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The effect of stocking density and enrichment on hair cortisol, hair dehydroepiandrosterone (sulphate) and their ratio in growing-finishing pigs

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HIGHLIGHTS

- The effect of stocking density and enrichment on chronic stress in pigs was studied.
- Hair cortisol, DHEA(S) and their ratio were used as a proxy for chronic stress.
- A lower stocking density resulted in less chronic stress in the pigs.
- More enrichment items did not have an effect on chronic stress.
- Sex affected hair cortisol, DHEA(S) and/or their ratio only sometimes.

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ABSTRACT

Understanding how husbandry practices affect chronic stress in growing-finishing pigs is essential to improve their welfare. The objective of this study was therefore to investigate the effect of two important practices, i.e., stocking density and enrichment, within different husbandry systems by studying concentrations of hair cortisol and hair dehydroepiandrosterone (sulphate) (DHEA(S)) and their ratio, as markers for chronic stress. Hereto, in six experiments, various organic and conventional systems were studied in which the stocking density and the level of enrichment varied. We found that a lower stocking density generally resulted in lower hair cortisol and DHEA(S) concentrations, but the effect of stocking density on the hair cortisol/DHEA(S) ratio was less clear. Access to enrichment only tended to result in higher DHEA(S) concentrations in one of the experiments. Furthermore, sex tended to affect or affected hair cortisol, DHEA(S) and/or the ratio only in some of the experiments. These results suggest that a lower stocking density is beneficial for growing-finishing pigs as they seemed to be less chronically stressed. That the enrichment items did not beneficially affect hair cortisol and DHEA(S) was likely due to the relatively small contrast between the control and enriched condition, as the pigs in the control condition already had access to straw. As not much studies have investigated hair DHEA(S) concentrations in pigs, more research is needed to get more insight of this hormone in relation to chronic stress and the effect of sex in pigs.

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1. Introduction

There are many different pig husbandry systems in the world. In the majority of countries in Europe the intensive indoor system is the dominant system, in which finishing pigs are kept at a high stocking density, with no outdoor access and with limited (variation in) pen enrichment (Ludwiczak et al., 2023). Such a husbandry system is common in Denmark and Poland. In the Southern part of Europe, e.g., in Italy, extensive husbandry systems with unlimited access to an outdoor paddock and with a lower stocking density are often observed (Rodríguez-Estévez et al., 2010; Vitali et al., 2021). These husbandry systems can vary in several parameters, such as the typical pig breed used at the farm, the type of feed provided to the animals, the stocking density, and the presence of enrichment (Ludwiczak et al., 2023). These factors are, however, often confounded with each other or difficult to disentangle to see clearly how each factor directly affects the pigs.

Stocking density and pen enrichment are factors that may directly affect the welfare of the animals. In Europe (EU), the minimal stocking density for pigs in intensive indoor systems is 0.30–0.65 m^2 depending on the pigs' weight, and these densities are doubled in organic systems and additional outdoor space is required (European Union 2009 and 2018). Pigs are hierarchical animals; thus, the more submissive animals may, for example, have limited access to a feeder compared to the more dominant pigs, or they are not allowed to rest. The higher the stocking density, the more difficult it will be for the submissive individuals to find their place in the group. On the other hand, a lower stocking density could lead to a decrease in competition between the animals and thereby overall animal welfare is expected to be improved (Fu et al., 2016; Ludwiczak et al., 2021). The amount and quality of enrichment also differ greatly between production systems, mainly since outdoor systems provide a variety of enrichments that are not accessible in indoor conditions. Enrichment is very important for pigs to have access to, as it could offer entertainment when provided in the form of objects (e.g., toys, chains, branches, wooden logs) or the possibility for (natural) rooting and foraging behaviour when provided as substrate on the floor (e.g., straw, soil). Lack of proper pen enrichment causes frustration and distress or boredom-like states, and can lead to tail biting (Averós et al., 2010; D'Eath et al., 2016; Larsen et al., 2018; Godyń et al., 2019). Thus, sufficient quantity and quality of materials allowing manipulation activities are required by the European Union (EU Council Directive, 2001). Unfortunately, the majority of welfare studies have so far focused on comparing completely barren indoor systems with enriched outdoor systems, rather than trying to determine the effect of stocking density within intensive and within extensive systems, as well as the impact of enrichment within a certain type of system (Pugliese et al., 2005; Maiorano et al., 2013; Lebret et al., 2015; Latoch et al., 2021).

Two physiological parameters that can be measured to determine the effect of stocking density and enrichment on (chronic) hypothalamus–pituitary–adrenal (HPA) axis activation (and by this be potential additional parameters of welfare) throughout the production life of the pig, are (hair) cortisol and dehydroepiandrosterone (sulphate) (DHEA(S)) (Moberg, 2000; Mormède et al., 2007; Carroll et al., 2018; Heimbürge et al., 2019; Pollock et al., 2021; Whitham et al., 2020). Cortisol and DHEA(S) are both components of the mammalian stress response system, which is partly regulated by the HPA axis. Cortisol makes glucose available to use as energy source in the stress response (Mormède et al., 2007; Whitham et al., 2020) and DHEA(S) is released concurrently with cortisol to minimize the negative effects of cortisol (Maninger et al., 2009; Kamin and Kertes, 2017; Whitham et al., 2020; Kayondo et al., 2025). In addition, the cortisol/DHEA(S) ratio is useful to be calculated, as it is an indicator of the functional status of the HPA axis (Kamin and Kertes, 2017; Bergamin et al., 2019; Whitham et al., 2020). Measuring cortisol, DHEA(S) and their ratio in non-invasively collected hairs is particularly valuable to retrospectively look at chronic stress (Russell et al., 2012; Bergamin et al., 2019; Heimbürge et al., 2019; Ghassemi Nejad et al., 2022).

