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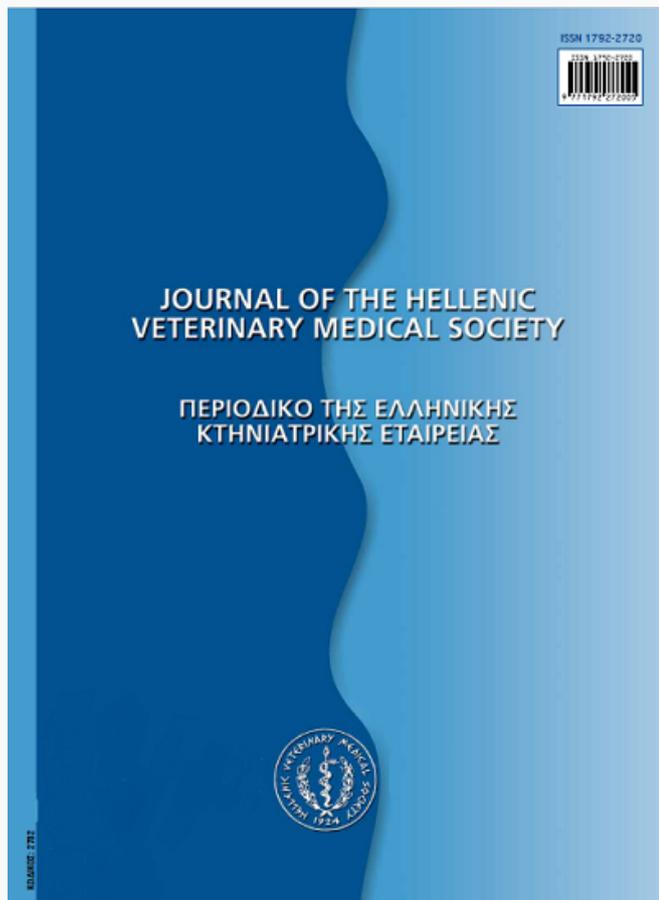
ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

Application of liquid chromatography coupled to high-resolution mass spectrometry to measure urinary cortisol in loose housed sows

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◼ Application of liquid chromatography coupled to high-resolution mass spectrometry to measure urinary cortisol in loose housed sows

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◼ Εφαρμογή υγρής χρωματογραφίας σε συνδυασμό με φασματογραφία μάζας υψηλής ανάλυσης στον προσδιορισμό της κορτιζόλης σε ούρα ομαδικά σταβλισμένων χοιρομητέρων

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ABSTRACT. Cortisol is the most common physiological parameter used to measure welfare in pigs. In field studies evaluating stress in individual pigs which are group housed, the collection of spontaneously voided urine is practical. The purpose of the study was to apply a liquid chromatography coupled to high-resolution mass spectrometry approach to observe the patterns of diurnal urinary cortisol excretion among loose sows of three herds. We applied the analytical method in spontaneously voided urine of thirty, repeatedly sampled within a day, multiparous sows of three Greek herds. We found the level of urinary cortisol being highest before morning feeding [geometric mean of urinary cortisol to creatinine ratio being 2.72 (95% confidence interval: 1.17, 6.30), 5.65 (3.15, 10.14) and 2.60 (1.50, 4.50) in sows of herds A, B, and C, respectively] and lowest at 19:00 h [0.56 (0.27, 1.18), 1.24 (0.74, 2.07), 0.88 (0.55, 1.44)]. However, the patterns of diurnal urinary cortisol excretion appeared different among herds.

