



# Effects of parity and litter size on cortisol measures in commercially housed sows and their offspring

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## ABSTRACT

Breeding sows are regularly exposed to on-farm stressors throughout the duration of their production period. The impact of such stressors may differ for primi- and multiparous sows, as sows could learn to cope with stressors as they gain experience with them. If parity affects stress in sows, it may also impact their prenatal offspring through differential maternal stress. In addition to parity, litter size is another potential factor involved in stress of sows and piglets. Larger litters may be a source of discomfort for gestating sows, while it can result in intra-uterine growth restriction of piglets. In the current study, we aimed to assess whether parity and litter size affect cortisol measures in breeding sows and their offspring. To do this, we measured salivary cortisol concentrations of 16 primiparous and 16 multiparous sows at three time points: 1) while sows were group housed, 2) after sows were separated from the group prior to moving to the farrowing unit and 3) after handling procedures. In addition, hair cortisol concentration was determined for the sows during late gestation and for their low birth weight ( $n = 63$ ) and normal birth weight ( $n = 43$ ) offspring on day 3 after birth, to reflect in-utero cortisol exposure. It was expected that if sows adapt to on-farm stressors, the more experienced, multiparous sows would show decreased stress responses in comparison to primiparous sows. However, we found a comparable acute stress response of primi- and multiparous sows to separation from the group. Handling procedures did not influence sows' salivary cortisol concentrations. Sows' hair cortisol concentration was positively correlated with litter size. Future research is needed to assess whether this finding reflects increased stress in sows carrying larger litters. Parity or litter size did not have a direct effect on their offspring's hair cortisol concentration. Larger litters did have a higher occurrence of low birth weight piglets. For these piglets, females had higher neonatal hair cortisol concentrations than males. Overall, our results indicate that breeding sows do not adapt to all on-farm stressors. In addition, litter size may influence HPA axis activity in both sows and piglets.

## 1. Introduction

Sows housed on commercial breeding farms are regularly exposed to external stressors during the multiple years of their production period. Common examples are unstable social hierarchies due to regular mixing of unfamiliar sows in group housing [31,56] and being handled by animal caretakers [42,58]. Such stressors will cause the animal to employ physiological and behavioural responses to maintain homeostasis [5]. While events such as negative social interactions and handling procedures are considered acute stressors (i.e., a temporary

disturbance in their environment), these events may also cause chronic stress when they are repeated regularly over time [7]. As (the absence of) stress and the ability to adequately respond to stressors are components of most definitions of animal welfare [4,37], increasing our understanding of sows' responses to stressors is important.

The impact of on-farm stressors may differ for primiparous sows (those that are on their first reproduction cycle) and older, multiparous sows, as primiparous sows are unfamiliar with many of the common husbandry procedures surrounding pig production. For example, young sows' salivary cortisol concentration is higher after a first confrontation

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with unfamiliar conspecifics, compared to subsequent mixing events [9,25,54,55]. As these sows gain experience with mixing events, they receive fewer skin lesions and perform less agonistic behaviors, indicating they learned to avoid unnecessary aggression while establishing a dominance hierarchy. These studies show that sows appear able to learn how to cope with certain acute stressors as they gain experience with them. However, only a limited number of on-farm stressors have been assessed in relation to sows' stress response.

Stress in sows is not only of interest for their own welfare, but also that of their offspring. Maternal stress during pregnancy exposes prenatal offspring to elevated circulating cortisol levels [27]. This has been associated with altered development and functioning of the HPA-axis in a variety of species, including pigs [32,35,38]. Piglets from experimentally stressed sows show an exaggerated HPA-axis response to various common stressors compared to piglets from less or non-stressed sows, such as mixing with unfamiliar animals [20,26], restraint [24] and tail docking [48]. These findings are supported by alterations in behaviour observed in prenatally stressed piglets, such as increased escape attempts during an open-field test [39]. Overall, these studies show that stress in sows could affect their piglets' stress responses later in life. If parity affects sows' responses to on-farm stressors, this could also influence their offspring.

In addition to potential effects of parity, litter size is another factor which may be related to stress in both sows and piglets. Selection for increased litter sizes has been very successful in domestic pigs [43]. It has been suggested that larger litters may result in the sow suffering from larger energetic and nutritional demands of the fetuses and increased physical discomfort, particularly during the later stages of pregnancy [47]. However, whether this is associated with increased stress for breeding sows is unclear. As litter size has been reported to increase after the first parity [17], such stress may be more relevant for multiparous sows. Litter size may influence stress responses in piglets as it relates to the occurrence of low birth weight (LBW; [47]). In larger litters, not all fetuses receive sufficient oxygen and nutrients for optimal development [57]. The resulting intrauterine growth restriction may affect postnatal HPA-axis functioning. For example, LBW piglets have higher plasma cortisol concentrations than normal birth weight (NBW) piglets throughout the first week of life [30], as well as an increase in adrenal size and stronger acute stress responses post-weaning [41].

