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# Neurological functioning and fear responses in low and normal birth weight piglets

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ABSTRACT

Low birth weight (LBW) piglets are an increasingly common occurrence on commercial pig farms, due to selection for sow fecundity. In humans, LBW is a known risk factor for impaired brain development, resulting in impaired neurological functioning and increased vulnerability to stressors. In pigs, the pre-weaning effects of LBW on neurological and emotional functioning are less well known. To assess neurological development, 60 LBW and 60 normal birth weight (NBW) piglets were subjected to a neurological examination at day one after birth. To assess fear responses, another 60 LBW-NBW pairs were compared in a human approach test (HAT) at three weeks of age. In the neurological exam, neonatal LBW piglets were found to be less likely to display a withdrawal reflex in response to a painful stimulus ($P = 0.022$) and showed impaired coordination during locomotion on a balance beam ($P = 0.030$). These findings suggest LBW piglets' increased neonatal mortality, often due to an impaired ability to compete over food or avoid crushing by the sow, could be influenced by a delayed neuromotor development. No effects of birth weight were found on behavioral responses in the HAT, such as latency to approach an unfamiliar human and rate of vocalizations. This suggests LBW and NBW piglets have similar levels of fear in this test. However, we also found indications that the fearfulness displayed by nursing piglets in the HAT is likely due to the temporary removal from the farrowing pen, instead of the presence of an unfamiliar human. Therefore, we can only conclude that LBW does not influence the behavioral response to this combination of stressors (i.e., social isolation from the sow and the piglets' litter mates, whilst being in a novel environment), as measured by the HAT. Future studies are required to assess whether fear responses to less salient on-farm stressors are affected by birth weight.

## 1. Introduction

On commercial pig farms, selection for sow fecundity has resulted in an increase in average litter size (Rutherford et al., 2013). Where the commercial pig's ancestor, the wild boar, produced litters of four to six piglets, sows on commercial farms now produce an average litter size of around 16 piglets (Rangstrup-Christensen et al., 2018; Rutherford et al., 2011). These larger litters place higher demands on the sow's uterine capacity, as each fetus requires sufficient space, nutrients and oxygen to properly develop (Père and Etienne, 2000; Wähner and Fischer, 2005). Intra-uterine growth restriction (IUGR) occurs when a sow cannot provide all fetuses with the requirements for optimal development, leading to piglets born with low birth weight (LBW). As larger litters have become more common, the occurrence of LBW piglets on commercial farms has also increased (Rutherford et al., 2013). Such piglets are at a greater risk for a variety of impairments, such as poorer thermoregulation (Herpin et al., 2002), and decreased overall vigor associated with lower food intake and a higher risk of crushing by the sow (Rutherford et al., 2013; Weary et al., 1996). This shows the sub-optimal development experienced by LBW piglets *in utero* may also negatively affect their postnatal functioning.

In humans, LBW is known to be a risk factor for impaired brain

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development. For example, LBW is associated with delayed neurological development (Arcangeli et al., 2012), with LBW children having poorer scores in a variety of neurodevelopmental domains, including motor skills (Savchev et al., 2013; Tosun et al., 2017). Children born with LBW are also at an increased risk of developing emotional disorders such as higher trait anxiety (Lahti et al., 2010). These findings of human studies are of interest, as both brain development and the IUGR leading to LBW are similar for humans and pigs. In both species, the growth spurt in brain development occurs from the late prenatal until the early postnatal period (Conrad et al., 2012; Dobbing and Sands, 1979). In humans, similar to pigs, IUGR can occur naturally due to insufficient supply of nutrients and oxygen (Cox and Marton, 2009). As the processes of brain development and IUGR are similar, it is expected that the outcomes found in human studies could occur in LBW pigs as well.

Assessing whether LBW is a risk factor for impaired brain development in pigs is of relevance for their welfare on commercial farms. First, if LBW pigs are more vulnerable to negative emotions, as has been found in humans, this could be a risk factor for negative animal welfare (Dantzer, 2002). Second, assessing neurological functioning of LBW piglets could provide a better understanding of the risk factors for their increased mortality (Rutherford et al., 2013). If LBW piglets suffer from delayed neurological development, it is possible that they are less able to appropriately respond to their environment.

In pigs, potential effects of LBW on neurological and emotional development have not yet been systematically addressed. There are indications that LBW affects early neurological functioning in pigs. LBW piglets show altered locomotion parameters compared to NBW piglets during the first days after birth, suggesting a difference in neuromotor development (Vanden Hole et al., 2018). Also, LBW piglets show reduced white matter development and brain myelination, suggesting altered brain connectivity (Radlowski et al., 2014; Vallet and Miles, 2012). However, studies on functional outcomes of such altered brain development are lacking. As for their emotional development, although multiple studies have compared emotional responses of LBW and normal birth weight (NBW) pigs, these studies have mostly focused on post-weaning effects (Gieling et al., 2014; Murphy et al., 2015; Poore and Fowden, 2003). Therefore, these studies tell us little about the emotional state of LBW piglets in the farrowing pen. The studies that have assessed pre-weaning emotional development have mostly relied on physiological measures of emotion and stress. For example, LBW piglets have increased cortisol production pre-weaning, which could indicate they are more stressed than their NBW siblings (Klemcke et al., 1993; Roelofs et al., 2018). However, to draw conclusions about LBW pigs' welfare, it is preferable to combine such physiological indicators with behavioral measures of emotion (Rutherford et al., 2013).

