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1 **Effects of stocking density and environmental enrichment on**
2 **behavior and fecal corticosteroids levels of pigs under commercial**
3 **farm conditions**

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22

23 **ABSTRACT**

24 In intensive pig farming of Western and Southern Europe, welfare concerns are still often
25 related to barren environments and crowded conditions. Pig producers need to balance the
26 requirements to improve welfare conditions at farm with practical considerations. The aim of
27 this study was to determine the extent to which the reduction of stocking density and the
28 provision of suspended pieces of hard wood as environmental enrichment have an influence
29 on both behavior and fecal corticosteroids concentration in commercially housed growing-
30 finishing pigs. A total of 640 growing pigs were arranged in a 2×2 factorial design with
31 stocking density (high, $1.0 \text{ m}^2/\text{pig}$ and low, $1.5 \text{ m}^2/\text{pig}$) and environmental enrichment (yes or
32 no) as factors. Ten replicate pens were allocated to each treatment. Observations of behavior
33 (instantaneous scan sampling) were made in each pen at 2-week intervals, when pigs were
34 from 15 to 31 weeks of age. Fecal samples were collected to determine corticosteroids
35 concentration in the 40 selected pens. The pigs housed in low density showed a higher
36 (although not significant) level of exploration of pen furniture than crowded pigs (10.11% vs.
37 8.53%, respectively; $P = 0.09$). Similarly, social interactions were observed more frequently
38 ($P \leq 0.001$) among the pigs in the enriched (10.27%) than in barren (6.69%) pens. The pigs
39 housed in barren pens had greater incidences of tail biting (barren: 1.35%, enriched: 0.42%; P
40 ≤ 0.01) and aggression (barren: 1.30%, enriched 0.61%; $P \leq 0.05$). Crowded pig in barren
41 pens spent less time moving (2.26%) compared to other treatments. Uncrowded pig in
42 enriched pens spent less time lying inactive (43.97%) and feeding (14.48%) compared to
43 other treatments. Fecal corticosteroids doubled their concentration from the first (56.74 ng/g)
44 to the last (108.10 ng/g) sampling date ($P \leq 0.001$). The crowded pigs showed higher ($P \leq$
45 0.001) concentration than the pigs housed in low stocking density (85.09 ng/g and 76.08 ng/g,
46 respectively). No differences were found in corticosteroids concentration between the pigs
47 housed in barren and enriched pens. To conclude, the reduction of stocking density modified
48 the pigs behaviors and reduced the fecal corticosteroids levels, highlighting an improvement

49 of welfare conditions. The provision of suspended pieces of hard wood modified the pigs
50 behaviors, but did not exert relevant effects on fecal corticosteroid levels.

51 **Key Words:** stocking density, environmental enrichment, fecal cortisol, behavior, welfare,
52 pig

ACCEPTED MANUSCRIPT

53 **INTRODUCTION**

54 In the last decades, application of technological innovations in agricultural sectors
55 and in animal production has led to more and more specialized techniques. However, the
56 derived beneficial aspects in terms of productivity have often been obtained at the expense of
57 behavioral needs and welfare of kept animals. The majority of growing-finishing pigs reared
58 in Southern and Western Europe are housed according to intensive farming conditions and
59 predominantly held in a barren environment. These environmental conditions limit the
60 expression of their species-specific behaviors (De Jonge et al., 1996; Edwards, 2010). Two of
61 the most important welfare concerns are related to high stocking densities and restriction of
62 social and locomotory activities. A reduction of space allowance has been associated with a
63 decline of production, a worsening of health status, and an increase in stressful and
64 uncomfortable conditions for the animals (Barnett, 2007). In growing and finishing pigs, a
65 reduction of space allowance is usually responsible for a decline of feed efficiency and a
66 worsening of weight gain (EFSA, 2005). Moreover, increasing level of aggression, reducing
67 exploratory activities and abnormal behaviors (e.g., tail and ear biting) can be observed while
68 increasing stocking density (Hörning, 2007).

69 Similarly, increases in behaviors like nosing and chewing penmates as well as in the
70 level of aggression have been shown in growing pigs reared in a barren environment (Beattie
71 at al., 2000). Commercially farming systems usually provide minimal stimulation to the
72 animals. Different types of environmental enrichments have consequently been proposed to
73 ameliorate the welfare conditions of intensive kept animals (Newberry, 1995). Concerning
74 pigs, the European Union (EU) legislation recognizes their needs to explore and manipulate,
75 and requires the use of materials that allow them to perform these activities (EU Directive
76 2008/120/EC). Among the proposed enrichments, straw seems to be very effective in
77 providing new stimuli for pigs allowing the containment of harmful social behaviors such as
78 tail and ear biting (Bracke et al., 2006). However, the proposed solutions or even law's
79 requirements are not always easily applicable in commercial production systems. For example,

80 straw and other substrates (e.g., woodshavings, mushrooms compost, peat, etc.) suggested as
81 environmental enrichments are not often compatible with most of the commercial pig farms in
82 Southern and Western Europe, where the use of slatted or partly slatted floor is still prevalent,
83 since their use would necessarily require substantial structural and operational changes to
84 manure handling systems. For this reason, in commercial housing systems the use of
85 alternative point-sources enrichments (e.g., chains, plastic balls, rubber tyres, etc.) has been
86 tested (van de Weerd and Day, 2009). Nowadays it's recognized that a successful enrichment
87 should be ingestible, destructible, deformable, chewable, and 'non-routable'.

