Steroids in pig hair and welfare evaluation systems: combined approaches to improve management in pig breeding?

Marta Montillo[1], Sara Rota Nodari[2], Tanja Peric[1*], Alessia Polloni[2], Mirco Corazzin[1], Cristina Bergamin[1], Anna Balestrieri[3], Alberto Prandi[1] and Antonella Comin[1]

[1]*Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, 33100 Udine, Italy.*
[2]*Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini', 25124 Brescia, Italy.*
[3]*Istituto Zooprofilattico Sperimentale del Mezzogiorno, 80055 Portici (NA), Italy.*

[*]*Corresponding author at: Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, 33100 Udine, Italy.*
E-mail: tanja.peric@uniud.it.

Veterinaria Italiana 2020, **56** (3), 177-184. doi: 10.12834/VetIt.1974.11885.1
Accepted: 23.03.2020 | Available on line: 31.12.2020

Keywords
Cortisol,
CReNBA,
Dehydroepiandrostero-ne-sulphate,
Hair,
Swine.

Summary
The aim of the present pilot study was to determine the allostatic load by analysing the hair cortisol and dehydroepiandrosterone-sulphate (DHEA-S) of commercial pigs belonging to different farms having good overall animal welfare according to the CREnBA (Centro di Referenza Nazionale per il Benessere Animale – Brescia, Italy) assessment protocol. The study was conducted on 86 pigs belonging to three farms with a grade higher than 8 on the CReNBA welfare evaluation system. Hair samples were taken by shaving on sows 1-10 days after weaning (Farms 1 and 2) and at the age of 8-10 months (Farm 3). The hormone concentrations were measured by radioimmunoassay. From the box plots, it is evident that the hair cortisol concentrations of animals were different between farms. Conversely, the variability of the hair DHEA-S concentration was similar between the F1 and F2 farms but much lower at F3. For all the variables considered (cortisol, DHEA-S and cortisol/DHEA-S ratio), F2 showed a higher hair concentration level than F1 ($P < 0.05$). The study's results indicate that the measurement of cortisol and DHEA-S in pig hair shows a different allostatic load between them, although the official welfare evaluation method assessed the farms as having good overall animal welfare.

Introduction

In recent years, pig welfare has become increasingly important, especially in intensive breeding, as evidenced by the European legislation (Council Directive 2008/120/EC). Based on the European Food Safety Authority scientific opinion (EFSA 2012), legislators have identified some objective parameters to evaluate pig welfare. These have also been adopted by the Italian National Reference Centre for Animal Welfare (Centro di Referenza Nazionale per il Benessere Animale – CReNBA, Brescia, Italy), which developed a welfare monitoring system based on animal-related measures (Nielsen *et al.* 2004, Bertocchi *et al.* 2018). This system considers the farm management, personnel, facilities and equipment status quo with the aim of increasing the transparency of animal husbandry practices on farms and guaranteeing compliance with Directive 2008/120/EC, which establishes the minimum standards for the protection of pigs (implemented in Italy by the Legislative Decree no. 122 of 7 July 2011). It is organized as a checklist that is recognized by the Italian Ministry of Agricultural, Food and Forestry Policies as an official tool for farm welfare evaluation (Note from the Italian Ministry of Health 0018297-24/07/2018-DGSAF-MDS-P).

These systems give an overall judgment of animal welfare at the farm level, but the selected parameters are not always effective when applied to conditions of chronic stress (Bacci *et al.* 2014). In fact, animal stress responses are linked to many parameters, both subjective and environmental; they depend on the nature, intensity and duration of the stressor (Bacci *et al.* 2014).

Exposure to chronic stressors leads to injurious effects on health and welfare, caused by an allostatic overload due to the continuous activation of the protective mechanisms of

an organism to maintain its homeostasis in response to environmental variations (McEwen 2003). Allostatic load mediators include dehydroepiandrosterone-sulphate (DHEA-S) and cortisol (Ali *et al.* 2016). Cortisol and DHEA-S are endogenous hormones produced by the adrenal cortex in response to the secretion of adrenocorticotropic hormone (ACTH) (Kroboth *et al.* 1999, Bacou *et al.* 2017). DHEA-S, which is the main circulating form and acts as a reserve pool (Skarlandtová *et al.* 2014), is also produced to a lesser degree in the gonads or placenta, and can be produced as neurosteroids in the central nervous system (Skarlandtová *et al.* 2014). The actions of cortisol are numerous and include the stress response, increasing blood sugar through gluconeogenesis, suppressing the immune system, and aiding with the metabolism of fat, protein and carbohydrates (Marieb and Hoehn 2010). DHEA-S is a precursor for the synthesis of anabolic and sexual steroids (Kroboth *et al.* 1999). It also reacts to higher corticotrophin-releasing hormone (CRH) and ACTH levels (Nieschlag *et al.* 1973), so it could be considered a marker of hypothalamic-pituitary-adrenal (HPA) axis activity (Maninger *et al.* 2010). DHEA-S also has anti-glucocorticoid properties; it acts as an "anti-stress" steroid and minimizes negative glucocorticoid effects (Maninger *et al.* 2009). In fact, several studies suggest that variations in DHEA-S levels are associated with chronic health disorders (Tagliaferro and Ronan 2001). Depletion of this hormone has been associated with the development of chronic disease in humans (Tagliaferro and Ronan 2001), and, moreover, experimental evidence strongly suggests that DHEA-S is closely linked to health maintenance (Tagliaferro and Ronan 2001). The plasma levels of DHEA-S change with age; the highest levels are present during young adulthood and they slowly and permanently decrease.