The aim of this study was to assess the effects of parity and litter size on stress responses in sows and their offspring. Acute stress responses of pregnant primi- and multiparous sows were measured as increase in salivary cortisol concentration after exposure to multiple on-farm stressors, specifically separation from the herd and handling procedures prior to farrowing. As an additional stress marker, hair cortisol concentration was measured during late pregnancy, reflecting longer term circulating cortisol levels [34]. After farrowing, hair cortisol concentration was also measured in neonatal piglets with LBW and NBW. Hair cortisol concentration in neonatal piglets reflects cortisol accumulation during prenatal development, which is influenced by both cortisol production of the piglet and exposure to maternal cortisol [28]. We expected primiparous sows to show an exaggerated acute stress response compared to multiparous sows, due to their lack of experience with on-farm stressors. Furthermore, we expected that a difference in stress between sows would result in a difference in hair cortisol concentration of their offspring. Finally, based on previous studies we expected LBW piglets to have elevated hair cortisol concentrations compared to NBW piglets [30,38].

## 2. Material & methods

### 2.1. Ethical note

All methods that demanded the handling of live animals were reviewed and approved by the local animal welfare body (Animal Welfare Body Utrecht) and were conducted in accordance with the

recommendations of the EU directive 2010/63/EU.

### 2.2. Animals

Sows (Yorkshire x Dutch landrace) were selected from the commercial pig breeding farm of Utrecht University. In total, hair and saliva samples were collected from 16 primiparous sows (first parity) and 16 multiparous sows (third parity and higher, average parity = 4–5) over a period of 7 weeks (July–August).

After the sows farrowed, up to four piglets were selected from each litter based on birth weight. From each litter, all piglets were weighed within 24 h after birth. One female and one male piglet with a birth weight closest to the litter average were selected as normal birth weight (NBW) piglets. Two additional piglets were selected as low birth weight (LBW) piglets if their birth weight was a minimum of 1 SD below the average birth weight of the study population, yielding a maximum birth weight of 1.05 kg. Furthermore, to ensure a difference in absolute birth weight between NBW and LBW piglets, LBW piglets were only selected if their birth weight was a minimum of 1 SD below the average litter birth weight. For one litter, no NBW males were available. Piglet selection resulted in 63 NBW piglets (32 females, 31 males) and 43 LBW piglets (26 females, 17 males).

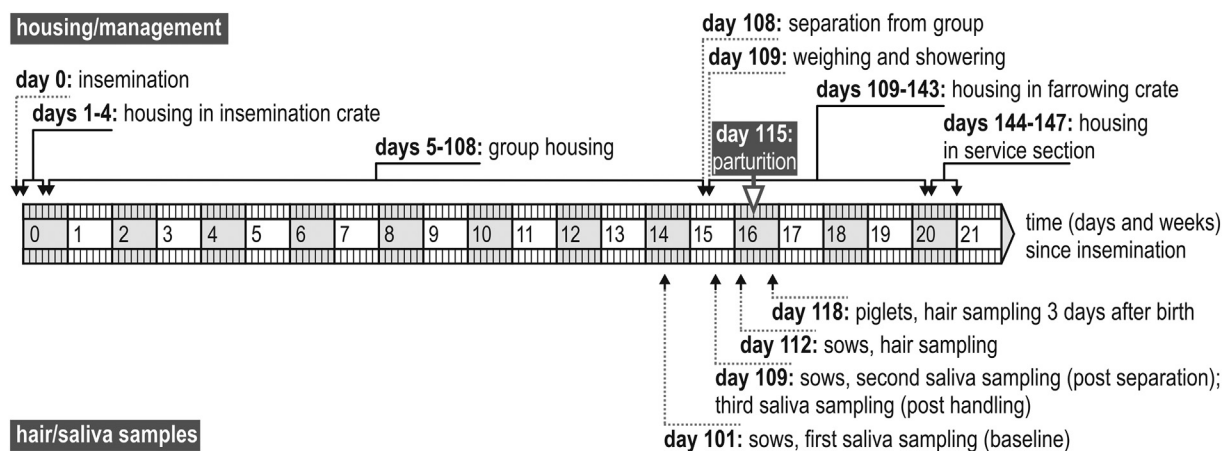
To assess whether LBW piglets are more common in larger litters, 60 additional litters (from sows that were not selected for the study) were weighed within 24 h after birth. Occurrence of LBW piglets was measured as proportion of piglets within a litter weighing < 1.05 kg at birth.

### 2.3. Housing and handling

During gestation, sows were group-housed in a barn measuring approximately 263 m<sup>2</sup>. The barn was naturally ventilated as long as indoor temperatures maintained at < 20 °C. Environmental temperatures ranged from 6.7 to 31 °C during the experiment. It contained two straw-bedded lying areas (87 m<sup>2</sup> each, separated by a 1.5 m high wall), a dunging area (71 m<sup>2</sup>) and a feeding area (18 m<sup>2</sup>) containing three walk-through electronic feeding stations (Intellitek ESF, Fancom B.V., Panningen, the Netherlands) set on a 24 h feeding cycle. Water was available ad libitum from five drinkers. Sows had access to a 53 m<sup>2</sup> outside area with concrete floor and ad libitum provision of grass silage. The herd consisted of a dynamic group with an average of 100 sows present in the barn, ranging in parity from gilts to 9th parity sows. Weekly transfers of animals consisted of a cohort of sows being removed from the herd approximately one week before their expected farrowing date and a similar number of sows being (re-)introduced to the herd approximately four days after insemination (see Fig. 1 for a timeline of a sow's reproductive cycle).