88 Together with behavioral analysis and other animal-based parameters (e.g., body
89 conditions, injuries, etc.), physiological measurements (i.e., hormonal) are also of particular
90 value in welfare assessment (Möstl and Palme, 2002). The hypothalamus-pituitary-adrenal
91 (HPA) axis is activated when animals react to stressful events (Moberg, 2000). The activation
92 leads to, among other things, an increased synthesis and release into the circulation of
93 glucocorticosteroids (Woodman, 1997). Quantification of glucocorticosteroids in blood
94 unfortunately requires capture, restraint and blood sampling. These tasks on animals result in
95 a rapid release of corticosteroids into the circulation making hormone blood levels of little use
96 in chronic stress studies. Consequently, during the past decade there have been increased
97 efforts to develop non-invasive sampling methods for corticosteroids and their metabolites
98 quantification in secreted or excreted material. Hormonal consequences of stressful conditions
99 have been studied in pigs (Mormède et al., 2007) and van de Weerd and Day (2009) reported
100 some studies concerning the effect of environmental enrichments on cortisol level in pigs.
101 However, analyses of the fecal concentration of corticosteroids and their metabolites as a
102 mean to non-invasively assess animal welfare have been poorly studied in this species (Palme,
103 2012).

104 Pig producers must balance the requirements to provide appropriate welfare
105 improvements with practical considerations: applicability in commercial practice, cost
106 implications, impact on performance and product quality, etc. The aim of the present study
107 was therefore to determine if a reduction of stocking density and the introduction of

108 suspended pieces of hard wood as environmental enrichment may affect behavior and fecal
109 corticosteroids concentration in growing-finishing pigs under commercial farm conditions.

110

111

112 **MATERIALS AND METHODS**

113 All procedures and treatments were in compliance with the ethical guidelines of the
114 International Society for Applied Ethology (ISAE, 2002) and with the European Directives
115 (2001/88/EC and 2001/93/EC) on the minimum standards for the protection of pigs.

116

117 **Animals and housing**

118 The study was held at a commercial pig unit for growers and finishers located in N-W
119 Italy (latitude: 44° 43' 28" N; longitude: 7° 48' 34" E; altitude: 545 m a.s.l.) from May to
120 October 2012. A total of 968 [(Landrace × Yorkshire) × Duroc] hybrid pigs of both sexes
121 (females and castrated males) were initially enrolled in the experiment. Due to the large
122 number, the pigs were acquired in two batches from the same supplier. The pigs were
123 previously reared under the same conditions. Briefly, all pigs were teeth clipped and partially
124 tail docked at approximately 3d of age; afterwards, they were weaned at 3 weeks of age. Prior
125 to being enrolled in the experiment, the pigs were exposed to the same transport. At farm
126 entry, pigs were 13 weeks of age with an average weight of 25±1.2 kg.

127 Animals were housed in two adjacent buildings. Each building consisted of one single
128 room containing 27 (+1 hospital pen) and 35 (+1 hospital pen) pens, respectively, equally
129 distributed at each side of a central corridor. The pens measured 2.98 m × 6.63 m and they
130 were equipped with concrete slatted floors, except for the feeding area, which was equipped
131 with solid concrete floor. Pens partitions, made of concrete blocks, were fenced to allow
132 visual contact among pigs in adjacent pens. The two buildings were equipped with an

133 automatically controlled natural ventilation system: adjusting the inlet and outlet vents
134 regulated the natural airflow through the buildings. Natural lighting was sufficient during the
135 whole experimental procedures. Artificial light was mainly used only during husbandry tasks
136 and to provide at least a light period of 12 h per day.
137 Pigs in both houses received the same dry pelleted diets (from 13 to 17 weeks of age: 19.0%
138 crude protein, 5.2% crude fiber, 1.1% lysine, 13.3 MJ of digestible energy (DE)/kg; from 17
139 weeks of age until slaughter: 17.8% crude protein, 4.6% crude fiber, 1.1% lysine, 13.4 MJ
140 DE/kg). Diets were automatically provided *ad libitum* every day at morning (approximately at
141 7 am) in a multiple space dry feeder. Water was freely available from two nipple drinkers per
142 pen.

143 All pigs were vaccinated against Aujeszky's disease according to laws prescription
144 (Italian Ministry of Health, 1997).

146 **Experimental treatments**

147 When growing pigs arrived at farm, they were randomly divided into the two
148 buildings. The pigs of the first batch were housed in the first building. This building had
149 space for 513 pigs, consisting of 27 pens housing 19 animals each. The resulting stocking
150 density was equal to 1.0 m²/pig (high stocking density, HD). Currently, it represents the EU
151 minimum space allowance for pigs over 110 kg live weight (EU Directive 2008/120/EC). The
152 pigs of the second batch arrived at farm one week after the first batch and they were housed in
153 the second building. This building had spaces for the remaining 455 pigs, consisting of 35
154 pens housing 13 animals each. In this case, the stocking density was equal to 1.5 m²/pig (low
155 stocking density, LD). This value is usually indicated in organic pig production as the
156 maximum stocking density in indoor housing (IFOAM, 2005).