The concentration of these steroids can be determined in different biological matrices, of which the most studied in the literature are blood (Cooper *et al.* 2009), saliva (Hillmann *et al.* 2008), urine (Pol *et al.* 2002), faeces (Möstl *et al.* 1999) and hair (Bacci *et al.* 2014, Trevisan *et al.* 2017). However, hair is currently the only matrix whose properties allow retrospective analysis and provide information over the medium and long terms. Hair collection is also simple and non-invasive, and samples are easy to store and transport. Another benefit of sampling hair is that the results are not subject to handling stress, as shaving the animals does not alter the hormone content of the hair in the same way that blood sampling can alter plasmatic hormone concentrations. As pointed out by Heimbürge and colleagues (Heimbürge *et al.* 2019) in a review, the use of hair hormone analysis requires some recommendations, such as the sampling of hairs from the same body region and of the same color; the consideration of the time delay between hormone incorporation and sampling of hair that depends on hair growth velocity, which may vary between species and body regions; the avoidance of external contamination; and the use of the "shave-reshave" method, if possible, to establish a precise timeframe. If a non-regrowth hair sample is used, one must consider that the pig hair follicles pass through one complete cycle per year and that the maximum percentage of the anagen phase occurs from August to January (Mowafy and Cassens 1976, Watson and Moore 1990). Although the precise mechanisms of incorporation into hair are still not fully understood, steroid hormones can only be incorporated into the growing hair shaft when the follicle is connected to the central blood supply (Davenport *et al.* 2006, Thom 2016). Several studies suggest that hair steroid concentrations are not subject to diurnal variation or acutely influenced by sweat (Stalder and Kirschbaum 2012, Grass *et al.* 2015), and that local follicular cortisol production may only marginally contribute to hair steroid concentrations *in vivo* (Grass *et al.* 2015). Thus, given the reliability and stability of this matrix, hair analysis is increasingly used in various fields for evaluating the activity of the HPA axis (Comin *et al.* 2012, Comin *et al.* 2013, Peric *et al.* 2017, Stradaioli *et al.* 2017, Caslini *et al.* 2016) and in behavioural studies (Dettmer *et al.* 2015, Montillo *et al.* 2019). In the literature, cortisol is the main steroid analysed in hair, but other steroids have recently been analysed such as dehydroepiandrosterone (DHEA) (Trevisan *et al.* 2017, Peric *et al.* 2017, Bergamin *et al.* 2019) and reproductive hormones (Peric *et al.* 2017, Dettmer *et al.* 2015, Bergamin *et al.* 2019, Cattet *et al.* 2017).

The aim of the present pilot study was to determine the allostatic load by analysing the hair cortisol and DHEA-S of commercial pigs belonging to different farms having good overall animal welfare according to the CREnBA assessment protocol.

Material and methods

Ethics

Hair samples were collected from commercial farms in Northern Italy that rear the heavy Italian pig to produce Prosciutto di Parma according to PDO (Protected Designation of Origin). Although hair sampling is non-invasive and is not a troublesome procedure, the study was carried out in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

Animals and study design

Three farms (F1, F2, and F3) given a grade higher than 8 (on a scale from 1 to 10) on the CReNBA evaluation system were selected. The CReNBA Welfare Assessment Sheet used to assess the farms is provided in the Supplementary Materials. Within these farms, from the same animals used for assessing the animal welfare, hair samples were collected. In particular, 86 pigs (25, 19, and 42 from F1, F2, and F3, respectively) were considered.

Farm 1 was a farrow-to-finish farm with 350 Hermitage sows with mean parity of 2.6 ± 2.8. Farm 2 was a farrow-to-finish farm with 626 Goland sows with a mean parity of 3.4 ± 3.1. Hair samples from Farms 1 and 3 were collected 1-10 days after weaning. Breeding management on both farms was a three-week batch farrowing system that led to natural oestrous synchronization by weaning. Farm 3 was part of a closed-cycle pig holding facility of 657 Duroc Anas x Dan AC pigs and animals were sampled at the end of the fattening cycle at the age of 8-10 months (23 females and 19 males).

On all the farms, pigs were reared according to the current Italian legislation, which implements Council Directive 2008/120/EC, laying down minimum standards for the protection of pigs. The environmental temperature was kept at around 25 °C.

Hair collection

All samples were collected in October 2016 to minimize the possible effect of season on hair cortisol concentrations, as described in Bacci and colleagues (Bacci *et al.* 2014). After being gently restrained, a patch of hair (20 x 30 cm) was collected from the dorsal area of the neck behind the ears of each pig, using surgical scissors to shave the hair close to the skin. Hair samples were kept in paper envelopes and stored in the dark at room temperature until analysis.

