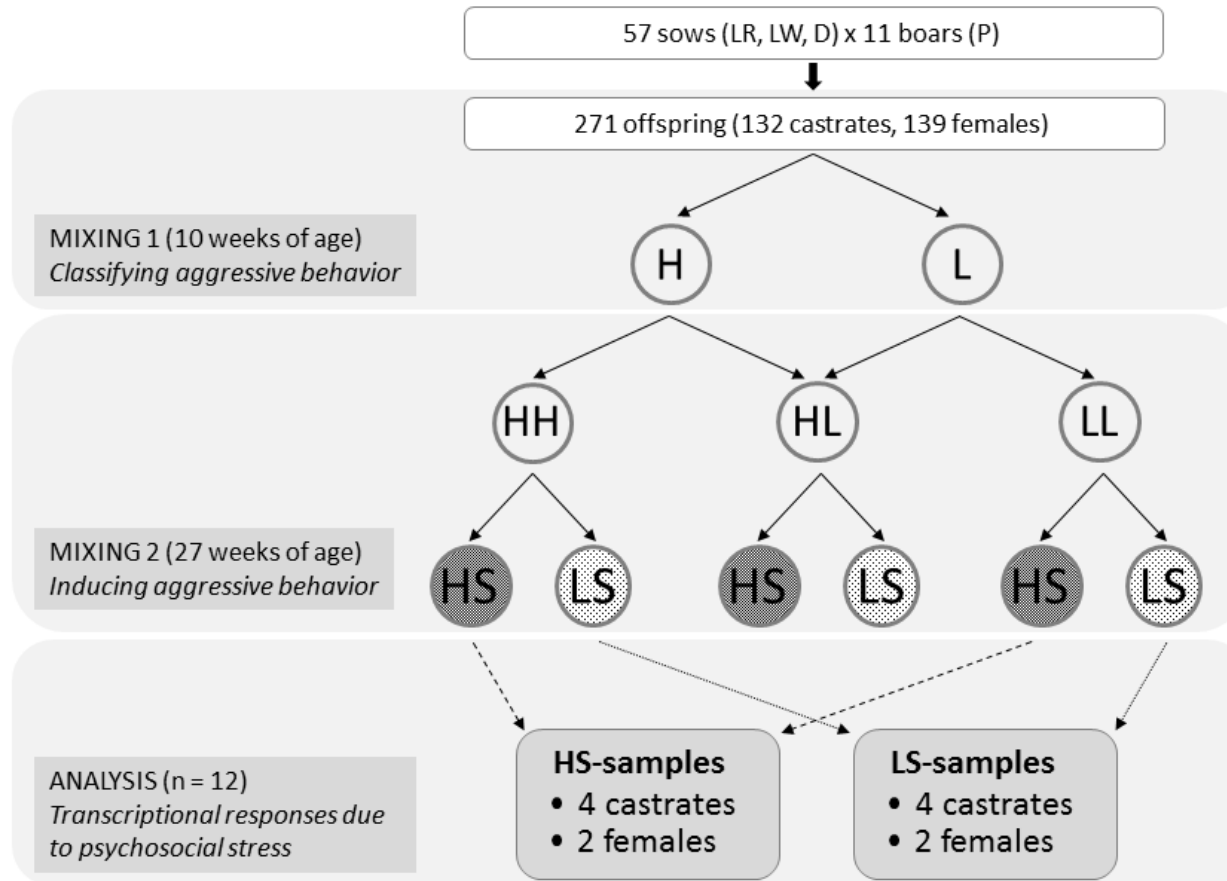
515 HS – High stressed; LS – Low stressed; P-value: significance of association between dataset and IPA-pathways; Benjamini-Hochberg multiple testing correction;

516 **Table 4: Comparison of microarray and quantitative PCR (qPCR) results for selected transcripts to verify microarray data.**

517

Gene symbol	Microarray			Real-Time PCR #			Correlation ##	
	p-value	expression	FC	p-value	expression	FC	coefficient	p-value
ALDH1B1	<0.001	HS < LS	3.01	<0.001	HS < LS	2.40	0.92	<0.001
ALDH9A1	<0.05	HS < LS	1.25	<0.01	HS < LS	1.47	0.93	<0.001
COMT	<0.05	HS < LS	2.02	<0.001	HS < LS	2.09	0.92	<0.001
DHCR24	<0.01	HS < LS	1.40	<0.01	HS < LS	1.48	0.93	<0.001
DHCR7	>0.10	HS < LS	1.15	>0.10	HS < LS	1.16	0.88	<0.001
HMGCR	>0.10	HS < LS	1.66	<0.05	HS < LS	1.67	0.85	<0.001
SQLE	<0.05	HS < LS	2.01	<0.05	HS < LS	1.84	0.90	<0.001
GALP	<0.10	HS < LS	4.26	<0.01	HS < LS	4.60	0.99	<0.001
IL1R1	<0.01	HS > LS	1.92	<0.05	HS > LS	2.00	0.57	<0.10

518 HS – High stressed; LS – Low stressed; FC - Fold change; # Values were calculated by factorial normalization on *RPL10* and *RPS11* expression values; ##
 519 correlation of normalized expression values was calculated by Spearman;



520

521

522

523

Figure 1: Experimental design used to analyze transcriptional responses due to different levels of psychosocial stress. The origin and composition of the profiled sampling groups is shown regarding their parental genetics, classified and induced aggressive behavior, and resulted stress levels. LR - Landrace; LW – Large White; D – Duroc; P – Pietrain; H – High lesion score; L – Low lesion score; HS – High-stressed; LS – Low-stressed;