Low birth weight and early-life iron deficiency in piglets

Post-weaning effects on cognition, development, and motivation

Alexandra Antonides

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Laag geboortegewicht en vroege ijzerdeficiëntie in biggen
Effecten op cognitie, ontwikkeling, en motivatie na het spenen
(met een samenvatting in het Nederlands)

Proefschrift
ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 september 2016 des middags te 4.15 uur

door
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I like pigs

Dogs look up to us
Cats look down on us

Pigs treat us as equals

~ Sir Winston Churchill

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Chapter 1

General introduction

Complex processes involved in growth and development make young animals vulnerable. Many developmental processes have a transient window of opportunity. An example is the development of speech in humans and song in birds, which are both sensitive to external influences during only a certain period of time (Doupe and Kuhl, 1999). If a baby or chick does not receive the right, or any, external auditory input during this sensitive period, it will never be able to form normal speech or song. Thus, some factors may influence developmental processes during a specific, critical time frame only, after which the effects of their presence or absence cannot be reversed. Such factors may be internal or external, and may occur prenatally (before birth), perinatally (around birth) or postnatally (after birth). Proper cognitive, physical and anatomical development depend on the correct orchestration of these processes and the factors influencing them.
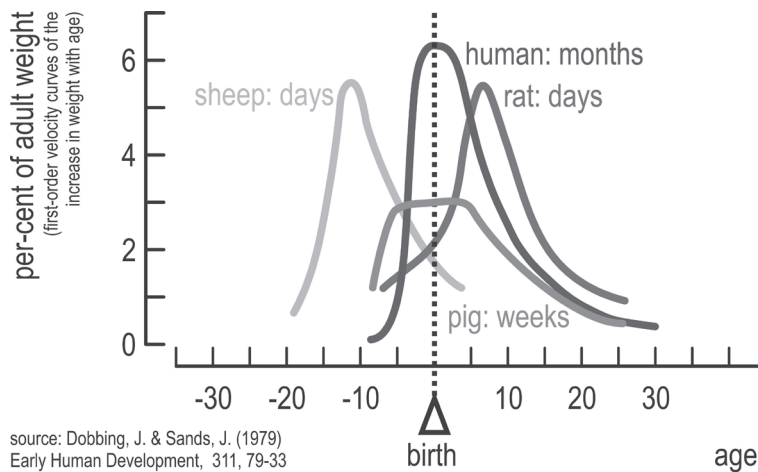
During early development, animals generally go through a growth spurt in which vital organs, such as the brain, develop quickly. This growth spurt occurs at different stages of development in different species (Figure 1). In rodents, brain development occurs mainly postnatally, whereas in humans it occurs perinatally. The developing brain of rats is believed to be comparable to that of a full-term newborn human baby, with regard to the degree of maturation, around postnatal day 12-13 (Romijn et al., 1991). Although widely used, rodent models for human conditions have recently raised concerns, as successful pre-clinical studies in rodents poorly translate to effective clinical use in humans (van der Worp et al., 2010; Macleod, 2011). Animals that more closely resemble humans are likely to yield more relevant study results (Festing and Altman, 2002). Large animal models may therefore show less discrepancies in study outcomes with humans.

Pigs as animal model in translational research

Pigs have recently received increasing attention as animal model species in translational research (e.g. Lind et al., 2007; Kobayashi et al., 2012). In comparison with rodents, the pattern and timing of brain development in pigs is more similar to that of humans (Dobbing and Sands, 1979; Conrad et al., 2012). In addition, the pig's brain anatomy more closely resembles that of humans. The pig has a relatively large brain that, like the human brain, has convolutions (gyri) and grooves (sulci) in its surface, whereas the rodent brain

has a smooth surface. In addition, pigs are social and intelligent animals which can be trained in complex cognitive tasks at a young age (Dilger and Johnson, 2010; Mendl et al., 2010). Piglets can easily be purchased from farms with well-documented life history, which makes them attractive animals to use in research.

The pig can thus serve as a large animal model to examine environmental effects during the neonatal period, when the brain is rapidly developing, on long-term cognitive and physical development. Studies with pigs may both complement, and fill the gap between human and rodent studies.

source: Dobbing, J. & Sands, J. (1979)
Early Human Development, 311, 79-33

Figure 1. The growth spurt in different animal species often used in translational research compared to that in humans.

Complications during early development

In developing countries, up to 15% of all human pregnancies end in miscarriage or stillbirth, showing that early development is vulnerable (Casterline, 1989). Extensive research has shown that maternal physiology and behavior during pregnancy can have profound effects on the development of the fetus. For example, maternal stress and anxiety during pregnancy increase the chance of spontaneous abortion, preterm labor, and malformations and growth restriction of the baby (Mulder et al., 2002). They also delay the baby's

development of motor and mental skills (Huizink et al., 2003). Perinatal complications, such as blood flow restriction and shortage of oxygen during birth, are major causes of growth retardation, cognitive disability, developmental delay and mortality in humans (van Handel et al., 2007). These complications all have been linked to lower neuropsychological performance in children at 7 years of age, with low birth weight having the strongest effect (Seidman et al., 2000).

Low birth weight (LBW) in human neonates − defined as a birth weight lower than 2500 g − born at term is primarily thought to be caused by placental insufficiency, causing intra-uterine growth restriction (Biri et al., 2007; Cox and Marton, 2009). LBW children show poorer neuro-developmental outcomes and poorer school performance at adolescence than normal birth weight (NBW) controls (Larroque et al., 2001; Arcangeli et al., 2012). Similarly, LBW in rats has negative effects on postnatal development and learning ability (Ogata et al., 1985; Saito et al., 2009).

Low birth weight in pigs

If multiple fetuses share the uterus, they may experience prenatal competition. The fetuses not only must share space, but also nutrients and oxygen supply. In livestock, prenatal stress caused by malnutrition has an impact on health and behavior in later life (Rutherford et al., 2012). While in humans the occurrence of twins and triplets is rather rare, pig litters consist of 14 piglets on average in European high-production countries. Strong selection for high production levels and ever-increasing litter sizes is causing this average to rise rapidly, with litters of more than 20 piglets becoming increasingly common. As a comparison: the wild boar, the ancestor of the commercial pig, generally produces litters of 4 to 6 piglets (Rutherford et al., 2011).

Not surprisingly, disadvantages and complications arise from the strong artificial selection for large litter sizes. Piglets from large litters may have undergone more limitations during fetal development than piglets from small litters, due to the stronger intrauterine competition for resources. Large litters result in intra-uterine crowding (IUC), which challenges the sow's capacity to nurture all piglets sufficiently (Prunier et al., 2010). IUC has negative effects on placental development, resulting in increased pre- and neonatal mortality and reduced

overall piglet viability. Moreover, IUC causes a decrease in blood flow, oxygen and nutrient availability per fetus (Père and Etienne, 2000). This reduces birth weight and pre-weaning weight gain, and increases birth weight variability within litters (Quiniou et al., 2002; Beaulieu et al., 2010). Thus, the incidence of LBW piglets increases with larger litter sizes.

LBW in piglets can be the cause of numerous welfare problems. LBW piglets are less responsive to their environment and have a higher risk to suffer from hypothermia, starvation and crushing by the sow than NBW piglets (Rutherford et al., 2013). In addition to their reduced survival probability, prenatal growth restriction may also influence brain development. As in commercial pigs being born with a LBW is increasingly common, they are a readily available animal model to study the effects of LBW on cognition and development. Although numerous studies have investigated the effects of LBW on production and welfare in pigs (e.g. Baxter et al., 2008), studies on cognitive performance of LBW pigs are scarce and inconclusive. Gieling et al. (2012) found that LBW piglets showed slightly lower working memory performance than NBW piglets. However, this effect disappeared with further training. The same LBW and NBW animals were then used in a conditional discrimination task by Murphy et al. (2013). Of the pigs that learned the task, the LBW animals – contrary to expectation – learned the task faster than the NBW animals. More research is needed to elucidate the effects of birth weight on cognitive performance and development.

Food motivation

Early-life competition among newborn piglets influences their survival chances: the smaller and weaker piglets must work harder for resources than their larger and stronger littermates. Fast intake of sufficient amounts of colostrum after birth is essential for early survival. Within a litter, piglets choose and defend one of the sow's teats, i.e. they establish a teat order, during the first seven days after farrowing. Teat competition is accompanied by aggression, and usually the piglets with higher birth weights win these fights (Scheel et al., 1977). The anterior teats are most preferred by piglets, as they yield more milk than the posterior teats (Rosillon-Warnier and Paquay, 1984). Smaller piglets must thus often settle for the less productive posterior teats. The strong early life competition, combined with the probably higher desire for energy intake, make it likely that LBW

piglets have a higher food motivation than NBW pigs. Food motivation in LBW piglets has, to our knowledge, not yet been investigated scientifically.

Iron deficiency

After birth, postnatal care and proper nutrition are of great importance for the survival of the neonate. Good nutrition is essential for its normal development, growth, and health. Nearly a third of the human world population suffers from one or more forms of malnutrition, which may lead to mental and physical impairments, and even death. In fact, around half of the deaths among children under the age of five years is associated with malnutrition (de Onis et al., 2006). The most common nutritional deficiency in humans is iron deficiency, affecting around two billion people worldwide (WHO, 2000; Ramakrishnan and Yip, 2002). Iron is required for many important biological functions in the body, and plays an especially important role in brain functioning (Youdim et al., 2010). The severity of impairments and which specific impairments are caused by iron deficiency, are dependent on the timing and duration of the deficiency. The most common period for iron deficiency in humans lies between zero and five years of age (McLean et al., 2009). Children who suffered from severe iron deficiency in infancy perform worse in motor, cognitive, social and emotional tasks compared to controls that received a balanced diet (Lozoff and Georgieff, 2006; Walter et al., 1989). Behavioral and developmental deficits in early-life iron deficient children are still found up to ten years after receiving a balanced, iron-sufficient diet (Lozoff et al., 2000). Impairments resulting from iron deficiency in rodents have been found specifically in the prefrontal cortex and hippocampus (Rao et al., 2012; Ranade et al., 2013). The hippocampus is a brain structure that is known to be involved in spatial learning and memory. Consequently, rats that suffered from early-life iron deficiency show reduced performance in behavioral tasks involving learning and memory (for example in the Morris water maze: Yehuda and Youdim, 1989). This was even found in adulthood after iron repletion, complementing the finding in humans that the adverse effects of early-life iron deficiency can be permanent (Bourque et al., 2008). These findings suggest that iron deficiency during early development causes irreversible impairments in brain structure and function. The impairments are believed to be due to rapid brain growth during early development and its associated high iron demand (Lozoff and Georgieff, 2006). Because of the differences in developmental and brain

morphology between rodents and humans, more and better suited animal models are needed to study the long-term cognitive effects of iron deficiency. Piglets may be especially suited to study the effects of iron deficiency: they have limited iron stores at birth, a high requirement of iron and a low external supply of iron (Starzyński et al., 2013). In addition, sow milk is low in iron content and does not meet the piglets' high iron requirements (Brady et al., 1978). In commercial pig farming, it is therefore common practice to provide piglets with an intramuscular injection of iron dextran on day 3 to 6 after birth (Svoboda and Drabek, 2005). Withholding this iron injection allows to induce and investigate the effects of naturally occurring iron deficiency in piglets.