Hair washing procedure and extraction

Before analyses, hair strands were washed as described by Bacci and colleagues (Bacci *et al.* 2014). Hair was than extracted according to the method described by Trevisan and colleagues (Trevisan *et al.* 2017). In brief, approximately 60 mg of trimmed hair was placed in a glass vial along with 3 mL of methanol, and 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, and the remaining residue was dissolved in 0.8 mL of 0.05 M phosphate-buffered saline (PBS) of pH 7.5.

Hair hormone analysis

Cortisol

The hair cortisol concentrations were determined as described by Bergamin and colleagues (Bergamin *et al.* 2019) using a solid-phase microtiter RIA assay for pigs developed in-house. The cross-reactivities of the anti-cortisol antibody with other steroids were as follows: cortisol, 100%; corticosterone, 1.8%; aldosterone, < 0.02%. The intra- and inter-assay coefficients of variation were 3.7% and 10.1%. The sensitivity of the assay for cortisol was 1.23 pg/well.

DHEA-S

A 96-well microtiter plate (OptiPlate; Perkin-Elmer Life Sciences, Waltham, MA, USA) was coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer (pH 9) and incubated overnight at 4 °C. The plate was then washed twice with RIA buffer (pH 7.4) and incubated overnight at 4 °C with 200 µL of the anti-DHEA-S serum diluted 1:800 (Bertin Bioreagent, Montigny le Bretonneux, France). Cross-reactivities of this antibody with other steroids are as follows: DHEA-S 100%, 4-Androsten-3,17-dione (4-androstenedione) 0.2%, 4-Androsten-17-ol-3-one (testosterone) < 0.01%, 5-Androsten-3-ol-17-one (dehydroepiandrosterone, DHEA) < 0.01%, 5-Androstan-3-ol-17-one (androsterone) < 0.01%. After washing the plate with RIA buffer, the standards (5-200 pg/well), the quality-control extract, the test extracts, and the tracer (DHEA-S; Perkin-Elmer Life Sciences, Waltham, MA, USA) were added, and the plate was incubated overnight at 4 °C. The bound hormone was separated from the free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 µL of scintillation cocktail, the plate was counted on a β-counter (Top-Count; Perkin-Elmer Life Sciences, Waltham, MA, USA).

The intra- and inter-assay coefficients of variation were 3.9% and 10.3%, respectively. The sensitivity of the assay was 0.58 pg/well.

Statistical analysis

The statistical analysis was performed using SPSS for Windows (1989-1997) and R software (R software 2017). The animal was treated as experimental unit. The normality of the data distribution was tested using the Shapiro-Wilk test, and the homogeneity of variances was assessed using Levene's test. The differences between hair hormone levels at the two farms rearing sows (F1, F2), and between gilts and barrows at F3 farm were tested using the Mann-Whitney U test.

Results

The cortisol and DHEA-S concentration, and the cortisol/DHEA-S ratio were similar between barrows and gilts at F3 farm (Table I), as well as the animals' weight (median, min, max; 200 kg, 160 kg, 220 kg; P > 0.05; data not reported in tables). Therefore, the gender effect was not considered in subsequent analyses for this farm.

Box plots of hair cortisol and DHEA-S concentrations of the animals at the three farms considered are shown in Figures 1a and 1b, respectively. In Figure 1a, it is evident that the variability of the hair cortisol concentrations of animals were different between farms. Indeed, the interquartile range (IQR), which is considered a measure of the data variability [calculated as the difference between the first (Q1) and third (Q3) quartile], is 50.54 pg/mg, 95.09 pg/mg, and 8.98 pg/mg for F1, F2, and F3, respectively. Moreover, at all the farms at least one outlier was detected. Conversely, the variability of the hair DHEA-S concentration (Figure 1b) was similar between F1 and F2 farms (13.42 pg/mg and 12.08 pg/mg of IQR, respectively), but much lower at F3 (4.60 pg/mg of IQR). Also for this hormone, at all the farms at least one outlier was detected. Finally, the IQR of the cortisol/DHEA-S ratio was 2.60, 4.94, and 1.29 for F1, F2, and F3, respectively (Figure 1c), with outliers found in F1 and F3.

The body weight of the sampled animals was similar between F1 and F2 (median, min, max; 200 kg, 120 kg, 250 kg; P > 0.05; data not reported in tables), as well as the animals' parity (P > 0.05). The median [min-max] hair cortisol concentrations recorded on the three farms (F1, F2 and F3) were 38.09 [2.81; 153.29], 78.86 [13.88; 459.72] and 17.51 [9.13; 44.20] pg/mg while for DHEA-S were 15.73 [7.86; 61.89], 20.71 [12.49; 75.37] and 7.78 [4.39; 23.88] pg/mg. The median [min-max] cortisol/DHEA-S ratio was 1.99 [0.323; 7.97], 3.18 [0.773; 14.03] and 1.91 [0.809; 6.55] pg/mg for F1, F2 and F3, respectively. For all the variables considered (cortisol, DHEA-S and cortisol/DHEA-S ratio), F2 showed higher hair concentration levels than F1 (P < 0.05; Figure 1).