157 Whilst half of the pens in both buildings were kept in their original configuration
158 (barren pens, BP), the other half was equipped with an environmental enrichment (enriched
159 pens, EP). The enrichments were realized on-farm and consisted of a cylindrical piece of hard

160 wood (*Robinia pseudoacacia* L.) suspended from its center on a chain at pig head level. The
161 wooden pieces were 35 cm in length and had a diameter of 6 to 10 cm. The wooden pieces
162 were replaced once during the experimental period depending on their wear. Each pen was
163 equipped with two environmental enrichments, placed on each side of the pen partitions.

164 External pens (in the corners of the buildings) as well as hospital pens were excluded
165 from the selection procedure. Of the remaining 54 pens, 40 were randomly selected and
166 followed during the experimental period (20 weeks). The percentage of males to females was
167 similar in each pen and did not vary across treatment. The selected pens were arranged in a 2
168 × 2 factorial design with 10 replications (pens) each: high density – barren pen (HD-BP), high
169 density – enriched pen (HD-EP), low density – barren pen (LD-BP), and low density –
170 enriched pen (LD-EP). Therefore, a total of 640 pigs were involved in the experimental
171 measurements.

172

173 **Data collection**

174 During the first two weeks after entry, the pigs were allowed to overcome the
175 transport's stress, and to habituate in the new surrounding and groups formation. Since pigs
176 housed in LD building arrived at farm with one-week interval than the pigs housed in HD
177 building, data collection in the two buildings was carried out on alternate weeks to ensure that
178 pigs were at the same age when data were collected. The same observers assessed all the pens.
179 When a pig was removed from one of the selected pens due to healthy problems or severe
180 injuries, no replacements were made to avoid disruption of the social structure within the
181 groups. However, a pig's removal from a pen determined a variation in the experimental
182 density. Therefore, at each sampling date, only pens with the initial stocking density (19
183 animals in HD and 13 animals in LD) were considered in the subsequent statistical analysis.

184

185

186 Behavioral measurements

187 Behavioral observations were carried out when pigs were from 15 to 31 weeks of age.
188 Instantaneous scan sampling of each pen was performed to determine the number of pigs
189 performing each activity provided in the predetermined ethogram (Table 1) adapted from Guy
190 et al. (2002a) and van de Weerd et al. (2006). The observer recorded the pigs' activities from
191 outside the pen. During the experimental period, behaviors were recorded at 2-week intervals
192 for 9 times, one day per each selected week. Pigs were observed during 3 periods (at 9 am, 11
193 am, and 1 pm) each observation day. Scan samples were repeated three times in the each
194 period with a 10-minute interval. All considered behavioral activities were mutually exclusive.

195

196 Measurements of fecal corticosteroids concentration

197 Feces collection was carried out at 2-week intervals and was always scheduled the
198 day before the behavioral measurements to avoid that other experimental tasks could affect
199 corticosteroids concentrations. For the determination of baseline fecal corticosteroids
200 concentration (FCC) of each pen, fecal samples were collected twice when pigs were 14
201 weeks of age.

202 FCC in pigs as an index of circulating cortisol has a 48-hour time lag to extraction
203 (Möstl et al., 1999). The distribution of corticosteroids concentration in pig's feces is not
204 homogeneous and thus the whole sample has to be collected and subsequently homogenized
205 prior to assay (Carlsson et al., 2007). After defecation, feces were sampled from the bedding
206 and immediately refrigerated to be transported to the laboratory, where samples were thawed
207 at -20°C until analysis.

208 To extract steroids from nonliquid matrices (such as dried solids) feces were
209 subjected to an organic phase extraction using ethanol; the use of ethanol is recommended as
210 a mean to completely solubilize the dried steroid because certain steroids have limited
211 aqueous solubility (Cook, 2012).

212 Extraction and determination of corticosteroids in the feces were carried out as
213 previously reported by Prola et al. (2013). Briefly, fecal samples were kiln dried at 55°C for
214 24 h, thoroughly crushed, and five aliquots of pulverized feces (0.20 g each) were put into
215 extraction tubes, which were then sealed with a Teflon cap. Next, 1 mL of ethanol (Sigma
216 Aldrich, St. Louis, MO, USA) for every 0.1 g of solid was added to each tube, and the
217 mixture was shaken vigorously for 30 min. Samples were centrifuged at $3,300 \times g$ for 15 min,
218 and the supernatant recovered in a clean tube for evaporation to dryness in a SpeedVac
219 (ThermoFisher Scientific, Waltham, MA, USA). Extracts were stored at -80°C . Extracted
220 samples were dissolved into 100 μL ethanol followed by at least 400 μL of kit Assay Buffer
221 (Arbor Assays, Ann Arbor, MI, USA), then they were vortexed and rested for 5 min twice to
222 ensure complete steroid solubility. FCCs were determined using a pan-specific cortisol
223 enzyme immunoassay kit (K003; Arbor Assays, Ann Arbor, MI, USA) validated for dried
224 fecal extracts. All analyses were repeated twice. It is uncertain to which extent native
225 molecules and immunoreactive metabolites of cortisol were quantified in the kit used.
226 Consequently we have used the terminology fecal corticosteroid concentration (FCC). Inter-
227 and intra-assay coefficients of variation were less than 10%. The test's sensitivity was
228 determined by measuring the least amount of hormone standard consistently distinguishable
229 from the zero concentration standard and was calculated to be 17.3 pg/mL.