A recent study showed that piglets withheld iron supplementation and fed an iron-deficient diet show impaired learning in a T-maze task (Rytych et al., 2012). However, the performance of these pigs was assessed during the period of iron deficiency treatment, and not during or after iron repletion.

Assessing cognitive performance using the holeboard task

In order to assess the effects of (adverse) early-life events on cognitive development, validated cognitive tests are needed. The spatial cognitive holeboard task has been validated as a suitable task to measure the acquisition and retention of complex spatial orientation (van der Staay et al., 2012). It consists of a large square arena containing a matrix of 4x4 possibly rewarded sites, in which four hidden rewards can be found (Figure 2, panel 1 and 2). The holeboard task is a free-choice maze, meaning that the animal is free to walk around and visit or revisit any site in whichever order the animals chooses. By keeping track of which sites are visited and revisited by the animal, two components of spatial memory can be distinguished simultaneously in the holeboard task: working and reference memory (van der Staay et al., 1990). Working memory is a form of short-term memory that is used during a specific trial (van der Staay et al., 2012) and that, once used, should be forgotten (Dudchenko, 2004), i.e. the short-term memory store must be reset at the end of a trial. This includes information stored about which sites an animal has already visited during a trial. Reference memory is a form of long-term memory that involves the general rules of a task, such as where to find hidden rewards and how to get access to these rewards (van der Staay et al., 2012). Information stored in reference memory is relevant across trials. Other measures in the holeboard task include

trial duration, which is the time needed to find all four rewards; and inter-visit interval, which is the average time between two hole visits. These measures may provide some information about the pig's speed of searching for the food rewards, which may be taken as measures of the pig's motivation.
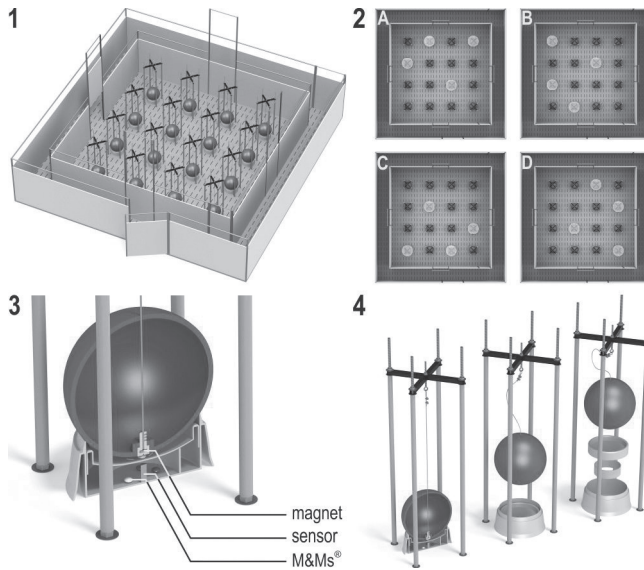

Figure 2. (1) The spatial cognitive holeboard for pigs. (2) Four patterns of baited holes, or configurations, used in the present studies. (3), (4) Constructional details of the holes. Each hole – a fool bowl with a false bottom under which three M&M's® chocolates are placed in order to mask odor cues – is covered by a red ball. Each food bowl is equipped with a sensor that sends a signal to the computer if the contact between the magnet in the ball is interrupted; i.e. when the pig lifts the ball with its snout (illustrations: Yorrit van der Staay).

The holeboard task for pigs is adapted from rodent studies (van der Staay et al., 2012) and adjusted for use with pigs (Arts et al., 2009; Gieling et al., 2012; Bolhuis et al., 2013). In the holeboard for pigs, the possibly rewarded sites, or 'holes', consist of plastic food bowls. These bowls are covered by a large plastic ball, that can be lifted by the pig with its snout in order to consume the reward. All holes contain a false bottom underneath which several rewards are always placed during trials, in order to prevent the pigs from finding the rewards by smell (Figure 2, panel 3 and 4).

After the animal has learned where the rewards can be found, the rewards can be placed in different locations. Pigs then must re-learn where the rewards

are hidden, allowing measurement of the response flexibility of an animal to adapt to a new situation, or new 'rules' within the task (i.e. where the rewards are hidden). When tested daily in the holeboard task, pigs show learning curves over time for all measures, in both the learning (or acquisition) and re-learning (or reversal) phase, which can be compared between treatment groups.

Aim and outline of the thesis

The aim of this thesis is to investigate the effects of LBW and early-life iron deficiency on post-weaning cognition, development and motivation in pigs. We both propose these studies to serve as animal models for these complications in humans, and to gain more knowledge about, and improve the welfare of, commercial pigs. The results of our studies may therefore benefit both human and animal research, and may improve pig husbandry practices.
We aimed to answer the following questions:

• Do LBW pigs show impaired cognitive development?
• Do LBW pigs have a higher motivation for food than their NBW siblings?
• Do pigs from large litters (in which the piglets, on average, have a lower birth weight) show emotional and cognitive impairments compared to pigs from small litters?
• Does early-life iron deficiency impair cognitive and physical development?

In line with previous findings in human and rodent studies, we expected to find detrimental effects of LBW, large litters and early-life iron deficiency on cognitive and physical development in pigs. With regards to motivation, we expected that LBW pigs would show higher food motivation than NBW pigs. The first part of this thesis focuses on the effects of birth weight and litter size on cognition, motivation and development. The second part of the thesis focuses on the effects of early-life iron deficiency on cognitive and physical development.

Part I: Low birth weight and litter size

In **Chapter 2**, we investigated learning and memory performance in the hole-board task in LBW and NBW pigs, using stricter selection criteria for LBW piglets than in previous research (Gieling et al., 2012; Murphy et al., 2013).

As cognitive development may be compromised in LBW piglets, we expected them to show cognitive impairments.

In **Chapter 3**, we studied food motivation in LBW and NBW pigs in two separate motivation tasks. The two tasks were a runway task and an operant wheel turning task.

In **Chapter 4**, we assessed whether litter size affects post-weaning emotionality in an open field test, and learning and memory performance in the holeboard task. Pigs from small litters were compared with pigs from large litters. The piglets were purchased from two suppliers which each breeds another pig line. Each supplier provided half of the piglet from small and large litters.

Part II: Iron deficiency

In **Chapter 5**, we assess the effects of withholding piglets an iron injection and feeding them an artificial, iron-deficient milk formula on blood parameters, growth, memory performance and brain histology. These animals were compared to piglets that received an iron injection and were fed an artificial, iron-sufficient milk formula. After weaning all animals were fed an iron-sufficient diet. Then, memory and learning in all animals was assessed in the holeboard task. In **Appendix I**, we further investigated the effects of iron deficiency on synaptic plasticity in the hippocampus of the piglets from the study described in Chapter 5. The aim of this study was to provide a link between iron deficiency and reduced synaptic plasticity as a cause of impaired cognitive functioning.

In **Chapter 6**, we studied the effects of withholding piglets an iron injection and feeding them sow milk as only nourishment until weaning. Sow milk is iron-deficient. In the study in Chapter 5, the piglets underwent early maternal deprivation, which may have acted as a confounding factor affecting the results. Therefore, the piglets now were left with the sow during iron deficiency treatment to rule out that early maternal deprivation confounded the results of the previous study. **Chapter 7** provides the general discussion of the results of all experiments described in this thesis.

Part I

Low birth weight and litter size

Chapter 2

Very low birth weight piglets show improved cognitive performance in the spatial cognitive holeboard task

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ABSTRACT

Low birth weight (LBW) is common in humans and has been found to cause lasting cognitive and developmental deficits later in life. It is thought that the primary cause is intra-uterine growth restriction due to a shortage of oxygen and nutrients supply to the fetus. Pigs appear to be a good model animal to investigate long-term cognitive effects of LBW, as LBW is common in commercially farmed breeds of pigs. Moreover, pigs are developmentally similar to humans and can be trained to perform complex tasks. In this study, we trained ten very low birth weight (vLBW) piglets and their ten normal birth weight (NBW) siblings in a spatial cognitive holeboard task in order to investigate long-term cognitive effects of LBW. In this task, four out of sixteen holes contain a hidden food reward, which allows measuring working memory (short-term) and reference memory (long-term) in parallel. Piglets were trained for 46-54 trials during the acquisition phase, followed by a 20-trial reversal phase in which a different set of four holes was baited. Both groups acquired the task and improved their performance over time. A mixed model repeated measures ANOVA revealed that vLBW piglets showed a better reference memory performance than NBW piglets in both the acquisition and reversal phase. Additionally, the vLBW piglets fell back less in working memory scores than the NBW animals when switched to the reversal phase. These findings are contrary to findings in humans. Moreover, vLBW pigs had lower hair cortisol concentrations than NBW pigs in flank hair at 12 weeks of age. These results could indicate that restricted intra-uterine growth causes compensatory mechanisms to arise in early development that result in beneficial effects for vLBW piglets, increasing their low survival chances in early-life competition.

Keywords: low birth weight, pigs, spatial cognition, memory, learning, hair cortisol

INTRODUCTION

Processes during fetal development are complex and therefore prone to disturbances and complications. Negative effects on fetal development during pregnancy can result in physical or neurological deficits and disorders later in life (Colletti, 1979). In humans, low birth weight (LBW) in babies born at term is a common phenomenon: the prevalence in developing countries ranges from 15 to 25% and is expected to be even higher as many births in such countries are not reported (Ramakrishnan, 2004). LBW in humans is defined as a weight less than 2500 g at birth and is thought to be primarily caused by intra-uterine growth restriction (IUGR) through a chronic shortage of oxygen and nutrients supply due to placental inefficiency (Biri et al., 2007; Cox and Marton, 2009). LBW has been linked to impaired cognitive function and various other deficits later in life. It is important to distinguish between LBW infants born preterm and at term (also called small for gestational age: SGA), because prematurity itself can already lead to cognitive deficits (Baar et al., 2009). SGA in humans is associated with impaired neurodevelopmental outcomes and poorer school performance, learning difficulties and attentional problems during adolescence (Larroque et al., 2001; O'Keeffe et al., 2003; Arcangeli et al., 2012). Moreover, SGA is linked to an overall volume reduction of the brain, a decrease in white matter in both the cerebrum and cerebellum, and a small reduction of cerebellar grey matter (Martinussen et al., 2009). These cognitive deficits and behavioral problems later in life associated with SGA or LBW at term make it a pressing issue for further research. Although long-term effects of LBW have been studied in human LBW babies and children, a suited model animal is needed to study the long-term effects of LBW on cognitive development in a more controlled manner.

