HP master thesis

# Effect of Birth Order on Performance and Affective State of Pigs

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# CONTENTS

# Effect of Birth Order on Performance and Affective State of Pigs

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In pigs, birth order is associated with higher pre-weaning mortality. However, knowledge on the effect of birth order on welfare of surviving piglets is limited. The aim of this study was to investigate the possible link between birth order and both piglet performance and affective state. Firstly, the following data was collected from 393 piglets: stillbirth rate, intactness of the umbilical cord, cyanosis, drinking time, birth weight, teat order, weaning and end weight. Secondly, an active-choice judgement bias test was performed with low birth order (n=10) and high birth order (n=10) piglets. During the preceding discrimination training, the pigs learned to associate two tone cues with either a high or low reward, provided at different locations. After training, ambiguous intermediate tones were introduced and the pig's choice of location was observed. Results showed that last-born piglets had a higher birth weight than middle-born piglets. They also drank from more caudal teats than first-born piglets. In the judgement bias test, low and high birth order piglets showed a similar learning rate, latency and optimistic bias. Before the first individual trial, salivary cortisol levels were highest in low birth order pigs. However, after the trial, levels had decreased to the high birth order pigs' level. Additional behavioral tests and further study of stress response are proposed for validation of these results.

Keywords: pigs, birth order, birth weight, asphyxia, colostrum, cognition, judgement bias, cortisol

## INTRODUCTION

When a pig is born, its performance later in life has already been partly determined. Factors which influence piglet survival and performance include the intra-uterine environment and colostrum intake. With an average of 13-15 piglets born per litter in the Netherlands (1), it is conceivable that these piglets are not all born under the same conditions. In fact, researchers agree that pigs which are born later in birth order have a higher chance of dying within the first days after birth (2–6). Death of piglets pre-weaning is an extensive problem in swine husbandry, with average live-born pre-weaning mortality rates in Europe ranging from 11 to 13% (4,7).

The first event of a piglet's life, parturition, is already more stressful for the last-born; it has to endure the most uterine contractions. Each contraction causes a decline in blood flow and reduces oxygen delivery to the fetus (8). Moreover, the number of broken umbilical cords increases with birth order (9). This is caused by the uterine contractions in combination with the longer distance which the cord of the last-born piglet, being in the most cranial part of the uterine horn, needs to stretch during parturition (9). The blood flow to the fetus is badly compromised when the umbilical cord is ruptured (8). Pigs are exceptionally susceptible to perinatal asphyxia compared to other species. As a result, a faltering oxygen supply can lead to irreversible brain damage (8,10,11). In humans, perinatal asphyxia is associated with motor alterations, such as cerebral palsy and seizures, and cognitive alterations, such as attention deficit hyperactivity disorder and mental retardation (12). Neonates with moderate neonatal encephalopathy, a consequence of perinatal asphyxia, show learning and cognition impairment later in life and seem to have more difficulty making friends (13). In addition, rats with perinatal asphyxia show impaired spatial learning and less interest in novel environments (14). It is thus plausible that last-born piglets show more cognitive impairments than their first-born siblings.

Last-born piglets are also at risk of receiving insufficient amounts of colostrum. Colostrum is important for energy uptake and the immune system. Because antibodies are unable to be transported through the diffuse epitheliochorial placenta of pigs, piglets have to acquire maternal antibodies by drinking colostrum (2). The quantity and quality of colostrum piglets receive is not equal, since the anterior teats secrete more colostrum with higher concentrations of IgA and IgG than the posterior teats (15,16) and the amount of protein and immunoglobulins decreases by 50% in the first six hours of suckling (4). Piglets nursing the anterior and middle teats have a greater average daily gain than those nursing the posterior teats (16,17). A significant effect of birth order on the intake of IgG has been found (4,18), albeit not in all studies (19). This effect has two possible explanations. Firstly, last-born piglets are the last to arrive at the teat, when the anterior teats have already been claimed and colostrum quality is decreasing. Secondly, asphyxiated piglets might have more difficulty moving to and finding the udder (8,11).

Animal welfare is traditionally described in terms of the Five Freedoms, formulated by the British Farm Animal Welfare Council (20): "1) Freedom from hunger, thirst, 2) freedom from discomfort, 3) freedom from pain, injury or disease, 4) freedom to exhibit normal behavior and 5) freedom from fear and distress." Definitions of animal welfare have evolved over the past decades; in the view that we use to describe animal welfare, two criteria are added. Firstly, the animal should be able to adequately adapt to negative stimuli. Secondly, the adaptation should enable the animal to reach a state which it perceives as positive (21).

The welfare of last-born pigs is likely to be compromised. They can experience hypoxia, hunger, weakness and, due to impaired intake of maternal antibodies, possibly sickness, while having no means for adaptation to these negative stimuli. Hypoxia results in mild to moderate welfare compromise, while hunger and sickness would lead to moderate to severe welfare compromise (22). Hypoxia, hunger and sickness can be measured, though sometimes indirectly. For example, the time to reach the udder and the weight and growth of a piglet can give an indication of hunger. Cortisol is also a useful indicator of welfare, since the HPA-axis is activated as reaction to stressors. However, to be able to draw conclusions on the mental state of pigs, a different approach is needed. A judgement bias test (JBT) can be used to give an indication of both short-term emotion and long-term mood (23).

JBTs have been used for a variety of animal species, including companion animals (24,25), farm animals (26,27), captive wild animals (28,29), rodents (30) and insects (31). The test is preceded by training in which the animal learns to associate one cue with a positive outcome and another cue with a negative outcome. After the training, the animal is confronted with a third, ambiguous cue that lies somewhere between the positive and negative cue. The reaction of the animal to this cue depends on their mood and personality (32). Animals in a positive mood or with a positive personality respond to the ambiguous cue as if they expect a positive outcome, which is called an 'optimistic' bias, while animals in a more negative mood or with a negative personality respond as if expecting a negative outcome, which is called a 'pessimistic' bias (32,33). Examples of factors that affect judgement bias of pigs are housing conditions (34) and birth weight (35).

The design of the JBT needs to be adapted to the specific abilities of the species. A JBT can be either a Go/No-Go task or an Active Choice task. In a Go/No-Go task, an animal has to respond only to the positive cue (36,37). In an Active Choice task, both the positive and negative cue requires a similar response, but in a different location (38). Cues can be spatial (23), visual (39), olfactory (31) or auditory (38). The positive cue is commonly associated with a food reward, while the negative cue can be associated with punishment, absence of reward or a less favorable reward (smaller or delayed reward) (33,40). In our research group, an Active Choice task with auditory cues and two rewards of different sizes has proven to be suitable for pigs (35,38,41,42).