230 According to the manufacturer, the cortisol kit presents the following cross reactivity:
231 100% with cortisol, 18.8% with dexamethasone, 7.8% with prednisolone, 1.2% with
232 corticosterone and 1.2% with cortisone. Serial dilutions (1:4, 1:8, 1:16, and 1:32) of fecal
233 samples were assayed to test for parallelism against the standard curve ($P < 0.05$ for all
234 assays). The mean recovery rate of cortisol added to dried feces was 96.7%.

235

236

237 **Statistical analyses**

238 For all the data analyses, the pen was the experimental unit. The pen was treated as a
239 random effect and nested within treatment. Data were analyzed as repeated measures mixed
240 models (REML) in SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) with stocking density (D),
241 environmental enrichment (E), and their interaction (D×E) as fixed effects. While analyzing
242 FCC, age of animals was also considered as fixed effect. Concerning behavior measurements,
243 data were first collated and percentage of each behavioral activity of the ethogram was
244 expressed as ratio of the total number of observations for the three observation moments of
245 the day. Normality of residuals was checked with graphical methods and Kolmogorov-
246 Smirnov test. Data, with the exception of 'Lying' behavior and FCC, were subjected to
247 LOGIT transformation to meet the assumptions of REML (homogeneity of variance,
248 normality of error and linearity), and then reanalyzed. Significance was declared at $P \leq 0.05$,
249 and statistical trend are considered as $P < 0.10$. Results of statistical analysis are reported as
250 estimate least-squares means. Results are always presented as untransformed data.

251

252

253 RESULTS

254 Behavioral activities

255 Table 2 presents the frequencies of the considered behavioral activities. The
256 mounting behavior was not analyzed because it was seen very rarely. The pigs spent the
257 majority (>50%) of the observation time lying on the floor pens. The second most observed
258 behavior was feeding activity, followed by exploration of pen furniture and social interactions.
259 The incidence of the other considered behaviors was under the 5% of the observation time for
260 scan samples.

261 The overall effect of stocking density showed a tendency just on exploration of pen
262 furniture. Difference in the percentages of exploring pen between the two stocking density
263 treatments approached significance (HD: 8.53%, LD: 10.11%; $P = 0.09$).

264 The presence of environmental enrichment significantly affected most of the
265 behavioral activities. Pigs in the enriched pens spent more time performing social positive
266 interactions than pigs in barren pens (EP: 10.27%, BP: 6.69%; $P \leq 0.001$). On the other hand,
267 the incidences of tail-biting (BP: 1.35%, EP: 0.42%; $P \leq 0.01$) and aggressive behavior (BP:
268 1.30%, EP: 0.61%; $P \leq 0.05$) were significantly greater in the pigs housed in the barren pens.

269 The percentage of time spent moving through the pen was significantly lower in the
270 pigs housed in high density and barren pens (HD-BP) compared to LD treatments, with HD-
271 EP pigs showing an intermediate value between LD treatments and pigs housed in HD-BP.
272 For low density and enriched pens (LD-EP), scan samples of behavior showed that the pigs
273 spent a larger percentage of observation time feeding if compared to all other treatments and
274 lower percentage of observation time lying, although it was not statistically different from the
275 value detected in the enriched pens of high density treatment.

276 HD and LD pigs spent similar percentages of observed time exploring the
277 environmental enrichment (4.23% and 4.35%, respectively). Stocking density and
278 environmental enrichment did not affect drinking and excreting (plus urinating) activities.

279

280 **Fecal corticosteroids concentration**

281 Unreliable results were obtained from the samples collected when the pigs were at 21
282 weeks of age and consequently they were not considered in the statistical analysis.

283 FCC baseline values were not different among treatments (HD-BP: 50.63 ng/g, HD-
284 EP: 47.78 ng/g, LD-BP: 48.27 ng/g, LD-EP: 47.95 ng/g). Furthermore, the baseline values
285 were not different with the concentration detected at the first sampling date.

286 Stocking density significantly ($P \leq 0.001$) affected the average level of corticosteroids
287 measured during the whole experimental period. In fact, while there were no differences in
288 FCC between the pigs housed in barren and enriched pens (82.03 ng/g and 79.14 ng/g,

289 respectively), the pigs housed with high stocking density showed higher FCC (85.09 ng/g) if
290 compared to the pigs housed in low stocking density pens (76.08 ng/g).

291 Figure 1 shows the FCC variations during the experimental period. FCC significantly
292 ($P \leq 0.001$) increased with the increasing of age and live weight of pigs: FCC at the last
293 sampling date shows almost double value (108.10 ng/g) if compared to the first sampling date
294 (56.74 ng/g). Furthermore, at the end of the experimental period, stocking density shows a
295 significant effect analyzing FCCs at each sampling date. At the second-last sampling date,
296 FCC levels detected in pigs housed in high stocking density (HD-BP: 110.03 ng/g and HD-
297 EP: 112.57 ng/g) were higher ($P \leq 0.01$) if compared to pigs housed in low stocking density
298 (LD-BP: 90.40 ng/g and LD-EP: 85.45 ng/g). Similarly, higher ($P \leq 0.05$) FCCs were
299 detected at the last sampling date in pigs housed in high density pens (HD-BP: 123.59 ng/g
300 and HD-EP: 111.15 ng/g) than in low density pens (LD-BP: 102.06 ng/g and LD-EP: 94.76
301 ng/g).