In the pig industry, litter size is increasing as a result of selective breeding. For example, in Denmark the average litter size increased from 11.9 piglets born alive in 2000 to 14.8 piglets in 2011 (Kondrup, 2013). The decline in birth weight of the piglets in a litter is around 40 g per additional piglet (Quiniou et al., 2002; Beaulieu et al., 2010). As a consequence of the larger litter sizes, the incidence of LBW piglets is increasing. Pigs can thus serve as an attractive animal model to study the effects of LBW on cognition and development. Additionally, they have relatively large brains and are physiologically – especially

in early development – more similar to humans than for example rodents, which are more commonly used as animal models for translational research (van der Staay, 2006). Moreover, pigs are highly social and intelligent animals and can be trained to perform complex cognitive tasks (Mendl et al., 2010). Recently, Gieling et al. (2012) studied the effects of LBW in piglets on long-term cognition, and found an indication that LBW might negatively affect cognitive development. LBW piglets had a retarded working memory performance compared to their NBW siblings at the start of the first reversal phase in a spatial cognitive holeboard task. However, these effects disappeared with further training, and no difference was found in the preceding acquisition phase. Furthermore, visual inspection of Figure 1a in Gieling's study suggests that reference memory scores for LBW animals were higher than the scores of their NBW siblings in both the acquisition and the first reversal phase. This impression, however, was not confirmed statistically. The same LBW and NBW animals were then used in a conditional discrimination task by Murphy et al. (2013). In that study, of the pigs that learned the task, the LBW animals learned the task faster than the NBW animals.

As the results from these studies are inconclusive with respect to the long-term cognitive effects of LBW in piglets, we repeated the study by Gieling et al. (2012) with stricter criteria to define LBW and tested the piglets at a younger age. Whereas Gieling et al. defined LBW as 1 SD below the average weight of a litter, we used piglet birth weight data of previous experiments to determine an upper weight limit of LBW as 1 SD below the average birth weight of nearly 500 piglets, resulting in an upper weight limit of 1050 g which we defined as a very low birth weight (vLBW). In the current study, we looked at learning and memory measures in vLBW and normal birth weight (NBW) piglets to investigate the effects of vLBW on long-term cognitive functioning. To this end, we used the spatial cognitive holeboard for pigs (Arts et al., 2009; Gieling et al., 2012). This is a free choice maze in which the animal is free to walk around and visit or revisit any site in the arena, in order to find multiple hidden rewards. By keeping track of which sites are visited and revisited by the animal, working and reference memory can be assessed (van der Staay et al., 1990), which are forms of short- and long-term memory, respectively (Dudchenko, 2004; Olton and Samuelson, 1976).

In addition, at the end of the experiment and after euthanasia, flank hair

samples from all animals were collected to determine hair cortisol concentration, which is increasingly used as a long-term biomarker for exposure to stress. Whereas cortisol concentrations in serum, saliva or urine samples are single time-point measurements which are strongly influenced by daily fluctuations, cortisol concentration in hair provides a measure for long-term or chronic stress over a prolonged time period (Russell et al., 2012). We expected that vLBW piglets would show impaired memory scores in the holeboard task compared to their NBW siblings, i.e. that they would reach lower memory scores and show longer trial durations. Additionally, we expected that vLBW animals would have higher hair cortisol concentrations as they face more (environmental and physical) challenges in early survival due to their developmental lag (e.g. compromised thermoregulation: Herpin et al., 2002).

MATERIALS AND METHODS

Ethics note

This study was reviewed and approved by the local ethics committee (DEC, DierExperimenten Commissie) and was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid suffering.

Subjects

Pigs ((Terra × Finnish landrace) × Duroc) born at the commercial pig breeding farm of the University Utrecht were selected. Twenty animals (ten pairs of NBW and vLBW siblings, each pair from a different litter: four pairs of female piglets, six pairs of male piglets) were selected based on their body weight measured on the day of birth. Selection occurred in two runs with one week in between to ensure that enough vLBW animals could be selected for the study. The vLBW animals were selected based on two criteria: a minimum of 1 SD below the average birth weight of the study population (based on the birth weights of 484 piglets; this yielded at cut-off of < 1050 g) and from a litter containing a minimum of 10 piglets. The NBW piglets were selected based on the average birth weight of the litter with the same sex as the selected vLBW piglet. Of all piglets, head size (snout to the back of the cranium) and total body length (snout to tail base) was measured on the day of birth to check for

asymmetrical growth as an additional measure for intra-uterine growth retardation (Amdi et al., 2013). In order to increase survival of the vLBW piglets during the first days, close monitoring and hand feeding of sow milk (once per day) were applied. NBW siblings were handled for the same amount of time. One male piglet of the vLBW group was euthanized due to lasting illness during the habituation period, thus the experiment was conducted with 19 piglets in total.

Housing

Selected piglets were transported to our research facility at age 4-7 days and were housed by birth weight class and age in groups of five animals each in four adjacent pens (1.25 × 2.50 m until 10 weeks of age, then until 12 weeks of age 2.50 × 2.50 m) containing sawdust bedding, straw and toys. Temperature was gradually decreased from 26 °C in the first weeks to 21 °C at the end of the study. During the first week, a heat lamp was suspended 1 m above each pen. A 12/12 hour light/dark cycle was applied with lights on at 7 a.m. A radio played continuously; slightly louder at daytime (7 a.m. to 4 p.m.) than at night. Water was provided ad libitum. Pigs were fed milk replacer (Milkiwean Yoghurt, Trouw Nutrition, Nutreco Global, The Netherlands) for the first four weeks. In addition to the milk replacer, commercial piglet feed was provided. Piglets were gradually weaned between three and four weeks of age, after which they were fed a balanced commercial pig feed.

Apparatus

The holeboard apparatus (Ossendrijver BV, Achterveld, The Netherlands) consisted of a 360 × 360 cm square arena with a 4x4 matrix of food bowls, surrounded by a small corridor (40 cm) with a slatted black synthetic floor (for details, see Figure 2 in Chapter 1 of this thesis). The synthetic walls were 80 cm high and had a steel bar on top (total height: 1 m). The apparatus was elevated 25 cm off the floor. The arena could be entered through four different guillotine doors, one on each side, which were operated from the outside using a string and pulley system. Pigs entered the holeboard through the main entrance and always turned left into the corridor until they found an open door, through which they entered the testing arena. Piglets inside the holeboard arena were able to see three surrounding walls of the stable and the pitched roof

containing one fluorescent tube, as well as the experimenter standing in the corridor directly right of the main entrance door. The experimenter avoided eye contact with the piglets during trials. Auditory extra-maze cues were the radio that was playing next to the experimenter and the waiting area where pen mates were housed during testing, which the piglet in the arena could hear. The waiting area was located in front and slightly left of the holeboard apparatus.

The food bowls were covered by red plastic balls which could be lifted by the piglets with their snout (JollyBall Dog Toy, ø 24 cm, 400 g), to prevent the piglets from finding the rewards based on vision. To ensure that the rewards were not found by smell, every food bowl contained three rewards (replaced daily) in a false bottom. The apparatus was cleaned at the end of each testing day and after a trial if an animal had defecated during a trial. Hole visits were automatically recorded using custom made software (Blinq Systems, Delft, The Netherlands). A visit was scored when a pig lifted the ball and the connection between the magnet in the ball and the sensor in the food bowl was broken. This signal was registered by an interface (LabJack) and sent to a PC. If the same ball was lifted again within 10 s and no other hole was visited in between, this was not counted as a revisit. A trial started when a pig entered the arena with both front legs and ended when a piglet found all four rewards or when the maximum time of 450 s was reached (whichever event occurred first), after which the piglet was allowed to leave the arena through the door closest to the main entrance door.

Training and testing

During the first three weeks after arrival into the new pens, all piglets were gradually habituated to the two experimenters, the hallway leading to the holeboard and the holeboard itself, in sessions of 10-30 minutes per day. Training occurred with mini marshmallows as reward, as these were easy to consume for the young animals. In the testing phase, M&M's® chocolates were used as reward. Holeboard testing started when all piglets had learned to lift balls with their snout in order to find rewards and were comfortable to enter the arena alone, which was at approximately seven weeks of age. Before testing, six habituation trials (two trials per day, three days in total) were conducted in which all 16 holes were baited with a food reward. Then, each animal was assigned

its own rewarded configuration, in which 4 of the 16 holes were baited. In total, four different configurations were used, in such a way that every hole was baited equally often. All piglets received two trials in close succession per day on the first four testing days (total: eight trials), after which they were tested in four massed trials per day. The entrance door was randomly assigned per trial by the software. After a predetermined learning criterion was reached (average reference memory score > 0.6 over the last four trials), which was after at least 46 acquisition trials and at most 54 trials, the animals moved to the reversal configuration. The reversal configuration was the 180° rotated pattern of the configuration used during the acquisition phase (A to C, B to D, C to A, B to D). All piglets received 20 reversal trials, thus in total all pigs received at least 74 trials. At the end of the experiment (at 12 weeks of age), all animals were euthanized by an intracardial injection with an overdose of pentobarbital (Euthasol®, AST Farma B.V. Oudewater, The Netherlands), after which brains were dissected and weighed.

Hair samples

At the end of the experiment and after euthanasia, hair samples (0.5 - 1 g) were taken with a trimmer from the left flank of each animal. Of each hair sample, 250 mg was washed, dried and ground with a bead beater for 30 minutes in steel micro vials containing three 1 mm steal beads. Thereafter, 50 mg of each powdered sample was collected in a micro-centrifuge tube. 1 ml methanol was added after which the samples were incubated at room temperature for 24 h with slow rotation to extract steroids. Of the extract, 0.6 ml was placed in a new tube and dried at 45 °C in a heating block overnight. The dried extracts were dissolved in 0.4 ml phosphate buffer. Cortisol concentrations were then determined in duplo using a Salivary Cortisol ELISA kit (Salimetrics LLC, PA, USA). As one vLBW animal was euthanized at 4 weeks of age due to lasting illness, the hair of another vLBW animal was unusable and the ground hair sample of one NBW animal was lost accidentally, cortisol concentrations in hair samples of 8 vLBW and 9 NBW pigs were determined.

Statistics

From the holeboard data the following measures were calculated after either all rewards were found or the maximum time of 450 s had elapsed, whichever

event occured first (van der Staay et al., 2012): (1) Reference memory (RM), a ratio that is defined by the number of visits and re-visits to the rewarded set of holes divided by the number of visits and re-visits to all holes; (2) Working memory (WM), a ratio defined by the number of visits that yield a food reward divided by the number of visits and re-visits to the rewarded set of holes; (3) Trial duration (TD), the time between entering the holeboard and finding all four rewards (when not all rewards were found the maximum trial duration of 450 s was recorded); (4) Inter-visit interval (IVI), the average time between two hole visits; (5) Latency to first visit (LFV) and latency to the first rewarded (LFR) visit; (6) Total visits (TV), unrewarded visits (URV) and rewarded visits (RV); and (7) Number of visits until 1st (Vfirst), 2nd (Vsecond), 3rd (Vthird) and 4th (Vfourth) reward found (Gieling, 2013). These variables include measures for both memory performance (RM, WM) and for motivation or speed (TD, IVI).