Therefore, within the Horizon2020 EU project mEATquality, we aimed to disentangle two factors characterizing the pig husbandry, i.e., stocking density and enrichment, within different husbandry systems by studying concentrations of hair cortisol and DHEA(S) and their ratio. Hereto, various organic and conventional systems were studied in the European countries Poland, Denmark, and Italy, in which the stocking density and enrichment varied within six experiments. We expected that a lower stocking density and access to enrichment items will result in lower hair cortisol and DHEA(S) concentrations.

2. Materials and methods

2.1. Animals and housing

2.1.1. Experiments Poland

In Poland, two experiments (Table 1) were performed, which both took place at standard commercial farms in the province of Wielkopolska, Poland. These experiments were carried out in accordance with the Local Ethical Committee for Experiments on Animals in Poznan, Poland, and the European Union (EU) directive 2010/63/EU for animal experiments.

Experiment 1 - A total of 72 tail-docked pigs (DanBred) were included in the experiment. The pigs were on average 30 kg (about 70–77 days of age) and allocated to two pens at the start of the experiment. Among them, 48 (20 females and 28 castrated males) were housed together in a control pen (C) with 1 m^2/pig, and 24 (10 females and 14 castrated males) in a pen with a lower stocking density (S) with 2 m^2/pig. Both pens had a concrete floor with straw. Water and feed (a dry-mash diet provided following Polish recommendations for growing and finishing pigs (Grela and Skomiał, 2020)) were offered ad libitum. Lights were on for 12 h per day, and temperature was set at 20–21 °C. The experiment lasted 80 days, from March to May 2023.

Experiment 2 - A total of 60 tail-docked pigs [(Polish LW x Polish L) x (Duroc x Pietrain)] were included in the experiment. The pigs were on average 25.5 kg (about 63–70 days of age) and allocated to two pens at the start of the experiment. Among them, 30 (13 females and 17 castrated males) were housed together in a control pen (C) with 1 m^2/pig (30 m^2 in total), and 30 (13 females and 17 castrated males) in a pen with a lower stocking density (S) with 2 m^2/pig (60 m^2 in total). Both pens had a slatted floor. Water and feed (a dry-mash diet provided following Polish recommendations for growing and finishing pigs (Grela and Skomiał, 2020)) were offered ad libitum. Lights were on for 12 h per day, and temperature was set at 20–22 °C. The experiment lasted 87 days, from August to December 2023.

2.1.2. Experiment Denmark

The experiment Table 1 took place at an experimental farm at AU-Viborg campus, Tjele, Denmark. The experiment was carried out in accordance with the Ministry of Food, Agriculture and Fisheries, The Danish Veterinary and Food Administration under act 474 of 15 May 2014 and executive order 2028 of 14 December 2020. A total of 410 pigs (DanBred; 210 females and 200 castrated males) were included in the experiment (Coutant et al., 2025). The pigs were on average 31 kg (about 77 days of age) and balanced for sex (females and castrated males), and were assigned to one of five treatments at the start of the experiment. Pigs were originally supposed to be not tail-docked, but due to issues in the rearing farm, only 21 % of the pigs had intact tails. These pigs were equally distributed over the five treatments. All pigs were housed on a partly-slatted floor with straw. Water and feed (a commercial dry-feed) were available ad libitum. Lights were on for 15 h per day. Temperature followed a standard curve; it was set at 21 °C upon arrival, and then it was gradually decreased to 17 °C from week 8 onwards. The experiment lasted 70–77 days, from August to October 2023.

The five treatments consisted of a control treatment, two stocking density treatments and two enrichment treatments. The pigs ($n = 107$) in the control treatment (C) were housed in six pens with 0.7 m^2/pig,

Table 1
Overview of the most relevant stocking density and enrichment aspects of each of the six pig experiments.

