The effects of ovarian biopsy and blood sampling methods on salivary cortisol and behaviour in sows

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Abstract

In reproductive physiology research, experimental animals are often subjected to stressful procedures, including blood sampling and biopsy. In this present study, presence of pain or distress induced by four different procedures was examined using a measurement of salivary cortisol levels and activity observations in sows. The treatments were: 1) PAL: The ovary was palpated through the rectum without snaring, 2) TUB: transvaginal ultrasound-guided biopsy of the ovary was conducted without snaring, 3) SNA: a soft rope snare was placed around the maxilla, 4) CAT: A soft rope snare was placed around the maxilla, and an intravenous catheter was inserted through the ear vein of the sows. Activities, social cohesion and other pain-related behaviour, and salivary cortisol concentrations were recorded. Salivary cortisol concentrations in CAT sows increased in response to the procedure ($P < 0.05$), whereas the other treatments did not trigger a significant response. The CAT sows had higher cortisol concentrations than the other groups for 10 min after initiation of the procedures ($P < 0.01$), and they maintained higher cortisol levels than the PAL and TUB groups 15 min post-treatment ($P < 0.05$). Furthermore, the CAT sows showed the highest

frequency of head shaking ($P < 0.001$) and trembling behaviour ($P < 0.05$) during the 1h post-treatment. Summarizing, the catheterization procedure might induce a short-term pain or stress response during and after the procedure in terms of pain-related behaviour and salivary cortisol status. We suggest that TUB might not cause appreciable pain or distress.

Additional keywords: pain behaviour; stress response; glucocorticoid; luteal tissue; catheterization

1. Introduction

Björkman et al. (2016) examined the use of a transvaginal ultrasound-guided biopsy technique (TUB) for the observation of the porcine corpus luteum together with its effects on reproductive performance. In the past, a biopsy of the corpus luteum in pigs could be completed only through anaesthesia and laparotomy (Ribeiro et al., 2007) or euthanasia (Conley and Ford, 1989), with attendant sampling limitations and welfare concerns. The TUB was designed to provide an alternative method, and possibly promote research gains and pig welfare. Consequently, Björkman et al. (2016) suggested that this could be extensively utilized for longitudinal studies to investigate corpus luteum mechanisms in pigs. However, it is not known whether a less invasive biopsy method still causes distress to the pig.

A nonsurgical catheterization procedure through the auricular vein is well accepted and used in numerous pig studies to collect frequent and sequential blood samples, minimizing the number of times needles are inserted, thereby avoiding vascular injury and pain and distress. The method has been verified as suitable for collecting blood samples through various studies also conducted in our research stations (Peacock, 1991; Peltoniemi et al., 1995; Tast et al., 2001; Virolainen, 2005; Yun et al., 2013). To date, however, the potential of the catheterization method to trigger a stress response,

particularly in situations when pigs are tethered during the procedure and when their necks and ears are bandaged during the sampling periods, has not been examined.

Measuring cortisol concentrations could be an effective method to assess pain and distress in animals, particularly if they are physically restrained for research purposes (Weary et al., 2006; Merlot et al., 2011). In lieu of a blood sample in which the stressful sampling methods could significantly impact the biological process under investigation, measuring cortisol concentrations in saliva represent a growing trend in welfare studies, and have been validated in numerous pig studies (Geverink et al., 1999; Hillmann et al., 2008; Cook et al., 2013). Because the saliva sampling method is non-invasive and less stressful compared with blood sampling, it might be possible to collect multiple cortisol samples for measuring distress in pigs (Cook et al., 2013). In addition, it is widely known that observations of pain-related behaviours and reduction in particular behaviours, such as normal activity, are also useful for pain assessment in pig research (e.g. Weary et al., 2006).

Many benefits accrue from using the TUB or catheterization methods, particularly for taking multiple samples and improving the welfare of experimental animals. However, it has not yet been determined if the procedures are accompanied by pain and stress in the animal. We hypothesized that both the TUB and the catheterization method would cause a similar short-term stress response in the experimental sows, considering the degree of invasiveness of the procedures involved. In this study, therefore, the short-term stress response of sows to TUB and catheterization was examined through changes in physiological and behavioural variables.