The six habituation trials preceding holeboard testing were analyzed in trial blocks using the mean of two trials, thus a total of three blocks (one trial block per day). Trial blocks of the actual holeboard testing were calculated using the mean of four trials, except for the first block, which was the mean of six trials. These data were otherwise analyzed in the same manner as the habituation trials. Of all animals, the first 46 acquisition trials thus divided into 11 trial blocks (block 1-11) were analyzed, yet not the extra acquisition trials that a piglet received when it had not yet reached the criterion of RM > 0.6. Of the reversal trials, all 20 trials were analyzed in blocks of 4 trials, thus divided into 5 trial blocks (block 12-16). The holeboard data analyses were performed for three different phases: acquisition, transition and reversal. The transition phase is the switch from the acquisition phase to the reversal phase, i.e. the last trial block of the acquisition compared to the first trial block of the reversal (block 11 to 12). This is a measure of the response flexibility of an animal: a large difference means that the animal faced difficulties to adapt to the new situation.

All data were analyzed using a mixed model to account for clustering of piglets within litters and repeated measurements within piglets. Fixed effects for holeboard data analysis were Birth weight (vLBW or NBW), Trial block and Birth weight by Trial block. For weekly weights, fixed effects were Birth weight, Week and Birth weight by Week. In all analyses, a random effect for litter was added, and the correlation of repeated measures within piglets was addressed using a different compound symmetry structure for the residuals per combination of lit-

ter and treatment (PROC MIXED, SAS Institute, Cary, NC, U.S.A.).

RESULTS

Cognitive holeboard performance

Table 1 shows the results of statistical analyses for all measures. As RM and WM are the most important measures of memory performance and TD and IVI the most important motivational measures, these four variables will be discussed in more detail.

Habituation trials

In the habituation trials (all holes baited, 6 trials preceding the testing phase) there was no difference in performance between the birth weight groups for Total number of hole visits ($F_{1,16}$ = 0.13; p = 0.7257) or Total rewards found ($F_{1,16}$ = 0.02; p = 0.8958). Within all subjects, there was no difference in Total number of hole visits ($F_{2,32}$ = 1.17; p = 0.1972), whereas there was an increase in Total rewards found over the three habituation days (Trial blocks effect: $F_{2,32}$ = 6.15; p = 0.0055). No interaction effects between Birth weight and Trial blocks were found for Total number of hole visits ($F_{2,32}$ = 0.27; p = 0.7642) or Total rewards found ($F_{2,32}$ = 1.21; p = 0.3129).

Working memory

Working memory (WM) performance increased for both birth weight groups in all three phases (acquisition, transition and reversal phase; Trial blocks effect; p = < 0.0001). WM scores did not differ between the two groups in the acquisition ($F_{1,16}$ = 0.19; p = 0.6675) nor in the reversal phase ($F_{1,16}$ = 0.49; p = 0.4956). In the transition phase, the NBW group fell back more in working memory scores than the vLBW group when starting the reversal phase ($F_{1,16}$ = 20.12; p = 0.0004). There was an interaction effect of Birth weight and Trial blocks on working memory performance in the acquisition phase ($F_{10,160}$ = 2.32; p = 0.0142). However, further analysis of the data revealed that this interaction was due to a difference in performance between the birth weight groups in trials 15-18 ($F_{1,262}$ = 3.83; p = 0.0515) and trials 39-42 ($F_{1,262}$ = 7.25; p = 0.0076), though not to an overall difference in performance between

NBW and vLBW piglets (Figure 1A). Similarly, the interaction effect between Birth weight and Trial blocks on working memory in the reversal phase ($F_{4,64}$ = 2.90; p = 0.0287) was found to be due to the lower performance of the NBW group in the first reversal block (trials 47-50; $F_{1,262}$ = 10.75; p = 0.0012), thus to the larger drop in working memory performance in the transition phase.

Reference memory

The vLBW piglets had higher reference memory scores than the NBW group (Figure 1A; Table 1) in the acquisition ($F_{1,16}$ = 11.58; p = 0.0036) as well as in the reversal phase ($F_{1,16}$ = 9.34; p = 0.0075). Reference memory scores also showed that the vLBW group learned faster than the NBW group (Birth weight by Trial blocks interaction) in both the acquisition ($F_{10,160}$ = 4.84; p = <0.0001) and the reversal phase ($F_{4,64}$ = 5.66; p = 0.0006). Both groups fell back in reference memory scores to chance performance (RM score = 0.25) in the transition from acquisition to reversal (Figure 1A), which resulted in a larger drop in reference memory scores of vLBW piglets as compared to NBW piglets ($F_{1,16}$ = 4.90; p = 0.0418). The NBW group made more total (TV) and unrewarded visits (URV) than the vLBW group in all phases, which is in line with the finding that reference memory scores were lower in the NBW group for all three phases (Table 1).

Trial duration and inter-visit interval

In both groups, the trial duration decreased over time in both the acquisition and reversal phase (Figure 1B). The average trial duration did not differ between the groups in any phase (Table 1: Birth weight effect). However, the vLBW group initially needed more time in the acquisition phase to complete a trial than the NBW group, therefore trial durations of vLBW pigs decreased more over time than in the NBW group ($F_{10,160}$ = 2.10; p = 0.0271). Trial durations in the transition from the acquisition to the reversal phase showed a trend that NBW piglets needed more time to complete the task than vLBW pigs starting the reversal phase (Figure 1B; $F_{1,16}$ = 4.48; p = 0.0503). The inter-visit interval (IVI) is a measure of how fast the animal searches for rewards, and may thus provide an indication of how motivated the animal is to complete the task. IVI did not differ between groups (Figure 1B; Table 1).

Growth

The NBW piglets had on average a higher birth weight than the vLBW piglets (Figure 2A; $t18 = 8.44$; $p < 0.0001$). Over the course of the experiment, the weights of the NBW group remained higher than those of the vLBW group (Figure 2B; $F_{1,17} = 4.65$; $p = 0.046$). Moreover, the vLBW piglets had a slower growth rate than the NBW piglets ($F_{11,187} = 3.50$; $p = 0.048$). The head size relative to the full body length on the day of birth did not differ between the groups ($t16 = -0.247$; $p = 0.808$).

Brain weights

The relative brain weight was calculated by dividing the brain weight by the total body weight at the end of the experiment (age 12 weeks). There was a strong trend that the NBW group had higher absolute brain weights than the vLBW group ($t8 = 2.30$; $p = 0.051$). The relative brain weights did not differ between the groups ($t8 = -1.18$; $p = 0.274$).

Hair cortisol concentrations

Figure 3 shows cortisol concentrations in flank hair at 12 weeks of age for the vLBW and NBW groups. Cortisol concentration in hair of the vLBW pigs was significantly lower than in hair of NBW pigs ($F_{1,8} = 23.61$; $p = 0.0013$).
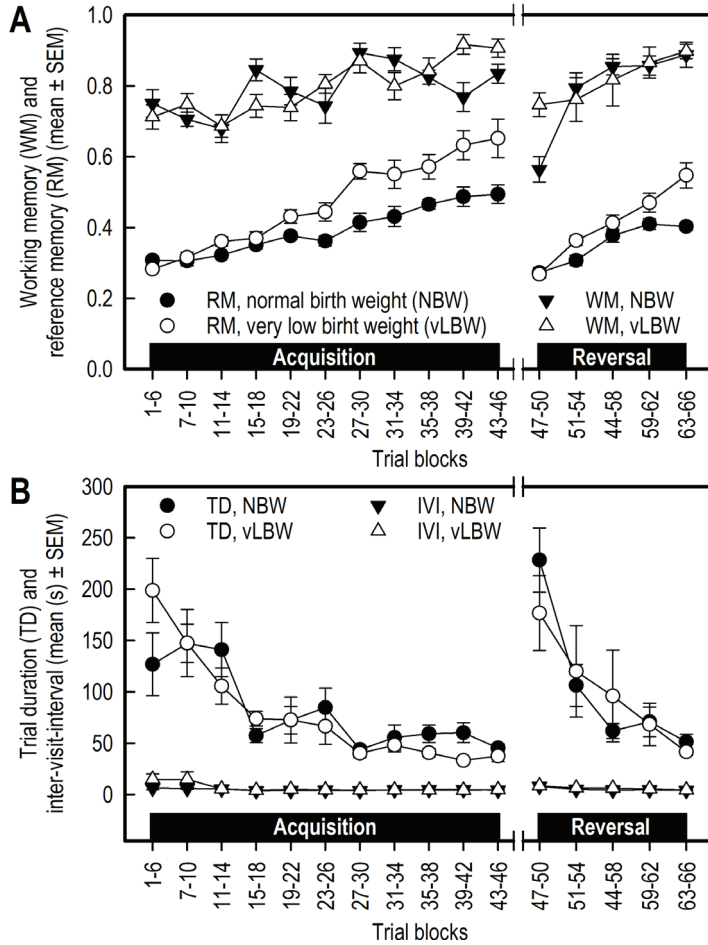
Figure 1. (A) Working and reference memory performance and (B) Trial duration and inter-visit interval of NBW (n=10) and vLBW (n=9) piglets in the spatial cognitive holeboard task during the acquisition phase (trials 1-46) and the reversal phase (trials 47-66).