The effect of birth order on the affective state of pigs has, to the authors' knowledge, not yet been studied. Moreover, the effect of birth order on piglet performance is not completely understood. The aim of this study was therefore to investigate 1) the possible effect of birth order on multiple variables indicative of viability, asphyxia, milk intake and growth, and 2) performance of first and last-born piglets in a judgement bias test.

# MATERIALS & METHODS

## Ethical Note

This study was reviewed and approved by the local ethics committee of Utrecht University.

## Piglet Performance

A timeline of the complete experiment is provided in Figure 1.

### Subjects

The birth of 29 litters with a total of 461 piglets [(Terra x Finnish Landrace) x Duroc] was attended on the commercial breeding farm of the Faculty of Veterinary Science in Utrecht. The inclusion criteria for the study were a full-term birth and a litter size of more than 10 piglets. Two litters were excluded due to litter size (n=4 and n=9). Included in the study were 393 piglets of which the birth order was known. Human intervention was necessary for one sow. The sow was given a sedative seven hours before parturition and an oxytocin injection after the second piglet was manually delivered.

### Data Collection after Birth

Directly after the birth of a live piglet, birth order, time of birth, intactness of the umbilical cord and presence of cyanosis were recorded. After measuring the drinking time, the piglet received an ear tag and was weighed. Drinking time was defined as the time it took a piglet to reach a teat and hold it for more than two seconds. Identification of the piglet had priority over drinking time, so when a piglet was in risk of being misidentified, the ear was tagged before drinking and drinking time was not recorded. This resulted in the recording of drinking time of 67 piglets. If the piglet had still not drunk after one hour, the piglet was brought to the teat and drinking time was set to 60 minutes. The other possible interventions were removing the membranes, clearing the airway and pulling the piglet away to prevent crushing by the sow. Moreover, some piglets were cross-fostered shortly after birth to create litters of roughly the same size, according to standard procedure at the breeding farm. When a piglet was stillborn, only birth order and time of birth was recorded. Additionally, pathological examination was performed on mummified piglets from one litter.

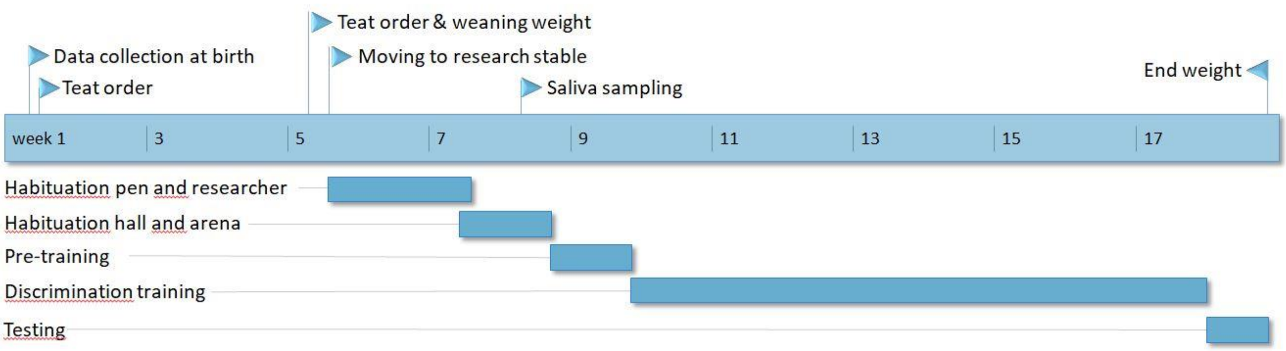


**Figure 1** | Experiment timeline. This shows the timeline of one piglet chosen for the JBT as an example. For piglets not chosen for the JBT, only data collection at birth, teat order and weaning weight were performed. Births of all JBT piglets took place in the same week. Length of discrimination training varies across pigs; the maximum length is shown.

These piglets were sent to the Veterinary Pathology Diagnostic Centre of Utrecht University directly after birth.

### Teat Order

In the first week after birth, the teat from which a piglet drank was observed for 10 first-born and 11 last-born piglets. The teat pairs were numbered from cranial to caudal. The observations were made during two drinking sessions on the same day. A drinking session was included when a maximum of two piglets were not drinking. In the week before weaning, teat order was observed for 18 first-born and 19 last-born piglets.

### Weaning and End Weight

Twenty piglets (chosen for the JBT) were weighed the day before weaning and at the end of the JBT (at approximately five months of age). One piglet was weighed at weaning but could not be identified at follow-up. This piglet was replaced by the next-born sibling for the JBT and weighing afterwards.

## Judgement Bias Task

### Subjects

Twenty pigs born in the same week were selected based on birth order and were moved to the research stables after weaning (at around four weeks of age). An overview of the subjects is given in Table 1. First and last-born piglets that had died before weaning were replaced by littermates, provided that the number of piglets born between the low and high birth order piglet was never less than nine.

| **Low birth order group**<br>*Gender distribution:*<br>*20% male* | | | **High birth order group**<br>*Gender distribution:*<br>*50% male* | | |
|---|---|---|---|---|---|
| ***Piglet*** | ***Litter*** | ***Birth order*** | ***Piglet*** | ***Litter*** | ***Birth order*** |
| 1 | 1 | 1 of 20 | 11 | 1 (2)* | 18 of 20 |
| 2 | 1 | 4 of 20 | 12 | 2 | 16 of 16 |
| 3 | 3 | 1 of 19 | 13 | 3 | 18 of 19 |
| 4 | 3 | 2 of 19 | 14 | 3 | 19 of 19 |
| 5 | 4 | 1 of 14 | 15 | 4 | 14 of 14 |
| 6 | 5 | 2 of 15 | 16 | 5 | 15 of 15 |
| 7 | 6 | 1 of 15 | 17 | 6 | 15 of 15 |
| 8 | 7 | 2 of 20 | 18 | 7 | 20 of 20 |
| 9 | 8 | 1 of 11 | 19 | 8 | 11 of 11 |
| 10 | 9 | 1 of 18 | 20 | 9 (7)* | 17 of 18 |

Table 1 | Pigs selected for the judgement bias test.
*The number in brackets corresponds to the litter in which the piglet was placed after cross-fostering.

### Housing

The pigs were placed in two straw-bedded pens of approximately 4x5 m. Each pen housed five low birth order and five high birth order piglets. Siblings were divided randomly over the two pens. The pens contained a covered nest area with plastic transparent slabs for insulation and heat lamps, which were removed after eight weeks. Enrichment in the form of toys (a chain with sticks and balls) was also present. The temperature in the stable varied between -6 and 32 $^{0}$C. The pigs received water ad libitum and were fed twice a day.