302

303 **DISCUSSION**

304 **Behavioral activities**

305 In the present study, since the pen size was constant among treatments, stocking
306 density decreased with increasing group size. Therefore, stocking density and group size
307 effects were confounded, and group size could have affected the obtained results. However, it
308 is worth to point out that several studies (EFSA, 2005; Schmolke et al., 2002; Street et al.,
309 2008; Turner et al., 2003) suggested that the influence of stocking density on pigs
310 productivity and behaviors (e.g., lying, tail biting, social interaction, etc.) seems to be
311 predominant on group size effect. Moreover, the same studies showed that no effects or
312 negligible effects were detected while comparing different group sizes (at the same stocking
313 density), especially if an adequate space allowance is provided to pigs.

314 In the current study, the pigs housed in HD pens ate less frequently than those housed
315 in LD-BP and LD-EP pens, the latter spending the highest detected level in feeding activity.
316 Similar results were reported by Street and Gonyou (2008). These authors hypothesized that
317 crowded conditions may be responsible for hindering feeder access. The same authors did not
318 ascribe the reduced feeding frequency to higher level of aggression: in fact, an increase in
319 competition at the feeder did not occur in crowded pigs and they observed a lack of difference
320 in injuries prevalence, which are indexes of aggressive behaviors. Furthermore, higher level
321 of aggression would be expected with restricted feeding: Baxter (1985) suggested that pigs in
322 stable groups could be aggressive when there is a feed competition because the resource is
323 limited. Similar considerations reported by Street and Gonyou (2008) might be partially
324 supported by the reduction of moving activity observed in HD-BP pigs of our study.
325 Concerning pig productive performance, no effects of stocking density or environmental
326 enrichment were observed: live weights of pigs at the end of the experimental period were
327 comparable (HD-BP: 158.6 kg; LD-BP: 161.9 kg; HD-EP: 165.2 kg; LD-EP: 165.6 kg). This
328 suggests that the pigs housed in HD pens probably compensated the reduced feeding
329 frequency through longer meals. The same feeding strategy was already reported by Wolter et
330 al. (2000): they suggested that crowded pigs ate fewer but longer meals than uncrowded pigs.
331 More recently, Jensen et al. (2012) expressed similar considerations, concluding that there is
332 no evidence that productivity can be improved by increasing space allowance of finishing
333 pigs.

334 Concerning the effect of stocking density, we detected a tendency on exploration of
335 pen furniture, with the pigs housed in low density showing a higher explorative level than
336 crowded pigs. Our results did not support previous results indicating that an increased space
337 *per se* without enrichment causes a reduction in locomotory and exploratory activities
338 (Whittaker et al., 2012). However, our study confirms the conclusion of the same authors:
339 enrichment plays a greater role in modifying behavior than space allocation did.

340 As suggested by Newberry (1995), an environmental enrichment represents any
341 modification of a barren environment aiming at improving biological functioning of captive
342 animals. A variety of studies exist on the effect of environmental enrichments in pigs'
343 behavior and welfare (see the review of van de Weerd and Day, 2009). As already discussed
344 above, although straw bedding has the highest potential to meet the criteria that define a
345 successful enrichment, it does not apply to the majority of pig farms due to the
346 incompatibility with current liquid-slurry handling systems. For this specific reason, marginal
347 or point-source enrichments have been tested. In the present study, the pigs housed in the
348 enriched pens showed more active behaviors (e.g., exploring, interacting, moving, etc.) than
349 the pigs housed in barren environment. However, only explorative behaviors towards
350 penmates were statistically different between barren and enriched housed pigs. Similar results
351 were reported by Guy et al. (2002b); in the same study, the pigs with an enrichment object in
352 their pen also exhibited more positive social interactions. Furthermore, some recent studies
353 (Tönepöhl et al., 2012; Telkänrantaa et al., 2014) highlighted that the provision of point-
354 source objects as minimal environmental enrichments in pigs could increase the level of
355 overall activity if compared to pigs housed in barren conditions. The results of our trial and
356 those of the above mentioned studies seem to contrast with the hypothesis that pigs reared in
357 barren environments have elevated level of motivation to explore and interact in comparison
358 to pigs reared in enriched pens (Stolba and Wood-Gush, 1980). However, an explanation to
359 these different results might be provided by the diversity and the amount of enrichment used.
360 As reported by van de Weerd et al. (2006), one of the main consequences of providing objects
361 as environmental enrichments is that pigs can easily lose interest on them. In this case, the
362 level of exploratory motivation decreases as pigs become familiar and they can redirect
363 inappropriate stimuli towards penmates. Our results suggest that the provided enrichment is
364 effective, since 'negative' behaviors (i.e., aggressive behavior and tail biting) were less
365 performed by the pigs housed in the enriched pens.