Experiment	Conventional/ Organic	Stocking density m^2/pig (no. pigs/pen)			Enrichment		
		C	S(1)	S2	C	E(1)	E2
Exp 1 Poland	Conventional	1 (48)	2 (24)	-	-	-	-
Exp 2 Poland	Conventional	1 (30)	2 (30)	-	-	-	-
Exp Denmark	Conventional	0.7 (18)	1.4 (9)	2.1 (6)	straw	straw, wood logs, feed bags, ropes	straw, roughage
Exp 1 Italy							
Batch 1	Organic	-	-	-	straw (indoors) concrete floor (outdoors)	straw, branches concrete floor, shade	-
Batch 2	Organic	-	-	-	straw (indoors) concrete floor (outdoors)	straw, branches concrete floor, shade	-
Exp 2 Italy							
Batch 1	Organic	2.4 (17)	6.8 (6)	-	-	-	-
Batch 2	Organic	2.4 (17)	6.8 (6)	-	-	-	-
Exp 3 Italy	Conventional	1.15 (12)	3.0 (12)	-	-	-	-

corresponding to 18 pigs per pen. The pigs ($n = 54$) in the first stocking density treatment (S1) were housed in another six pens with 1.4 m^2/pig, corresponding to nine pigs per pen, and the pigs ($n = 36$) in the second stocking density treatment (S2) were housed in six pens with 2.1 m^2/pig, corresponding to six pigs per pen. Pigs ($n = 106$) in the first enrichment treatment (E1) were housed in another six pens with 0.7 m^2/pig, corresponding to 18 pigs per pen. Enrichment consisted of wood logs, empty paper feed bags, and ropes. On Mondays, each pen was provided with three wood logs. The logs were removed on Wednesday, and three paper bags were given. On Thursday, the left-over bags were removed and three new paper bags were provided. On Fridays, the left-over bags were removed and three ropes were hung on the pen fixtures. The ropes were removed on the following Monday, and the same rotation started again. Pigs ($n = 107$) in the second enrichment treatment (E2) were also housed in six pens with 0.7 m^2/pig, corresponding to 18 pigs per pen. Here, enrichment consisted of spreading maize-based roughage directly on the floor of the concrete area of the pen at 1100 h each day. In the first week of the experiment, 2 kg of roughage was given per pen. This was increased to 3 and 4 kg in the second and third week, respectively. From the fourth week until the end of the experiment, 5 kg roughage was given to each pen.

2.1.3. *Experiments Italy*

In Italy, three experiments (Table 1) were performed. Experiment 1 and 2 took place at a private organic farm in the province of Cuneo, Italy, and experiment 3 was performed at the experimental farm of the Council for Agricultural Research and Economics (CREA) in the province of Modena, Italy. These experiments were carried out in accordance with the European Union (EU) directive 2010/63/EU for animal experiments.

Experiment 1 - Two batches of 68 undocked pigs per batch (Fomeva K-line x Topigs TN70) were included in the experiment. The pigs were on average 60 kg and 71 kg (150 and 185 days of age) in the first and second batch, respectively, and allocated to two pens at the start of a batch. Among them, 34 were housed in two control pens (C) and 34 in two pens with extra enrichment (E) consisting of branches (vine shoots from the on-farm organic plantation) indoors and shade in the East facing outdoor run (shade was created by a net (1 m^2/pig) that hung above the outdoor run). In each pen, 17 pigs were housed, balanced for sex (females and castrated males). All pens had a sloped floor with straw inside and a concrete floor in the outdoor run. Inside, pigs had a space of 1.36 m^2/pig and outside of 1.05 m^2/pig. Water and feed (a liquid feed provided following Italian recommendations for growing and finishing pigs) were available ad libitum. Lights and temperature were set according to farm procedures. The first batch lasted 60 days, from February to April 2023. The second batch lasted 57 days, from October to November 2023.

Experiment 2 - Two batches of 64 undocked pigs per batch (Fomeva K-line x Topigs TN70) were included in the experiment. The pigs were on average 32 kg and 38 kg (116 and 132 days of age) at the start of a batch in the first and second batch, respectively. Of these 64 pigs, 34 pigs were housed in two control pens (C) with 1.36 m^2/pig indoors and 1.05 m^2/pig outdoors, and 30 pigs in five pens with a lower stocking density (S) with 3.85 m^2/pig indoors and 2.98 m^2/pig outdoors. In the two C-pens, 17 pigs were housed and in the five S-pens, six pigs were housed, balanced for sex (females and castrated males). All pens had a sloped floor with straw inside and a concrete floor in the outdoor run. Water and feed (a liquid feed provided following Italian recommendations for growing and finishing pigs) were available ad libitum. Lights and temperature were set according to farm procedures. The first batch lasted 90 days, from April to July 2023. The second batch lasted 75 days, from December 2023 to February 2024.

Experiment 3 - A total of 48 undocked pigs (Italian Duroc x Large White) were included in the experiment. The pigs were on average 68 kg at the start of the experiment (145 days of age). Of these 48 pigs, 24 pigs were housed in two control pens (C) with 1.15 m^2/pig indoors and 24 pigs in two pens with a lower stocking density (S) with 3.0 m^2/pig indoors. In each pen, 12 pigs were housed, separated by sex (females and castrated males). Pigs were housed intensively in pens with a solid floor. Water was available ad libitum and feed (a commercial dry feed for growing and finishing pigs) was provided twice a day. Lights and temperature were set according to farm procedures. The experiment lasted 70 days, from August to October 2023.

2.2. *Hair sampling*

At each institute, hair (bristles) was sampled from the dorsal skin area behind the nape of the neck by means of a razor. All pigs had the same coloured hairs (i.e., pinkish) in this area. Sampling was performed while the pigs were constrained in a scale or corridor, as part of the standard weighing procedure prior to slaughter (i.e., at about 6 months of age). Each sample (weighing at least 120 mg) was put in a paper envelope with the pig ID code, and then sealed and stored in the dark at room temperature before being sent to the laboratory for analysis at the University of Udine, Udine, Italy.