The present study aimed to assess neurological functioning and emotional development in LBW piglets. We hypothesized that as the IUGR responsible for LBW may impair brain development, it would also negatively affect pigs' postnatal neurological and emotional responses. For an assessment of neurological functioning, we performed a neurological examination based on veterinary diagnostic protocols. This examination consisted of various tests of behavioral abnormalities, reflexes and proprioception. In addition, we assessed piglets' coordination and balance during locomotion to collect further information on their motor skills. For an assessment of emotional development, we compared behavioral responses of LBW and NBW piglets in a human approach test (HAT). Behaviors such as latency to approach an unfamiliar human and vocalization rate were used as indicators of fear (Forkman et al., 2007; Murphy et al., 2014). Based on results from human studies, we expected LBW piglets to display a delayed neurological development, as indicated by poorer outcomes in the neurological examination. In the HAT, we expected LBW piglets to show increased fear-related behaviors compared to NBW piglets.

## 2. Materials & 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

Pigs [(Yorkshire x Dutch Landrace) x Duroc] were selected from the commercial pig breeding farm of Utrecht University. For the neurological examination, 60 LBW-NBW sibling pairs (29 female and 31 male pairs) were selected from 28 different litters. For the human approach test (HAT), a separate set of 60 LBW-NBW sibling pairs (32 female and 28 male pairs) was selected from 39 different litters. From each litter, all piglets were weighed on the day of birth. A piglet was selected as LBW if it met three criteria: 1) a birth weight of at least 1 SD below the litter average, 2) a birth weight of at least 1 SD below the study population average, which yielded a maximum birth weight of 1050 g for LBW piglets, and 3) born in a litter of at least 10 piglets. For each LBW piglet, a NBW sibling was selected based on two criteria: 1) the same sex as the LBW piglet, and 2) a birth weight closest to the litter average.

Selected piglets were housed with their own litter and sow in a farrowing pen (2.4 × 1.8 m), in which the sow was restrained in a centrally positioned farrowing crate (1.90 × 0.85 m). The floor of the pen was partially solid with floor heating for the piglets, and partially slatted for waste disposal (cast iron slatted floor, 1.80 × 1.17 m, 8 mm slat width, 8 mm slot width). In addition, each pen was equipped with a heat lamp located in a corner of the pen, above the solid flooring. This corner of the pen floor was covered with saw dust, to create a resting area for the piglets. Temperature inside the farrowing unit was maintained at 24 °C until the piglets were approximately one week old, after which it was reduced to 20 °C. Piglets were provided with supplemental feed according to supplier's recommendations, starting with milk replacer (Milkiwean BabyMilk, Trouw Nutrition, Nutreco N.V., Amersfoort, the Netherlands) when they were two to three days old. Sows were fed twice daily with standard pregnant sow pellets (Lacto, De Heus Voeders B.V., Ede, the Netherlands), according to supplier's recommendations. Water was available *ad libitum* for both piglets and sow. To improve survival of piglets in larger litters, cross-fostering was regularly applied, but only when piglets were two to four days old to ensure colostrum intake.

All selected piglets were weighed and ear-tagged on the day of birth. Piglets selected for neurological examination were tested at 1–2 days old. Therefore, they did not experience any additional husbandry procedures prior to being examined. Piglets selected for the HAT were tested at approximately three weeks of age (all piglets were 20–22 days of age at time of testing). The additional husbandry procedures they were subjected to before testing consisted of tail docking and an iron dextran injection of 1 ml, containing 200 mg Fe/ml (MS FerroPig, Schippers Export B.V., Bladel, the Netherlands) at 3 days of age.

### 2.3. Neurological examination

#### 2.3.1. Balance beam apparatus

Balance and coordination were assessed using a balance beam apparatus based on similar set-ups used by Friess et al. (2009, 2007) and Sullivan et al. (2013). The balance beam consisted of a 120 cm wooden beam covered with a thin rubber mat as a walking surface (Fig. 1). Unlike the set-up used by Sullivan et al. (2013), the beam was not inclined at an angle as this would have made the task more muscle strength dependent. As LBW piglets may suffer from impaired muscle development (e.g. Berard et al., 2010), using an inclined beam would increase the potential confound of muscle strength. Additionally,

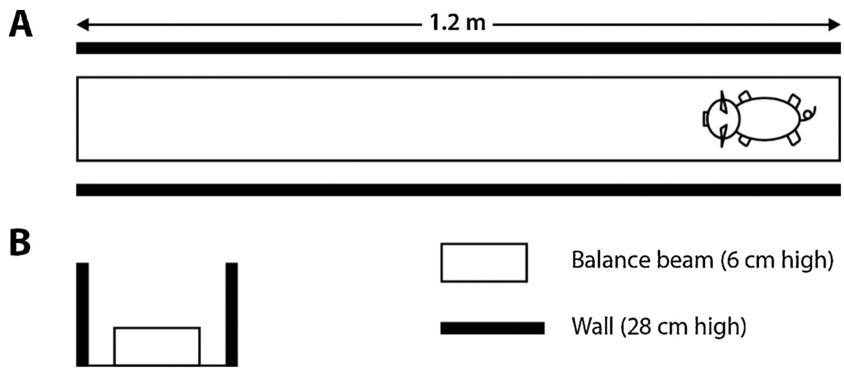


**Fig. 1.** Schematic overview of the balance beam apparatus, with top view (A) and front view (B).

instead of using a single beam width for all piglets, beam width was selected based on the size of the piglets. Using a single beam width would have confounded results by providing less of a challenge for smaller piglets. Three beams of different widths were used, with the selected beam being approximately 5 cm wider than the piglet's shoulder width (measured as distance between widest points of the shoulders). Piglets with a shoulder width < 6 cm were tested on a 10 cm wide beam, piglets with a shoulder width of 6–7 cm were tested on a 12 cm wide beam, and piglets with a shoulder width ≥ 8 cm were tested on a 15.5 cm wide beam. Wooden board walls were positioned alongside, but not touching, the balance beam. Each wall was placed approximately 2.5 cm away from the beam. This created a narrow space alongside each side of the beam where a piglet could safely misstep without falling. The balance beam was cleaned daily. Additionally, it was rinsed after a piglet soiled it during testing.