Discussion

The CReNBA welfare monitoring system gives an important assessment of a farm's compliance with the minimum requirements for the rearing of pigs (Directive 2008/120/EC).

In this pilot study, all three farms had a high CReNBA scoring even if, as observed from the box plots, both the hormones and their ratio showed different value distributions between the farms. The hair hormone concentrations recorded imply a difference in their allostatic load. Both cortisol and DHEA-S are

Table I. *Median [min-max] of hair hormone concentrations (pg/mg) and cortisol/DHEA-S ratio recorded for barrows and gilts in Farm 3 (F3).*

	Gender		*P*-value
	Barrow	Gilt	
Cortisol	17.36 [9.13; 33.62]	17.65 [10.53; 44.20]	0.658
DHEA-S	8.03 [4.39; 14.29]	7.30 [4.63; 23.88]	0.752
Cortisol/DHEA-S	1.91 [0.81; 4.81]	1.91 [0.96; 6.55]	0.930

DHEA-S = dehydroepiandrosterone-sulphate.

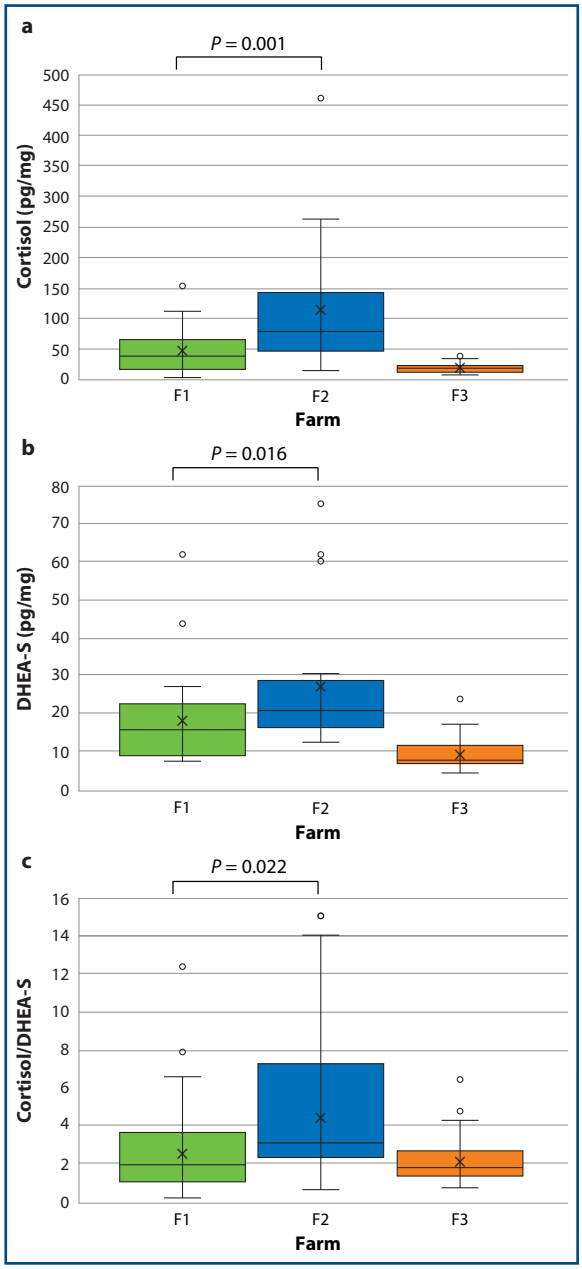

Figure 1. *Hair cortisol (**a**) and DHEA-S (**b**) concentrations, and hair cortisol/DHEA-S ratios (**c**), recorded on three pig farms (F1, F2, F3).* The box represents the interquartile range, in which the horizontal line represents the median, × represents the mean, and the small circle identifies outliers.

Veterinaria Italiana 2020, **56** (3), 177-184. doi: 10.12834/VetIt.1974.11885.1

considered indicators of allostatic load (McEwen 2003, Charney 2004) and resilience (Peric *et al.* 2017, Russo *et al.* 2012), and their ratio is believed to be an index of the anabolic/catabolic balance (Qiao *et al.* 2017). The ratio can be important in determining how an individual's HPA axis is functioning (Buckham Sporer *et al.* 2008, Guilliams and Edwards 2010, Saczawa *et al.* 2013). Since in mammals DHEA has anabolic and anti-glucocorticoids effects and, thus, protects against the catabolic effects of cortisol (Kalimi *et al.* 1994, Labrie *et al.* 2006), a common procedure used to simultaneously test the impact of both hormones is the ratio between cortisol and DHEA (Qiao *et al.* 2017, Goodyer *et al.* 1998, Hechter *et al.* 1997). It has been observed in different studies (Gallagher *et al.* 2006, Wolkowitz *et al.* 2001) that under acute stress stimulus, concentrations of DHEA increase as those of cortisol rise. Under conditions of chronic stress, DHEA remains unchanged or progressively diminishes while cortisol increases or does not change. This condition results in an elevated cortisol/DHEA ratio (Wolkowitz *et al.* 2001) that, in a scenario of allostatic overload, when demands exceed the resources available to meet the needs (McEwen and Wingfield 2003), could be expected to be found.