### Apparatus

The judgement bias apparatus (Figure 2) consisted of a test arena (3.6 x 4.2 m) with two identical goal-boxes, an antechamber and a start-box (1.2 m$^2$). Entrance from the start-box to the antechamber and access to the goal-boxes could be controlled by pulley-operated guillotine doors. The goal-boxes contained a food bowl with a false bottom. M&M's® (chocolate candies) were used as reward. They were placed in the false bottoms to avoid scent discrimination between the goal-boxes. The food bowls were covered with plastic balls to mask the reward from view. Tone cues were generated using Online Tone Generator[1] and played on speakers (Logitech, Lausanne, Switzerland) placed on the outer wall of the arena, between the two goal-boxes.

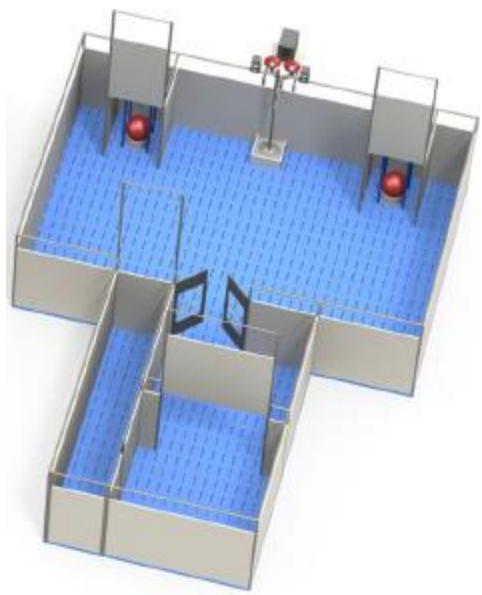

**Figure 2** | Judgement Bias test apparatus. Illustration by Yorrit van der Staay.

[1] http://onlinetonegenerator.com/

### Habituation

First, the pigs were habituated to the pen and the researcher for approximately two weeks, until they all approached the researcher voluntarily. Next, they were habituated to the hallway that led to the test arena, then to the arena itself. The habituation in the test arena started with free exploration in groups of five. Rewards were placed in both goal-boxes. When all pigs could lift the balls of the goal-boxes, group size was gradually decreased until the pigs were comfortable on their own. They then had to perform eight forced trials: they started from the start-box and only one goal-box was open per trial. The number of trials per session (corresponding to one day) was increased to 10 and then 12.

### Pre-training

After 3.5 weeks of habituation, tone cues were introduced. Tones with a frequency of 200 Hz and 1000 Hz were used as positive and negative tones, i.e. corresponding to a high or low reward, respectively. Which tone was positive and which negative was counterbalanced across animals, just as the location of the rewards (left or right goal-box). The low reward consisted of one M&M and the high reward of four M&Ms. The rewards were increased to three and eight M&Ms for four pigs, because they showed little interest in the low reward of a single M&M. The pigs performed forced trials in which only one goal-box was open per trial. The tone corresponding to the open goal-box started when the pig was in the start-box and stopped when the pig lifted the ball. Each pig performed six positive and six negative trials in pseudo-random order for four days. Then, open choice trials were performed in which both goal-boxes were open, but only the goal-box corresponding to the tone was baited with M&Ms. When the pig lifted the ball of the right goal-box, it was rewarded verbally and with a clicker. When it made a wrong choice, the wrong goal-box was closed and the pig could still visit the other goal-box. These open choice trials were executed for two days, after which discrimination training started.

### Discrimination Training

A session started with three forced trials as described above, of which two were always negative. This was followed by 10 open trials (5 positive and 5 negative in pseudo-random order) with both goal-boxes open. A right choice was still rewarded with voice and clicker, but the difference with the open choice trials was that both goal-boxes were closed after a wrong choice and the pig had to return to the start-box without a reward. Every fifth session, the first six open trials were replaced by open choice trials. In addition, every 10th session, the right goal-box was empty for one positive and one negative trial and was only rewarded with voice and clicker. This way, the pigs could learn that a right choice was not always rewarded with M&Ms. Discrimination training was performed until the pig reached the criterion of four right choices in both the positive and negative trials for three consecutive sessions, with a maximum of 35 sessions.

### Testing

Four testing sessions followed discrimination training when a pig had reached the criterion or after 35 training sessions. A test session was equivalent to discrimination training, save the addition of three ambiguous trials, inserted in the test session at trial 6, 11 and 16. During those trials, one of the following ambiguous tones was played: a middle tone, a near-negative tone (between the middle and negative tone), or near-positive tone (between the middle and the positive tone). The ambiguous tones were of frequencies at equal intervals between the negative and positive tone on a logarithmic scale: 299.97 Hz, 447.21 Hz and 668.74 Hz. Which frequency corresponded to the near-negative or near-positive valence depended on which tone the pig had learned to associate with a high or low reward. During the 6th trial, the middle tone was played, while the order of the near-positive and near-negative tone at the 11th or 16th trial changed every session. The valence of the trials preceding the ambiguous trials (positive or negative) was counterbalanced across sessions to neutralize the effect of prior trials on judgement bias. During the ambiguous trials, the goal-boxes were empty. However, all choices were rewarded with voice and clicker to prevent the pigs from associating the new tones with the absence of a reward.

## Salivary Cortisol

Saliva samples were collected before and directly after the pigs' first individual habituation trial. Sampling started at approximately 10 pm with the first pen. Saliva was collected by letting the pigs chew on a cotton swab (150 mm x 4 mm WA 2PL; Heins Herenz, Hamburg, Germany) until thoroughly moistened. The swabs were placed in collection tubes (Salivette, Sarstedt, Germany), put on ice for transportation, and then centrifuged at approximately 3500 g for 10 minutes at 10 $^0$C. Samples were stored at -20 $^0$C until analysis. A Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics BV, The Hague, the Netherlands) was used to determine cortisol concentration in duplo.

## Statistical Analyses

Statistical analyses were performed using R (R C ore Team, 2014). The significance threshold was set at $p = 0.05$.

### Piglet Performance

For analyses of the number of stillbirths, damaged umbilical cords, piglets born inside the membranes and piglets with cyanosis, piglets were divided into two groups (first and second half) based on birth order relative to total litter size. Freshly stillborn and mummified piglets were included in litter size. In case of odd-numbered litter sizes, the median piglet was classified as second half. Results were analyzed using Fisher's exact test.