Each week, a cohort of six to 10 sows was separated from the main herd for farrowing. From a cohort, all available primiparous sows were selected along with an equal number of multiparous sows. Due to limited availability of primiparous sows, five cohorts were required to collect data on 16 primi- and 16 multiparous sows. Sows were separated from the main herd on the day before they were moved to the farrowing unit. Separation occurred after feeding, with the electronic feeding station giving selected sows access to a pen adjacent to the main herd's facilities. All selected sows were housed together in the separation pen until being moved to the farrowing unit. This separation pen measured 35 m<sup>2</sup> and contained a concrete floor without bedding. Water was available from one drinker. Separation from the herd usually occurred approximately 12–16 h prior to moving to the farrowing unit. On the day of relocation, the sows were showered in groups of three to seven animals (shower room measured 7.3 m<sup>2</sup>) and individually weighed. Sows were then escorted to the farrowing unit.

The farrowing unit consisted of a mechanically ventilated, thermostatically controlled room containing 10 farrowing pens. Temperature inside the farrowing unit was maintained at 24 °C until



**Fig. 1.** Schematic overview of a reproductive cycle of commercially housed sows, as applied during the experiment. Time points of handling procedures (housing/management) and sample collection (hair/saliva samples) are included.

one week post-farrowing, after which it was maintained at 20 °C. Each pen (2.4 × 1.8 m) was fitted with a centrally positioned farrowing crate (1.8 × 0.6 m). Pens had partially slatted floors with floor heating for the piglets. Sows were fed twice-daily, with water available ad libitum. Each sow was provided with a length of rope as chewing substrate.

#### 2.4. Saliva samples

Saliva samples were collected from each sow at three time points: (1) a baseline sample collected while sows were group-housed with the main herd, two weeks prior to expected parturition, (2) a post-separation sample, collected while sows were housed in the separation pen prior to relocation to the farrowing unit, and (3) a post-handling sample, collected after sows were showered and weighed on their way to the farrowing unit (Fig. 1).

All baseline and post-separation samples were collected between 8:30 and 9:30 in the morning. All post-handling samples were collected between 9:00 and 10:00 in the morning, approximately 20 min after a sow was showered and weighed, to allow for the peak in salivary cortisol response develop (e.g. [33]). Saliva was collected by letting each sow chew on two cotton swabs (Cotton Swabs 150 mm × 4 mm WA 2PL; Heinz Herenz, Hamburg, Germany) until they were sufficiently moistened. Swabs were centrifuged using saliva collection tubes (Salivette, Sarstedt, Germany) at around 3524 g for 10 min at 10 °C. Saliva samples were stored at −20 °C until salivary cortisol concentration was determined in duplo using a Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics BV, The Hague, the Netherlands) with an analytical sensitivity of 0.2 µg/dL. Cross-reactivity of the antibody with other steroids is as follows: prednisolone 76%, 11-deoxycortisol 11.4%, cortisone 0.98%, corticosterone 0.94%, triamcinolone 0.13%, dexamethasone 0.04%, aldosterone 0.03%, progesterone 0.02%. Samples were randomly distributed amongst plates, balanced for parity and timepoint. Intra-assay and inter-assay coefficients of variation (CV) were 3.8 and 9.3%, respectively.

#### 2.5. Hair samples

Sow hair samples were collected three days before expected parturition (Fig. 1). Hair was taken from the left dorsal flank region of each sow with a disposable razor (using a new razor for each sample). Piglet hair samples were collected three days after birth (Fig. 1). Hair was taken from both flanks to ensure sufficient material for cortisol analysis. Hair cortisol concentration was determined based on the protocols by Davenport et al. [12] and Moya et al. [36]. Hair samples were washed twice for 3 min in 10 mL (sows) or 5 mL (piglets) of isopropanol and dried for 5 days in a clean protected hood. Approximately 35 mg of the

clean, dry hair was then ground with a bead beater (TissueLyser II, QIAGEN Benelux B.V., Antwerp, Belgium) for a minimum of 2 × 15 min at 30 Hz, in 2 mL tubes (Eppendorf Safe-Lock, Eppendorf Nederland B.V., Nijmegen, the Netherlands) containing three 2.3 mm steel beads (BioSpec, Lab Services B.V., Breda, the Netherlands). The exact amount of hair per sample that was processed was recorded so cortisol concentrations could be corrected for sample weight. One mL of methanol was added to ground samples and they were incubated for 24 h with slow rotation to extract corticosteroids. 0.6 mL of the extract was dried using a vacuum centrifuge. The dried extracts were then dissolved in 0.3 mL phosphate buffer. A Salimetrics Salivary Cortisol ELISA kit with an analytical sensitivity of 0.01 µg/dL was used to determine hair cortisol concentrations in duplo. This kit has previously been validated for determining hair cortisol concentrations [12,36]. Cross-reactivity of the antibody with other steroids is as follows: dexamethasone 19.2%, prednisolone 0.57%, corticosterone 0.21%, 11-deoxycortisol 0.16%, cortisone 0.13%, triamcinolone 0.09%, 21-deoxycortisol 0.04%, progesterone 0.02%, testosterone 0.01%. There is no detectable cross-reactivity with 17α-hydroxyprogesterone, 17β-estradiol, DHEA, transferrin and aldosterone. Intra-assay and inter-assay CV were 3.6 and 23.0%, respectively. The higher inter-assay CV implies plate-to-plate variation (i.e. different plates produced different cortisol concentrations for the same sample). To avoid an influence of inter-assay CV on group comparisons, samples were balanced across plates for parity (sows) and birth weight and sex (piglets).