		Between subjects			Within subjects			Interaction		
		Birth weight			Trial blocks			Birth weight X Trial blocks		
Measure	Phase	F	df	P≤	F	Df	P≤	F	df	P≤
Habituation										
TV	Hab	0.13	1,16	0.7257	1.17	2,32	0.1972	0.27	2, 32	0.7642
REW	Hab	0.02	1,16	0.8958	6.15	2,32	**0.0055**	1.21	2, 32	0.3129
Acquisition (Acq), transition (Trans), reversal (Rev)										
WM	Acq	0.19	1, 16	0.6675	7.32	10, 160	**<0.0001**	2.32	10, 160	**0.0142**
	Trans	20.12	1, 16	**0.0004**	42.07	1, 16	**<0.0001**	2.81	1, 16	0.1130
	Rev	0.49	1, 16	0.4956	12.38	4, 64	**<0.0001**	2.90	4, 64	**0.0287**
RM	Acq	11.58	1, 16	**0.0036**	49.33	10, 160	**<0.0001**	4.84	10, 160	**<0.0001**
	Trans	4.90	1, 16	0.0418	115.85	1, 16	**<0.0001**	8.27	1, 16	**0.0110**
	Rev	9.34	1, 16	**0.0075**	51.44	4, 64	**<0.0001**	5.66	4, 64	**0.0006**
TD	Acq	0.79	1, 16	0.3870	22.43	10, 160	**<0.0001**	2.10	10, 160	**0.0271**
	Trans	4.48	1, 16	0.0503	109.14	1, 16	**<0.0001**	0.03	1, 16	0.8650
	Rev	0.43	1, 16	0.5214	28.14	4, 64	**<0.0001**	0.36	4, 64	0.8341
LFV	Acq	1.01	1, 16	0.3302	2.48	10, 160	**0.0085**	1.01	10, 160	0.4351
	Trans	0.18	1, 16	0.6781	0.01	1, 16	0.9172	0.00	1, 16	0.9595
	Rev	1.27	1, 16	0.2764	0.97	4, 64	0.4289	0.68	4, 64	0.6080
LFR	Acq	0.00	1, 16	0.9741	12.20	10, 160	**<0.0001**	1.81	10, 160	0.0635
	Trans	1.50	1, 16	0.2390	36.44	1, 16	**<0.0001**	0.82	1, 16	0.3796
	Rev	0.05	1, 16	0.8313	11.10	4, 64	**<0.0001**	1.97	4, 64	0.1104
IVI	Acq	0.85	1, 16	0.3704	7.20	10, 160	**<0.0001**	1.69	10, 160	0.0876
	Trans	0.02	1, 16	0.8958	8.23	1, 16	**0.0111**	0.53	1, 16	0.4760
	Rev	0.06	1, 16	0.8109	6.23	4, 64	**0.0003**	0.44	4, 64	0.7827
TV	Acq	16.25	1, 16	**0.0010**	28.86	10, 160	**<0.0001**	1.94	10, 160	**0.0428**
	Trans	9.00	1, 16	**0.0085**	113.95	1, 16	**<0.0001**	1.48	1, 16	0.2416
	Rev	9.60	1, 16	**0.0069**	40.31	4, 64	**<0.0001**	1.54	4, 64	0.2008
URV	Acq	15.32	1, 16	**0.0012**	38.42	10, 160	**<0.0001**	2.00	10, 160	**0.0366**
	Trans	7.25	1, 16	**0.0160**	133.53	1, 16	**<0.0001**	0.86	1, 16	0.3668
	Rev	9.73	1, 16	**0.0066**	51.51	4, 64	**<0.0001**	1.02	4, 64	0.4014
RV	Acq	2.25	1, 16	0.1527	7.04	10, 160	**<0.0001**	2.07	10, 160	**0.0299**
	Trans	14.05	1, 16	**0.0018**	16.30	1, 16	**0.0010**	3.70	1, 16	0.0723
	Rev	1.21	1, 16	0.2875	6.07	4, 64	**0.0003**	2.96	4, 64	**0.0263**
REW	Acq	2.00	1, 16	0.1760	4.13	10, 160	**<0.0001**	1.81	10, 160	0.0626
	Trans	0.11	1, 16	0.7417	6.61	1, 16	**0.0205**	0.11	1, 16	0.7417
	Rev	0.08	1, 16	0.7831	5.65	4, 64	**0.0006**	0.60	4, 64	0.6654
Vfirst*	Acq	2.23	1, 16	0.1549	5.79	10, 160	**<0.0001**	1.08	10, 160	0.3788
	Trans	0.02	1, 16	0.8938	32.91	1, 16	**<0.0001**	0.55	1, 16	0.4674
	Rev	3.53	1, 16	0.0786	6.85	4, 64	**0.0001**	1.40	4, 64	0.2456
Vsecond*	Acq	5.79	1, 16	**0.0285**	15.94	10, 160	**<0.0001**	2.47	10, 160	**0.0088**
	Trans	0.12	1, 16	0.7340	59.46	1, 16	**<0.0001**	0.39	1, 16	0.5400
	Rev	1.63	1, 16	0.2194	27.05	4, 64	**<0.0001**	0.74	4, 64	0.5650
Vthird*	Acq	16.18	1, 16	**0.0010**	27.72	10, 160	**<0.0001**	1.88	10, 160	0.0522
	Trans	2.28	1, 16	0.1508	87.78	1, 16	**<0.0001**	0.03	1, 16	0.8735
	Rev	12.79	1, 16	**0.0025**	29.42	4, 64	**<0.0001**	0.44	4, 64	0.7773
Vfourth*	Acq	10.49	1, 16	**0.0051**	29.94	10, 160	**<0.0001**	1.80	10, 160	0.0647
	Trans	3.17	1, 15	0.0950	80.47	1, 15	**<0.0001**	0.08	1, 15	0.7811
	Rev	4.87	1, 15	**0.0433**	27.49	4, 60	**<0.0001**	0.27	4, 60	0.8946

WM = working memory, RM = reference memory, TD = trial duration, LFV = latency first visit, LFR = latency first reward, IVI = inter-visit interval, TV= total visits, URV = unrewarded visits, RV = rewarded visits, REW = number of rewards found, Vfirst, Vsecond, Vthird, Vfourth = number of visits before the first, second, third, fourth reward was found.

*: For further information about the operational definitions of these variables, see Gieling, 2013, pp. 173–176

Table 1. Performance of vLBW and NBW piglets in the spatial cognitive holeboard task during the habituation phase, and during the acquisition, transition, and reversal phase.
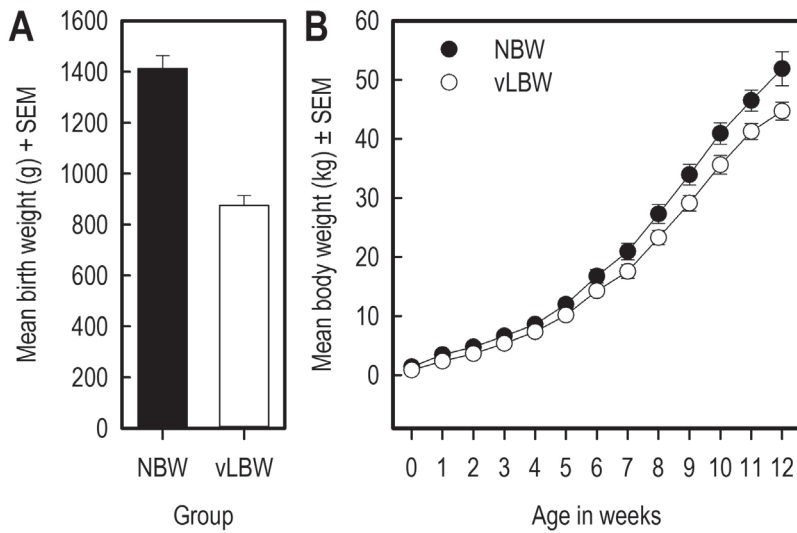
Figure 2. Weights and growth of the piglets. (A) The birth weights of the vLBW and NBW piglets in grams. (B) The body weights of the piglets in kilograms over the course of the experiment.
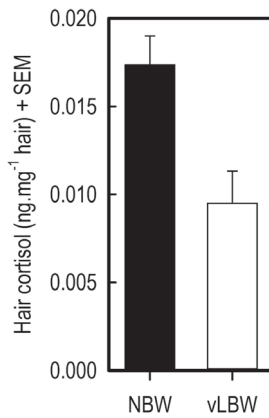

Figure 3. Hair cortisol concentrations in flank hair of 12-week-old NBW (n=9) and vLBW (n=8) piglets.

DISCUSSION

The aim of this study was to investigate long-term cognitive performance of very low birth weight piglets compared to their normal birth weight siblings. As human studies show that LBW children born at term show (neuro)developmental impairments later in life (e.g. O'Keeffe et al., 2003), we expected to find deficits in learning and memory performance in vLBW piglets.

Our results confirm previous findings that young piglets are able to acquire the holeboard task. Memory scores improved and latencies declined over the course of the experiment for all piglets. Although we attempted to test the piglets in our study at a younger age than previous studies using the holeboard task for pigs (Arts et al., 2009 (9 weeks); Haagensen et al., 2013 (6-7 weeks, minipigs); Gieling et al., 2014 (7-8 weeks)), the piglets were not able to perform the task until they were approximately 7 weeks of age. It can therefore be assumed that a certain level of physical and mental development needs to be achieved in order to perform the holeboard task, which the piglets reach at about 7 weeks of age. Moreover, it appears that the animals are not comfortable to be alone in the holeboard arena before that age.

The body weights of the vLBW group remained lower than those of the NBW group throughout the experiment. Thus, vLBW piglets did not show compensatory growth, which is in line with previous studies that have looked at the effects of birth weight on growth performance in pigs (Gondret et al., 2005; Rehfeldt and Kuhn, 2006).

Improved cognitive performance

Performance in the holeboard task was opposite to what we expected and differs from findings in human studies. In the current study, vLBW piglets showed a faster acquisition and reached a higher performance level in the holeboard task than the NBW animals. Previous studies conducted by Gieling et al. (2012) and Murphy et al. (2013) on the cognitive effects of LBW in pigs used less strict criteria when selecting LBW pigs, and their results on the effects of LBW on long-term cognition were inconclusive. In the present study, stricter selection criteria for LBW animals were applied – the most prominent being a lower birth weight limit as criterion for vLBW – which may explain

why results were supported statistically in the present study, whereas they were not in previous studies on cognitive effects of LBW in pigs. The average weight difference between the LBW and NBW pigs was 549 g in the study of Gieling et al., whereas it was 539 g in our study. However, when comparing the birth weights of all piglets of that study to the current study, there is a 631 g difference in average birth weight between piglets used. This results in lower absolute birth weights of the piglets selected for the current study and thus more extreme low birth weights in our vLBW group than in the LBW group of Gieling et al., which may explain the differences in our findings.

Although we have fed the vLBW additional sow milk by hand feeding them in the first days after birth, we do not expect our findings on cognitive performance to be due to this difference. Hand feeding these vLBW animals was often unsuccessful as the animals struggled and did not ingest much of the milk they were offered, which was once a day in the first 4 days. We furthermore expect that NBW animals in general ingest more sow milk due to their stronger chances in teat competition. All animals were removed from the sow after 4 to 6 days, after which all animals received the same amounts of milk replacer. Thus, we do not expect that the small amount of additional feeding to the vLBW animals had any effects on cognitive results in the holeboard task. LBW pigs may have developed mechanisms to compete with their larger siblings in order to increase their low survival chances. It is possible that growth restriction due to (mild) intra-uterine hypoxia or ischemia in vLBW pigs causes a process called brain preconditioning to occur, in which pre- or early postnatal sublethal stressors induce protection against other future stressors or injuries (for a review, see Stetler et al., 2014). Although this is mere speculation, the results of the current study do indicate that the occurrence of LBW in pigs probably involves or triggers other mechanisms than LBW in humans, as our results are opposite to findings in human LBW or SGA infants. Such compensatory mechanisms as brain preconditioning resulting from intra-uterine stress could make the animal able to cope with stress and competition better , which would be advantageous for vLBW pigs competing for resources in large litters. Humans do not have this early postnatal competition, as they are usually born with only one or two babies at a time. Thus, the difference in effects of LBW on long-term cognition between humans and pigs may be due to the difference in early-life competition for resources.

Another possible explanation for the fact that LBW animals performed better may be that they are more strongly motivated to obtain a food reward than the NBW animals. Measures in the holeboard task that can provide an estimation of the motivation of the animals are the latency of the first visit, inter-visit interval, and the number of rewards found (van der Staay et al., 2012). The birth weight groups did, however, not differ for these measures. It may still be interesting to further investigate motivation for food rewards in LBW and NBW pigs in future studies, using more specific tests designed to measure motivation.

Previous studies defined LBW in piglets as 2 SD (Cooper, 1975) or 2.5 SD (Gondret et al., 2005) below the average weight, whereas we used 1 SD below the average weight of a large study population. These stricter criteria, however, do require more intensive postnatal care of the piglets as survival chances are low in animals with a vLBW. Reduced vigour and thermoregulation are amongst the main causes of neonatal mortality in vLBW piglets (Tuchscherer et al., 2000; Herpin et al., 2002). Thus, extra care is needed in order to increase survival chances of vLBW piglets. In order to provide the required extra care and allow close monitoring of vLBW piglets, intensive care units similar to those used for human neonates have been developed (Lennon et al., 2011). In the set-up of the current study, the available infrastructure could not provide this extra care. It may be useful to use stricter criteria for LBW animals in future studies and raise the pigs under intensive care conditions.