In Poland, for experiment 1, 23 pigs (12 females and 11 males) from the control pen and all pigs ($n = 24$, 10 females and 14 males) from the pen with decreased stocking density were selected for hair sampling. For experiment 2, 24 pigs (12 females and 12 males) from the control pen and 24 pigs (12 females and 12 males) from the pen with decreased stocking density were selected for hair sampling. In Denmark, four to nine pigs (balanced for sex) from each pen and for each treatment (C: $n = 36$, S1: $n = 46$, S2: $n = 36$, E1: $n = 32$, E2: $n = 44$) were selected for hair sampling. In Italy, for experiment 1, per batch 24 control pigs (12 per pen, balanced for sex) and 24 pigs (12 per pen, balanced for sex) from the enriched treatment were selected for hair sampling. For experiment 2, in batch 1, 24 control pigs (12 per pen, balanced for sex) and 24 pigs (4–5 per pen, balanced for sex) from the lower stocking density treatment were selected for hair sampling. In batch 2, 30 control pigs (15 per pen, balanced for sex) and all 30 pigs (six per pen, balanced for sex) from the lower stocking density treatment were selected for hair sampling. For experiment 3, all 48 pigs were selected for hair sampling.

2.3. Hair cortisol and DHEA(S) analysis

A first, hairs were washed and extracted as described by Bergamin et al. (2019). Washing with isopropanol is essential to minimize the risk of extracting steroids deposited on the surface of the hairs through e.g., sebum. For extraction, 60 mg of hair was placed in a glass vial along with 3 mL of methanol. The vials were incubated at 37 °C for 16 h. Next, the liquid in the vial was evaporated to dryness at 37 °C under an airstream suction hood. The remaining residue was dissolved in 0.8 mL of ELISA buffer (50 mM phosphate buffer, pH 7.4, 0.4 % BSA, 0.5 M NaCl).

Subsequently, the concentrations of cortisol and dehydroepiandrosterone (sulphate) (DHEA(S)) were measured using an in-house ELISA, as described by Falco et al. (2023). In brief, microplates were coated with anti-rabbit-IgG antibody. After an overnight incubation, the plates were washed five times with washing buffer. Aliquots of cortisol or DHEA-S standards, quality-control extract, and hair test extracts were added to the microplate wells. The cross-reactivities of the anti-cortisol antibody with other steroids were: cortisol 100 %, cortisone 4.3 %, corticosterone 2.8 %, 11-deoxycorticosterone 0.7 %, 17-hydroxyprogesterone 0.6 %, dexamethasone 0.1 %, progesterone, 17-hydroxypregnenolone, DHEAS, androsterone sulphate, and pregnenolone < 0.01 %. The cross-reactivities of the DHEA(S) antibody with other steroids were: DHEA-S 100 %, 5-androsten-3-ol-17-one (dehydroepiandrosterone, DHEA) 76.6 %. Anti-cortisol or anti-DHEA(S) antibody diluted 1:32,000 and 1:80,000, respectively, in ELISA buffer was added along with the cortisol- or DHEA-S-peroxidase conjugate diluted 1:6000 or 1:10,000, respectively, in ELISA buffer. Plates were incubated overnight and then washed five times in washing buffer to remove any unbound cortisol or DHEA(S). The amount of bound conjugate was quantified by adding the chromogenic substrate. The plates were incubated for 30 min in the dark at room temperature (23 °C). The reaction was stopped with 2 M H_2SO_4. Absorbance was read at 450 nm using a plate reader (EnSight Multimode Plate Reader, Perkin-Elmer Life Science, Boston, USA). The sensitivities of the assays were 9.4 and 5.4 pg/mL for cortisol and DHEA(S), respectively. Hormone concentrations of both cortisol and DHEA(S) were expressed as pg/mg given that entire hairs were extracted. The cortisol/DHEA(S) ratio was calculated by dividing cortisol by DHEA(S) and multiplying the outcome by 100.

2.4. Statistical analysis

SAS (SAS 9.4, SAS Institute Inc.) was used for statistical analyses. Per experiment (the two batches in the Italy experiments 1 and 2 were treated as separate experiments), hair cortisol, hair DHEA(S), and the cortisol/DHEA(S) ratio were analysed using general linear models with treatment (i.e., stocking density (C, S(1), S2), or enrichment (C, E(1), E2)), sex (males and females) and the interaction between treatment and sex as fixed effects. Pen was included as a random effect nested within the fixed factors where appropriate (Denmark experiment and Italy experiments 1 and 2). Prior to analyses, cortisol, DHEA(S), and/or the cortisol/DHEA(S) ratio were log transformed to obtain normally distributed residuals.

All results are presented as untransformed means ± SE, and $P < 0.05$ was regarded as significant and $0.05 < P \leq 0.10$ as a tendency. Significant interactions were further investigated with post hoc pairwise comparisons using the differences of the least square means with Tukey correction.