#### 2.6. Statistical analysis

All statistical analyses were performed using R statistical software, version 3.4.2 (R [45]). For linear mixed models, package nlme [40] was used. For each mixed model the random effect structure was assessed using Restricted Maximum Likelihood (REML) estimation. Final selection of random effect structure was based on Akaike's information criterion (AIC). Using the selected random effect structure, different fixed effect structures were assessed using Maximum Likelihood (ML) estimation. Selection of the final model was based on AIC. When a fixed effect did not improve the fit of a model (i.e. AIC was not lower after inclusion of the variable), it was taken as indication that this fixed effect was not of importance in explaining the data. Such fixed effects were excluded from the model. Statistical significance was set at  $P < .05$ . Effect size was calculated as Pearson's  $r$ . Unless indicated otherwise, results are presented as mean ± SD.

##### 2.6.1. Sows

Average litter size of primi- and multiparous sows was compared using Welch's  $t$ -test. The effect of parity on sows' hair cortisol

concentrations was analyzed using Welch's *t*-test. The effects of potential on-farm stressors and parity on sows' salivary cortisol concentrations were analyzed using a linear mixed model. The full model included Parity, Time-point and their two-way interaction as fixed effects and random intercepts for Cohort to account for a random effect of group composition during separation from the main herd. Salivary cortisol concentrations were  $\log_{10}$  transformed to improve the distribution of residuals. Saliva samples from two sows (1 primiparous and 1 multiparous) were insufficient for cortisol analysis. Therefore, salivary cortisol analysis was performed on the remaining 30 samples. Correlation analysis was performed between different cortisol measures (baseline salivary cortisol concentration, increase in salivary cortisol concentration from baseline to post-separation and hair cortisol concentration) and litter size. Spearman's rho was used for correlation analysis as baseline salivary cortisol concentrations and increase in salivary cortisol concentrations were not normally distributed. Correlation coefficients were calculated for all sows combined, and primi- and multiparous sows separately.

### 2.6.2. Piglets

Average birth weight of LBW and NBW pigs was compared using Welch's *t*-test. A linear mixed model was used to analyze potential effects on piglet hair cortisol concentration. The full model included Birth weight category (LBW or NBW), Sex, Litter size, Sow parity, Sow hair cortisol concentration, Sow baseline salivary cortisol concentration, Sow salivary cortisol increase (calculated as the increase in salivary cortisol from baseline to post-separation) and all two-way interactions as fixed effects. Random effect structure consisted of random intercepts for Litter. A reciprocal transformation was used on piglet hair cortisol concentrations to improve distribution of residuals.

To assess whether LBW piglets occur more frequently in larger litters, proportion of LBW piglets within litters was compared. Litter size (LS) was categorized in four categories: 13 total born or less ( $LS_{\leq 13}$ ), 14–16 total born ( $LS_{14-16}$ ), 17–18 total born ( $LS_{17-18}$ ), 19 total born or more ( $LS_{\geq 19}$ ). Total number of piglets born included all piglets born alive and all (appearing) normally developed piglets born dead. Fully or partly mummified piglets were excluded from litter size as these did not participate in intrauterine crowding [47]. As proportion LBW piglets was not normally distributed, a Kruskal-Wallis test (from package *pgirmess* [18]) was used to compare categories. To further assess if there was an increase of the proportion of LBW piglets with increasing litter size, a Jonckheere trend test (from package *clinfun* [50]) was used.

## 3. Results

### 3.1. Sows

#### 3.1.1. Litter size

Primiparous sows produced smaller litters than multiparous sows (primiparous:  $13.69 \pm 3.36$  piglets, multiparous:  $17.38 \pm 2.45$  piglets;  $t_{27.41} = -3.55$ ,  $P = .0014$ ,  $r = 0.56$ ).

#### 3.1.2. Salivary cortisol

Removing factors from the full model did not improve AIC, therefore the full model was used in final analysis. Separation from the main herd caused an increase in salivary cortisol concentration in all sows compared to baseline (Time-point effect:  $F_{2,80} = 57.46$ ,  $P < .0001$ ,  $r = 0.44$ ; Fig. 2). Salivary cortisol concentration in post-handling samples were comparable to baseline. Salivary cortisol concentrations were higher for primiparous sows than multiparous sows at all time points (Parity effect:  $F_{1,80} = 4.13$ ,  $P = .0455$ ,  $r = 0.14$ ; Fig. 2). Visual inspection of the data suggests primiparous sows had a stronger increase in salivary cortisol in response to separation from the main herd compared to multiparous sows. However, no interaction effect of parity and sample were found, suggesting primi- and multiparous sows did not