Growth retardation and (a)symmetry

The total body length and head size measured on the day of birth were used to check for asymmetrical growth as an additional measure for intra-uterine growth retardation (IUGR). Asymmetrical IUGR is the most common form of IUGR in humans (70%) and is a sign of head or brain sparing in the third trimester of pregnancy, whereas symmetrical IUGR is thought to find its onset much earlier in the course of pregnancy (Lin et al., 1991). Severe IUGR piglets have been shown to have larger relative brain weights than mild IUGR piglets, and these in turn have larger relative brain weights than normal birth weight piglets (Amdi et al., 2013). In the current study, a strong trend was found that the NBW piglets had larger absolute brain weights than the vLBW piglets. This is a consequence of the larger total size of the NBW piglets. How-

ever, the relative size of the head did not differ between the vLBW and NBW groups on the day of birth, ruling out that asymmetrical growth has occurred in the vLBW animals. This is an indication that growth retardation in our vLBW piglets had an early onset in the course of pregnancy and brain sparing did not occur in these animals, i.e. that the present vLBW piglets are not modelling IUGR. In a study investigating indicators of neonatal survival in piglets, birth weight was shown to be a critical factor with respect to mortality in live-born piglets (Baxter et al., 2008). However, regarding mortality in still-born piglets, shape and size of the piglets (as measured by ponderal index and body mass index) appeared to be better indicators for survival. Piglets showing asymmetrical IUGR thus have a high prevalence of prenatal mortality. This might explain why we did not find any asymmetrical growth in the vLBW piglets that were available for selection in our study.

Hair cortisol concentrations

Significantly lower hair cortisol concentrations (HCC) in flank hair of vLBW than NBW piglets at 12 weeks of age were found. This implies that the vLBW animals experienced less stress over the course of their lives. For example, in humans, traumatized patients with PTSD had higher HCC than controls without PTSD symptoms, and in both groups the number of traumatic life events positively correlated with HCC (Steudte et al., 2011). In dogs, salivary cortisol concentrations measured in their home environment positively correlated with HCC (Bennett and Hayssen, 2010). Similarly, HCC in rhesus macaques correlated with saliva samples taken from animals that were trained for saliva collection (Davenport et al., 2006). These studies show that hair is a reliable medium for measuring long-term basal cortisol concentrations.

The difference in HCC between vLBW and NBW pigs may be due to the difference in performance between the two groups, as making more mistakes in the task and thus performing worse may have caused slightly more distress in the NBW animals over time than in the vLBW animals. As the animals were tested in the holeboard task from age 7 to 12 weeks, after which the hair was collected, stress levels are likely to be at least partially influenced by the effects of holeboard testing. Another plausible explanation is that the NBW animals experienced more stress due to less space per pig they had available in their home pen. Home pens measured the same for all groups of five animals (1.25

× 2.50 m until 10 weeks of age, then until 12 weeks of age 2.50 × 2.50 m); while the NBW animals were significantly heavier – and thus larger – than the vLBW pigs during the entire course of the experiment. Moreover, one vLBW animal was euthanized at 4 weeks of age, thus one vLBW group was housed with four pigs in their home pen. This reduced space per pig in the home pen for NBW animals may have caused elevated stress levels in the NBW pigs as compared to the vLBW animals. In future studies comparing stress levels in pigs, housing and space per pig should thus be taken into account.

An alternative explanation is that vLBW pigs are somehow less affected by stressors than NBW pigs. Human small for gestational age (SGA) infants show a blunted stress response to a pain stimulus, which is thought to be due to intrauterine-induced alteration of the hypothalamus-pituitary-adrenal axis (Schäffer et al., 2009). This phenomenon may also occur in vLBW piglets and explain the lower HCC levels in the hair of vLBW piglets found in the current study. However, further research on stress and stress responses in vLBW pigs is needed to test this hypothesis.

CONCLUSION

In conclusion, our results do not corroborate findings in humans, suggesting that other mechanisms may be underlying the occurrence of (v)LBW in humans than in pigs. These results may therefore not provide information that can be used in the investigation of vLBW occurrence and its effects in humans. They raise, however, a whole new set of questions about which mechanisms may be causing and influencing vLBW and its effects in pigs. Since we found an increased cognitive performance and reduced stress levels in vLBW as compared to NBW animals, it can be speculated that the conditions that lead to vLBW in pigs may trigger beneficial compensatory mechanisms, which may either arise pre- or early postnatally. As piglets face strong early postnatal competition, especially in large litters where vLBW occurs the most, such compensatory mechanisms can improve survival chances in vLBW animals. Looking further into these mechanisms is necessary in order to elucidate why effects of LBW are different between pigs and humans, which in turn might generate knowledge that can benefit both piglet welfare and improve animal husbandry practices.

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Chapter 3

Testing post-weaning food motivation in low and normal birth weight pigs in a runway and operant conditioning task

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ABSTRACT

Low birth weight (LBW) pigs face more welfare challenges than their normal birth weight (NBW) siblings. Understanding the underlying mechanisms of cognitive and learning abilities in these pigs may help to improve their welfare. Early competition in life over resources combined with the higher need for nutrient intake make it likely that LBW pigs have a higher motivation for food than NBW pigs. This study aimed to compare the motivation to obtain food rewards between LBW and NBW pigs, using variable numbers of rewards in two separate tasks; a runway and an operant conditioning task (the nose wheel task). Ten pairs of littermates were used. From each litter, one LBW piglet (mean birth weight ± SEM: 854 ± 33 g) and one NBW piglet (1332 ± 53 g) was selected. Pigs were tested in the runway task at 12 weeks of age and the nose wheel task at 19 weeks of age. Both tasks consisted of a baseline phase (two rewards), a high reward phase (eight rewards) and an extinction phase (no rewards). Statistical analyses using mixed models showed that NBW animals left the start box faster than LBW animals in the high reward phase in the runway task. However, their run time in this phase was not shorter and no other birth weight effects were found in any other phase or measure in this task nor in the nose wheel task. All animals decreased their run time in the runway task between the baseline phase and high reward phase, and increased their run time in the extinction phase. Likewise, in the nose wheel task, all animals reached a higher number of total rewards gained and spent a lower percentage of time away from the feeder in the high reward phase compared to the baseline phase. Additionally, they showed a decrease in motivation during the extinction phase. Our results indicate that there is no difference in motivation to obtain food rewards between LBW and NBW pigs. However, both the results of the runway and the nose wheel task show a phase effect between the baseline, high reward and extinction phase. This is in accordance with the underlying theory that animals have a higher motivation for resources that are more desired. Therefore, we show that both tasks are sensitive enough to measure motivation for food rewards in pigs, and are consequently useful to study factors influencing pig motivation.

Keywords: motivation, pigs, birth weight, runway, operant conditioning

INTRODUCTION

In current pig husbandry, there is a strong selection for high sow fecundity. This has resulted in an average litter size increase of 0.25 piglets per year (Hazeleger et al., 2007). Consequently, there is an increased birth weight variation within litters (Rutherford et al., 2013). The mean birth weight of a litter decreases with approximately 35 gram for each piglet additionally born in a litter (Quiniou et al., 2002). This causes an increased incidence of piglets that are born with a low birth weight (LBW).

LBW in piglets has several negative consequences for their survival and welfare. Pigs experience both pre- and postnatal competition for resources. Colostrum intake as soon as possible after birth is essential for piglet survival, which is difficult for LBW piglets as they need to compete with their larger siblings for teats. Teat competition is accompanied by aggression, and usually the piglets with higher birth weights win these fights (Scheel et al., 1977). LBW piglets are slower to respond to their environment than normal birth weight (NBW) piglets, causing increased early mortality risks due to crushing by the sow (Baxter et al., 2008). Additionally, LBW piglets have lower body reserves and therefore have a higher risk to suffer from hypothermia and starvation (Herpin et al., 2002). Moreover, weight gain is reduced lifelong in LBW piglets (Douglas et al., 2013).

Understanding the underlying mechanisms of cognitive abilities in pigs may help to improve their welfare (Gieling et al., 2011a). Although many studies have investigated the effects of LBW on production and welfare (e.g. Baxter et al., 2008), to our knowledge, no studies have yet examined the effects of LBW in pigs on food motivation.

There are some indications that LBW pigs have an increased motivation for food. (Murphy et al., 2013) found that LBW piglets learned a discrimination task faster than NBW pigs, hypothesized to be due to higher (food) motivation. The results of another study on LBW pigs' cognitive functioning showed that LBW piglets outperformed their NBW siblings in a spatial cognitive holeboard task (Chapter 2, also published in Antonides et al., 2015a). The authors argued that this may have been due to increased motivation for food.

In studies on both rats and humans it has been found that LBW alters feeding preferences. LBW rats have a higher overall food intake (Vickers et al., 2000). LBW (in humans: small for gestational age, SGA) individuals who experienced prenatal nutrient restriction showed a higher preference for fatty diets (Lussana et al., 2008) and SGA women favor carbohydrates over proteins in their diet (Barbieri et al., 2009). It has been suggested that restricted nutrient supply and a stressful prenatal environment prepare the body for the postnatal environment by adjusting metabolic patterns that favor energy storage (Gluckman and Hanson, 2004). In livestock, prenatal stress caused by malnutrition has an impact on health and behavior later in life (Rutherford et al., 2012). Hair cortisol as a long-term measure for stress can provide an indication of the differences in the stress physiology between LBW and NBW piglets.

The early competition over resources, combined with the possible higher need for energy intake, make it likely that LBW piglets have a higher motivation for food (rewards) than NBW pigs. The current study aimed to compare the motivation to obtain food rewards between LBW and NBW pigs. To this end, we subjected ten LBW pigs from different litters and their ten NBW siblings to two food motivation tasks. Different tools can be used to induce a cost for a desired resource in order to measure motivation (Kirkden and Pajor, 2006). In the first task, food motivation was assessed in a runway. In the second task, the pigs' motivation was tested using a nose wheel in a feeding station as a cost mechanism to gain access to the reward (da Silva et al., 2012).

We expected to find a higher food motivation in LBW pigs than in NBW pigs in both tasks. As animals show a higher motivation for more desired resources (Kirkden and Pajor, 2006), we expected all pigs to show increased motivation for a higher number of rewards, and a decrease in motivation when no rewards were offered (extinction). Additionally, we measured hair cortisol as a long-term measure for stress. In accordance with previous findings in LBW pigs' hair cortisol (Chapter 2, also published in Antonides et al., 2015a), we expected the LBW pigs to have lower hair cortisol values than NBW pigs.

MATERIALS AND METHODS

Ethical note

This study was reviewed and approved by the local ethics committee (DEC, DierExperimenten Commissie) and is in accordance with the recommendations of the EU directive 86/609/EEC.