2. Materials and methods

The study procedure was reviewed and approved by the Animal Experiment Board (ELLA) in Finland, permission ESAVI/3331/04.10.03/2011. The experiment was conducted on a commercial pig farm registered as an experimental research station in Vihti, southern Finland during 2015.

71 2.1. Animals and management

72 Sows were housed in groups, approximately twenty per group pen (20 × 5 m), where they were
73 allowed ad libitum access to water from a nipple drinker, and were fed a standard pregnancy diet
74 twice a day (08:00/16:00) via an automatic liquid feeding system. One day prior to the procedures,
75 all the sows included in the experiment were randomly paired, and each pair was separately housed
76 in pens (2.5 × 2.5 m). Both group and individual pens consisted of a solid concrete floor with
77 abundant straw as bedding material. Before the procedures, each sow was individually transported
78 to the operation stall located in the corridor outside the room. The operation stall comprised a
79 conventional steel crate, a feeding trough and a rubber mat on the floor. After the treatments, the
80 sows were returned to the individual pen, staying with their original pen mates, and their behaviour
81 was monitored for one hour thereafter. The sow transportation both ways, between the pen and the
82 operation stall, was done gently within one minute, by a trained staff member.

83 2.2. Experimental design

84 A total of 32 weaned sows (Finnish Yorkshire × Large White; parity 3.9 ± 0.6) were randomly
85 selected for the experiment, approximately four days after weaning. The sows were assigned to four
86 treatments: 1) Ovarian palpation (PAL; N = 8): The ovary of the sows was palpated through the
87 rectum without snaring, 2) Biopsy (TUB; N = 8): TUB of the ovary of the sows was conducted
88 without snaring, 3) Snaring (SNA; N = 8): A soft rope snare was placed around the maxilla of the
89 sows, 4) Catheterization (CAT; N = 8): A soft rope snare was placed around the maxilla, and an
90 intravenous catheter was inserted through the ear vein of the sows. Sows in the PAL and SNA
91 groups were designed as control groups of the TUB and CAT sows, respectively. This experimental
92 setup was used to evaluate the separated stress factor induced by the TUB and catheterization per
93 se, while standardising other stressors, i.e., palpating and snaring. The sows of the PAL and TUB
94 groups were treated on the same day, and the sows of the SNA and CAT groups were treated on the
95 following day. All procedures, including the TUB and catheterization, lasted for approximately 10

96 min per sow and they were performed at the prescribed time between 1200 and 1500 h. The timing
97 and duration of the procedure according to the treatments were considered to standardize cortisol
98 circadian patterns on the proceeding day. One sow from TUB had to be excluded from data analysis
99 as we failed to collect a biopsy from the sow.

100 2.2.1. Ovarian palpation (PAL) and transvaginal ultrasound-guided biopsy (TUB)

101 After sows were transported to the operation stall, faeces were removed from the rectum, and the
102 vulva was washed three times with a Povidone-Iodine solution (7.5% Betadine, Leiras Oy, Helsinki,
103 Finland). The ovary of the sows in the PAL group was palpated through the rectum for
104 approximately 10 min. The sows in both PAL and TUB groups were not snared during the process.
105 The TUB procedure was thoroughly described by Björkman et al. (2016). Briefly, the Tru-Cut
106 biopsy needle (16-gauge diameter, Zamar, Ultimate, PMO16-25, Poreč, Croatia), consisting of an
107 inner needle with a 1 cm specimen notch and an outer cutting cannula, was inserted into a needle
108 guide (Length 18 cm; DBSE12X Biopsy kit, Esaote SpA, Maastricht, The Netherlands), which was
109 placed onto a 6.8 MHz micro-convex array probe (Length 30 cm; Endocavity probe, SE3123,
110 Esaote SpA, Maastricht, The Netherlands). The probe was connected to an ultrasound device
111 (MyLabOne Vet, Esaote SpA, Maastricht, The Netherlands), and placed into the vagina towards the
112 uterine cervix. Simultaneously, the ovary was palpated through the rectum and relocated towards
113 the caudal end of the cervix and the ultrasound probe. After the ovary appeared on the ultrasound
114 screen, the biopsy needle was placed through the vaginal wall and the ovarian surface into the
115 ovary. When in place, the inner needle was pushed about 1 cm into the ovarian tissue, followed by
116 the outer cannula, which cut and trapped ovarian tissue inside the notch.