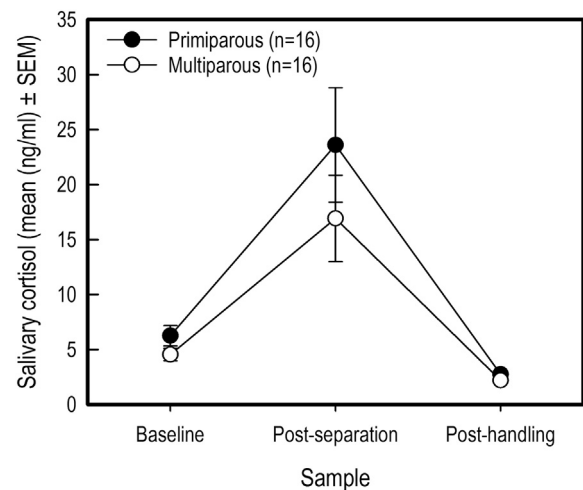


Fig. 2. Average salivary cortisol concentrations of primi- and multiparous sows. Baseline samples were collected in group-housing, post-separation samples were collected after separating sows from the main herd prior to moving to the farrowing unit and post-handling samples were collected after sows were showered and weighed.

differ in their acute stress response (Parity x Time-point interaction:  $F_{2,80} = 0.01$ ,  $P = .9909$ ).

#### 3.1.3. Hair cortisol

No effect of parity was found on hair cortisol concentrations of sows (primiparous:  $36.23 \text{ pg/mg} \pm 8.97$ , multiparous:  $35.90 \text{ pg/mg} \pm 4.86$ ;  $t_{23.10} = 0.13$ ,  $P = .8983$ ).

#### 3.1.4. Correlation analysis

Sows' baseline salivary cortisol concentration was negatively correlated with the increase in salivary cortisol concentration after they were removed from the main group ( $r_s = -0.37$ ,  $P = .0420$ ; Fig. 3A; Table 1). This suggests sows with a higher baseline salivary cortisol concentration displayed on average a smaller increase in salivary cortisol after exposure to a stressor. A positive correlation was found between baseline salivary cortisol concentration and hair cortisol concentration for primiparous sows only ( $r_s = 0.54$ ,  $P = .0422$ ; Table 1). No other correlations between cortisol measures were found (Table 1).

Litter size was found to be positively correlated with sows' hair cortisol concentration ( $r_s = 0.36$ ,  $P = .045$ ; Fig. 3B; Table 1). This positive correlation was also found when analyzing multiparous sows separately ( $r_s = 0.61$ ,  $P = .0116$ ; Fig. 3B; Table 1). No other correlations between litter size and cortisol measures were found (Table 1).

### 3.2. Piglets

#### 3.2.1. Birth weight

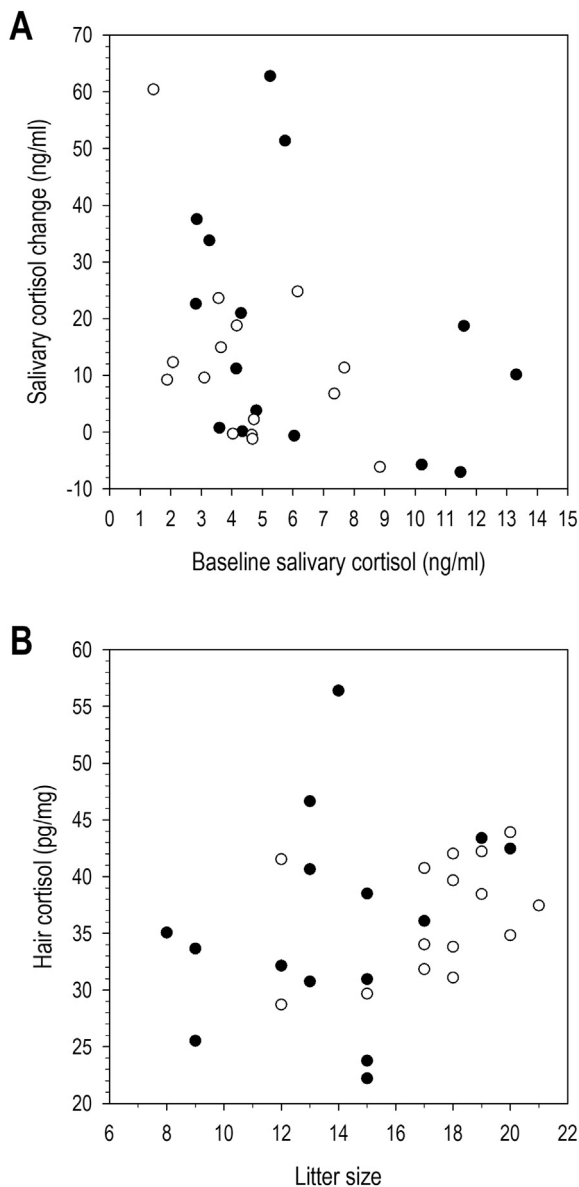
LBW piglets had on average a lower birth weight than NBW piglets (LBW:  $0.89 \pm 0.14 \text{ kg}$ , NBW:  $1.38 \pm 0.19 \text{ kg}$ ;  $t_{103.09} = -15.75$ ,  $P < .0001$ ,  $r = 0.84$ ).

#### 3.2.2. Hair cortisol

All factors describing the sows' stress response (Sow hair cortisol concentration, Sow baseline salivary cortisol concentration and Sow salivary cortisol increase), as well as Litter size and Sow parity, did not improve the fit of the model based on AIC either independently or as two-way interactions and were therefore excluded from the final model. The factors Birth weight and Sex were of importance to the model according to AIC. Therefore, the final model consisted of Birth weight, Sex, and their interaction as fixed effects.