Animals and housing

Ten NBW and ten LBW pigs [Duroc × (Terra × Finnish landrace)], born in conventional farrowing crates on the commercial pig breeding farm of Utrecht University were selected. Ten litters in which at least 10 piglets were born were used, born in two batches of five litters each in two successive weeks, to ensure that enough LBW piglets could be selected. All piglets of each litter were weighed on the day of birth, including all stillborn piglets and piglets that died shortly after birth. From each litter, one LBW piglet was selected, with a maximum weight of 1 SD below the average birth weight of 484 piglets weighed in previous experiments. This yielded a maximum weight of 1050 g for the LBW piglets. Moreover, the selected piglets were at least 1 SD below the average birth weight of the litter they were born into. The lightest piglet of the litter which met both criteria and that was healthy and lively was selected from each litter. The selected NBW sibling was the piglet with a birth weight closest to the average birth weight of the litter. Preferably, NBW and LBW pigs with the same sex were selected per litter. For one pair, no NBW sibling with the same sex as the selected LBW pig close to the birth weight average was born. Therefore, the final selection consisted of five pairs of males, four pairs of females and one male-female pair. For two pairs, the originally selected NBW piglet was crushed by the sow. Selection of the new NBW piglet was based on the weight closest to the average of the litter on week four instead of day one, since birth weight information of the remaining NBW pigs in the litter was not saved at the time. As the weight of LBW piglets remains lower compared to NBW piglets throughout life (Douglas et al., 2013), we assumed that these two pigs did not have a LBW. To check for asymmetrical growth as an indicator of intra-uterine growth retardation (IUGR), head size (snout to back of cranium) and total body length (snout to tail base) were measured on the day of birth (Amdi et al., 2013).

Selected piglets were weaned at four weeks of age and housed in groups of five of the same age, with NBW and LBW piglets housed separately. This was done to avoid strong competition for hierarchy within the pen. Since the NBW and LBW pigs were thus separated, littermates were divided over groups and not housed together. Groups were housed in four similar pens (ca. 5 m × 6 m) with concrete flooring, with straw bedding and toys. Each pen contained a covered piglet nest containing a rubber mat and a thick layer of sawdust and straw bedding. A heat lamp was suspended approximately 1 m above ground in the piglet nest to avoid chilling of the piglets. The heat lamps were removed at 14 weeks of age, by which time the pigs were no longer observed lying under the lamps or showing huddling behavior. Lights in the stable were on between 7:30 a.m. and 4:30 p.m. Ambient temperature in the stable was recorded daily during the experiment and ranged from -1° C to 14 ° C. The stable was naturally ventilated (no heating aside from the above-mentioned heat lamps). Water and feed was provided ad libitum, except for two hours prior to testing when pigs were mildly food deprived by removing all feed. This was done to prevent saturation before testing. Feed was offered in a large feeding trough (2.5 m × 0.3 m) to avoid feeding aggression. Radios played continuously at the pens and in the testing area at a moderate volume to prevent sudden background noises from startling the pigs during testing. Pigs were weighed on the day of birth and then weekly from week 4 until week 20. Each task was performed in a separate testing area with an adjacent waiting area.

After weaning and prior to testing, all pigs were habituated to the experimenters, waiting areas and testing areas for approximately one hour per day. The waiting and testing areas were located in the same building as the home pens. At the age of 12 weeks, all pigs were tested in the runway task. At the age of 19 weeks, pigs were tested in the nose wheel task (progressive ratio task). At the age of 22 weeks, all pigs were transported to a commercial slaughterhouse.

The runway task

In the runway task, pigs needed to walk or run from a start box to a goal box to obtain food rewards (M&M's® chocolates). The runway (6 m × 1 m) had two boxes (1 m × 1 m) on each side that served either as start box or goal box in each run (Figure 1). The boxes had an upward sliding door (width: 1 m) allowing access to the runway. Two experimenters operated the doors from outside

the runway, using a rope and pulley system. Which experimenter operated which box was alternated daily. Both boxes contained a reward box (40 cm × 40 cm) with a concrete food bowl in it. The grey synthetic walls of the runway and boxes were 80 cm high.

Pigs were gradually habituated and trained in the runway. This was initially done in pairs to minimize stress and, once the pigs did not show escape attempts or freezing behavior anymore, pigs were trained individually. During these training sessions, pigs entered the runway and were offered two rewards in each food bowl, which they could approach at will. Once the pigs had learned where to find the reward, the upward sliding doors were closed as soon as the pigs had entered the box, and opened again when the pigs had consumed the rewards. At 12 weeks of age, when all pigs successfully completed runs and showed no fear of the sliding doors, formal testing started.

Before a session started, a pig was led into the runway and entered the first start box (first start box was alternated daily). To motivate pigs to enter the start box, they were rewarded with the same amount of rewards they would receive in each run that day (two or eight, see below). The door of the other box was kept closed to direct the pig to the correct start box. As soon as the pig entered the start box, the sliding door was lowered. After the rewards were consumed, both sliding doors were opened and a session started. Each session consisted of four consecutive runs. A run consisted of a piglet leaving the start box and entering the goal box where the rewards were consumed. When a pig reached the food bowl of the goal box, both sliding doors were closed. The experimenter operating the start box of that session refilled the food bowl with rewards for the next run. As soon as all rewards in the goal box were consumed, both doors were opened again for the next run. After completing four runs (one session), the door of the last goal box was opened and the piglet was allowed to leave the runway through a door on the side (Figure 1). Pen mates were housed in the adjacent waiting area during testing. Each session, pigs of a pen were tested in the same order (pen and pig order were randomized daily). All pigs received three sessions (i.e. 12 runs) per day.
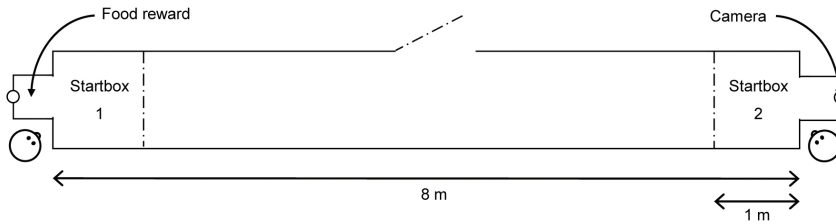
Figure 1. Schematic overview of the runway task with two start boxes, (upward sliding) doors indicated with dotted lines and on both ends of the runway an experimenter operating the upward sliding doors, standing outside the runway.

Phases in the runway task

The runway task was performed in five different phases, over seven (working) days. In the first phase (baseline, three days, nine sessions in total), two rewards were used in each run. In the second phase (high reward, two days, six sessions in total), the number of rewards was increased to eight, while all other test procedures remained the same. For the third phase (obstacles, one day, three sessions), obstacles were added to the runway to increase the difficulty to complete a run. In this phase, eight rewards were given in each run. The four obstacles were: a yellow garbage bin weighed down with bricks, two small feeding stations placed upside down, and a chair placed upside down. Only the chair completely blocked the runway, thus pigs had to climb over it. In the fourth phase (re-baseline, one day, three sessions), the obstacles were removed and two rewards were given per run. The re-baseline phase was added to 're-set' the motivation of the pigs before the extinction phase, as their motivation could have otherwise been influenced by the obstacles being removed from the runway in the extinction phase.

In the fifth and last phase (extinction, one day, two sessions), no rewards were offered. The extinction phase consisted of two instead of three sessions, as pigs failed to complete the runs, indicating rapid extinction of their motivation.

Recorded data in the runway task

All sessions were recorded with two camcorders (JVC Adixxion, GC-XA1) that were mounted 1 m above each goal box. Behaviors were scored from the recordings frame-by-frame with accuracy to a tenth of a second, using Microsoft Windows Movie Maker. Each of the observers scored half of the videos.

In addition, in order to calculate the inter-rater reliability using Spearman's rank correlations test (Martin and Bateson, 1993) an overlap of 15% of all videos (of different phases) was scored independently by both observers. Inter-rater reliability was very high ($r > 0.99$; $p < 0.01$).

Recorded measures were: (1) leave time: the time in seconds elapsed between retraction of the pig's head from the food bowl and leaving the start box with all four legs; (2) run time: the time in seconds between leaving the start box with all four legs and the snout reaching the food bowl in the goal box; and (3) session duration: the time in seconds elapsed between leaving the start box with four legs in the first run and the snout reaching the food bowl in the goal box in the last run. When a run lasted more than 60 s, pigs were encouraged by the experimenters to reach the goal box and the maximum run time of 60 s was scored. When the maximum time was scored for more than two consecutive runs, a session was ended. This was done in two sessions of one NBW pig, for which missing values were assigned in the remaining runs.

The nose wheel task

At the age of 19 weeks, all pigs were tested for their motivation to work for a food reward in the nose wheel task. To this end, a nose wheel feeding station was used (developed by Verbakel B.V., The Netherlands), as described by da Silva et al. (2012). The nose wheel feeding station was a custom-made feeder with a rotation disc. In order to receive a reward, a pig needed to push the rotating disc 360° in an upward direction with their snout. M&M's® chocolates were used as reward, and were delivered manually into the feeder by the experimenter standing next to the feeding station, after the required number of rotations was completed. An automated loud click-sound indicated that the required number of rotations was completed and that the pig would receive a reward. The feeding station was located in a testing room measuring 3 m × 2 m, where the pig could move freely during the experiment. Testing was done using a progressive ratio schedule. This means that each time a reward was received, the number of required wheel turns was automatically increased with one rotation (by a connected computer) in order to gain the next reward, starting at one rotation for the first reward.

All pigs were trained to operate the nose wheel feeding station in a fixed ratio

schedule and then in a progressive ratio (PR) schedule. Testing started when all pigs could operate the nose wheel successfully to gain rewards. Success was determined by rewards being earned by the pigs six consecutive times (with the last reward requiring six full turns of the wheel).

Phases in the nose wheel task

The nose wheel task was conducted in three different phases divided over five testing days. In the first phase (baseline, two days) two M&M's® chocolates were used as reward. In the second phase (high reward, two days) eight M&M's® were used as reward. In the last phase (extinction, one day) all testing procedures remained the same, but no reward was given. During the extinction phase, the loud click-sound still indicated that the required number of rotations was performed.

Recorded data in the nose wheel task

Durations in the nose wheel task were recorded live during trials by two experimenters using stopwatches. A trial started when a pig first touched the rotation disk with its snout. The following measures were recorded or calculated: (1) time away from feeder (s): the time during a trial that the pig was not operating the feeding station (head turned away from the feeding station); (2) number of times rewarded: the number of times the pig was rewarded for completing the required number of wheel turns during a trial; (3) trial time (s): the time that elapsed between when the rotation disc was first touched and when a pig was away from the feeder for longer than 120 s, or after the maximum time of 900 s had elapsed; (4) average time per turn (s): the average time for one complete rotation of the nose wheel disc, calculated as (trial time − time away from feeder) / number of completed rotations. For example, when the number of times rewarded = 6, then the number of completed rotations was (1 + 2 + 3 + 4 + 5 + 6) = 21; and (5) percentage of time away from feeder, calculated as (time away from feeder / trial time) × 100.