3. Results

3.1. Stocking density experiments

A lower stocking density resulted in lower hair cortisol concentrations in all experiments except the third experiment in Italy (Table 2) and this was significant for these experiments, except for batch 1 of the second experiment in Italy (Table 2). This stocking density effect was, however, dependent on sex in the Denmark experiment (stocking density x sex interaction, $P = 0.05$). Post hoc analysis showed that hair cortisol concentrations were lower in both females and males in S1 compared to the control situation. In addition, hair cortisol concentrations were lower in the S1 males compared to both S2 males and females (C females: 6.2 ± 0.6 vs. C males: 7.6 ± 0.7 vs. S1 females: 3.9 ± 0.3 vs. S1 males: 3.3 ± 0.2 vs. S2 females: 5.8 ± 0.8 vs. S2 males: 5.3 ± 0.4 pg/mg). Furthermore, sex tended to affect and affected hair cortisol concentrations of the pigs in experiment 1 and 2 in Poland, respectively. Females had lower concentrations than males (Table 2). Finally, the interaction between stocking density and sex also affected the hair cortisol concentrations of the pigs in experiment 3 in Italy ($P = 0.008$). Females had lower concentrations than males in the control situation, but no other differences were found between the groups (C females: 5.3 ± 0.3 vs. C males: 8.7 ± 1.4 vs. S females: 8.7 ± 2.4 vs. S males: 6.0 ± 0.6 pg/mg).

A lower stocking density resulted in significantly lower hair DHEA(S) concentrations in all experiments except the second experiment in Poland (Table 3). This stocking density effect was, however, dependent on sex for the Denmark experiment (stocking density x sex interaction, $P = 0.02$). Post hoc analysis showed that hair DHEA(S) concentrations were lower in both females and males in S1 and S2 compared to the control situation, but no other differences were found between the groups (C females: 62.4 ± 5.6 vs. C males: 96.0 ± 26.8 vs. S1 females: 20.0 ± 1.9 vs. S1 males: 17.3 ± 2.3 vs. S2 females: 26.8 ± 1.2 vs. S2 males: 27.1 ± 1.9 pg/mg). Furthermore, sex tended to affect and affected hair DHEA(S) concentrations of the pigs in batch 1 of the second experiment in Italy and the second experiment in Poland, respectively. Females had lower concentrations than males (Table 3).

The cortisol/DHEA(S) ratio was affected by stocking density in experiment 2 in Poland, in the Denmark experiment, and in batch 2 of the second experiment in Italy (Table 4). In the latter two experiments, the cortisol/DHEA(S) ratio was lower in the control than in the lower stocking density situation (Table 4). For the second experiment in Poland, this effect was dependent on sex (stocking density x sex interaction, $P = 0.03$). Post hoc analysis showed that the cortisol/DHEA(S) ratio was lower in the males in the lower stocking situation compared to the other groups and the other groups did not differ from each other (C

Table 2
Means ± SE of hair cortisol concentrations (pg/mg) for pigs housed at different stocking densities (Control, S(1), S2) and for female (F) and castrated male (M) pigs.

Experiment	Stocking density				Sex		
	Control	S(1)	S2	P-value	F	M	P-value
Exp 1 Poland	3.6 ± 0.3	2.1 ± 0.2	-	<0.001	2.7 ± 0.3	3.0 ± 0.3	0.09
Exp 2 Poland	3.3 ± 0.2	2.7 ± 0.1	-	0.02	2.8 ± 0.2	3.2 ± 0.1	0.02
Exp Denmark	6.9 ± 0.4	3.6 ± 0.2	5.5 ± 0.5	<0.001	5.2 ± 0.3	5.2 ± 0.3	0.85
Exp 2 Italy							
Batch 1	9.1 ± 1.3	5.8 ± 0.6	-	0.12	7.1 ± 0.7	7.8 ± 1.4	0.89
Batch 2	6.5 ± 1.7	3.2 ± 0.2	-	0.02	3.8 ± 0.3	5.9 ± 1.7	0.12
Exp 3 Italy	7.0 ± 0.8	7.3 ± 1.2	-	0.88	7.0 ± 1.2	7.4 ± 0.8	0.34

Table 3
Means ± SE of hair dehydroepiandrosterone (sulphate) (DHEA(S)) concentrations (pg/mg) for pigs housed at different stocking densities (Control, S(1), S2) and for female (F) and castrated male (M) pigs.

	Stocking density				Sex		
Experiment	Control	S(1)	S2	*P*-value	F	M	*P*-value
Exp 1 Poland	77.1 ± 6.2	45.0 ± 4.6	-	<0.001	61.7 ± 6.1	59.9 ± 6.6	0.82
Exp 2 Poland	19.9 ± 3.5	22.2 ± 1.9	-	0.12	19.0 ± 3.4	23.1 ± 1.9	0.03
Exp Denmark	79.2 ± 13.8	18.7 ± 1.5	26.9 ± 1.1	<0.001	35.0 ± 3.1	44.3 ± 9.3	0.74
Exp 2 Italy							
Batch 1	255.3 ± 23.0	94.4 ± 5.8	-	0.02	165.6 ± 21.9	185.0 ± 25.5	0.06
Batch 2	555.1 ± 92.7	95.0 ± 9.9	-	<0.001	292.4 ± 76.3	357.7 ± 80.3	0.29
Exp 3 Italy	41.7 ± 1.9	34.4 ± 1.7	-	0.006	38.8 ± 1.9	37.3 ± 2.0	0.55

Table 4
Means ± SE of the hair cortisol/DHEA(S) ratio for pigs housed at different stocking densities (Control, S(1), S2) and for female (F) and castrated male (M) pigs.