Female piglets had a higher average hair cortisol concentration than male piglets (Sex effect:  $F_{1,71} = 4.17$ ,  $P = .0449$ ,  $r = 0.28$ ; Fig. 4). Hair





**Fig. 3.** Correlation between baseline salivary cortisol concentration and post-stressor salivary cortisol change (A) and between litter size and hair cortisol concentration (B) for primiparous (closed circles) and multiparous sows (open circles).

cortisol concentration differed for LBW and NBW piglets (Birth weight effect:  $F_{1,71} = 4.66$ ,  $P = .0342$ ,  $r = 0.28$ ; Fig. 4). No sex-dependent effect of birth weight on hair cortisol concentration was found (Birth weight  $\times$  Sex interaction:  $F_{1,71} = 2.19$ ,  $P = .1430$ ). However, visual inspection of the data suggests the found birth weight effect was mostly due to the higher cortisol concentration of LBW females (Fig. 4).

### 3.2.3. Birth weight and litter size

Mean proportion of LBW piglets differed across different litter size categories ( $H_3 = 14.26$ ,  $P = .0026$ ), with an increasing proportion of LBW piglets with increasing litter size ( $J = 2085$ ,  $P = .0003$ ). Average proportion of LBW piglets was  $10.65\% \pm 16.91$  in  $LS_{\leq 13}$  litters,  $16.73\% \pm 12.42$  in  $LS_{14-16}$  litters,  $18.42\% \pm 14.98$  in  $LS_{17-18}$  litters and  $26.75\% \pm 15.41$  in  $LS_{\geq 19}$  litters.

## 4. Discussion

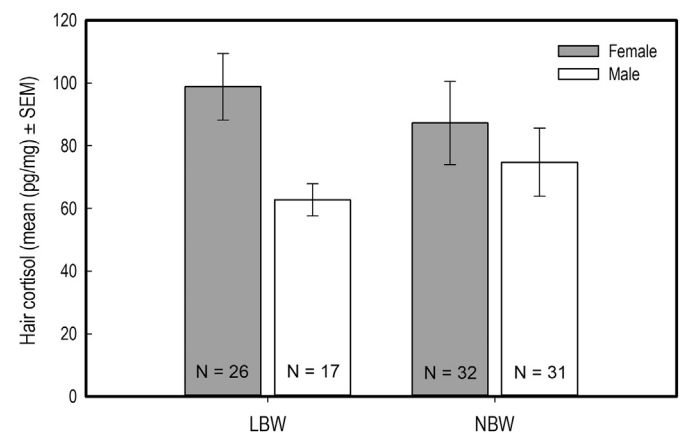
In this study, we assessed potential effects of parity and litter size on

**Table 1**

Spearman rank correlations and their associated  $P$  values for all sows combined (All), multiparous sows only (Multi) and primiparous sows only (Primi).

			Baseline salivary cortisol	Salivary cortisol increase	Hair cortisol	Litter size
Baseline salivary cortisol	All	$r_s$	–	<b>–0.37</b>	0.27	–0.17
		$P$	–	<b>0.0420</b>	0.1681	0.3826
	Multi	$r_s$	–	<b>–0.44</b>	–0.05	–0.03
		$P$	–	<b>0.0972</b>	0.8775	0.9212
Salivary cortisol increase	Primi	$r_s$	–	<b>–0.41</b>	<b>0.54</b>	–0.18
		$P$	–	0.1297	<b>0.0422</b>	0.5228
	All	$r_s$	–	–	–0.07	–0.18
		$P$	–	–	0.7120	0.3553
Hair cortisol	Multi	$r_s$	–	–	0.12	–0.15
		$P$	–	–	0.6961	0.6188
	Primi	$r_s$	–	–	–0.15	–0.01
		$P$	–	–	0.5756	0.9796
Litter size	All	$r_s$	–	–	–	<b>0.36</b>
		$P$	–	–	–	<b>0.045</b>
	Multi	$r_s$	–	–	–	<b>0.61</b>
		$P$	–	–	–	<b>0.0116</b>
	Primi	$r_s$	–	–	–	0.19
		$P$	–	–	–	0.4868
	All	$r_s$	–	–	–	–
		$P$	–	–	–	–
	Multi	$r_s$	–	–	–	–
		$P$	–	–	–	–
	Primi	$r_s$	–	–	–	–
		$P$	–	–	–	–

Correlations printed in bold have associated probabilities  $< 0.05$ . Correlations printed in italics have associated probabilities  $0.05 < P < 0.10$ .



**Fig. 4.** Average hair cortisol concentrations of LBW and NBW piglets at three days after birth.

stress responses in commercially housed sows and their offspring. No differences in cortisol measures between primi- and multiparous sows were found. Both primi- and multiparous sows showed an acute stress response to separation from the group prior to being moved to farrowing crates, suggesting sows do not adapt to this stressor. A positive correlation was found between litter size and hair cortisol concentration, with multiparous sows having larger average litter sizes than primiparous sows. In these larger litters, piglets with low birth weight (LBW) were more common. Furthermore, female piglets showed an increased neonatal hair cortisol concentration compared to males, with this difference being more pronounced in LBW piglets. No other effects of parity, litter size or maternal cortisol measures were found on piglet hair cortisol concentration.