366 Levels of aggression available in the literature are highly variable. There are several
367 factors that can affect the level of aggression in pigs. Pigs are social animals and their social

368 groups are based upon dominance hierarchy. It is commonly agreed that, when unfamiliar
369 pigs are brought together, the formation of a hierarchy order is establish within 24 or 48 hours
370 (Deen, 2010). Samarakone and Gonyou (2007) tested difference in productivity and
371 aggression between group sizes of 18 and 108 pigs per pen. ‘Social negative’ behaviors
372 (including aggression and tail biting) did not differ between the two groups, but they
373 progressively decreased over the following 48 hours after group formation: the percentage of
374 time spent fighting varied from 3.5-4.5%, detected at group formation, to 1.0-1.5% after two
375 days. Although finishing pigs are usually in stable social groups, there are still several factors
376 that can affect their aggression level: breed, sex, amount and quality of available space,
377 amount of feed and feeding distribution, etc. (Deen, 2010). In an extensive study concerning
378 the application of the Welfare Quality® in growing pigs housed in intensive conditions
379 (Temple et al., 2011), the authors detected an averaged level of “negative social interaction”
380 equal to 3.6% of all pigs behaviors. Mattiello et al. (2003) observed behaviors of heavy pigs
381 reared at different space allowances in three housing systems. They found an overall level of
382 ‘social negative’ interaction around 2-3% of total observed behaviors. On the other hand, the
383 levels of aggression detected in our study are higher than results previously published in other
384 comparable studies. For example, Bolhuis et al. (2006) studied the effects of rearing and
385 housing environment on behavior of finishing pigs. Examining the results of the “barren”
386 pens only, the aggression levels ranged from 0.05 to 0.38% of the observed behaviors. More
387 recently, Camerlink et al. (2012) detected mean level of aggression equal to 0.18% of
388 observation time in finishing pigs. As the authors suggested, the stable situation and to avoid
389 mixing unfamiliar pigs helped that aggression hardly occurred in their study. On the basis of
390 the above-mentioned data, our results are in line with other studies. The experimental
391 conditions might have contributed to maintain this level of aggression among pigs.

392 The activity of enrichment exploration was not influenced by stocking density;
393 similar results were obtained in a previous trial where pen size was not found to influence toy
394 use (Apple and Craig, 1992).

395 Recently, Tönepöhl et al. (2012) firstly used a piece of wood on a chain as
396 environmental enrichment for pigs. Pigs housed in the enriched pens were allowed to
397 manipulate either a plastic star on a chain or a piece of wood on a chain. These authors
398 reported that pigs in enriched pens were less inactive and even only point-source enrichments
399 may exert positive effects on animal welfare. Similar conclusions have been more recently
400 reported by Telkänranta et al. (2014) that also supported that suspended pieces of wood may
401 be promising environmental enrichments for pigs.

402

403 **Fecal corticosteroids concentration**

404 Limited available spaces as well as barren environments were widely shown to
405 adversely affect adrenocortical hormones, with consequent well-being reduction (SVC, 1997;
406 Möstl et al., 1999). The concentration of cortisol in blood depends on the species: pigs
407 showed baseline levels ten times higher than cows, and more than twice higher in response to
408 a stressor (Mormède et al., 2007). Furthermore, the same authors outlined that it is sufficient
409 to expose a pig to a novel environment to significantly increase blood cortisol. Whittaker et al.
410 (2012) reviewed the effect of space on pig's welfare. They reported that gilts housed in group
411 with low space allowance (1 m²) showed increased plasma corticosteroids concentration
412 compared to groups with higher space allowance (2 and 3 m²), with consequent negative
413 effects on reproductive performance. van de Weerd and Day (2009) reported that, while
414 higher levels of plasma cortisol were shown in pigs housed in crowded pens compared with
415 uncrowded ones, there was no difference in plasma cortisol concentrations between enriched
416 and barren pens. This is in agreement with the results obtained in the present study on fecal
417 corticosteroids.

418 By contrast, unchanged levels of basal free cortisol concentration were reported in
419 fattening pigs housed in pens with different space allowance, and even lower levels were
420 detected in gilts with reduced space compared to control group (Mormède et al., 2007). More

421 recently, Marco-Ramell et al. (2011) compared physiological parameters of pigs housed at
422 different stocking densities. Differently from what we detected, these authors observed that
423 serum cortisol was not altered in higher density (0.25 m²/pig vs. 0.50 m²/pig) but it is worth
424 mentioning that pigs were involved in a quite short trial (i.e., 26 days).

425 However, available results on the effects of enrichment objects in pigs are still
426 unclear (van de Weerd and Day, 2009).

427 Assessments of corticosteroids, their metabolites, and other stress sensitive molecules
428 in feces are increasingly used to monitor the stress of animals (Cook, 2012). Besides the
429 added advantage of allowing non-invasive and easy sampling, the analysis of these
430 compounds in feces can be a particularly useful indicator of chronic, long-term stress since
431 they provide an estimation of cortisol secreted during a time period rather than a point value
432 detected in blood samples (Millspaugh and Washburn, 2004). As reported by Palme (2012),
433 in the last decade an increasing literature has been carried out on fecal cortisol/corticosterone
434 metabolites measurement in farmed animals; however, very few studies investigated it on pigs.

435 Cortisol metabolites in cattle feces were shown to increase after transport and after
436 adrenocorticotrophic hormone administration (Palme et al., 1999). Similarly, Lexen et al.
437 (2008) concluded that the measurement of fecal cortisol metabolites could be used as a
438 parameter to monitor adrenocortical activity in sheep during shearing and transport. The use
439 of fecal cortisol to assess stress levels over long-term conditions in horses was also suggested
440 by Hughes et al. (2010). A reduced level of fecal corticoid metabolites in mink observed
441 during nine months was detected in the presence of increased environmental complexity
442 (occupational materials) (Hansen et al., 2007).