The distributions of values obtained in this study suggest different levels of allostatic load that may have been given not only by the individual welfare status but also by the different biological statuses, the different management strategies and environmental challenges at the farms, though the objective of this study has not been to identify which of these is the cause of non-uniformity. At present, however, there is still the need to identify threshold values for the detection of allostatic overload in order to set up strategies to control it. Chronic or repeated acute stresses can have cumulative negative health consequences for that originate from a high level of allostatic load for the organism. Both acute and chronic stress can be quantified by measuring the changes in physiological parameters such as heart rate, blood pressure, and levels of various metabolic hormones, but it can be difficult to discriminate between acute, chronic, or diurnal variations (Lee *et al.* 2015). The hair hormone concentration is assumed to be a retrospective marker of integrated hormone secretion over longer periods of time. Comparisons with literature data, however, must be done bearing in mind some peculiarities related to the bristles, which, like other hair samples, provide the integral of the concentrations of steroids referable to a time period of weeks or months, depending on whether the shave-reshave method was used for sampling or not. Hair growth in pigs, as in other species, occurs in cycles (anagen, catagen, and telogen phases). As reported by Mowafy and Cassens (Mowafy and Cassens 1976) and Watson and Moore (Watson and Moore 1990), pig hair follicles pass through one complete cycle per year and the seasons have a marked effect on the activity of the hair follicle. The anagen phase is about four months long and is more prominent during the autumn and winter. For the remaining period and until the end of the summer, the anagen phase is strongly reduced and marks the start of the moulting of the telogen follicles. So, the bristles collected in this study from sows 1-10 days after weaning in October started with a new cycle and the sample collected included hair already in the anagen phase, characterized by maximum hormonal incorporation. Indeed, even if the mechanisms of cortisol incorporation are not yet fully understood, steroid hormones can only be incorporated into the growing hair shaft when the follicle is connected to the central blood supply (Davenport *et al.* 2006, Thom 2016). Both elevated cortisol and cortisol/DHEA-S ratios can be explained by the presence in the sample of new-growth hair that incorporates the high hormone concentrations triggered by the progression of the lactation at that time. According to the lactation period, Weary and colleagues (Weary *et al.* 2002) described a system in which both sows and piglets are allowed to go to a communal area from day 12 (sows) or 14 (piglets) of lactation onwards. At the end of lactation (day 27), these sows spent over 14 h per day away from their piglets and nursed approximately 40% less than control sows in farrowing crates. Using a shave-reshave method and considering the hair retrospective features for hormone concentrations, Bacci and colleagues (Bacci *et al.* 2014) also recorded the highest hair cortisol concentrations just after weaning at the time of pregnancy diagnosis.

The statistical differences in the hormone concentrations and their ratios between the two farms rearing sows could also be seen in the breed differences. Lower hair cortisol concentrations were found by Peric and colleagues (Peric *et al.* 2013) in crossbreed F1 compared to Holstein-Friesian heifers, while in pigs only transgenic pigs were compared to their conventional close relatives (Martelli *et al.* 2014). From a management perspective and in light of reducing antibiotic use, this finding could be helpful for choosing the best breed for each farm and increasing animal robustness, as well as for the evaluation of animal responses to specific environments, housing systems and management schemes. The concept of robustness refers to the combination of high production potential and resilience to stressors in an individual, which enables sustained productivity under a wide variety of environmental conditions (Knap 2005). The HPA axis plays a central role in the trade-off between production and robustness (Mormède and Terenina 2012), and some authors (Bacou *et al.* 2017) have suggested the possibility of increasing robustness

through the genetic selection of animals based on the activity of their HPA axis. Hair could be a valid matrix for this purpose.

In entire male and female fattening pigs of 12 weeks of age (Antonides *et al.* 2015, Casal *et al.* 2017), the mean hair cortisol concentrations (19.30 ± 0.63 pg/mg; around 15.00-20.00 pg/mg) were close to the values recorded in our study, while in a study carried out on older animals (21 weeks of age) the concentrations recorded were even higher (pigs with no tail lesions: 42.20 ± 3.29 pg/mg; non-lame pigs: 43.07 ± 2.69 pg/mg) (Carroll *et al.* 2018). To our knowledge, no studies were carried out on older fattening pigs, which are usually characteristic of heavy pig farming. The highly standardized process of pig fattening that requires similar ages and weights between animals could explain the similar activity of the HPA axis between the sampled individuals. Observing the box plot, we see a narrow range of cortisol and DHEA-S values. As this is the first study to evaluate DHEA-S hair concentrations, it could form the basis for further evaluations considering this hormone and the ratio between cortisol and DHEA-S. As the fattening pigs were 8-10 month old at the time of hair sampling and we used a non-reshaving method, the hair hormones deposited in the hair shaft collected in October had not yet finished their cycle (Mowafy and Cassens 1976) and the sample collected should be mostly hair in the anagen phase, characterized by maximum hormonal incorporation.