For analyses of drinking time and teat order, a division into three groups (beginning, middle and end) was made based on birth order relative to total litter size. For analysis of birth weight, only the fist, middle and last-born piglets were used. Prior to analyses, a $\log_{10}$-transformation was performed on drinking time and a square root-transformation on teat order. Drinking time, teat order, birth weight, weaning weight and end weight were analyzed using a linear mixed-effect model (lme in R), using the following method: firstly, the best random effects were selected. This was done by comparing models with different random effects and with all explanatory variables as fixed effect using the Akaike Information Criterion (AIC). The model with the lowest AIC was selected. Possible random effects for all models were litter, piglet identity and piglet identity nested in litter. Secondly, fixed effects were chosen. Maximum likelihood models with different combinations of variables as fixed effects and with the selected random effect were created. Again, AIC was used to select the best model. Thirdly, residual plots were visually inspected to check assumptions. If a variable was not included in the best model, no effect of this variable on the outcome variable could be demonstrated.

For drinking time, the best model included litter as random effect and birth order as fixed effect. For teat order, piglet identity was used as random effect and birth order as fixed effect. For birth weight, litter was used as random effect and birth order and total number of piglets as fixed effects. For both weaning and end weight, models with litter as random effect and birth order group and gender as fixed effects were used.

### Judgement Bias Task

The following variables were calculated:

- **Sessions until criterion**, i.e. number of sessions necessary to reach the criterion;
- **Correct choices**, i.e. average number of correct choices per block of three training sessions, forming a learning curve;
- **Optimistic choice (OC) percentage**, i.e. the percentage of choices for the goal-box normally containing the large reward;
- **Latency to respond**, i.e. time from the pig's first step out of the start-box until one of the balls was lifted.

Results from one pig were excluded from analysis of the learning curve because it had performed less than 13 trials per session in the beginning of discrimination training. Session 34 and 35 were also not included in the learning curve because of the low number of piglets that were still in the discrimination training phase. Test results from the pigs that had not reached the criterion were included in analysis of OC and latency because they their test performance was comparable with the pigs that had reached it.

Sessions until criterion, correct choices, OC and latency were analyzed using a linear mixed-effect model, using the aforementioned method. The model for sessions until criterion included litter as random effect and location as fixed effect. For correct choices, piglet identity was used as random effect and session block as fixed effect. The best model for OC had piglet identity as random effect and cue type as fixed effect. Latency to respond was analyzed using pig identity as random effect and gender, cue type and gender*cue type as fixed effects. The interaction between gender and cue type was further analyzed by running the model on separate datasets for each cue type.

Additionally, the effect of repeated testing on OC percentage was analyzed by creating a model with piglet identity as random effect and cue type and session (first two or last two) as fixed effects.

### Salivary Cortisol

Cortisol concentrations were $log_{10}$-transformed before analysis. In the linear mixed-effect model for salivary cortisol, litter was a random effect and sample, birth order, gender, birth order*sample, and gender*sample were fixed effects. Interactions were further analyzed by running the model on separate datasets per sample type, sex and birth order group.

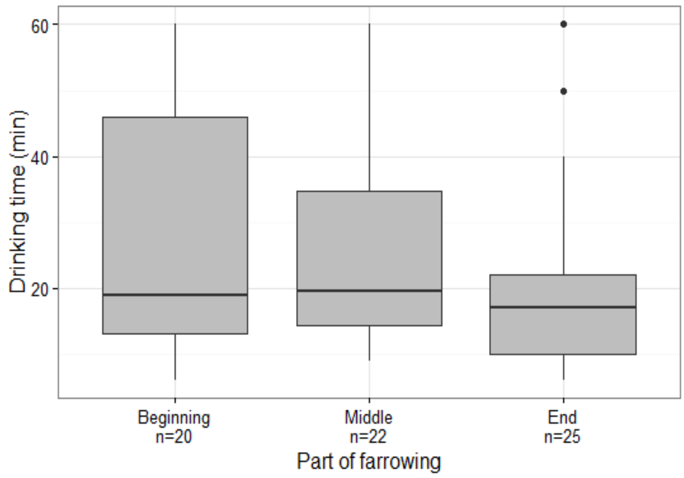


**Figure 3** | Drinking time of piglets born in the beginning, middle and end of farrowing.

# RESULTS

## Piglet Performance

### Descriptive Results

The 27 sows included in the study had a mean parity of 4 ± 2.6 (range 1 to 9) and a mean litter size of 17 ± 3.0 (range 11 to 23). The average time between birth of the first and last piglet was 211 ± 133 minutes (range 92 to 699), with an average birth interval of 14 ± 17 minutes (range 0 to 155). Total stillbirth rate was 11.3%, consisting of freshly stillborn (4.7%) and mummified (6.6%) piglets. Nineteen piglets (6.0%) were born with a broken umbilical cord, three piglets (0.9%) with a knot in the umbilical cord and five piglets (1.6%) inside the placenta. Cyanosis or paleness was found in four piglets (1.2%). Mean birth weight was 1.30 ± 0.32 kg (range 0.42 to 2.20 kg, n=337). Mean drinking time was 24 ± 16.1 minutes (range 6 to 60 min, n=67). The maximum drinking time of 60 minutes was recorded for five piglets. Teat order was recorded twice on the same day. The percentage of piglets that drank from the same teat twice was 67% in the first week and 90% in the week before weaning. One last-born piglet did not drink during both drinking sessions.

### Statistical Results

No significant difference was found in the numbers of stillbirths, piglets born inside membranes, damaged umbilical cords or cyanosis between the first and second half of farrowing (Table 2). No significant difference in drinking time between piglets born in the beginning, middle and end of farrowing could be detected ($F = 2.4$, $p = 0.1$) (Figure 3). Figure 4 illustrates that last-born piglets drank from more caudal teats than first-born piglets ($F = 5.4$, $p = 0.04$). Last-born piglets were shown to have a higher birth-weight than middle-born piglets ($t=2.7$, $p=0.01$) (Figure 5). At weaning and at the end of JBT training, the difference between the weight of low and high birth order piglets was not significant (Birth order group: $F = 2.3$, $p = 0.4$; $F = 2.3$, $p = 0.2$) (Figure 6).