443 Royo et al. (2005) published one of the few papers on fecal cortisol in pigs, studying
444 the effect of repeated housing in metabolic cages on fecal excretion of cortisol. Cortisol level
445 increased in feces at the first stay in metabolic cage, but not in the following visits. The
446 authors suggested that fecal cortisol could be used as a measure of acute stress.

447 To the best of our knowledge, it is the first time that the assessment of fecal
448 corticosteroids levels has been used to evaluate long-term stress in pigs under commercial

449 farm conditions. Our results on fecal corticosteroids confirm those previously reported on
450 plasma cortisol, which appeared to be unaffected by enrichment objects (van De Weerd and
451 Day, 2009).

452 The results of the presents study seem to suggest that an increasing stocking density
453 strongly affects fecal corticosteroids concentration and modified some behavioral activities of
454 growing-finishing pigs. On the other side, the provision of point-source enrichment-objects
455 seems to affect pig's behaviors but not the corticosteroids concentrations in feces. A possible
456 answer to such difference may be found in the extremely complex mechanisms that regulate
457 the overall response to stress at the physiological, hormonal, and behavioral level.

458 Any change, event, or modification in the rearing environment represents external
459 stimuli for animal. The organism responds to the homeostasis's perturbation (i.e., stress) to
460 return system to equilibrium. According to intensity and duration of stimuli, the stress
461 response can be both beneficial and detrimental to the organism. From a hormonal point of
462 view, stress elicits the activation of the HPA axis causing the release of corticosteroids in
463 blood (Mormède et al., 2007). For this reason, corticosteroids plasma levels are used as index
464 of stress. Environmental enrichment induced a rise in plasma corticosteroids concentration in
465 rats (Moncek et al., 2004) and horses (Fureix et al., 2013). However, there is a lack of
466 agreement and knowledge about the effects of environmental enrichment on plasma
467 corticosteroids and there are contrasting results in the available literature. Young (2003)
468 reported a reduction of plasma cortisol among physiological evidences to support that an
469 environmental enrichment works properly. On the contrary, as we already mentioned, van de
470 Weerd and Day (2009) detected no effects of environmental enrichments on plasma cortisol
471 of pigs housed in barren and enriched pens. Therefore, it's difficult to hypothesize a
472 significance and which results we would have obtained in our study by analyzing plasma
473 cortisol. For this reason, as already suggested by many authors (see for example Fureix at al.,
474 2013), we used fecal samples rather than plasma in order to avoid bias caused by sampling
475 procedures and to assess chronic stress.

476 As expected, differences in behavioral activities were detected between the pigs
477 housed in barren and enriched pens. Therefore, our results confirm what previously reported
478 in literature. Moreover, our results support the hypothesis that the provision of a suspended
479 piece of hard wood is an effective environmental enrichment for growing-finishing pigs.

480 On the other side, density showed a strong effect on corticosteroids levels of pigs.
481 This is not an unexpected result since the assessment of corticosteroids in pig's feces allows
482 the evaluation of chronic stress (Cook, 2012). In fact, the corticosteroids difference between
483 the pigs housed in the two stocking density increase during the experiment, and it became
484 significant at the end of the productive cycle. Some, but non-negligible, effects of stocking
485 density were also observed on behaviors. The statistical analysis showed an effect of density
486 on exploration of pen furniture. As we already reported in the manuscript, we probably did
487 not detect a significant effect on aggression level due to the experimental conditions (e.g.,
488 mixing unfamiliar pigs was avoided).

489

490 CONCLUSION

491 Stocking density and environmental enrichments constitute two aspects that can be
492 modified by pig producers at farm level. In this study, a reduction of stocking density
493 determined modifications in pigs behaviors and a significant reduction in fecal corticosteroids
494 levels, highlighting an improvement of animal welfare conditions. When considering
495 marginal environmental enrichments, the biggest challenge for point-source enrichment
496 objects is to ensure that the enrichments are practical and effective. Suspended pieces of hard
497 wood in the growing-finishing pigs modified their behaviors, but did not exert relevant effects
498 on fecal corticosteroid levels.

499 Finally, we can conclude that, when considering enrichment and density effects on
500 pig welfare at farm level, it is advantageous to detect simultaneously behavioral and
501 physiological parameters because they may provide different information of the same

502 complex mechanism, and, therefore they may both contribute in the assessment of pig welfare
503 at farm level.

504

505

506

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512 manuscript drafting were mainly carried out by the first author, with the help and inputs from
513 all coauthors. The study includes only noninvasive procedures (i.e., behavioral observations
514 of the pigs and feces collection), without any alteration in the regular husbandry and
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517

518 **Conflict of interest**

519 The authors declare that there is no conflict of interest.

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ACCEPTED MANUSCRIPT

1 Figure captions

2 Figure 1. Effects of stocking density and environmental enrichment on corticosteroids
3 concentration (ng/g) in pigs feces (** $P \leq 0.01$; * $P \leq 0.05$). Different letters (a, b)
4 represent significant differences among treatments for each sampling date ($P \leq 0.05$).
5 HD-BP, high density-barren pen; HD-EP, high density-enriched pen; LD-BP, low
6 density-barren pen; LD-EP, low density-enriched pen.