117 2.2.2. Snaring (SNA) and catheterization through the auricular vein (CAT)

118 The sows in CAT and SNA were caught with a soft rope snare placed around the maxilla to
119 provide restraint for 10 min while being catheterized or just snared, respectively. The dorsal surface

of the ear was cleaned (7.5% Betadine, Leiras Oy, Helsinki, Finland) and disinfected (Desinfektol P, Berner Oy, Helsinki, Finland). The catheterization method was detailed previously (Virolainen, 2005; Yun et al., 2013). A tourniquet was tied around the base of the ear and a 13-gauge intravenous catheter (Intraflon2, Vygon, Ecouen, France) was inserted into the auricular vein, and a 50 cm long vinyl tube (OD/ID of 1.5 × 1.0 mm, Steri-products, Australia) was threaded through the catheter into the jugular vein. The 13-gauge catheter was removed, and an 18-gauge blunted needle hub was inserted into the end of the vinyl tubing. A stopper was then inserted into the needle hub to prevent blood backflow. The end of tube with the stopper was stored in a Velcro pouch attached to the nape of the sow's neck. The neck together with the folded-ear was bound twice with a bandage.

2.3. Sample collection and assays

Five saliva samples from each sow were collected on synthetic swabs (Salivette® Cortisol, Sarstedt, Nümbrecht, Germany). The swabs were fixed with forceps and placed around the back teeth for approximately one minute by a trained researcher. If necessary, the researcher induced the sow to chew the swab by gently rubbing. In cases where sows were not able to move their jaws due to snaring, the researcher wiped around the back teeth with the swab to obtain the sample. The first and the last saliva samples from the sow were collected in the individual pens, and the other samples were collected at the operation stalls within over five minute intervals (Table 1). All saliva samples were collected in ice-chilled tubes, and centrifuged for 10 min at 1000 × g. The samples were stored at -20 ℃ for subsequent analysis of cortisol.

Table 1. Scheme for saliva sampling from sows during palpation (PAL), biopsy (TUB), snaring (SNA) and catheterization (CAT).

Time (min)	PAL (n=8)	TUB (n=7)	SNA (n=8)	CAT (n=8)
-5	Sampling at the individual pen			
	Sows are transported to the operation stall			

0	Sampling at the operation stall			
	Procedures initiate according to the treatments			
5	Sampling 5 min after the initiation of procedures			
10	Sampling 10 min after the initiation of the procedure	Sampling immediately after collecting biopsy	Sampling immediately after releasing a snare	Sampling immediately after releasing a snare
	Sows are transported to the individual pen			
15	Sampling at the individual pen			

Concentrations of salivary cortisol were analyzed in duplicate with a commercial radioimmunoassay kit (ImmuChem^{TM} CT cortisol kit, MP Biomedicals, Orangeburg, NY, USA) using a modified RIA method for saliva. Briefly, a calibration curve (from 0.5 to 50 ng/ml) suitable for saliva samples was made by diluting the highest calibrator of the kit with phosphate-buffered saline (pH 7.5). 200 µl of each saliva sample or diluted calibrator was added to antibody coated tubes and incubated with 1 ml of 125I-labelled cortisol solution at 37 ℃ for 45 min. The tubes were decanted and counted in a gamma counter. Parallelism between undiluted and 4-fold diluted saliva samples was 99%. The quantification limit of the cortisol assay was 0.5 ng/ml. The intra- and inter-assay coefficients of variation were 6.5-9.7% and 9.7-12.1%, respectively.