Table 2 | Prevalence of stillbirths, births inside membranes, damaged umbilical cords and cyanosis.

| | **Birth order group** | | **NA** | **p** |
|---|---|---|---|---|
| | *First half* | *Second half* | *(total = 393)* | |
| *Stillborn* | 22 (11.3%) | 24 (11.2%) | 38 | 1 |
| *Freshly stillborn* | 9 (4.6%) | 10 (4.7%) | | |
| *Mummified* | 13 (6.7%) | 14 (6.5%) | | |
| *Born inside membranes* | 1 (0.5%) | 4 (1.8%) | 38 | 0.38 |
| *Damaged umbilical cord* | 10 (6.8%) | 12(7.4%) | 137 | 1 |
| *Cyanosis/paleness* | 1 (0.6%) | 3 (1.8%) | 129 | 0.62 |

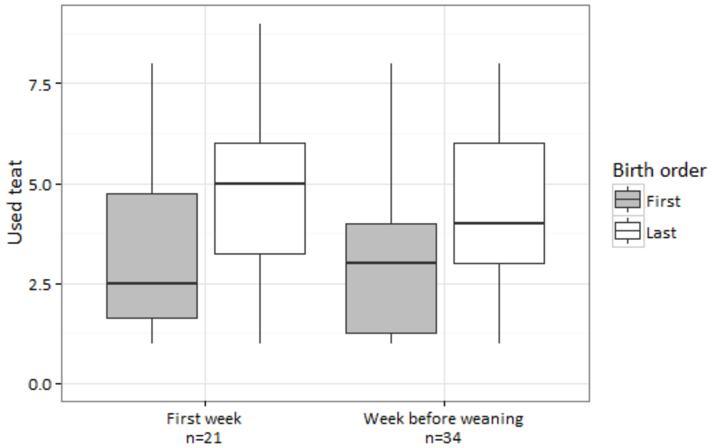


**Figure 4** | Teat order of first and last-born piglets during the first week and the week before weaning.

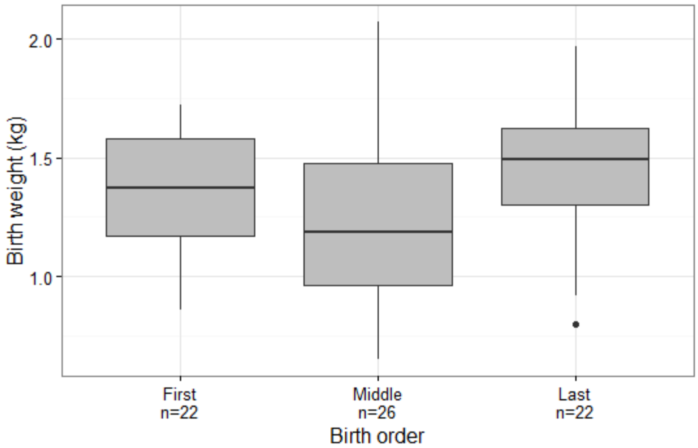


**Figure 5** | Birth weight of first, middle and last-born piglets.

## Judgement Bias Task

### Discrimination training

Six piglets from the low birth order group and eight from the high birth order group reached the criterion within 35 sessions. Birth order group did not affect the number of sessions required to reach the criterion (mean ± SD: low 24.2 ± 2.4, high 24.1 ± 2.8), nor did the location of the high reward (Location: F = 5.8, p = 0.053). Figure 7 shows the learning curves of low and high birth order pigs in the discrimination training phase. The number of correct choices increased with test sessions (Session: F=50, p < 0.001). Low and high birth order pigs did not show a difference in learning rate.

### Optimistic Choice Percentage

Birth order group did not affect optimistic bias (Figure 8). When cue type was more similar to the positive cue, optimistic bias increased (Cue: F=212, p<0.0001). Optimistic choice percentage did not differ significantly between the first and last two test sessions (F= 0.16, p = 0.7).

### Latency to Respond

The latency to respond was affected by cue type and gender (Figure 9). Latency decreased when cue type was more similar to the positive cue (Cue: F = 10.9, p < 0.0001). Moreover, males showed a higher latency to respond to the negative cue than females (Gender: F = 8.7, p = 0.009). No effect of birth order on latency was found.

## Salivary Cortisol

Results from salivary cortisol analysis are shown in Figure 10. LBO piglets had higher pre-stressor levels of cortisol than HBO piglets (Birth order: F = 12, p = 0.006), but this difference was absent in post-stressor samples (birth order: F = 3, p = 0.09). This was caused by a decrease of cortisol levels in LBO piglets (Sample: F = 15, p = 0.002) together with an increase of cortisol levels in HBO piglets (Sample: F = 8.2, p = 0.02). Females had higher pre-stressor levels of cortisol than males (Gender: F = 32, p = 0.0002). However, this gender effect was not present in post-