The evaluation of hormone concentrations as markers of the allostatic load allows to obtain an overall data about the herd hormonal status as well specific information regarding each animal evaluated. Differently, the CREnBA welfare assessment protocol informs farmers about the general scoring of the production system including animal-based measures, which remains an important starting point for ensuring minimum requirements.

Conclusions

In conclusion, the study's results indicate that, although the official welfare evaluation method assessed the farms as having good overall animal welfare, the measurement of cortisol and DHEA-S in pig hair showed a different allostatic load between them. Thus, the hair hormone evaluation can be a reliable complementary tool for obtaining individual information regarding steroid concentrations over a medium-long period even if further research is needed to determine the cause of non-uniformity between farms. Hair analysis is an animal-friendly sampling technique that does not harm the animal and has great potential for use by official veterinarians and farmers at the farm and slaughter stages as a simple and reliable tool to assess the overall allostatic load. Thus, monitoring the concentrations of HPA axis mediators would provide the opportunity for prevention of, or earlier intervention in, stress-related chronic illnesses. An important step in future research would be to define the threshold values of hair hormone concentrations for the detection of allostatic overload in pigs.

References

Ali O.S., Badawy N., Rizk S., Gomaa H. & Saleh M.S. 2016. Allostatic load assessment for early detection of stress in the workplace in Egypt. Open Access Maced. *J Med Sci*, **15**, 493-498.

Antonides A., Schoonderwoerd A.C., Nordquist R.E. & van der Staay F.J. 2015. Very low birth weight piglets show improved cognitive performance in the spatial cognitive holeboard task. *Front Behav Neurosci*, **9**, 43.

Bacci M.L., Nannoni E., Govoni N., Scorrano F., Zannoni A., Forni M., Martelli G. & Sardi L. 2014. Hair cortisol determination in sows in two consecutive reproductive cycles. *Reprod Biol*, **14**, 218-223.

Bacou E., Haurogné K., Mignot G., Allard M., De Beaurepaire L., Marchand J., Terenina E., Billon Y., Jacques J., Bach J.M., Mormède P., Hervé J. & Lieubeau B. 2017. Acute social stress-induced immunomodulation in pigs high and low responders to ACTH. *Physiol Behav*, **169**, 1-8.

Bergamin C., Comin A., Corazzin M., Faustini M., Peric T., Scollo A., Gottardo F., Montillo M. & Prandi A. 2019. Cortisol, DHEA, and sexual steroid concentrations in fattening pigs' hair. *Animals (Basel)*, **9**, E345.

Bertocchi L., Fusi F., Angelucci A., Bolzoni L., Pongolini S., Strano R.M., Ginestreti J., Riuzzi G., Moroni P. & Lorenzi V. 2018. Characterization of hazards, welfare promoters and animal-based measures for the welfare assessment of dairy cows: elicitation of expert opinion. *Prev Vet Med*, **150**, 8-18.

Buckham Sporer K.R., Weber P.S.D., Burton J.L., Earley B. & Crowe M.A. 2008. Transportation of young beef bulls alters circulating physiological parameters that may be effective biomarkers of stress. *J Anim Sci*, **86**, 1325-1334.

Carroll G.A., Boyle L.A., Hanlon A., Palmer M.A., Collins L., Griffin K., Armstrong D. & O'Connell N.E. 2018. Identifying physiological measures of lifetime welfare status in pigs: exploring the usefulness of haptoglobin, C- reactive protein and hair cortisol sampled at the time of slaughter. *Ir Vet J*, **71**, 8.

Casal N., Manteca X., Peña R., Bassols A. & Fàbrega E. 2017. Analysis of cortisol in hair samples as an indicator of stress in pigs. *J Vet Behav*, **19**, 1-6.

Caslini C., Comin A., Peric T., Prandi A., Pedrotti L. & Mattiello S. 2016. Use of hair cortisol analysis for comparing population status in wild red deer (*Cervus elaphus*) living in areas with different characteristics. *Eur J Wildl Res*, **62**, 713-723.

Cattet M., Stenhouse G.B., Janz D.M., Kapronczai L., Anne Erlenbach J., Jansen H.T., Nelson O.L., Robbins C.T. & Boulanger J. 2017. The quantification of reproductive hormones in the hair of captive adult brown bears and their application as indicators of sex and reproductive state. *Conserv Physiol*, **5**, cox032.

Charney D.S. 2004. Psychobiological mechanisms of resilience and vulnerability: Implications for successful adaptations to extreme stress. *Am J Psychiatry*, **161**, 195-216.

Comin A., Peric T., Corazzin M., Veronesi M.C., Meloni T., Zufferli V., Cornacchia G. & Prandi A. 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. *Livest Sci*, **152**, 36-41.

Comin A., Veronesi M.C., Montillo M., Faustini M., Valentini S., Cairoli F. & Prandi A. 2012. Hair cortisol level as a retrospective marker of hypothalamic-pituitary-adrenal axis activity in horse foals. *Vet J*, **194**, 131-132.

Cooper T.A., Roberts M.P., Kattesh H.G. & Kojima C.J. 2009. Effects of transport stress, sex, and weaning weight on postweaning performance in pigs. *Profess Anim Sci*, **25**, 189-194.