#### 2.3.2. Protocol

Piglets were subjected to multiple tests of neurological functioning, based on veterinary diagnostic protocols (Constable et al., 2017; DeLahunta et al., 2015). Only vital piglets were examined, i.e. piglets which actively participated in feeding from the sow and performed independent locomotion. Examination took place with the piglet placed on an exam table (set-up adjacent to the farrowing pen) covered with a thin rubber mat as a walking surface, unless indicated otherwise. Neurological tests were performed in order of supposed increasing discomfort for the piglet, to minimize effects of stress. These were, in order of testing:

1 **Assessment of abnormal behaviors** which are considered major clinical signs of nervous dysfunction. These behaviors have previously been scored in pigs as signs of neurological impairment (gait abnormalities: Andersen et al., 2016; LeBlanc et al., 1993; Priestley et al., 2001; involuntary muscle contractions: Richter et al., 1995; nystagmus: Geraldo Neto et al., 2013; Satas et al., 1997), and/or are indicative of dysfunction in highly preserved brain regions such as the brain stem and cerebellum (Butler and Hodos, 2005) (abnormal posture). Presence of the following symptoms were scored:
  a Involuntary muscle contractions, defined as spasmodic twitching movements of head and/or limbs, more violent convulsions of part or all of the body, or muscle tremors (repetitive twitching of muscles) – scored by observing piglet in farrowing pen
  b Gait abnormalities defined as circling, dragging of limbs, ataxia (swaying, limb crossing, exaggerated increased/decreased range of movement), or compulsive walking into objects – scored by observing piglet's locomotion in farrowing pen
  c Abnormal (head) posture, defined as continuous deviation of the head and neck from the axial plane or tilting of the head while standing
  d Pathological nystagmus, defined as uncontrolled, repetitive movements of the eyeball(s) (assessed using a penlight)

2 **Assessment of menace response** as a sign of cranial nerve function. This response has previously been described for pigs (Nordquist et al., 2017; Setlakwe and Johnson, 2017) and other precocial species including horses, sheep and goats (Enzerink, 1998; Raoofi et al., 2011). The menace response consists of closing of the eyelids after registering the rapid approach of an object:
  a Closing of the eyelid was provoked by rapidly stabbing a finger towards the piglet's eye, without making contact. The test was then repeated for the other eye. Absence or presence of the menace response was scored.

3 **Assessment of proprioception** as a sign of peripheral nervous system function. Proprioception can be assessed by invoking an animal's postural responses. These responses, required for maintenance of posture (i.e., upright position) during standing and locomotion, develop early in precocial species such as pigs (Fox, 1964; Muir, 2000). While assessment of proprioception has not been previously described for pigs, it has been included in the neurological examination of a variety of rodent species (Mancinelli, 2015; Snow et al., 2017):
  a A *wheelbarrow test* was performed by raising the piglet's front limbs off the ground and forcing it to walk backwards while balancing on its hind limbs. It was scored whether the piglet stepped backwards to keep its balance. The wheelbarrow test was then repeated by making the piglet walk forwards while balancing on its front limbs.
  b A *hopping response test* was performed by raising three of the piglet's limbs and gently pushing it laterally in the direction of the supporting limb. It was scored whether the piglet hopped on the supporting limb to maintain its balance. The hopping response test was repeated for each of the front and hind limbs.
  c The *righting response* was tested by placing the piglet recumbent on its side. It was scored whether the piglet immediately corrected to an upright position.

4 **Assessment of coordination and balance** during locomotion as a sign of overall motor control, including both central and peripheral nervous system function. A balance beam was used to assess motor deficits, as has previously been described for pigs (Friess et al., 2009, 2007; Sullivan et al., 2013):
  a The piglet had to run across the balance beam three times in close succession. Each misstep (piglet placing foot next to the beam) was scored. After a misstep, the piglet was placed back onto the beam to continue its run. Piglets were encouraged to move towards a litter mate placed near the end of the beam.

5 **Assessment of withdrawal reflex** as a sign of spinal nerve function. Assessment of this reflex has previously been described for pigs (Baars et al., 2013; Nordquist et al., 2017). The withdrawal reflex consists of withdrawal of a limb after application of a noxious stimulus:
  a The coronary band of the claw of each hind limb was pinched using only the examiner's fingers. Presence or absence of rapid

limb withdrawal was scored. In addition, presence or absence of crossed extensor reflex, i.e. extension of the opposite limb, was scored.

Examiners performing the neurological examination were not blinded to the birth weight category of piglets, as examination started in the farrowing pen where piglets were housed in the presence of their litter mates. Therefore, size differences between LBW and NBW siblings were immediately noticeable to the examiners. However, by basing the neurological examination on a protocol describing behavioral responses which could be scored objectively (e.g. responses such as missteps on the balance beam and performing a righting response do not rely on interpretation of the examiner), any potential bias due to unblinded examiners was minimized.