Keywords: Liquid chromatography coupled to high-resolution mass spectrometry, Swine, Urinary cortisol

ΠΕΡΙΛΗΨΗ. Ο προσδιορισμός της στάθμης της κορτιζόλης είναι η φυσιολογική παράμετρος που χρησιμοποιείται συχνότερα στην εκτίμηση της ευζωίας των χοίρων. Για την αξιολόγηση της καταπόνησης (stress) των χοίρων που σταβλίζονται ομαδικά, η συλλογή δειγμάτων ούρων κατά την ούρηση των ζώων είναι εύκολη και πρακτική. Σκοπός της εργασίας αυτής ήταν η εφαρμογή υγρής χρωματογραφίας σε συνδυασμό με φασματογραφία μάζας υψηλής ανάλυσης για την περιγραφή της καμπύλης απέκκρισης κορτιζόλης στη διάρκεια της ημέρας σε ούρα ομαδικά σταβλισμένων χοιρομητέρων. Ο προσδιορισμός της κορτιζόλης έγινε σε δείγματα ούρων που συλλέχθηκαν κατά τη διούρηση από 30 χοιρομητέρες τριών ελληνικών εκτροφών. Από κάθε ζώο συλλέχθηκαν επαναλαμβανόμενα δείγματα την ίδια ημέρα. Βρήκαμε ότι η στάθμη της κορτιζόλης στα ούρα ήταν υψηλότερη πριν το πρωινό τάισμα των ζώων [ο γεωμετρικός μέσος όρος του λόγου της στάθμης της κορτιζόλης προς τη στάθμη της κρεατινίνης ήταν 2.72 (95% εμπιστοσύνης: 1,17, 6,30), 5,65 (3,15, 10,14) και 2,60 (1,50, 4,50) στις χοιρομητέρες των εκτροφών Α, Β, και Γ, αντιστοίχως] και χαμηλότερη στις 7:00 μ.μ. [0,56 (0,27, 1,18), 1,24 (0,74, 2,07), 0,88 (0,55, 1,44)]. Οι καμπύλες ημερήσιας απέκκρισης της κορτιζόλης στα ούρα των χοιρομητέρων διέφεραν σημαντικά μεταξύ των τριών εκτροφών.

Λέξεις ευρετηρίασης: Υγρή χρωματογραφία σε συνδυασμό με φασματογραφία μάζας υψηλής ανάλυσης, χοίροι, στάθμη κορτιζόλης στα ούρα.

INTRODUCTION

Cortisol is the most common physiological parameter used to measure welfare in pigs (Terlouw et al., 1997). To overcome the stress induced by blood sampling itself, methods for measurement of cortisol in pig urine or saliva have been developed. Urine steroid analysis may provide an integrated measure of cortisol production over a period of time (Mormède et al., 2007). In field studies evaluating stress in individual pigs which are group housed, the collection of spontaneously voided urine is more practical than the collection of saliva. Although glucocorticoids and their metabolites in urine are present in very low concentrations in a complex matrix, ultra-high performance liquid chromatography (U-HPLC) coupled to mass spectrometry has been proven suitable to enable sensitive detection of glucocorticoids in urine (Touber et al., 2007). The current trend within the field of mass spectrometric techniques is full scan high resolution MS analysis using ToF (time of flight) instruments (Wicklund

et al., 2008), Fourier Transform Ion Cyclotron Resonance (Marshall, 2000) or Fourier Transform Orbitrap MS (De Clercq et al., 2013). Because of pressure from welfare organizations as well as food distribution chains promoting good animal welfare to consumers, group sow housing systems are mandatory in the European Union from January 2013 onwards (EU Directive 2001/88/EC). Changes in several aspects of management accompanying adaptation from individual stalls to loose sow systems (Nielsen, 2008) are required in order to improve welfare and maintain sow productivity. Cortisol excretion data can be used as a welfare indicator when the stressor is known to negatively affect welfare (Fraser, 2008). Repeated stress stimulates the adrenals to produce cortisol (Otten et al., 2004). Its increase may reduce the number of liveborn piglets, their birth and weaning weight and increase the number of stillbirths and mummified piglets (Melchior et al., 2012). Therefore, the purpose of the study was to apply a liquid chromatography coupled to high-resolution mass spectrometry approach to observe the patterns of

diurnal urinary cortisol excretion among mid-pregnancy, multiparous, loose sows of three herds.