	Stocking density				Sex		
Experiment	Control	S(1)	S2	*P*-value	F	M	*P*-value
Exp 1 Poland	4.8 ± 0.3	5.4 ± 0.5	-	0.47	4.5 ± 0.3	5.6 ± 0.5	0.08
Exp 2 Poland	19.8 ± 1.4	14.1 ± 1.6	-	0.001	17.8 ± 1.7	16.1 ± 1.6	0.25
Exp Denmark	11.4 ± 1.0[a]	24.8 ± 2.2[b]	20.6 ± 1.5[b]	0.01	18.8 ± 1.5	20.1 ± 1.8	0.56
Exp 2 Italy							
Batch 1	3.9 ± 0.4	6.8 ± 0.8	-	0.24	5.7 ± 0.8	5.0 ± 0.6	0.22
Batch 2	1.9 ± 0.5	3.8 ± 0.2	-	0.001	2.7 ± 0.3	3.0 ± 0.5	1.00
Exp 3 Italy	17.2 ± 2.1	21.7 ± 3.6	-	0.11	18.8 ± 3.7	20.0 ± 2.0	0.15

[a,b] Values within a row with different superscript letters differ significantly at $P < 0.05$.

females: 18.5 ± 1.7 vs. C males: 21.1 ± 2.9 vs. S1 females: 17.2 ± 2.9 vs. S1 males: 11.1 ± 0.9). Sex only tended to affect the cortisol/DHEA(S) ratio in the first experiment in Poland. Females tended to have a lower cortisol/DHEA(S) ratio (Table 4).

3.2. Enrichment experiments

No effect of enrichment on hair cortisol (Table 5), hair DHEA(S), and the cortisol/DHEA(S) ratio was found, except a tendency in DHEA(S) concentrations in batch 1 of the first Italy experiment. Here, DHEA(S) concentrations tended to be higher in the enriched pigs than in the control pigs (Table 6). Sex only tended to affect the cortisol/DHEA(S) ratio in the experiment in Denmark and in batch 1 of the first Italy experiment. In both experiments, males tended to have a higher cortisol/DHEA(S) ratio than females (Table 7). The interaction between enrichment and sex was not significant in all experiments ($P > 0.05$, data not shown).

Table 5
Means ± SE of hair cortisol concentrations (pg/mg) for pigs housed under different enriched (Control, E(1), E2) conditions and for female (F) and castrated male (M) pigs.

	Enrichment				Sex		
Experiment	Control	E(1)	E2	P-value	F	M	*P*-value
Exp Denmark	6.9 ± 0.4	6.8 ± 0.3	6.8 ± 0.5	0.96	6.5 ± 0.3	7.1 ± 0.4	0.12
Exp 1 Italy							
Batch 1	5.6 ± 0.4	6.1 ± 0.6	-	0.76	5.5 ± 0.5	6.1 ± 0.6	0.34
Batch 2	5.2 ± 0.2	6.6 ± 0.4	-	0.22	5.9 ± 0.4	5.9 ± 0.3	0.74

4. Discussion

In this study, we aimed to disentangle the effect of stocking density and enrichment within different husbandry systems by studying concentrations of hair cortisol and DHEA(S) and their ratio. Hereto, various organic and conventional systems were studied in Poland, Denmark, and Italy, in which stocking density and enrichment varied within six experiments. We expected that a lower stocking density and access to enrichment items would result in lower hair cortisol and DHEA(S) concentrations. Generally, a lower stocking density indeed resulted in lower hair cortisol and DHEA(S) concentrations. The effect of stocking density on the cortisol/DHEA(S) ratio was less straightforward. Access to enrichment items, however, barely affected the concentrations of hair cortisol and DHEA(S) and their ratio. Furthermore, sex affected hair cortisol, DHEA(S), and/or the ratio in some of the experiments.

4.1. Stocking density

Generally, a lower stocking density resulted in lower hair cortisol concentrations in all experiments, except for the third Italy experiment. Lower hair cortisol concentrations at lower stocking densities suggest that these pigs were less (chronically) stressed, and with that may have had better welfare compared with the pigs housed at higher stocking densities, and this is in line with previous studies (Heimbürge et al., 2019; Morgan et al., 2021). Why the pigs in the third Italy experiment were not found to have lower hair cortisol concentrations at a lower stocking density is not clear. This experiment did notably differ from the other experiments in including heavier and older pigs and housing the female and male pigs separately. However, as this was the case for both stocking density conditions, these differences seem unlikely to explain this result. Perhaps other (unknown) factors, such as breed, that were different in this experiment compared to the other experiments or a combination of these factors, contributed to finding similar hair cortisol concentrations in the third Italy experiment (Russell et al., 2012; Heimbürge et al., 2019, 2020; Otten et al., 2020; Wiechers et al., 2021; Ghassemi Nejad et al., 2022; Peric et al., 2023; Levallois et al., 2024). Moreover, it is puzzling why in the Denmark experiment lowering the stocking density from 0.7 to 1.4 m^2/pig led to lower hair cortisol concentrations, but lowering the stocking density to 2.1 m^2/pig resulted in similar concentrations as for 0.7 m^2/pig. At present we cannot explain this result, but it may have to do with differences in feed intake (Coutant et al., 2025, submitted; Sell-Kubiak et al., submitted).