2.4. Behavioural observations

All sows were video-recorded continuously for one hour after the procedures to monitor activities, social cohesion and other pain-related behaviour. Internet protocol (IP) cameras (Niceview NICECAM420WL, Niceview Corp.) were mounted in each pen. The sequence output was recorded using IP-camera software (Blue Iris v.2.64, Perspective Software Corp.). The display resolution was 640 × 480 pixels, and the frame rate was 2 FPS.

The CowLog v.2.0 (Hänninen and Pastell, 2009) behavioural recording program with a trained observer was used for data analyses. The durations of activities were monitored, including standing

or walking, sitting, and sternal and lateral recumbence: (1) Standing or walking: standing on four feet or moving legs while standing, (2) Sitting: both front legs are straight while sitting, (3) Sternal recumbence: the sow lies on the sternum with udder concealed, (4) Lateral recumbence: the sow lies on the lateral with udder exposed and head, hip bone and shoulder touches the ground. Furthermore, the duration of social cohesion was assessed based on the distance between two sows in a pen. When the distance between the heads of two sows in the individual pen was approximately greater than sow mean body length, it was termed 'isolation'. In addition, 'desynchronization' was defined as when sows showed different activities or poses against their pairs in the pen. Four parameters selected from the literature (Hay et al., 2003; Noonan et al., 1994) were used to evaluate specific normal (i.e., sniffing) or pain-related (i.e., rubbing, trembling and head shaking) behaviour in sows after treatment: (1) Sniffing: The snout close to the floor with the head down, (2) Rubbing: Rubbing or scratching the body against the pen walls, (3) Trembling: Shivering as if cold, (4) Head shaking: Shaking head rapidly from side to side. These behaviours were represented by their occurrences during one hour after treatment.

2.5. Statistical analysis

Statistical processing of all data was done in SAS v.9.4 (SAS Institute Inc., NC, USA, 2012). Significant differences between treatment means were determined by LSD application, and set at $P < 0.05$, and tendencies were determined if $P > 0.05$ and $P < 0.10$. An individual animal represented an experimental unit.

A mixed model, using treatment as a fixed effect and pair as a random effect, was fitted to the data for analysis of all the behaviour observations and cortisol concentrations according to the treatments. We used multiple comparison procedures to analyse all the data, since the experimental procedure was the sole fixed variable whilst housing, transportation and confining in the operation stall were standardized between the treatments. Thereafter, post-hoc analyses using the Kenward-Rogers procedure were performed to compare cortisol concentrations after treatment for CAT vs.

184 PAL and TUB, and SNA vs. CAT, PAL and TUB sow groups, where significant differences were
185 found. Repeated measures with an 'unstructured' model were used to evaluate cortisol
186 concentrations before and after treatment.

187 Spearman rank correlation (r_s) coefficients were used to examine interactions between the salivary
188 cortisol concentrations and behavioural observations in post-treatment sows. Pearson correlation (r)
189 was applied to determine the parity effect on the salivary cortisol concentrations of the sow.

190

191 ## 3. Results

192 ### 3.1. Saliva cortisol concentrations

193 The average cortisol concentrations for all sows were 3.0 ± 0.3, 3.2 ± 0.3, 3.8 ± 0.3, 3.9 ± 0.3 and
194 4.4 ± 0.5 (LS mean ± SEM) ng/ml, according to the timing of treatments, i.e. -5, 0, 5, 10 and 15 min
195 from the initiation of treatment, respectively, and negatively correlated with parity of the sow (r = -
196 0.27, $P < 0.001$).