MATERIALS AND METHODS

Animals

Thirty multiparous sows (Landrace × Yorkshire; 3 groups of 10 sows each), 45-60 days-in-pig, belonging to three different Greek herds, were used. They were, indoor, operating on weekly farrowing schedules, farrow-to-finish herds, comprising 330 (A), 160 (B) and 800 (C) sows, respectively, with Danbred (A, B) and Hermitage (C) genetics. The sows were loose in static groups of 10 with free-access to non-locking stalls, for a period of at least 15 days before sample collection, in pens designed to conform to the regulations of the Directive. They were automatically fed a total of 2.6-2.8 kg DM per sow daily of typical dry sow diets containing 12.7 MJ ME and 4.9% crude fiber/kg DM given either in one meal at 07:00 h (B and C) or split in half and offered in two meals at 07:00 and 16:00 h (A). Herd visits and samplings were conducted in May 2014.

Sampling

Animals were accustomed to entrance of staff into the pen and to the sampling procedure during the two days preceding sampling. On sampling day, spontaneously voided mid-stream urine were collected from all sows early in the morning approximately 30-45 min before feeding at 07:00 (thereafter indicated as 06:30 sampling), and again at 13:00 and 19:00 h in herds B and C. In herd A an additional sampling was performed at 15:30 h before the second meal (Table 1). Samples were taken using cups (300 ml) attached to the end of a pole (1.5 m), which allowed collection from a distance. Sampling of all sows in the pens lasted from 10 to 40 minutes. From each sample, approximately 30 ml of urine were put into tubes and stored in insulated containers with dry ice until transport to the laboratory on the same day, where they were stored at -80 °C until analysis.

Cortisol measurement

Measurement of urine creatinine was used to correct for urine dilution (Hay et al., 2000; Pardue et al.,

1987). Standards of cortisol and the internal standard cortisol-d_4 were purchased from Sigma-Aldrich (St. Louis, USA). Primary stock solutions were prepared in ethanol at a concentration of 200 μg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at a range of 0.1 – 10 μg mL^{-1}. Reagents were of analytical grade when used for extraction purposes and obtained from VWR International (Merck, Darmstadt, Germany). For UHPLC-HRMS applications, reagents were of LC-MS Optima grade and obtained from Fisher Scientific (Loughborough, UK). The analytical extraction and purification of urine samples was performed according to De Clercq et al. (2013). Briefly, a five mL aliquot of urine was spiked with the internal standard (cortisol-d_4) to obtain final concentration levels of 10 μg L^{-1}. Next, a twofold liquid-liquid extraction with pure tert-butyl methylether was performed. The organic phases were collected, pooled and dried under a gentle stream of nitrogen at a temperature of 50 °C. The residue was dissolved in 100 μL solvent and transferred to a vial for UHPLC-HRMS analysis. Analysis was performed by UHPLC-Orbitrap mass spectrometry [6] and validated according to CD 2002/657/EC. The chromatographic separation was achieved by using an Accela UHPLC system (Thermo Fisher Scientific, San José, USA), equipped with a Nucleodur Isis C18 column (1.8 μm, 100 mm x 2 mm, Macherey-Nagel, Düren, Germany). The binary solvent system consisted of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) (80/20, v/v). The percentage of acetonitrile was increased to 25% in 1 min, and held there for 5.0 min. Next, a linear increase to 95% B in 1 min was performed, and further up to 100% in 1 min and held there for 2.0 min. High-resolution mass spectrometric analysis was performed on an Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, USA), equipped with a heated electrospray ionization probe (HESI-II), operating in a polarity switching mode. The resolution was set at 50,000 FWHM at 1 Hz and a scan range of m/z 150-650 was chosen. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San José, USA). Eight-point calibration curves in urine samples were used for quantification of the different compounds. The samples were fortified with concentrations, ranging from 0.50 to 75 ng mL^{-1} for cortisol and cortisone.