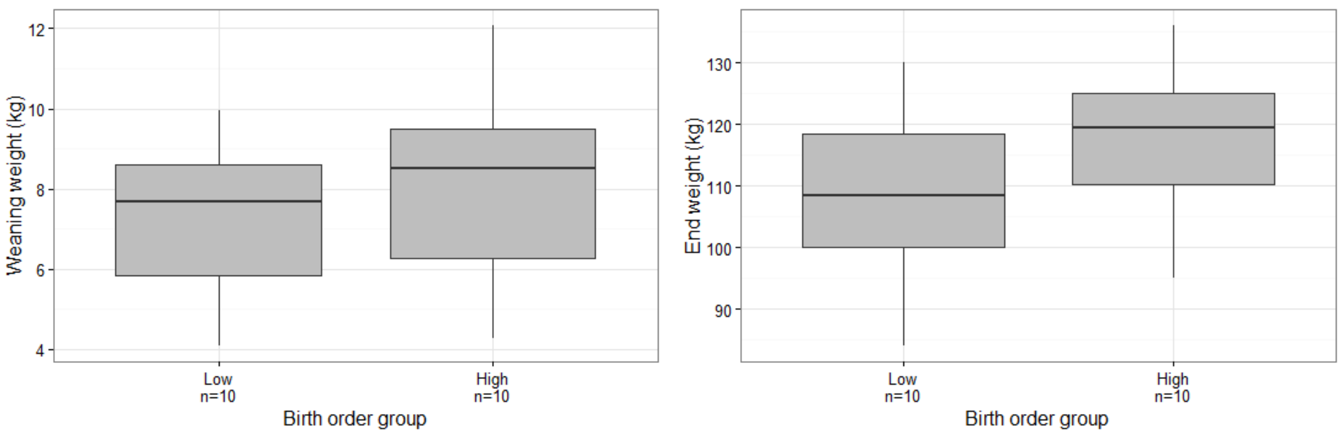


**Figure 6** | (A) Weaning and (B) end weight of low and high birth group pigs

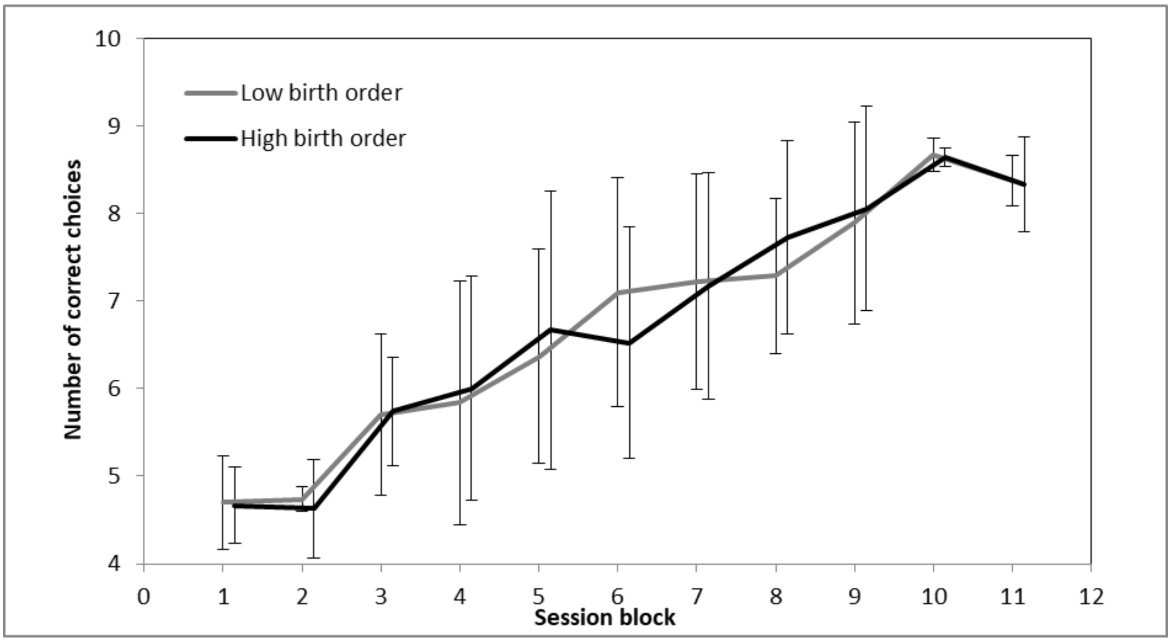


**Figure 7** | Number of correct choices per block of three sessions (mean ± SD) of low and high birth order pigs.

stressor samples (Gender: F = 0.3, p = 0.6). After performance of an individual trial, cortisol levels decreased in females (Sample: F = 5.2, p = 0.03) while remaining unaltered in males (Sample: F = 4.2, p = 0.08).

# DISCUSSION

This study used a non-invasive method to examine the effect of birth order on the performance and welfare of pigs. Last-born piglets were expected to have experienced a more difficult birth in addition to more competition during drinking, resulting in a more negative affective state. Of the variables measured in weaning piglets, birth order affected only teat order in the present study. Moreover, results from an active-choice JBT did not support the hypothesis: no effect of birth order on learning rate, optimistic bias or latency to respond was found.

## Piglet Performance

### Stillbirth

Although other research has shown that stillbirth rate and either birth order (9,43) or the duration of farrowing (44) are positively correlated, the present study did not find this relationship. This could be due to the limited sample size. Interestingly, the total stillbirth rate of 11.3% in the present study is higher than the range of 6.2% to 9.2% in other studies (5,9,43,44). The high number of mummified piglets, 6.7% versus approximately 2% in other studies (5,9), accounts for this difference. Although pathological examination was performed, no cause has been identified.

### Asphyxia

To obtain an indication of asphyxia in a non-invasive way, intactness of the umbilical cord and presence of cyanosis were measured. A broken umbilical cord is one of the most important causes of asphyxia (43), while cyanosis in new-borns is a consequence of oxygen deprivation (45). In this study, no effect of birth order on either condition could be demonstrated. Langendijk et al. (2018) also found no relationship between birth order and intactness of the umbilical cord (43), whereas Rootwelt et al. (2012) did (9). In the latter study, the same division into three birth order groups as the present study was made. The reason for the difference between birth groups in the current study not reaching significance could be the lower sample size. This is especially true for the measurement of cyanosis, because of the low observed prevalence. Secondly, the intactness of the umbilical cord was only recorded for live-born piglets. Since damage to the umbilical cord increases the chance of stillbirth (43), this could have affected the results. Lastly, the performance

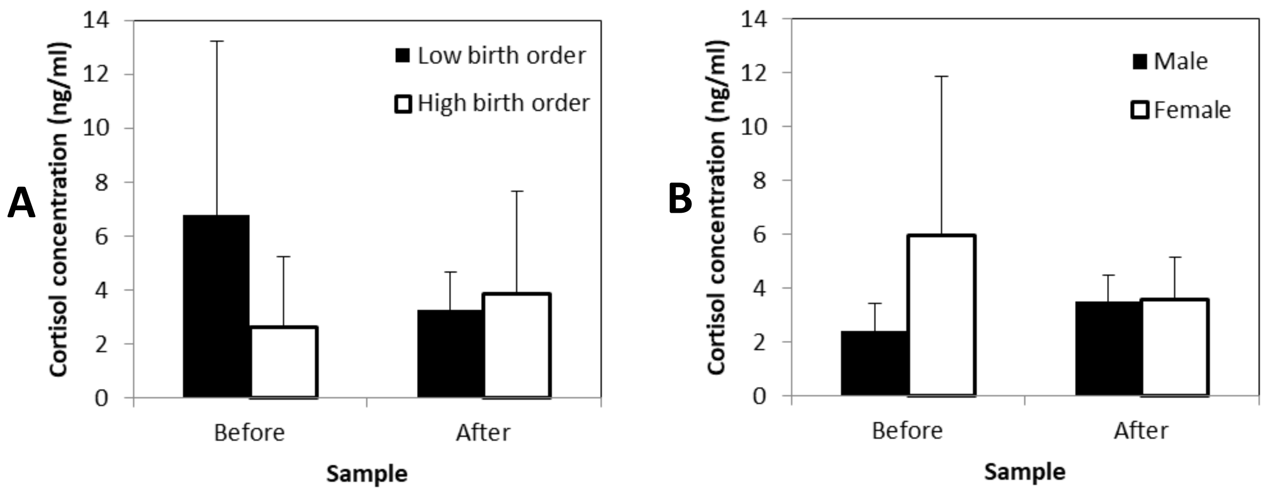


**Figure 10** | Salivary cortisol levels of (A) Low and high birth order pigs and (B) males and females. Mean ± SD

of cyanosis scoring by inexperienced observers increases the unreliability.