197 The catheterization brought about an increase in saliva cortisol concentrations of the sows ($P <$
198 0.05, Figure 1), while the cortisol levels associated with the other methods were not significantly
199 changed during the procedures ($P > 0.10$, Figure 1). The cortisol concentrations in CAT sows were
200 significantly greater than in sows in the other treatments for 10 min after initiation of the procedures
201 ($P < 0.01$, Table 2). Post-hoc comparisons showed that after CAT sows were returned to the
202 individual pens post-treatment, they still had higher saliva cortisol concentrations than non-snared
203 sows, i.e., PAL and TUB ($P < 0.05$, for both). However, saliva cortisol concentrations from the
204 sows in the SNA group were not significantly different to those of the PAL, TUB, or CAT sow
205 groups after treatment ($P > 0.10$). Sows in the TUB group tended to have higher cortisol
206 concentrations than those in the other groups before the experimental treatments, but the

concentrations did not rise during and after the procedures compared with the other methods (Figure 1, Table 2).

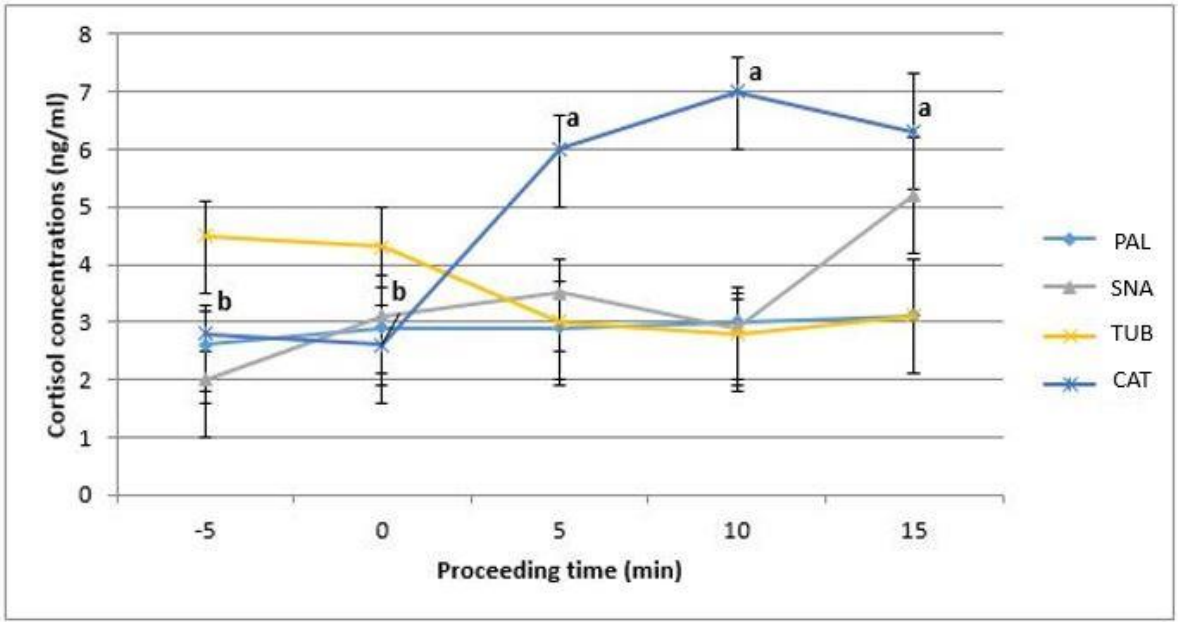

Figure 1. LS means and SE of sow saliva cortisol concentrations according to the experimental methods, i.e., palpation (PAL), snaring (SNA), biopsy (TUB) and catheterization (CAT), are plotted to demonstrate short term changes during the proceeding periods (total n=31). Different letters (a,b) indicate that the cortisol levels in the CAT treatment were significantly different according to the proceeding time ($P < 0.05$).

Table 2. Salivary cortisol concentrations in sows submitted to four different treatments: palpation (PAL), biopsy (TUB), snaring (SNA) and catheterization (CAT)[1].