### 4.1. Acute stress response in sows

Primi- and multiparous sows showed a comparable increase in

salivary cortisol concentration after a change in their (social) environment. Compared to baseline, salivary cortisol concentrations were higher after sows had spent time in a small subgroup of six to 10 animals, while separated from the main herd prior to moving to the farrowing crates. No effect of handling procedures was found, with salivary cortisol concentrations after handling being comparable to baseline.

These results suggest that the separation of sows from the main herd may cause an acute stress response, with the increase in salivary cortisol reflecting an HPA response to a stressor [33]. The stressor experienced during separation from the herd appears to be mostly of a social nature: the sows are forced to interact with a randomly selected group of conspecifics in a smaller pen with little (structural) enrichment material, without the possibility to avoid each other. These components are known to be stressful to sows. When housed in a large dynamic group, not all sows regularly interact with each other [15], suggesting a confrontation with randomly selected individuals can result in the establishment of a dominance hierarchy and its associated aggression [1,51]. In addition, the separation pen does not allow sows to perform avoidance behaviour, which is an important aspect of non-aggressive maintenance of social relationships [19,52]. This explains why overcrowding in a smaller pen has been shown to lead to increased aggression and plasma cortisol concentration in sows [21,61]. Social stress in a barren separation pen may have been exacerbated by the absence of enrichment items such as straw to distract the sows from negative social interactions [13,53].

It is possible that the reported increase in salivary cortisol concentration after separating sows from their herd was a result of factors other than an acute stress response. Basal cortisol levels have a circadian rhythmicity, with peaks in cortisol concentrations at late morning and early afternoon [46]. All saliva samples from the present study were collected between 8:30 and 10:00 in the morning. More specifically, baseline and post-separation samples were collected between 8:30 and 9:30 AM. The cortisol increase within this time frame in our sows is much larger than previously reported circadian fluctuations (e.g. [23,46]), making it unlikely that it was a result of circadian rhythm alone. Another possibility is that post-separation samples contained higher cortisol concentrations as they were collected a week later than the baseline samples. In humans, cortisol levels increase during the final stages of pregnancy [11,49]. In sows, baseline cortisol production throughout pregnancy is more difficult to assess due to the potential confounding influence of changes in housing conditions during late pregnancy (i.e. moving from group-housing to farrowing pens; [2]). While this means knowledge of the effects of gestational stage on cortisol production in breeding sows is limited, we can assume that such effects were not responsible for the increase in salivary cortisol in the present study. If that had been the case, post-handling samples (collected on the same day as the post-separation samples) would also have contained a higher cortisol concentration than the baseline samples. This was not the case, as post-handling samples and baseline samples showed a very similar cortisol level. To strengthen our findings, future research combining physiological markers of stress with behavioural observations are encouraged. Particularly, studies comparing separation pens containing different environmental elements (e.g. structural enrichment to allow avoidance of conspecifics such as barriers [6], straw) in combination with behavioural observations (e.g. of aggressive interactions) will allow assessment of which (combination of) elements are responsible for sows' on-farm stress response.

Handling procedures prior to sows' move to the farrowing crates (i.e. showering and weighing) did not cause an increase in salivary cortisol. Post-handling salivary cortisol concentrations of both primi- and multiparous sows were comparable to baseline level as measured in group housing. This suggests that handling by the animal caretakers was not experienced as stressful in our study. Previous studies have shown that human-animal interactions can be a source of stress for sows [42,58]. However, this is likely dependent on the caretaker's

disposition. For example, positive changes in caretaker's attitude towards pigs have resulted in a decrease in pigs' fearful behaviour [22]. Our study confirms that human-animal interactions are not necessarily a source of stress for sows. It is important to note that the handling procedures assessed in our study did not consist solely of contact with caretakers. Sows were showered, weighed and escorted to the farrowing unit. Showering of pigs is frequently applied in lairage, prior to slaughter, and is considered a means of distracting the pigs and decreasing abnormal behaviour [59,60]. Therefore, it is possible that showering the sows lowers their stress, possibly compensating for any negative effects from handling by the caretakers. This could be assessed by future studies assessing the impact of separate components of handling procedures.

#### 4.2. No effect of parity on cortisol measures

To further our knowledge of sows' potential for adaptation to stress, cortisol measures of primi- and multiparous sows were compared. Primiparous sows had on average higher salivary cortisol concentrations but showed a comparable increase in cortisol to multiparous sows after a stressor. Primi- and multiparous sows also had comparable hair cortisol concentrations. Based on the reported rate of hair growth for commercial breeding sows of ~0.7 cm/month [2] and considerable variation in hair length between individuals (own, non-systematic observations), we can assume that the cortisol concentrations found in these samples reflect a period of up to two months prior to collection (excluding the last 2 weeks to account for the length of hair within the dermis; [2]). This suggests that primi- and multiparous sows had comparable average circulating cortisol levels during the last weeks of group housing prior to moving to the farrowing pens. Together, our results do not support a systematic effect of parity on sows' stress responses. Rather, the comparable acute stress response for primi- and multiparous sows suggests that sows do not adapt to the stress of staying in the separation pen prior to farrowing.