Statistical analysis

For statistical analyses, the cortisol/creatinine ratio was calculated, in order to account for differences in urine production among sows, and transformed to its natural logarithm (LCC) for accommodating regression assumptions. Within each herd, the LCC were compared among sampling times by the model

$$Yjm = \mu + \alpha j + Am + ejm$$

where μ is the expected mean value of the dependent variable, α_j is the effect of the j_{th} sampling time, A_m is the random effect of the m_{th} sow and e_{jm} is the error term. Models' fit were graphically assessed by comparing the observed to the predicted values (Rabe-Hesketh and Skrondal, 2008). Analyses were conducted in Stata 13.1 (Stata Statistical Software. College Station, TX) and evaluated for significance at P<0.05.

RESULTS

One sow in farms A and B and three sows in farm C did not give adequate urine on one sampling occasion each. The geometric means of the cortisol/creatinine ratio by sampling time and farm are in Table 1. In herd A, the means of LCC did not differ between 06:30 and 13:00 ($P > .05$), between 06:30 and 15:30 h ($P > .05$) or between 13:00 and 15:30 h

($P > .05$); they decreased at 19:00 h (all pairwise $P < .001$). In herd B, the means of LCC were decreasing (06:30 vs 13:00 h, $P < .01$; 13:00 vs 19:00 h, $P < .01$) and in herd C, they decreased ($P < .05$) between 06:30 and 13:00 h but not between 13:00 and 19:00 h ($P > .05$); they were lower ($P < .001$) at 19:00 compared to 06:30 h.

DISCUSSION

In this study we sampled spontaneously voided urine of loose pregnant sows in three herds with different genetics and similar feed composition in order to study the diurnal patterns of urinary cortisol. We analyzed the urine with an analytical methodology with high analytical sensitivity and specificity. We only studied thirty sows in three herds because: 1) repeated sampling within a day of spontaneously voided urine is labor intensive and 2) to validly compare patterns among herds, sampling of animals in the different herds should be done in a relative short time-period since urinary cortisol exhibits seasonal rhythms and is affected by environmental factors such as temperature and humidity (Melchior et al., 2012). In loose housed sows, cortisol levels may rise shortly after mixing of the animals in groups but are lowered after the establishment of hierarchy (Jansen et al., 2007). Thus, we conducted the samplings more than 15 days after the

Time	Sampling	06:30		13:00	15:30		19:00
	Feeding		07:00			16:00	
Farm							
A		2.72[a]	Yes	1.68[a]	2.44[a]	Yes	0.56[b]
		(1.17, 6.3)		(1.05,2.67)	(1.68,3.55)		(0.27,1.18)
B		5.65[a]	Yes	2.70[b]	NS	No	1.24[c]
		(3.15,10.14)		(1.9,3.86)			(0.74,2.07)
C		2.60[a]	Yes	1.50[b]	NS	No	0.88[b]
		(1.5,4.5)		(0.83, 2.7)			(0.55, 1.44)

Means with different superscripts within farms differ ($P < .05$)
NS not sampled

Table 1. Geometric means (95% CI) of the diurnal urinary cortisol/creatinine ratio of thirty, group housed, multiparous Landrace × Yorkshire sows of three Greek farms (10 sows from each farm).

static groups were formed. Pigs are species with diurnal habits, with plasmatic levels of glucocorticoids (including cortisol) being naturally higher in the early morning and decreasing throughout the day (Mormède et al., 2007). In accordance, we found the level of urinary cortisol being highest before morning feeding and lowest at 19:00 h in all herds, a result reassuring the adequacy of the analytic technique employed. However, the within herd patterns of diurnal urinary cortisol excretion were different. In herd A the higher morning level of urinary cortisol remained similar for at least 9 h and was found to decrease at the sampling performed 3h after the second meal. In herd B urinary cortisol decreased gradually throughout daytime and in herd C it decreased from 06:30 to 13:00 h but not from 13:00 to 19:00 h, although there were numerical differences (Table 1). In summary, for assessing welfare of loose sows, measuring urinary cortisol with the liquid chromatography coupled to high-resolution mass spectrometry approach appears valid and useful. Researchers should expect, and may choose to control in the design of their studies, among herds differences in the urinary cortisol of sows because excretion data may vary due to pulsatility, influence of managerial and animal related factors such as temperature, humidity, feeding schedules and physiological state of animals.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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