### 2.4. Human approach test

#### 2.4.1. Arena

The arena used for the HAT measured 2 m x 1 m and was created by using two synthetic barriers (1.5 m high) as walls in a corridor with a concrete floor. The arena could be entered by temporarily removing one of the barriers. The arena was divided into three segments using black cloth tape (1.5 cm wide): a back and middle segment measuring 0.5 m x 1 m each and a front segment measuring 1 m x 1 m (Fig. 2). A camera (Sony HDR-AS50, Sony Europe Ltd, Weybridge, Surrey, UK) positioned on top of one of the synthetic barriers recorded the entire arena continuously during testing.

#### 2.4.2. Testing

Test procedure for the HAT was based on the methodology described by Janczak et al. (2003), with each piglet being individually exposed to an unfamiliar person. A piglet was taken from the farrowing pen and placed in the back segment of the test arena, situated in a room next to the farrowing unit (each piglet was carried to the test arena by a person not participating in the HAT). Here, the piglet was left for one minute to get accustomed to the test arena (habituation), after which a person that was unfamiliar to the piglet entered. The person crouched down in the front segment, leaning against the back wall of the arena, and remained there for three minutes. The unfamiliar person did not encourage the piglet to approach and/or make contact, i.e. they stayed motionless and silent. The piglet stayed in the arena with the unfamiliar person for a duration of three minutes, resulting in a total test duration (habituation and HAT) of four minutes.

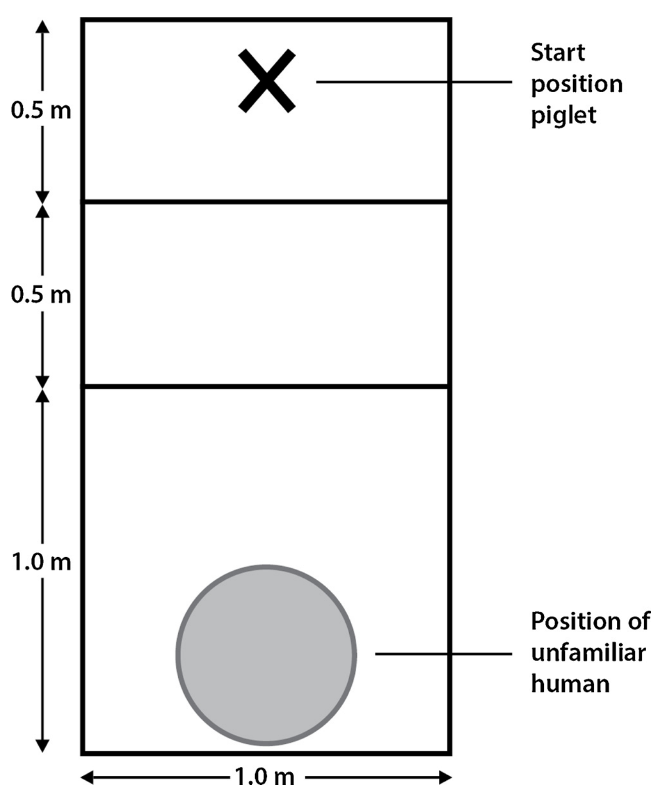


**Fig. 2.** Overview of the arena used for the human approach test, showing division of arena into three segments: front segment with position of unfamiliar human, middle segment, and back segment with start position of piglets.

#### 2.4.3. Behavioral variables

Based on the methodology described by Marchant Forde (2002), the following behavioral variables were scored in the HAT:

- Total number of **line crossings** (a crossing was scored as soon as both front limbs were placed in a new segment)
  - Scored separately for habituation and HAT
- Total number of **vocalizations**, as well as separate scores for number of **low-pitched grunts** and number of **high-pitched screams**
  - Scored separately for habituation and HAT
- **Latency to approach**, i.e. the time in seconds elapsed between the unfamiliar person entering the arena and the piglet approaching within 0.5 m of the human by placing both front limbs in the front segment where the human resided. Piglets which did not approach the unfamiliar human were given a score of 180 s.
- **Latency to touch**, i.e. the time in seconds elapsed between the human entering the arena and the piglet making physical contact. Piglets which did not make physical contact with the unfamiliar human were given a score of 180 s.
- **Time in back segment** and **time in closest segment**, scored as the proportion of time the piglet was scored as being in the segment furthest away from the unfamiliar human and the proportion of time the piglet was scored as being in the segment containing the unfamiliar human, respectively (Fig. 2).
- **Frequency of contact**, scored as total number of times the piglet made physical contact with the human

All behavioral variables were scored based on video recordings. Examiners scoring videos were blinded to the pigs' birth weight category. However, body weight at three weeks old varied considerably amongst LBW piglets, resulting in a number of smaller piglets being easily recognizable as having LBW.

### 2.5. Statistical analysis

All statistical analyses were performed using R statistical software, version 3.4.2 (R Core Team, 2017). For exact McNemar's test, package exact2 × 2 was used (Fay, 2010). For linear mixed models, package lme4 (Bates et al., 2015) was used. Type III tests were used to test significance of fixed effects of linear mixed models. Statistical significance was set at $P < 0.05$. Unless indicated otherwise, results are presented as mean ± SEM. Normality of data distribution was assessed using Shapiro-Wilk test for normality (for t-tests) or visual inspection of Q-Q plot of residuals (for linear mixed models). Furthermore, assumptions of linearity and homoskedasticity for linear mixed models were assessed using visual inspection of residual plots.