Previous studies have shown that sows do adapt to social stressors. However, these studies have frequently based their results on experimental designs with social environments that are quite different from sows' actual circumstances on a commercial farm (e.g. smaller group sizes of 2–8 animals, different physical environment [9,25,54,55]). Our study assessed acute stress in sows in on-farm conditions, whereas previous studies applied experimental conditions (minimizing confounding effects of elements besides the stressor of interest). This has resulted in a combination of potential stressors being responsible for sows' stress response in our study (e.g. contact with unfamiliar animals, decrease in space allowance, lack of enrichment). Additionally, on-farm exposure to separation from the group occurs only once per reproductive cycle. Perhaps the long time-interval between successive exposures is too long to facilitate adaptation. Previous studies showing adaptation to social stressors used intervals of several days [9,25,54] or weeks [55] between repeated exposures. A negative relationship between inter-stressor interval and adaptation to the stressor has previously been shown in rats [14]. Our study shows that on-farm stressors (i.e. presented as a combination of stressors with a long time-interval between repeated exposures) can remain stressful, even as sows gain experience with them.

We found limited correlations between salivary cortisol measures and hair cortisol concentration. Only for primiparous sows were baseline salivary cortisol concentrations and hair cortisol concentrations positively correlated. Such a lack of correlation between salivary and hair cortisol levels has previously been reported for male growing pigs [8]. These findings are likely due to the large difference in time period reflected by the different samples. In humans, hair and salivary cortisol levels only correlate when saliva samples over a longer time period were included in analysis [11]. Correlation analysis also revealed a negative correlation between baseline salivary cortisol concentration and increase in salivary cortisol after a stressor, suggesting sows with

higher baseline cortisol concentrations show a smaller increase in cortisol after a stressor. This finding is supported by factor analysis of cortisol responses in humans, where baseline cortisol loaded negatively on a factor also containing cortisol increase [29].

#### 4.3. Litter size

The multiparous sows in our study produced larger litters than the primiparous sows. This was expected based on previous research showing that litter size increases with parity until it reaches a peak around the sow's fourth litter [3,17]. We found a positive correlation between litter size and the sow's hair cortisol concentration. This suggests that sows carrying a larger litter had higher levels of circulating cortisol during the last weeks of pregnancy. Whether this increase in hair cortisol reflects a higher level of stress in sows carrying larger litters cannot be stated based on this finding alone. While it has been suggested that larger litters may increase a sow's daily discomfort [47], studies examining the potential effects of litter size on sows' stress during gestation are lacking. A study with mice has shown increased behavioural signs of anxiety in females carrying a larger litter [10]. As selection for increased litter size is ongoing on commercial farms, future studies examining the potential effects of larger litters on sows' (physiological and behavioural) stress responses are encouraged.

For the piglets, we found no general effect of litter size on neonatal hair cortisol concentration. This suggests litter size does not influence HPA-axis functioning of prenatal piglets. Comparable baseline and post-stressor salivary cortisol concentrations have previously been found for NBW piglets from small ( $\leq 13$  piglets) and large ( $\geq 18$  piglets) litters [16]. However, we did find that LBW piglets are more common in larger litters, with the proportion of LBW piglets and litter size being positively correlated. This finding is in support of previous studies reporting an increased incidence of LBW piglets in larger litters [44,47]. Also, LBW piglets had a significantly lower birth weight than NBW piglets. As birth weight is the main read-out parameter of intra-uterine growth restriction in pigs [62], this result suggests that the LBW piglets assessed in our study were most likely affected by suboptimal prenatal conditions.

We found effects of both birth weight and sex on piglets' hair cortisol concentration. Female piglets had an increased hair cortisol concentration compared to males, and this difference between sexes was exaggerated for the LBW piglets. This finding is corroborated by earlier studies of sex differences in HPA axis activity in LBW piglets. For example, female LBW piglets have increased adrenal weight and plasma cortisol concentration at three days after birth [30]. There has also been a report of a general effect of LBW on postnatal acute stress response, with LBW piglets showing exaggerated HPA axis function [41]. However, the authors also reported that it is possible that different mechanisms are responsible for the development of enhanced HPA axis activity of female and male LBW pigs. For example, a relationship between plasma cortisol concentration during an acute stress response and adrenal size at the age of three months (prior to puberty) was found for male, but not female, LBW pigs. Therefore, our findings are not necessarily predictive of differences in stress experienced by female and male LBW piglets at a later age. Rather, they are an additional indication that HPA axis development in pigs may be subject to sex-specific mechanisms.

#### 4.4. Conclusion

A comparable acute stress response to separation from the group prior to farrowing was found for primi- and multiparous sows. This finding suggests that sows do not adapt to this stressor as they gain on-farm experience. A positive correlation between foetal litter size and sow hair cortisol concentration was found. Further research is required to assess whether larger litters are a source of stress for breeding sows. No general effect of litter size was found on piglets' neonatal hair

cortisol concentration. However, larger litters contained more LBW piglets. For these piglets, females had higher hair cortisol concentrations than males. We found no evidence of an effect of maternal stress on piglet hair cortisol concentration. However, as all sows in our study showed similar cortisol levels, we were unable to properly assess differential effects of maternal stress. Our results add to the knowledge of sows' stress responses in a commercial farm setting. Future studies comparing stress responses of sows experiencing different housing conditions (and associated stressors) are encouraged.

#### Author contributions

FJS, RN and SR contributed to conception and design of the study. JRH, LG and SR contributed to data acquisition. SR performed statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision and have read and approved the submitted version.

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None.

#### Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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