The hair DHEA(S) results followed the hair cortisol results for four of the six experiments, which is in line with the mechanism of the (chronic) stress response system (with less release of cortisol and other glucocorticoids, less DHEA(S) release is needed to counteract their negative effects (Kamin and Kertes, 2017; Montillo et al., 2020; Whitham et al., 2020)). In the second Poland experiment, hair DHEA(S) concentrations were not lower at the lower stocking density condition, while hair

Table 6
Means ± SE of hair dehydroepiandrosterone (sulphate) (DHEA(S)) concentrations (pg/mg) for pigs housed under different enriched (Control, E(1), E2) conditions and for female (F) and castrated male (M) pigs.

Experiment	Enrichment				Sex		
	Control	E(1)	E2	*P*-value	F	M	*P*-value
Exp Denmark	79.2 ± 13.8	122.3 ± 38.2	145.5 ± 30.0	0.87	114.9 ± 20.3	120.2 ± 26.8	0.44
Exp 1 Italy							
Batch 1	131.3 ± 6.0	177.1 ± 15.0	-	0.09	163.6 ± 15.1	144.8 ± 8.4	0.24
Batch 2	55.4 ± 2.9	42.4 ± 2.2	-	0.22	50.4 ± 2.7	47.4 ± 3.1	0.89

Table 7
Means ± SE of the hair cortisol/DHEA(S) ratio for pigs housed under different enriched (Control, E(1), E2) conditions and for female (F) and castrated male (M) pigs.

Experiment	Enrichment				Sex		
	Control	E(1)	E2	*P*-value	F	M	*P*-value
Exp Denmark	11.4 ± 1.0	15.1 ± 3.2	10.6 ± 1.1	0.84	10.6 ± 0.8	13.8 ± 1.9	0.06
Exp 1 Italy							
Batch 1	4.6 ± 0.6	3.5 ± 0.3	-	0.50	3.5 ± 0.3	4.5 ± 0.6	0.07
Batch 2	9.9 ± 0.6	19.6 ± 4.1	-	0.22	12.7 ± 1.4	16.7 ± 4.1	0.81

cortisol concentrations were, and in the third Italy experiment, hair DHEA(S) concentrations were lower in the lower stocking density condition, while here hair cortisol concentrations were not lower. We do, however, not have an obvious explanation for this difference. It also remains unclear why in the Denmark experiment lowering the stocking density from 0.7 to 1.4 m^2/pig led to lower hair DHEA(S) concentrations, but lowering the stocking density to 2.1 m^2/pig resulted in similar concentrations as for 1.4 m^2/pig. At the same time, lower (hair) DHEA (S) concentrations have been found in (neurologically) ill and in chronically stressed individuals (Maninger et al., 2009; Kamin and Kertes, 2017; Peric et al., 2017; Trevisan et al., 2017; Whitham et al., 2020; Scollo et al., 2025). The generally lower hair DHEA(S) concentrations in the pigs in the lower stocking densities which were assumed to suffer less from chronic stress are in this light remarkable. As there are not that many studies yet about hair DHEA(S) in pigs, more research is needed to better understand which factors affect hair DHEA(S) and with that how to correctly interpret higher and lower hair DHEA(S) concentrations (Kamin and Kertes, 2017; Bergamin et al., 2019; Pollock et al., 2021; Whitham et al., 2020; Kayondo et al., 2025; Scollo et al., 2025).

The hair cortisol/DHEA(S) ratio was found to be higher in the lower stocking density conditions in five of the six experiments, and this was significant for the Denmark experiment (with similar ratios for both the 1.4 and 2.1 m^2/pig stocking density) and the second batch of the second Italy experiment. The hair cortisol/DHEA(S) ratio was also significantly affected by stocking density in the second Poland experiment, but here the ratio was lower in the lower stocking density condition. Higher (hair) cortisol/DHEA(S) ratios – which may indicate HPA axis dysfunctioning - have been found in individuals suffering from (neurological) illnesses or chronic stress (Maninger et al., 2009; Sollberger and Ehlert, 2016; Kamin and Kertes, 2017; Whitham et al., 2020; Kayondo et al., 2025; Scollo et al., 2025). Therefore, the (numerically) higher hair cortisol/DHEA(S) ratios in the pigs in the lower stocking densities which were assumed to suffer less from chronic stress are peculiar and against expectation. It could be that these higher ratios found in this study are due to that the hair DHEA(S) concentrations decreased more strongly than the hair cortisol concentrations in the lower compared to the higher stocking density, resulting in a higher ratio. If so, the generally higher hair cortisol/DHEA(S) ratios found in the pigs at the lower stocking densities are likely not related to chronic stress, but merely a result of the absolute numerator and denominator values. Indeed, Sollberger and Ehlert (2016) advocate to not only present the ratio of two the variables, but also the original two variables. Further research is warranted to be able to properly (biologically) interpret higher and lower hair cortisol/DHEA(S) ratios (Kamin and Kertes, 2017; Bergamin et al., 2019; Whitham et al., 2020; Kayondo et al., 2025).