#### 2.5.1. Birth weight

Average birth weight of LBW and NBW piglets was compared separately for piglets selected for the neurological examination and HAT using a dependent *t*-test to account for sibling pairs. For piglets tested in the HAT, average body weight of LBW and NBW piglets at three weeks old was also compared.

#### 2.5.2. Neurological examination

For the neurological examination, all variables that describe the presence of a response (i.e. outcomes of abnormal behaviors and responses, presence of reflexes) were analyzed using an exact McNemar's test to account for sibling pairs.

Average number of missteps on the balance beam of LBW and NBW piglets was compared using a linear mixed model with Birth Weight as fixed effect. Random effect structure consisted of crossed random intercepts for Litter (to account for selection of multiple piglets from the same litter) and Beam width.

#### 2.5.3. Human approach test

All variables for the HAT were scored by two observers, with one observer scoring 90% of all video recordings and one observer scoring 10%. To assess inter-rater reliability, 15% of all video recordings (i.e. video recordings of 18 piglets) were scored by both observers. Inter-rater reliability was determined using intraclass correlation coefficients (ICC) for all scored variables. ICCs were based on a mean-rating (k = 2), absolute-agreement, 2-way mixed-effects model.

The effects of birth weight on behavioral variables scored during the human approach test were assessed using linear mixed models with the factors Birth Weight, Sex and their interaction as fixed effects. Random effect structure consisted of random intercepts for Litter to account for testing multiple piglets from the same litter. Initial analyses also included a piglet's cross-fostering status, to assess whether having been cross-fostered affected its behavior during the HAT. However, as this factor did not have a significant effect on any behavioral variables, it was excluded from the final analysis.

A dependent *t*-test was used to compare time spent in back segment with time spent in front segment, to assess whether piglets spend relatively more time closest to or furthest from the unfamiliar human. Average number of line crossings per minute and the frequency of vocalizations per minute (for total vocalizations, high-pitched screams and low-pitched grunts) during habituation and HAT were compared to assess effects of the presence of a human on these behaviors. These variables were assessed using dependent *t*-tests, with the exception of frequency of screams per minute. For this variable, the differences between paired scores did not follow a normal distribution. Therefore, frequency of screams per minute during habituation and HAT was analyzed using a Wilcoxon signed-rank test.

## 3. Results

### 3.1. Neurological examination

#### 3.1.1. Birth weight

LBW piglets selected for neurological examination had on average a lower birth weight than NBW piglets (LBW: 0.76 kg ± 0.02, $n$ = 60; NBW: 1.32 kg ± 0.02, $n$ = 60; $t_{59}$ = -25.50, $P <$ 0.001).

#### 3.1.2. Neurological tests

In most neurological tests, all piglets from both birth weight groups (LBW: $n$ = 60; NBW: $n$ = 60) displayed a normal response:

- No piglets displayed abnormal behaviors, with the exception of 1 NBW piglet with tremors.
- Menace response was absent in all piglets.
- Palpebral reflex was present in all piglets.
- All piglets successfully performed front limb wheelbarrowing.
- All piglets displayed a hopping response for all four limbs.
- Righting response was present in all piglets.
- No piglets displayed crossed extensor reflex.

Five out of 60 LBW piglets failed to successfully perform hind limb wheelbarrowing, while none of the 60 NBW piglets failed to perform this response. Based on an exact McNemar's test, this difference between groups was not significant ($P$ = 0.063). LBW piglets did have a higher occurrence of absent withdrawal reflex (LBW: 13 piglets without withdrawal reflex, NBW: 4 piglets without withdrawal reflex; $P$ = 0.022). There were two NBW-LBW sibling pairs with both piglets failing to show the reflex, two sibling pairs with only the NBW piglet failing to show the reflex and 11 sibling pairs with only the LBW piglet failing to show the reflex.

Balance beam performance of each piglet was assessed using one of three possible balance beam widths, depending on the piglet's shoulder width. The narrowest and widest beam were exclusively used by LBW ($n$ = 28) and NBW ($n$ = 26) piglets respectively. The intermediate beam width was used for piglets of both birth weight categories: 32 LBW piglets and 34 NBW piglets. LBW piglets made on average more missteps on the balance beam (LBW: 3.23 ± 0.29, $n$ = 60; NBW: 1.85 ± 0.18, $n$ = 60; $X^2(1)$ = 4.71, $P$ = 0.030).

### 3.2. Human approach test

#### 3.2.1. Inter-rater reliability

ICCs for behavioral variables scored during the HAT were ≥0.95, indicating excellent inter-rater reliability (Koo and Li, 2016).

#### 3.2.2. Birth weight

LBW piglets selected for the HAT had on average a lower birth weight than NBW piglets (LBW: 0.86 kg ± 0.01; $n$ = 60, NBW: 1.43 kg ± 0.02, $n$ = 60; $t_{59}$ = -22.90, $P <$ 0.001). This difference in body weight was still present when the piglets were tested at three weeks of age (LBW: 3.70 kg ± 0.15, $n$ = 60; NBW: 6.28 kg ± 0.17, $n$ = 60; $t_{59}$ = -17.45, $P <$ 0.001).