### Colostrum and Milk Intake

Colostrum and milk intake play an important role in piglet performance. Piglets can receive less colostrum than their siblings by either drinking later (4) or from more caudal teats (15,16). In this study, the time it took piglets to drink for the first time did not differ between birth order groups. However, even when time from birth until drinking is the same, last-born piglets still begin drinking later than first-born piglets simply because they are born later, on average 3.5 hours after the first-born. Moreover, a first-born can choose the best teat before the last piglet is born. In the present study, last-born piglets drank from more caudal teats than first-born piglets. Interestingly, teat order was not reflected in weaning weight, although piglets nursing the cranial teats were expected to show a greater average daily gain (16,17). In the first week, one third of piglets changed their preferred teat between two subsequent drinking sessions. In the week before weaning, this was decreased to 10%, indicating that a stable teat order develops after the first week. This is in accordance with previous findings (46). It should be noted that some piglets were cross-fostered before the first observation of teat order. The effect of cross-fostering on teat order is yet undetermined.

### Piglet Weight

The average birth weight of the piglets in this study is comparable with other recent studies (47–49). Middle-borns were shown to have a lower birthweight than last-borns, while the birthweight of first-borns did not differ significantly from middle or last-borns. Rootwelt et al. (2012) compared three birth order groups and found a comparable distribution of birth weight along the groups (9). They observed an additional significant difference between birth weight of the first and middle birth order group. Beaulieu et al. (2010) discovered a positive correlation between birth rank (i.e. relative birth order) and birth weight (50). Taken together, it seems likely that middle-born piglets have the lowest birth-weight and last-born piglets the highest. Being heavier at birth is beneficial for piglet survival (2,51). At weaning, no significant difference between the weight of first and last-born piglets has been found in the present study. Other studies reported that higher birth order (49) or birth rank (50) is linked to higher weaning weight.

## Judgement Bias

### Learning

Fourteen pigs reached the criterion of 80% right choices in both the positive and negative trials during three consecutive sessions. This success rate is lower than previous studies with the same test design (35,38,41). Of the six pigs that did not reach the criterion, one pig made 80% right choices once, two pigs 3 times and three pigs 5 times, only not on consecutive days. Because of this, it was decided to test all pigs after 35 sessions. Pigs that did not reach the criterion responded equally well in response to

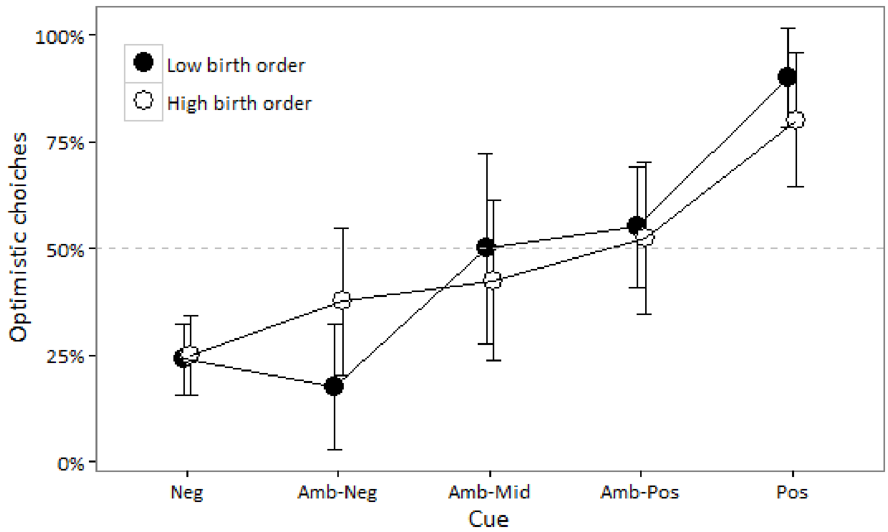


**Figure 8** | Optimistic choices of low and high birth order pigs

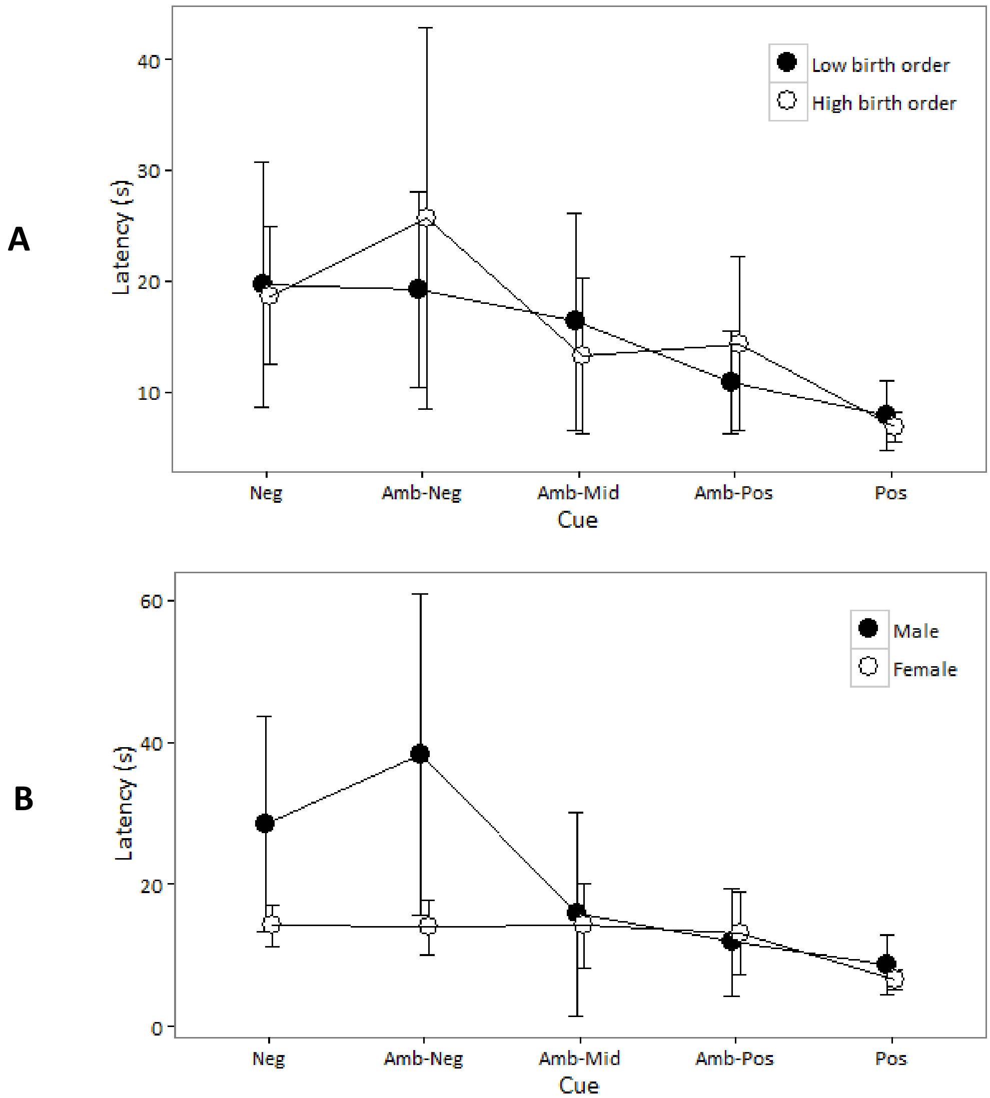


**Figure 9** | Latency to respond of **(A)** low and high birth order pigs and **(B)** males and females.

the trained cues, supporting the notion that they had learned to discriminate between the high and low tone.