	PAL	TUB	SNA	CAT	*P* value
n	8	7	8	8	
Cortisol concentration (ng/ml)					
-5, 0 min [2]	2.7 (0.5)	4.4 (0.6)	2.6 (0.5)	2.7 (0.5)	0.09
5, 10 min [3]	2.9 (0.6) [b]	2.9 (0.6) [b]	3.2 (0.6) [b]	6.5 (0.6) [a]	< 0.001
15 min	3.1 (1.0)	3.1 (1.0)	5.2 (1.0)	6.3 (1.0)	0.07

[1]All treatments proceeded between 0 and 10 min per sow.

[2]Repeated measures carried out LS Mean (SE) from -5 to 0 min according to the sampling scheme.

221 [3]Repeated measures carried out LS Mean (SE) from 5 to 10 min according to the sampling scheme.

222 [a,b] Different superscript letters indicate that there were significant differences between variables (P
223 < 0.001).

224

225 3.2. Behavioural observations

226 The SNA sows tended to sit for longer in the pen, and had longer desynchronized action against
227 their pen mates than the sows in other treatments (P = 0.09 and P < 0.05, respectively, Table 3). The
228 TUB sows tended to spend longer time for lateral laying down that the other sows (P = 0.09, Table
229 3).

230 During the 1 h post-treatment, sows in PAL showed a higher frequency of sniffing behaviour than
231 sows in SNA or CAT (P < 0.01, Table 3). The frequency of sniffing was a negatively correlated
232 with saliva cortisol in the post-treatment sows (r_s = -0.28, P < 0.01, Table 3).

233 The highest frequency of head shaking was recorded for the CAT sows followed, respectively, by
234 SNA and PAL sows (P < 0.001, Table 3), and TUB sows did not differ from SNA or PAL sows (P
235 > 0.10). The CAT sows also showed higher frequency of trembling behaviour than the other treated
236 sows (P < 0.05, Table 3). The frequencies of head shaking, trembling, and rubbing behaviour were
237 correlated with saliva cortisol concentrations in posttreatment sows (r_s = 0.36, P < 0.001; r_s = 0.35,
238 P < 0.001; r_s = 0.22, P < 0.05, respectively).

239 Table 3. Effects of palpation (PAL), biopsy (TUB), snaring (SNA) and catheterization (CAT) on
240 activities, social cohesion and other specific normal or pain-related behaviour during 1 h after the
241 procedures in sows (n=31). [1]

	PAL	TUB	SNA	CAT	*P* value
n	8	7	8	8	
Activities, sec / 1 h					
Standing/walking	828.8 (257.0)	583.4 (274.8)	1011.0 (257.0)	677.0 (257.0)	0.68

Sitting	29.9 (14.5)	19.1 (15.5)	62.0 (14.5)	10.6 (14.5)	0.09
Sternal recumbence	2363.1 (286.4)	1634.9 (306.1)	1789.1 (286.4)	2051.1 (286.4)	0.33
Lateral recumbence	296.1 (315.8)	1391.2 (333.3)	737.9 (315.8)	833.6 (315.8)	0.07
Social cohesion, sec / 1 h					
Isolation	1313.4 (440.0)	1949.9 (475.1)	1787.3 (440.0)	1737.0 (440.0)	0.78
Desynchronized	655.5 (410.9)	1192.9 (429.2)	1968.6 (410.9)	1006.6 (410.9)	< 0.05
Specific behaviour, frequency / 1 h					
Sniffing	6.4 (0.8) [a]	4.4 (0.9) [ab]	3.4 (0.8) [b]	2.0 (0.8) [b]	< 0.01
Rubbing	0.1 (0.2)	0.0 (0.2)	0.0 (0.2)	0.6 (0.2)	0.11
Trembling	0.0 (0.1) [b]	0.0 (0.1) [b]	0.0 (0.1) [b]	0.4 (0.1) [a]	< 0.05
Head shaking	0.1 (0.5) [c]	0.6 (0.5) [bc]	1.8 (0.5) [b]	3.3 (0.5) [a]	< 0.0001

[1]Values represent LS means (SE) of behaviour observations

[a,b] Different superscript letters indicate that there were significant differences between variables ($P < 0.05$).