#### 3.2.3. Behavioral variables

No effects of Birth Weight, Sex or their interaction were found on any of the behavioral variables scored during the HAT (Table 1). No difference was found between the time piglets spent in the furthest segment and time spent in the segment where the human resided (time spent in furthest square: 67.78 s ± 3.37, time in closest square: 73.12 s ± 3.13; $t_{119}$ = -0.90, $P$ = 0.368). Average number of line crossings per minute decreased when the human entered the arena (habituation: 7.63 ± 0.47, HAT: 5.20 ± 0.23; $t_{119}$ = 5.42, $P <$ 0.001). Average number of vocalizations per minute increased when the human entered the arena (habituation: 36.23 ± 2.29, HAT: 42.84 ± 2.30; $t_{119}$ = -3.41, $P <$ 0.001). Separate analysis of screams

**Table 1**
Mean ( ± SEM) behaviour of low birth weight and normal birth weight piglets in the human approach test, including measures scored during habituation phase. No significant differences between birth weight groups were found.

| Variable | Phase | LBW ($n$ = 60) | NBW ($n$ = 60) |
|---|---|---|---|
| Line crossings (per minute) | Hab | 7.57 ± 0.60 | 7.70 ± 0.73 |
| | HAT | 5.16 ± 0.33 | 5.24 ± 0.33 |
| Vocalization frequency (per minute) | Hab | 37.73 ± 3.31 | 34.73 ± 3.19 |
| | HAT | 43.66 ± 3.06 | 42.02 ± 3.47 |
| Grunt frequency (per minute) | Hab | 31.53 ± 2.34 | 27.33 ± 2.08 |
| | HAT | 28.97 ± 1.78 | 25.57 ± 1.78 |
| Scream frequency (per minute) | Hab | 6.18 ± 2.08 | 7.37 ± 1.74 |
| | HAT | 14.68 ± 2.32 | 16.73 ± 2.45 |
| Latency to approach (s) | HAT | 48.65 ± 5.46 | 51.65 ± 5.91 |
| Latency to touch (s) | HAT | 56.73 ± 5.76 | 61.87 ± 6.22 |
| Time in back segment (s) | HAT | 69.13 ± 4.19 | 66.43 ± 5.31 |
| Time in closest segment (s) | HAT | 74.03 ± 4.32 | 72.20 ± 4.56 |
| Frequency of contact (total) | HAT | 6.43 ± 0.42 | 6.42 ± 0.40 |

Abbreviations: LBW, low birth weight, NBW, normal birth weight, Hab, habituation; HAT, human approach test.

and grunts showed this increase in vocalization was due to an increase in average number of screams per minute (habituation: 6.78 ± 1.35, HAT: 15.71 ± 1.68; $Z = -6.28$, $P < 0.001$), with average number of grunts per minute being comparable for habituation and HAT (habituation: 29.43 ± 1.57, HAT: 27.27 ± 1.26; $t_{119} = 1.65$, $P = 0.102$).

## 4. Discussion

In the present study we assessed the effect of birth weight on neurological and emotional development in pigs. To do this, we compared the performance of low birth weight (LBW) and normal birth weight (NBW) piglets in a battery of neurological tests and a human approach test (HAT). Based on findings of delayed neurological development in LBW children (Arcangeli et al., 2012; Savchev et al., 2013; Tosun et al., 2017) and previous neurological assessments in pigs (Radlowski et al., 2014; Vallet and Miles, 2012; Vanden Hole et al., 2018), we expected LBW piglets to have lower scores in their neurological examination. This expectation was partially confirmed, with NBW piglets being more likely to display a withdrawal reflex in response to a painful stimulus and having improved coordination and balance compared to LBW piglets. In the HAT, we expected LBW piglets to show increased fear responses compared to NBW piglets, due to an increased vulnerability to negative emotions, as has been found in humans (Lahti et al., 2010). However, no differences were found between LBW and NBW piglets for any of the behaviors scored during the HAT.

It is important to note that the present study is based on the assumption that LBW piglets have suffered intra-uterine growth restriction (IUGR), as suggested by previous studies of LBW and IUGR in humans. In humans and pigs, IUGR occurs naturally due to an impaired supply of nutrients and oxygen to the developing fetus(es) (Cox and Marton, 2009; Hunter et al., 2016). This can negatively impact development during the later stages of pregnancy, when placental function cannot keep up with the increasing demands of the fetus(es). For both humans and pigs, this late prenatal period is also when the peak of brain development occurs (Conrad et al., 2012; Dobbing and Sands, 1979). In humans, there are multiple read-out parameters of IUGR, including LBW, serial observations of *in utero* growth and assessment of relative brain size as a sign of the so-called ‘brain-sparing effect’, where placental insufficiency leads to prioritized brain development (Iughetti et al., 2017; Pollack and Divon, 1992). In pigs, not all these measures are practically feasible. *In utero* observations would require longitudinal individual identification of each fetus. Furthermore, while measures of brain size such as head morphology have been suggested as markers for IUGR in pigs (Amdi et al., 2013), we have found them to be confounded by breed-specific differences in head shape, as most commercially kept pigs are crossbreds (own, non-systematic observations). Therefore, LBW appears to currently be the best available measure of IUGR in pigs. However, this means that the piglets assessed in the present study may represent a heterogenous population, consisting of both LBW piglets which have suffered IUGR and fully developed but constitutionally smaller piglets whose brain development has not been negatively affected *in utero*.

### 4.1. Neurological development

We subjected a large sample of neonatal piglets to a neurological examination at day one after birth. This allows us to describe some general features of early functional neurodevelopment in pigs, in addition to our comparison of LBW and NBW piglets.