Davenport M.D., Tiefenbacher S., Lutz C.K., Novak M.A. & Meyer J.S. 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen Comp Endocrinol, 147, 255-261.

Dettmer A.M., Rosenberg K.L., Suomi S.J., Meyer J.S. & Novak M.A. 2015. Associations between parity, hair hormone profiles during pregnancy and lactation, and infant development in rhesus monkeys (*Macaca mulatta*). *PLoS One*, **14**, e0131692.

EFSA. 2012. Scientific Opinion on the use of animal-based measures to assess welfare in pigs. *EFSA J*, **10**, 2512.

Gallagher P., Leitch M.M., Massey A.E., McAllister-Williams R.H. & Young A.H. 2006. Assessing cortisol and dehydroepiandrosterone (DHEA) in saliva: effects of collection method. *J Psychopharmacol*, **20**, 643-649.

Goodyer I.M., Herbert J. & Altham P.M. 1998. Adrenal steroid secretion and major depression in 8- to 16-year-olds, III. Influence of cortisol/DHEA ratio at presentation on subsequent rates of disappointing life events and persistent major depression. *Psychol Med*, **28**, 265-273.

Grass J., Kirschbaum C., Miller R., Gao W., Steudte-Schmiedgen S. & Stalder T. 2015. Sweat-inducing physiological challenges do not result in acute changes in hair cortisol concentrations. *Psychoneuroendocrinology*, **53**, 108-116.

Guilliams T.G. & Edwards L. 2010. Chronic stress and the HPA axis: clinical assessment and therapeutic considerations. *Standard*, **9**, 1-12.

Hechter O., Grossman A. & Chatterton R.T. Jr. 1997. Relationship of dehydroepiandrosterone and cortisol in disease. *Med Hypotheses*, **49**, 85-91.

Heimbürge S., Kanitz E. & Otten W. 2019. The use of hair cortisol for the assessment of stress in animals. *Gen Comp Endocrinol*, **270**, 10-17.

Hillmann E., Schrader L., Mayer C. & Gygax L. 2008. Effects of weight, temperature and behaviour on the circadian rhythm of salivary cortisol in growing pigs. *Animal*, **2**, 405-409.

Kalimi M., Shafagoj Y., Loria R., Padgett D. & Regelson W. 1994. Anti-glucocorticoid effects of dehydroepiandrosterone (DHEA). *Mol Cell Biochem*, **131**, 99-104.

Knap P. 2005. Breeding robust pigs. *Austr J Experim Agric*, **45**, 1-11.

Kroboth P.D., Salek F.S., Pittenger A.L., Fabian T.J. & Frye R.F. 1999. DHEA and DHEA-S: A review. *J Clin Pharmacol*, **39**, 327-348.

Labrie F., Luu-The V., Martel C., Chernomoretz A., Calvo E., Morissette J. & Labrie C. 2006. Dehydroepiandrosterone (DHEA) is an anabolic steroid like dihydrotestosterone (DHT), the most potent natural androgen, and tetrahydrogestrinone (THG). *J Steroid Biochem Mol Biol*, **100**, 52-58.

Lee D.Y., Kim E. & Choi M.H. 2015. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. *BMB Rep*, **48**, 209-216.

Maninger N., Capitanio J.P., Mason W.A., Ruys J.D. & Mendoza S.P. 2010. Acute and chronic stress increase DHEAS concentrations in rhesus monkeys. *Psychoneuroendocrinology*, **35**, 1055-1062.

Maninger N., Wolkowitz O.M., Reus V.I., Epel E.S. & Mellon S.H. 2009. Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). *Front Neuroendocrinol*, **30**, 65-91.

Marieb E.N. & Hoehn K. 2010. Human anatomy & physiology. San Francisco, California, Pearson Benjamin Cummings, 9th ed., 1264 pp.

Martelli G., Sardi L., Stancampiano L., Govoni N., Zannoni A., Nannoni E., Forni M. & Bacci M.L. 2014. A study on some welfare-related parameters of hDAF transgenic pigs when compared with their conventional close relatives. *Animal*, **8**, 810-816.

McEwen B.S. 2003. Interacting mediators of allostasis and allostatic load: towards an understanding of resilience in aging. *Metabolism*, **52**, 10-16.

McEwen B.S & Wingfield J.C. 2003. The concept of allostasis in biology and biomedicine. *Horm Behav*, **43**, 2-15.

Montillo M., Caslini C., Peric T., Prandi A., Netto P., Tubaro F., Pedrotti L., Bianchi A. & Mattiello S. 2019. Analysis of 19 minerals and cortisol in red deer hair in two different areas of the Stelvio National Park: a preliminary study. *Animals (Basel)*, **9**, E492.

Mormède P. & Terenina E. 2012. Molecular genetics of the adrenocortical axis and breeding for robustness. *Domest Anim Endocrinol*, **43**, 116-131.

Möstl E., Messmann E., Bagu E., Robia C. & Palme R. 1999. Measurement of glucocorticoid metabolite

concentrations in faeces of domestic livestock. *Zentralbl Veterinarmed A*, **46**, 621-631.