4. Discussion

Our hypothesis for this present study was refuted because the catheterization procedure triggered more evident short-term responses in the sows compared with the other procedures, including the TUB. Non-surgical cannulation methods have been widely used for sequential blood collection in clinical research in pigs to minimize stress, pain and anxiety during sampling against inserting the needle multiple times. Zanella and Mendl (1992) reported that a similar technique for jugular catheterization in sows did not affect salivary cortisol concentrations between 1 and 2 h post-treatment. Moreover, other evidence also demonstrated that the identical method using the jugular vein in young pigs tended to increase plasma cortisol concentrations 1 h post-treatment, but had no effect subsequently (Carroll et al., 1999). Nevertheless, to the best of our knowledge, there are only

few studies reporting on the impact of the procedure on cortisol levels in pigs. The catheterization procedure in the current study was performed within 10 min, and caused an increase in sow salivary cortisol levels in the meantime. Handling or snaring sows for catheterization might not be the sole reason for the effect on cortisol levels during the procedure, as those factors in the present study were standardized with the treatment for the snaring group. On the other hand, for instance, additional factors, such as inserting vinyl tubes or bandaging the neck of a folded ear, could contribute to elevation of the salivary cortisol levels in sows during the procedure. Glucocorticoids are known to take time to adjust a new situation (De Boer et al., 1988; Geverink et al., 1999). This may support the finding that higher salivary cortisol levels in catheterized sows were recorded even when they were returned to recover in the individual pen. However, based on the previous findings, it can be assumed that the elevated salivary cortisol caused by catheterization may not last longer than one hour (Carroll et al., 1999; Zanella and Mendl, 1992). Nonetheless, further studies are needed to examine potential factors affecting cortisol levels during this procedure and their effects in the long term period, and to establish the most suitable time for collecting consistent blood samples through the cannula.

A previous study examined the technical and practical aspects of the TUB technique for luteal tissue sampling, identifying several issues including effects of weight and age on success rate of biopsy and their impacts on reproductive performance (Björkman et al., 2016). In spite of a number of practical advantages from the technical aspect, the current study examined possible pain or distress induced by the TUB that could represent a welfare problem. In contrast to our current findings, Geverink et al. (1999) showed that a shot biopsy through a cannula to obtain a muscle sample increased saliva cortisol concentrations in gilts compared with the situation before the procedure, and also tended to cause a greater cortisol response in gilts compared with non-treated gilts 30 min after the procedure. Moreover, in the same study, they established that more flinching and rubbing behaviour occurred in gilts in the biopsy group in response to the procedure (Geverink

et al., 1999). This also contrasted with our findings, showing that obvious acute pain or distress was not observed in the TUB sows over the short term, and that other post-operative pain-related behaviour in the TUB sows did not differ from that for non-treated sows over 1 h post-treatment. One possible explanation could be that the genital organs, including the ovary, have no somatic nerve supply and thus not sensory receptors compared to muscle tissue (König and Liebich, 2004). Therefore, visceral pain could only be triggered by rapid stretching of the capsula surrounding the organ, i.e. ovary, which occurred rarely during the TUB procedure (Björkman et al., 2016). Furthermore, several studies demonstrated that transvaginal puncture of the ovary did not affect behavioural and physiological signs of acute stress in heifers and cows (Lauffenburger et al., 1999; Chastant-Maillard et al., 2003; Petyim et al., 2007). Consequently, on the basis of the current findings which demonstrated no short-term treatment impact on cortisol or pain-related behaviour, the results suggest that the TUB process for luteal tissue collection may not cause substantial short-term pain or distress to the sow when it compared with the PAL process. In addition, the study by Björkman et al. (2016) already showed that the long-term complications with the TUB procedure rarely observed in sows.