First, all pigs successfully completed (most) tests of proprioception. These tests forced the pigs to use postural reactions to maintain their balance. To our knowledge, the present study is the first to describe tests of proprioception for pigs. However, the ability to coordinate the movement of joints and muscles to maintain posture is known to develop quickly after birth in precocial species (Fox, 1964; Muir, 2000). As pigs are a precocial species, we expected (unimpaired) piglets to successfully perform postural adjustments in response to a disturbance of balance. Based on our findings, such postural reactions develop within the first day of life in pigs. We found no difference in proprioception between NBW and LBW piglets. However, it is important to note that as the tests of proprioception used in the present study have not previously been validated for pigs, it is possible that these tests are unsuitable to detect a difference in neurological development between NBW and LBW piglets. As it is uncertain how sensitive the applied tests are, a (mild) postural impairment in LBW piglets could have gone undetected. To further validate these tests of proprioception, future studies comparing performance of healthy piglets to those with postural deficits are recommended.

None of the piglets, LBW or NBW, displayed a menace response, that is, piglets did not respond to the sudden approach of an object towards the eye by immediate closure of the eyelids. This finding can be explained by the fact that the menace response is a learned response which develops over time. In other precocial species such as sheep, goats and horses, the menace response only develops after multiple days (horse: ˜9 days; Enzerink, 1998; sheep: ˜8 days, goat: ˜14 days; Raoofi et al., 2011). While assessment of the menace response has previously been described for pigs (Nordquist et al., 2017; Setlakwe and Johnson, 2017), the present study is the first to apply it to piglets of only a few days of age. To assess whether development of this response is delayed in LBW piglets, daily follow-up assessments over the first weeks of life are necessary.

Functional withdrawal reflexes were tested for by pinching the skin-claw intersection of a hind limb, a methodology that has previously been applied to pigs (Baars et al., 2013). Most piglets displayed a withdrawal reflex (i.e. rapid withdrawal of the limb) in response to a painful stimulus. This implies the methodology used is suitable for younger piglets as well as post-weaning pigs as tested by Baars et al. (2013). We found more LBW piglets than NBW piglets failed to perform this response, suggesting development of the withdrawal reflex is either delayed or impaired in LBW pigs. To distinguish between the two, longitudinal studies comparing the neurological development of LBW and NBW piglets are required. Either way, a (temporary) absence of functional withdrawal reflexes can result in piglets being unable to appropriately respond to painful stimuli.

Piglets' coordination and balance during locomotion were assessed using a balance beam. This methodology was based on previous studies assessing motor performance in pigs (Friess et al., 2009, 2007; Naim et al., 2010; Sullivan et al., 2013). We found LBW piglets to make more missteps on the beam compared to NBW piglets. This could be another indicator of delayed neurological development in LBW pigs, but potential confounding influences on our results must also be considered. First, multiple studies have reported that LBW pigs have fewer muscle fibers compared to NBW pigs (Beaulieu et al., 2010; Berard et al., 2010; Rehfeldt and Kuhn, 2006). This makes it possible that muscle strength is a confounding factor when assessing functional neuromotor performance in LBW and NBW pigs. To minimize the influence of muscle strength on balance beam performance, we used a level beam, instead of an inclined beam as reported by Sullivan et al. (2013). While this made the task less reliant on muscle strength, it remains a confound when assessing locomotion. Second, a piglet's body size likely affected the difficulty of the balance beam task, with smaller piglets having a relatively wider walking surface in relation to their stance width. To avoid LBW piglets having an advantage due to their smaller body size, we opted for using different beam widths for different sized piglets as opposed to the single beam width used in previous studies (Friess et al., 2009, 2007; Naim et al., 2010; Sullivan et al., 2013). By doing this, a more comparable task difficulty between LBW and NBW piglets was ensured. However, it is important to note that certain piglets still had the advantage of a relatively wider walking surface. Due to practical limitations, only three different beam widths were used. As a result, piglets with a range of different stance widths were tested on the same beam size, with the smallest piglets tested on each beam having an easier task compared to the larger piglets tested on that beam. Ideally, future studies apply a larger range of beam widths, minimizing differences in task difficulty.

When comparing the neurological assessment of LBW and NBW piglets, results of two tests suggest that LBW delays neurological development. Piglets with LBW were less likely to display a withdrawal reflex and showed impaired performance on the balance beam compared to NBW piglets. Based on these findings alone, we cannot state which specific neurological functions are delayed. Previous studies assessing the effects of LBW in pigs have also reported either functional outcomes, such as altered locomotion (Vanden Hole et al., 2018) or general impairments in brain development, such as decreased white matter development and reduced myelination of the cerebellum and brain stem (Radlowski et al., 2014; Vallet and Miles, 2012). Further studies are required to establish which exact (neurological or physical) impairments are responsible for the differences between LBW and NBW piglets found in the present study.

Irrespective of the underlying causes for the reported impairments associated with LBW, the observed functional deficits can have welfare consequences for pigs on commercial farms. Our findings of delayed development of withdrawal reflex and coordinated locomotion both suggest that LBW piglets may have a reduced ability to appropriately respond to their environment during the neonatal stage. This has also been suggested by studies reporting on LBW piglets' increased mortality. For example, LBW piglets consume less colostrum than NBW piglets (Devillers et al., 2007; Le Dividich et al., 2017, 2005), likely because they take longer to reach the udder and have more difficulty competing with their siblings over access to a teat (Rooke and Bland, 2002; Scheel et al., 1977). Another main cause of increased neonatal mortality in LBW pigs is crushing by the sow (Rutherford et al., 2013). A previous study has shown that LBW piglets spend more time in close proximity to the sow compared to NBW piglets (Weary et al., 1996). It is possible that LBW piglets are unable to move away from the sow quickly enough to avoid crushing. Both obtaining sufficient colostrum and avoiding crushing by the sow involve locomotor abilities. Risk of crushing could be further increased by a delayed or less vigorous response to pain. Perhaps the impairments reported in the present study are (in part) responsible for LBW piglets' increased mortality in the farrowing pen.