Overall, it seems that the effect of a lower stocking density on hair cortisol, DHEA(S), and their ratio is more clearly present when less pigs are put in a pen compared to increasing pen size, but more research is needed to confirm this and to understand why it works in this way. In addition, the observed effects of stocking density on the hair hormones may not only be caused by the different amount of space the animals had, but also from a different group dynamics between the animals within each stocking density (Estevez et al., 2007). Furthermore, we did not observe a clear difference in effect of stocking density between the more conventional systems and the organic system used. This may imply that pigs still benefit from a lower stocking density even in systems where the standard density is already relatively low.

4.2. Enrichment

More enrichment items did not lead to different concentrations of hair cortisol, hair DHEA(S) and the cortisol/DHEA(S) ratio, except for a tendency for higher hair DHEA(S) concentrations in the first batch of the first Italy experiment. We had expected to find more effects of having more enrichment items in the pen. Possibly, the contrast between the control and enriched condition was not sufficiently large to find changes in hair cortisol, hair DHEA(S) and their ratio, as in all experiments here the control condition included straw (which is considered to be excellent enrichment (Studnitz et al., 2007; Luo et al., 2020)) and the enriched condition was only topped with items such as ropes and branches. In other studies in which the stocking density was kept similar between both conditions, hair cortisol concentrations were found to be lower in the more enrichment condition (Casal et al., 2017; van der Staay et al., 2017; Morgan et al., 2019; Espejo-Beristain et al., 2022; Lee et al., 2024; 2025), or there was no effect of enrichment on hair cortisol concentrations (Morgan et al., 2019; Nannoni et al., 2019; Bučková et al., 2022; Luo et al., 2022; Lagoda et al., 2023). These contrasting results could indeed have been due to a difference in the type and/or amount of enrichment items provided. For instance, Nannoni et al. (2019) compared pens with a metal chain with pens with wooden logs, while van der Staay et al. (2017) compared pens with a thick layer of straw, balls, footballs and wooden sticks on the floor, and plastic chewing sticks and balls attached to chains with pens with only a chain, one ball and two chewing sticks. Luo et al. (2022), however, also had a large contrast in enrichment between pens, but did not find any effects on hair cortisol. This may be explained by the pigs being relatively short (i.e., 4 weeks) in both housing conditions or by the concentration of cortisol in hairs being differently contaminated by a different level of dirtiness in the enriched and barren pens (Otten et al., 2020; Luo et al., 2022). So, likely, the exact type, amount and duration of the enrichment item(s) given, determines whether there is an effect on hair cortisol concentrations. Hair DHEA(S) and the hair cortisol/DHEA(S) ratio were not mentioned above as - to the authors' knowledge - this is the first study that looked at the effect of enrichment on hair DHEA(S) and the hair cortisol/DHEA(S) ratio.

4.3. Sex

Effects of sex on hair cortisol, hair DHEA(S) and their ratio were not present in each stocking density and enrichment experiment, but the effects of sex (in interaction with stocking density) found, may suggest that the castrated male pigs were overall more chronically stressed and/or suffered more in the higher stocking density condition compared with the female pigs. This suggestion is, however, mainly based on the hair cortisol concentrations as we feel too little is known about what higher or lower hair DHEA(S) concentrations mean in growing-finishing pigs. One other study also found an effect of sex on hair cortisol, hair DHEA and their ratio in pigs (Bergamin et al., 2019). There, gilts had higher hair cortisol, but lower hair DHEA concentrations and a higher ratio than barrows. In other studies, hair cortisol, hair DHEA-S and/or their ratio were not found to be different between (castrated) male and female pigs (Heimbürge et al., 2020; Montillo et al., 2020; Parois et al., 2022; Nadlučnik et al., 2024). Following Heimbürge et al. (2019), we therefore conclude that the effect of sex on hair cortisol, hair DHEA(S) and their ratio is inconclusive. This is likely due to a variety of factors that are different between (castrated) males and females such as body condition, behaviour and gonadal and adrenal hormone metabolism and development (Kamin and Kertes, 2017; Bergamin et al., 2019; Heimbürge et al., 2019, 2020; Whitham et al., 2020).

5. Conclusion

A lower stocking density is beneficial for growing-finishing pigs as they seemed to be less chronically stressed, as shown by the generally lower hair cortisol and DHEA(S) concentrations. The effect of stocking density on the hair cortisol/DHEA(S) ratio was less obvious. Access to enrichment items barely affected the concentrations of hair cortisol and DHEA(S) and their ratio. This was likely due to the relatively small contrast between the control and enriched condition as the growing-finishing pigs in the control condition already had access to straw as enrichment. Sex affected hair cortisol, DHEA(S) and/or the ratio only in some of the experiments. As only few studies investigated hair DHEA(S) concentrations in pigs, more research is desired to get a better understanding of this hormone in relation to chronic stress in pigs and to the sex of the pigs.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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