There is no obvious explanation for the somewhat higher salivary cortisol concentrations for the TUB group compared with the others before the stressor application. Previous evidence suggests that salivary cortisol has a circadian rhythm that could be influenced by heredity, age, gender and time of the day (Hillmann et al., 2008; Larzul et al., 2015; Ruis et al., 1997). Despite such factors as breed, parity and body weight being randomly distributed, the time of sampling might have changed slightly among treatments in the current experiment. All experimental sows in the research farm were adapted to a fixed time for feeding at 0800 and 1600 h. The clock time of sampling, i.e. between 1200 and 1500 h, was therefore designed to reduce this potential confounding variables, as it is suggested that the expectation of feeding could affect the cortisol concentrations (Hillmann et al., 2008). There might also be a risk that different sampling days according to the treatment could

306 be an additional factor for the confounding consequences. However, this experimental setup,
307 conducting the PAL and TUB groups on the same day and the others on the following day, may
308 allow us to better standardize cortisol circadian patterns, since they were designed as controls and
309 treatments, respectively.

310 Sows screamed when snared to the crate, and resisted the tightening rope. However, our present
311 results showed no short-term differences in their salivary cortisol levels compared with those of the
312 non-snared sows but palpated through rectum. Furthermore, our finding that a slight increase in the
313 cortisol levels of the snaring sows after the procedure was not significant compared with the other
314 groups seems to be in agreement with previous findings (Soede et al., 2007; Merlot et al., 2011).
315 We therefore suggest that a snaring challenge might not induce a significant stress response in terms
316 of salivary cortisol status when applied in the short term. Probably our results would have been
317 clearer if the sows were not treated in any way, but we could not have separated between effects of
318 palpating and snaring. Furthermore, the stress response seen in the treatments with palpating was
319 very mild. The stress response of control sows without any treatments would not likely have been
320 different to those other groups.

321 Irrespective of handling or management systems, Strawford et al. (2008) found that there were
322 more scratches to the body in the younger sows, as they are attacked more often by the older sows
323 in group housing system, and that this could result in increasing cortisol levels in the younger sows.
324 Therefore, our findings that the cortisol concentrations increased with younger parity might be due
325 to the stress induced by being attacked by the older sows in the pen.

326 The current finding of a negative interrelationship between sniffing behaviour and salivary cortisol
327 levels could support the suggestion that reduced exploration behaviour might be associated with
328 pain in castrated pigs (Hay et al., 2003). In addition, reduction in activities is commonly associated
329 with animal in pain (Hay et al., 2003; Morton and Griffiths, 1985), and such animals were more
330 often isolated and desynchronized with their littermates (Arnold, 1985). The present study revealed

that the duration of sow activities and social cohesion regarding their partner 1 h post-treatment did not differ among treatments, with the exception of the snaring group, which showed higher tendencies for sitting and desynchronizing behaviour compared with the other group. However, the catheterized sows in this research often exhibited more trembling and head shaking, which could be considered to indicate pain, as reported previously (Morton and Griffithis, 1985; Noonan et al., 1994). Furthermore, the proven interrelationship between such pain-related behaviour and salivary cortisol levels in the current study also suggests that the catheterization procedure might cause pain and stress to sows during the procedure or within one hour post-treatment.

5. Conclusions

Present study confirmed that salivary cortisol was associated with some specific behaviour response, indicating pain or distress. We found that the transvaginal ultrasound-guided biopsy of luteal tissue in sows did not induce an acute pain or stress response during the procedure, but the non-surgical catheterization method performed in the present study could increase salivary cortisol concentrations and frequencies of pain-related behaviour in sows. Present experiment demonstrated that snaring might not cause an increase in cortisol levels during the process. Nonetheless, it cannot preclude the possibility that the catheterization procedure was not the sole reason for increased salivary cortisol levels and frequencies of pain-related behaviour during the procedure. We might also not expect that the trend towards higher cortisol levels last longer than an hour after the catheterization procedure. Further studies therefore are needed to investigate the causal relationship between the catheterization procedure and practical acute pain or distress, and to establish the optimal time for obtaining uniform blood samples through the cannula.

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