### 4.2. Human approach test

We used a HAT to elicit LBW and NBW piglets' fear responses to the presence of an unfamiliar human, as an assessment of the potential effects of birth weight on pigs' emotional responses. Human interaction paradigms such as the HAT are a commonly used method to assess fearfulness in different species of farm animals, including pigs (Forkman et al., 2007; Murphy et al., 2014). In the HAT, behavioral measures such as latency to approach an unfamiliar human, line crossings, and vocalizations are scored as indicators of the pigs' fearfulness during the test (e.g. Marchant Forde, 2002). Furthermore, pigs in a more negative emotional state are expected to show different behavioral responses than pigs in a more positive emotional state. For example, it has been reported that pigs in enriched housing conditions are faster to approach an unfamiliar human in the HAT compared to pigs housed in barren conditions (Reimert et al., 2014).

LBW and NBW piglets did not differ for any of the behavioral measures scored during the HAT. They were equally fast to approach an unfamiliar human, spent an equal amount of time near the human, were comparable in their exploration of the test arena (scored as line crossings) and had comparable rates of vocalizations, both low-pitched grunts and high-pitched screams. These findings suggest similar levels of fearfulness for LBW and NBW piglets during the HAT. This is in contrast to a study showing that LBW children are more likely to develop anxiety disorders (Lahti et al., 2010). Previous studies with pigs have also found LBW to be associated with increased anxiety (indicated by a higher rate of vocalization during an open field test) and an exaggerated acute stress response (Gieling et al., 2014; Poore and Fowden, 2003). It is possible these different results are related to a different salience of stressors applied during tests. Perhaps both LBW and NBW piglets in our study displayed a maximum stress response to the combination of a novel environment, isolation from the sow, and isolation from their littermates. There are indications that previous studies which reported a difference between LBW and NBW piglets may have applied less salient stressors. For example, results of these studies were based on older animals which had already been weaned (Gieling et al., 2014; Poore and Fowden, 2003). As piglets increase in age, their (vocalization) response to isolation from the sow decreases in intensity (Iacobucci et al., 2015; Weary et al., 1999; Weary and Fraser, 1997). Another study found LBW piglets to respond more strongly to a novel object, which is also likely to be less salient as a stressor (Litten et al., 2003). We suggest that future studies focus on behavioral responses to relevant on-farm stressors, as these would provide a better indication of LBW piglets' vulnerability to situations they might encounter on a commercial farm.

It is possible that we did not find the expected differences in behavioral responses during the HAT because they may not be valid reflections of the pigs' fearfulness. For example, latency to approach an unfamiliar human in the HAT is assumed to reflect fear, where less fearful pigs will be more likely to approach the human. However, such approaches could also be motivated by aggression or a desire for social support from the human (Murphy et al., 2014; Waiblinger et al., 2006). Line crossings do not always correlate with other fear-related variables in behavioral tests such as the elevated plus maze or light/dark test (Andersen et al., 2000). Rather, it seems that this measure is often confounded by the general activity level of an animal, independent of emotion (Murphy et al., 2014). Vocalization appears to be the behavioral response which is least likely to be confounded in the HAT. The majority of vocalizations we observed during the HAT were grunts with low tonality and high-pitched screams. Similar findings have been reported for a HAT performed with adult pigs (Marchant et al., 2001). Such vocalizations in pigs represent contact calls to (re-) establish social contact with group mates and calls to communicate a current stressed state, respectively (Manteuffel et al., 2004). For example, high-pitched screams are elicited in response to social isolation or painful procedures (da Silva Cordeiro et al., 2013; Düpjan et al., 2008; Weary and Fraser, 1995). Based on the presence of screams during the test (and its increasing frequency as testing progressed) we can assume that we were successful in eliciting an emotional response in our piglets using the HAT.

We found that piglets increased their rate of high-pitched screams after the human entered the test arena, as compared to the habituation phase prior to the HAT, whereas no change in the frequency of grunts was found. This could suggest that piglets became more fearful in the presence of an unfamiliar human (Manteuffel et al., 2004). However, if this were the case, it would also be expected that the piglets would avoid the human. We compared the proportion of time piglets spent in the segment furthest away from the human's position in the test arena to the time spent in the segment containing the human. No difference was found, suggesting the piglets did not actively avoid being near the unfamiliar human. Rather, the increase in the frequency of screams could be due to stress caused by other conditions inherent to the HAT, such as being in an unfamiliar environment and being isolated from the sow for a prolonged period. Such conditions are known to cause stress (and associated vocalizations) in piglets (Hötzel et al., 2011; Iacobucci et al., 2015). Based on the current study, the main stressor provided by the HAT in nursing piglets is the isolation from the sow and their familiar environment, and not the presence of an unfamiliar human.

## 5. Conclusion

We found LBW piglets displayed a delay in neurological development, particularly in neuromotor control. As piglets rely on locomotion and appropriate responding to painful stimuli to obtain food and avoid crushing by the sow, this result suggests neurological functioning may be associated with the increased neonatal mortality found for LBW piglets. In the HAT, LBW and NBW piglets displayed similar behavioral responses, suggesting birth weight does not predict fear responses in this test. However, it is possible that the HAT does not represent a relevant stressor for commercially housed nursing piglets. Therefore, future studies comparing fear responses of LBW and NBW piglets to on-farm stressors are encouraged.

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## Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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