Mowafy M. & Cassens R.G. 1976. Hair growth in the domestic pig. Quantitative aspects. *J Am Leather Chem Assoc*, **71**, 71-78.

Nielsen B.H., Angelucci A., Scalvenzi A., Forkman B., Fusi F., Tuyttens F., Houe H., Blokhuis H., Sørensen JT., Rothmann J., Matthews L., Mounier L., Bertocchi L., Richard M., Donati M., Nielsen P.P., Salinim R. deGraafm S., Hildm S. Messorim S., Nielsenm S.S., Lorenzi V., Boivin X. & Thomsen P.T. 2014. External scientific report on the use of animal based measures for the assessment of dairy cow welfare. EFSA supporting publication, EN-659.

Nieschlag E., Loriaux D.L., Ruder H.J., Zucker J.R., Kirschner M.A. & Lipsett M.B. 1973. The secretion of dehydroepiandrosterone and dehydroepiandrosterone sulphate in man. *J Endocrinol*, **57**, 123-134.

Peric T., Comin A., Corazzin M., Montillo M., Canavese F., Stebel M. & Prandi A. 2017. Relocation and hair cortisol concentrations in New Zealand white rabbits. *J Appl Anim Welf Sci*, **20**, 1-8.

Peric T., Comin A., Corazzin M., Montillo M., Cappa A., Campanile G. & Prandi A. 2013. Short communication: hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers. *J Dairy Sci*, **96**, 3023-3027.

Peric T., Corazzin M., Romanzin A., Bovolenta S., Prandi A., Montillo M. & Comin A. 2017. Cortisol and DHEA concentrations in the hair of dairy cows managed indoor or on pasture. *Livest Sci*, **202**, 39-43.

Pol F., Courboulay V., Cotte J.P., Martrenchar A., Hay M. & Mormède P. 2002. Urinary cortisol as an additional tool to assess the welfare of pregnant sows kept in two types of housing. *Vet Res*, **33**, 13-22.

Qiao S., Li X., Zilioli S., Chen Z., Deng H., Pan J. & Guo W. 2017. Hair measurements of cortisol, DHEA, and DHEA to cortisol ratio as biomarkers of chronic stress among people living with HIV in China: known-group validation. *PLoS ONE*, **12**, e0169827.

RCoreTeam. 2017. (Computer software). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing. Available online: https://www.R-project.org (accessed on 13 April 2019).

Russo S.J., Murrough J.W., Han M., Charney D.S. & Nestler E.J. 2012. Neurobiology of resilience. *Nat Neurosci*, **15**, 1475-1484.

Saczawa M.E., Graber J.A., Brooks-Gunn J. & Warren M.P. 2013. Methodological considerations in use of the cortisol/DHEA(S) ratio in adolescent populations. *Psychoneuroendocrinology*, **38**, 2815-2819.

Skarlandtová H., Bičíková M., Neužil P., Mlček M., Hrachovina V., Svoboda T., Medová E., Kudlička J., Dohnalová A., Havránek Š., Kazihnítková H., Máčová L., Vařejková E. & Kittnar O. 2014. Are there any differences between stress hormone levels in non-stress conditions and in potentional stress overload (heart catheterisation) in sows? *Physiol Res*, **63**, 733-741.

SPSS® Statistical package for social science SPSS. 1989-1997. (Computer software). Advanced Statistics 7.5. Chicago, IL, USA, SPSS Inc.

Stalder T. & Kirschbaum C. 2012. Analysis of cortisol in hair: state of the art and future directions. *Brain Behav Immun*, **26**, 1019-1029.

Stradaioli G., Peric T., Montillo M., Comin A., Corazzin M., Veronesi M.C. & Prandi A. 2017. Hair cortisol and testosterone concentrations and semen production of *Bos taurus* bulls. *Ital J Anim Sci*, **16**, 631-639.

Tagliaferro A.R. & Ronan A.M. 2001. Physiological levels and action of dehydroepiandrosterone in Yucatan miniature swine. *Am J Physiol Regul Integr Comp Physiol*, **281**, R1-R9.

Thom E. 2016. Stress and the hair growth cycle: cortisol-induced hair growth disruption. *J Drugs Dermatol*, **15**, 1001-1004.

Trevisan C., Montillo M., Prandi A., Mkupasi E.M., Ngowi H.A. & Johansen M.V. 2017. Hair cortisol and dehydroepiandrosterone concentrations in naturally *Taenia solium* infected pigs in Tanzania. *Gen Comp Endocrinol*, **246**, 23-28.

Watson S.A. & Moore G.P. 1990. Postnatal development of the hair cycle in the domestic pig. *J Anat*, **170**, 1-9.

Weary D.M., Pajor E.A., Bonenfant M., Fraser D. & Kramer D.L. 2002. Alternative housing for sows and litters: Part 4. Effects of sow-controlled housing combined with a communal piglet area on pre- and post-weaning behaviour and performance. *Appl Anim Behav Sci*, **76**, 279-290.

Wolkowitz O.M., Epel E.S. & Reus V.I. 2001. Stress hormone-related psychopathology: pathophysiological and treatment implications. *World J Biol Psychiatry*, **2**, 115-143.