- 1 Table 1. Definitions of behavior for scan animal samples adapted from Guy et al. (2002) and
2 van de Weerd et al. (2006)

Behavior	Description
Feeding	Pig stands in front of feeder with head lowered in feed hopper
Drinking	Pig stands, either with mouth touching or holding nipple drinker, or with snout in water bowl
Excreting or urinating	Pig stands in process of excreting or urinating
Exploring pen furniture	Pig stands and actively sniffs, noses, bites or chews floor and any part of the pen furniture
Examining enrichment	Pig stands and actively sniffs, noses, bites or chews the environmental enrichment
Social activity	Pig stands or lies and noses, lick or nibbles any part of a pen-mate's body
Aggressive behavior	Pig violently bites or knocks another group member with his head
Tail-biting	Pig holds a penmate's tail in its mouth and bites it
Mounting	Pig stands or attempts to stand, with front legs on back of another group member
Moving	Pig walks, trots or runs around the pen
Lying	Pig lies motionless on side or sternum with eyes closed

3

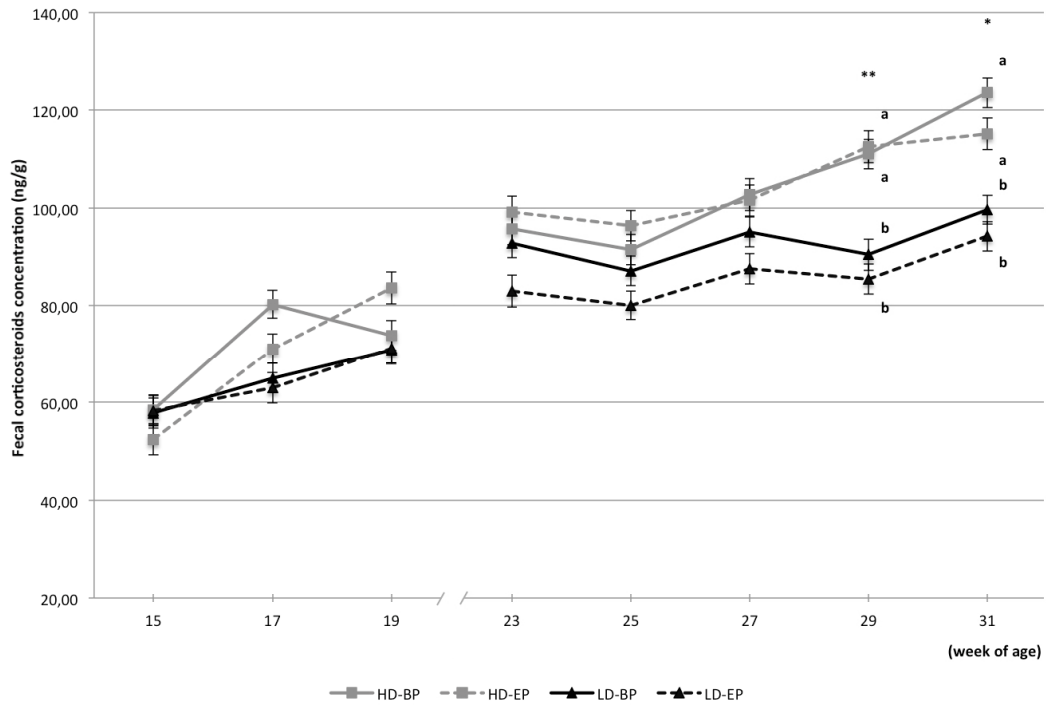
1 Table 2. Effects of stocking density and environmental enrichment on frequency of pigs behaviors (% of total observation time)

Behaviors	High density		Low density		Effects		
	Barren pen	Enriched pen	Barren pen	Enriched pen	D	E	D×E
	HD-BP	HD-EP	LD-BP	LD-EP			
Feeding	10.89 ^c	11.25 ^c	12.71 ^b	14.48 ^a	*	*	*
Drinking	4.81	5.17	4.38	5.92	ns	ns	ns
Excreting or urinating	2.13	2.15	1.98	2.61	ns	ns	ns
Exploring pen furniture	7.92	9.31	9.17	10.97	0.09	ns	ns
Examining enrichment	-	4.23	-	4.35	ns	-	-
Social activity	7.43	9.74	5.98	10.81	ns	***	ns
Aggressive behavior	1.33	0.64	1.28	0.59	ns	*	ns
Tail-biting	1.41	0.56	1.29	0.29	ns	**	ns
Moving	2.26 ^b	2.53 ^{ab}	2.98 ^a	3.16 ^a	*	ns	0.07
Lying	59.67 ^a	52.44 ^{ab}	59.33 ^a	43.97 ^b	**	***	0.06

2 ¹ Significance of effects of stocking density (D), environmental enrichment (E), and their interaction (D×E) is indicated; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq$

3 0.05; tendency $P < 0.10$; ns, not significant.

4 ² a, b, c: different letters at the same row means significant difference within treatments ($P \leq 0.05$).



Highlights

- We evaluated how to ameliorate pigs' welfare under commercial farm conditions
- We considered behavior and fecal corticosteroid concentration as welfare indicators
- Reducing stocking density modified behavior and reduced fecal corticosteroids level
- The provision of suspended pieces of wood in pens box modified pigs behavior
- The same piece of wood did not exert relevant effect on fecal corticosteroids level