### Optimistic Choices

Pigs responded optimistically to the intermediate ambiguous tone in 52% of trials. This optimistic bias is lower than Roelofs et al. (2017b) and Murphy et al. (2015) reported, but higher than Murphy et al. (2013) (35,38,41). Because the pigs are believed to have experienced good welfare in the relatively enriched housing, a higher optimistic bias was expected. The lack of optimistic bias might be explained by the heat: the temperature in the stable suddenly increased to a maximum of 32 $^0$C (due to weather conditions) around the time of testing. Although welfare implications of heat stress in pigs have not been studied, heat is presumed to negatively affect the emotional state.

Pigs reacted significantly faster as cues became more similar to the positive cue, showing that they preferred the high reward. This was also found in previous studies (35,38). Remarkably, males were slower to respond to the negative cue than females. Other studies found no difference between latencies of males and females (38,52).

One of the obstacles of the JBT is loss of ambiguity when pigs perform multiple test sessions (33). Roelofs et al. (2017b) reported a decrease in optimistic choice percentage when comparing the first and last two test sessions, suggesting the pigs learned that the ambiguous tones were unrewarded (38). To prevent this effect of repeated testing, both partial reinforcement (unrewarded correct choices) during training and secondary reinforcement (voice and clicker) was used in the present study. Neave et al. (2013) successfully used a partial reinforcement schedule during training of dairy calves (53), while Keane et al. (2014) used a clicker as secondary reinforcement in their study with grizzly bears (28). The combination of both methods is also successful in the present study, since no effect of repeated testing on optimistic choice percentage was found.

## Salivary Cortisol

The relationship between birth order and cortisol levels has not been studied before. The present study provided complex results. Prior to their first individual habituation trial, low birth order pigs had higher cortisol levels than high birth order pigs. The higher levels in low birth order pigs could suggest this group experienced more stress than high birth order pigs. Another explanation is that chronic stress in high birth order pigs alters the response to acute stressors, leading to decreased cortisol levels (54). One study revealed that pigs in barren environments have lower cortisol levels than pigs in enriched environments (55). They suggested that long-lasting decreased welfare weakens the circadian rhythm. However, this does not explain the drop in cortisol levels of low birth order pigs after a stressor.

The increase of corticosteroids after a stressful event initiates after some minutes and is best measured at least 10 minutes after first exposure (54). In this study, post-stressor samples were taken within 10 minutes of the pig's entry into the test arena. Post-stressor cortisol levels may therefore not reflect the reaction to the individual trial. Rather, it might reflect the reaction to moving from the stable to the hallway prior to the trial, which the pigs may not experience as stressful. Another explanation for the decrease in cortisol levels is that the pigs experience the short isolation as an agreeable break from stressful social interactions. The pigs were never really isolated, since they could hear and smell the others, which is sufficient for social support (56). During the habituation trial, the pigs received a food reward, which might also make this a positive experience. It is possible that the cortisol levels of low birth order pigs and not of high birth order pigs decreased, because those of the latter group were already low.

In addition, the present study found a difference in stress response between males and females. Cortisol levels of females were higher before the trial, but decreased during the trial while those of males slightly increased. Roelofs et al. (2018) found no sex difference in cortisol levels of pigs before and after a holeboard task (57). However, in humans, boys show a stronger cortisol response to stress than girls (58,59).

## Limitations and Recommendations for Further Studies

During farrowing, multiple interventions were performed to prevent suffering of piglets. By doing so, some piglets' lives were saved, such as of those born inside the membranes. This might have influenced the results, since these piglets would otherwise not have been included. Furthermore, bringing piglets to the teat after an hour influenced drinking time and possibly teat order; however, this was done with only five piglets.

Because attendance at birth, selection of piglets and the JBT were performed by the same person, the birth order group in which the pigs belonged was known, allowing for observer bias. Moreover, the M&Ms in the false bottoms were not replaced daily. This may have made scent discrimination of the wrong and correct goal-box possible. However, pigs did not seem to react differently or make more mistakes on days when the M&Ms were replaced.

Piglets were intentionally divided into relative birth order groups rather than using the absolute birth order. This way, the last-born piglet of a litter of 10 does not fall into the same category as the middle-born of a litter of 20. However, birth order is not to be seen independent of litter size. To account for this, litter size was included as possible fixed effect in the different models, but it was not included in the final models because it decreased model quality. A second way of taking litter size into account was an inclusion criterion of a litter size of 10 piglets. This number was chosen to maintain a large enough sample size, but it is lower than the average litter size (1). If the sample size of the study were higher, the minimum litter size could be increased. The JBT could then also be performed with ten first and ten last-born pigs, exclusively.

In this study, all pigs were housed in enriched environments. Group housing with straw bedding is believed to enhance piglet welfare (60). Pigs housed in enriched environments indeed show more optimistic bias than pigs in barren environments (47). Enriched housing conditions might have diminished the difference in affective states between first and last-born pigs. In the case of more aversive conditions, last-born piglets might have more difficulty coping, resulting in more negative affective states. Performing a JBT with pigs in poorer living conditions would therefore be a valuable addition.

Although a JBT is shown to be a valuable method of studying emotion, the sensitivity of JBTs to very small differences in affective states is questionable. To further validate the results of this study, JBT results should be complemented by other tests measuring emotions (40), such as behavioral observation, novel object tests or human interaction tests (33). For a better understanding of the stress response in low and high birth order pigs, determination of cortisol levels should be repeated with at least 10 minutes between stressor and post-stressor sampling. Also, a different stressor could be chosen, such as group mixing (61) or restraint with a nose sling (62).

## CONCLUSION

This study shows that piglets born later in birth order drink from more caudal teats and are therefore at risk of receiving less colostrum and milk. Results from a judgement bias test revealed no difference in affective state between first and last-born pigs. For conclusive results, conducting additional behavioral tests with piglets from large litters in poorer living conditions is proposed. Moreover, measurement of cortisol levels provided complex results which need to be studied further.

## AUTHOR CONTRIBUTIONS

YS, RN and SR contributed to study design. YS and YO acquired the data. YS performed statistical analysis and wrote the first draft of the manuscript. RN revised the manuscript and has read and approved the submitted version.

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