Hair samples for measuring cortisol

At 12 weeks of age, hair samples (0.5 - 1 g) were taken from the left flank of each animal with a trimmer. To this end, animals were briefly restrained with a pig snare. Of each hair sample, 250 mg was washed, dried and ground with a bead beater for 30 min in 2 ml Eppendorf tubes containing three 1 mm steal

beads. Thereafter, 50 mg of each powdered sample was collected in a micro-centrifuge tube. One ml methanol was added after which the samples were incubated at room temperature for 24 h with slow rotation to extract steroids. Of the extract, 0.6 ml was placed in a new tube and dried at 45°C in a heating block overnight. The dried extracts were dissolved in 0.4 ml phosphate buffer. Cortisol concentrations were then determined in duplicate, using a Salivary Cortisol ELISA kit (Salimetrics LLC, PA, USA). Because not enough hair for analysis could be collected from one NBW animal, analyses were performed on data of ten LBW and nine NBW pigs.

Statistical Analyses

All data were analyzed using the statistical software program SAS (version 9.4, SAS Institute, Cary, NC, USA). First, residuals of all variables were tested for normality using the Shapiro-Wilk test (SAS POC UNIVARIATE). Variables expressing latencies or durations were log10-transformed to meet the normality assumption. In all mixed model analyses, a random effect for litter was added to account for clustering of piglets within litters.

The effects of birth weight on the growth curves were analyzed with a mixed model ANOVA to account for repeated measures, with the fixed effects Birth weight (LBW or NBW), Week, and their interaction (SAS PROC MIXED). The effects of birth weight on head length in cm, full body length in cm, head length as percentage of full body length and cortisol concentration in hair samples at 12 weeks of age were analyzed using a mixed model ANOVA with the fixed effect Birth weight.

The runway task analyses
The mean values for leave time and run time were calculated for each pig over all runs of one day. Session duration was averaged over all sessions per day. The runway data analyses were performed for the five different phases: baseline, high reward, obstacles, re-baseline and extinction. Effects of birth weight on all variables were analyzed using a mixed model ANOVA per day (starting on the third baseline day). Additionally, changes over the three baseline days were analyzed. Differences between the third baseline day and the high reward phase, the baseline and re-baseline phase and between the re-baseline and extinction phase were analyzed. Fixed effects for these analyses were Birth

weight, Day and their interaction.

For the obstacle and extinction phase, effects on leave time and run time were analyzed over the different sessions. For the obstacle phase, this was done to see if the pigs decreased their run time over sessions. For the extinction phase, this was done to see if animals became less motivated in the second session. For these analyses, fixed effects were Birth weight, Session and their interaction.

The nose wheel task analyses

Data from the nose wheel task were analyzed using a mixed model ANOVA with fixed effects Birth weight, Day and their interaction. To assess possible learning effects over the days, effects during the baseline phase (two days) and the high reward phase (two days) were analyzed. Furthermore, effects of the transition between the phases were analyzed. To this end, the second baseline day was compared to the first high reward day, and the second high reward day to the extinction phase (one day).

The total trial duration was not normally distributed, as data were cut off with a maximum trial duration of 900 s, which was reached in 85% of all trials. All trials were therefore categorized as trial duration < 900 s (score 0), or as maximum trial duration reached = 900 s (score 1). The data were then analyzed using the Fisher exact probability test.

RESULTS

Birth weights and growth

NBW piglets had higher birth weights (Figure 2A; $F_{1,7}$ = 120.58; p < 0.001), larger heads ($F_{1,7}$ = 37.49; p < 0.001; mean ± SEM: NBW 11.00 ± 0.19 cm; LBW 9.83 ± 0.12 cm) and larger bodies at birth ($F_{1,7}$ = 23.35; p = 0.002; NBW 34.31 ± 0.63 cm; LBW 31.17 ± 0.45 cm) than LBW piglets. The head size relative to the full body length did not differ between the groups ($F_{1,7}$ = 0.32; p = 0.590; NBW 32.12 ± 0.74%; LBW 31.60 ± 0.61%). The weights of LBW pigs remained, on average, lower than those of NBW pigs during the experiment (birth weight: $F_{1,303}$ = 262.61; p < 0.001; Figure 2B). Furthermore, NBW pigs gained weight faster than LBW pigs (birth weight by week interaction: $F_{17,303}$ = 6.06; p < 0.001).

The runway task

In the high reward phase, NBW pigs had a shorter leave time (birth weight: $F_{1,9}$ = 9.10; p = 0.010) than LBW pigs (Figure 3A). Leave time tended to decrease in NBW pigs but not in LBW pigs in the transition from the baseline to the high reward phase (birth weight by day interaction: $F_{1,27}$ = 4.12; p = 0.050). This is in line with the difference between birth weight groups in leave time found in the high reward phase. No other birth weight effects within or between phases were found in this task.

Phase effects

During the baseline phase (three days), both run time (day: $F_{2,45}$ = 4.98; p = 0.010) and session duration (day: $F_{2,45}$ = 3.91; p = 0.030) decreased for all animals. There was no change in leave time during the three baseline days. No difference was found between phases in the baseline and re-baseline phase for leave time, run time and session duration for all animals. In the high reward phase, all animals decreased their run time (day: $F_{1,27}$ = 6.25; p = 0.020; Figure 3B) and session duration (day: $F_{1,27}$ = 13.62; p = 0.001) compared to the last baseline day. The day effects found within the baseline phase were smaller than the phase effects. Between the re-baseline and extinction phase, all ani-
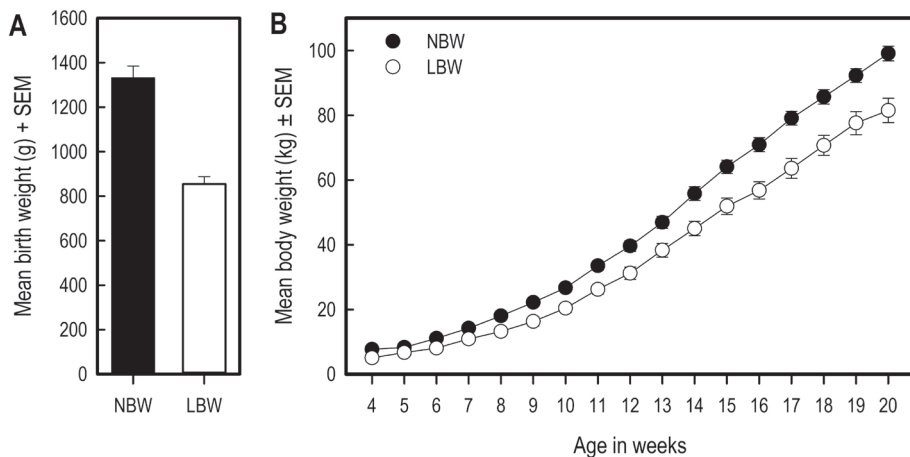

Figure 2. Birth weights and growth of the pigs. (A) The birth weights of the low birth weight and normal birth weight piglets in grams; (B) The mean body weight in kilograms of all pigs from weaning at four weeks until 20 weeks of age.

mals increased their leave time (day: $F_{1,27}$ = 15.53; p < 0.001; Figure 3A), run time (day: $F_{1,27}$ = 71.02; p < 0.001; Figure 3B) and, consequently, their session duration (day: $F_{1,27}$ = 10.87; p = 0.003).

Session effects
During the obstacle phase, all animals decreased their leave time (session: $F_{2,45}$ = 4.61; p = 0.010), run time (session: $F_{2,45}$ = 8.62; p < 0.001) and session duration (session: $F_{2,45}$ = 5.51; p = 0.007) over the three sessions.
During the extinction phase, all pigs increased their leave time (session: $F_{1,27}$ = 6.10; p = 0.020), run time (session: $F_{1,27}$ = 13.65; p = 0.001) and session duration (session: $F_{1,27}$ = 13.38; p = 0.001) over the two sessions.

The nose wheel task

In the nose wheel task, no birth weight effects or interaction effects were found on any measure (Figure 4).

Phase effects
The percentage of time spent away from the feeder was lower on the second baseline day for all animals compared to the first baseline day (day: $F_{1,27}$ = 8.30; p = 0.008). The number of times rewarded and the average time per turn did not differ between baseline days (Figure 4).

After the transition to a high reward, all animals showed a decrease in the percentage of time spent away from the feeder (day: $F_{1,27}$ = 48.03; p < 0.001) and reached a higher number of times rewarded (day: $F_{1,27}$ = 39.10; p < 0.001). Average time per turn did not change in this transition.

Between the first and second high reward day, percentage of time spent away from the feeder did not change. However, the number of times rewarded increased (day: $F_{1,27}$ = 12.12; p = 0.002) and the average time per turn decreased for all animals (day: $F_{1,27}$ = 19.57; p < 0.001).

After the transition from a high reward to no reward (extinction), all pigs spent a higher percentage of time away from the feeder (day: $F_{1,27}$ = 227.27; p < 0.001). Furthermore, they reached a lower number of times rewarded, i.e. completed less required wheel turns (day: $F_{1,27}$ = 69.72; p < 0.001) and used
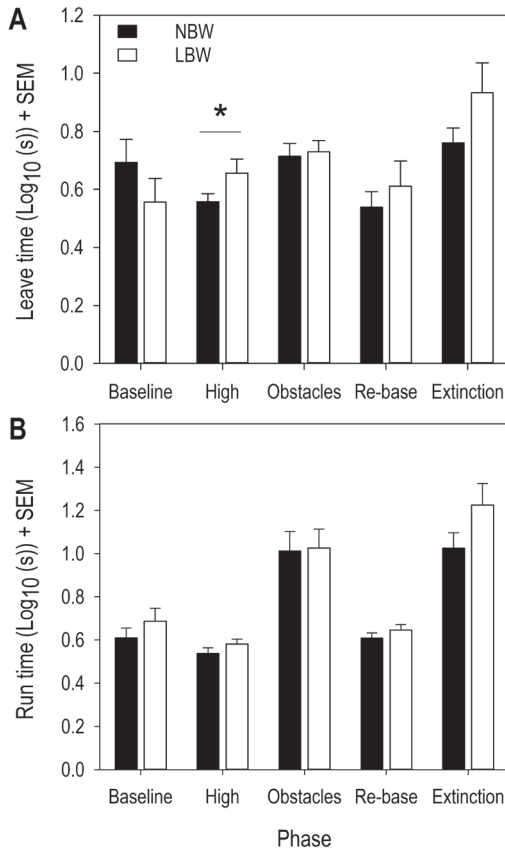
Figure 3. Overview of the results in the runway task for all five phases (in order of testing: baseline, high reward, obstacles, second baseline and extinction) for the low and normal birth weight pigs. Note: only the third day of the baseline phase is depicted in the graph. (A) Leave time in seconds; (B) Run time in seconds.

less time per turn (day: $F_{1,27} = 6.56$; p = 0.020).

There was no birth weight effect on the number of pigs that reached the total trial duration in any phase.

Hair samples

No birth weight effect was found on hair cortisol concentration ($F_{1,8} = 0.12$ p = 0.740; Figure 5).
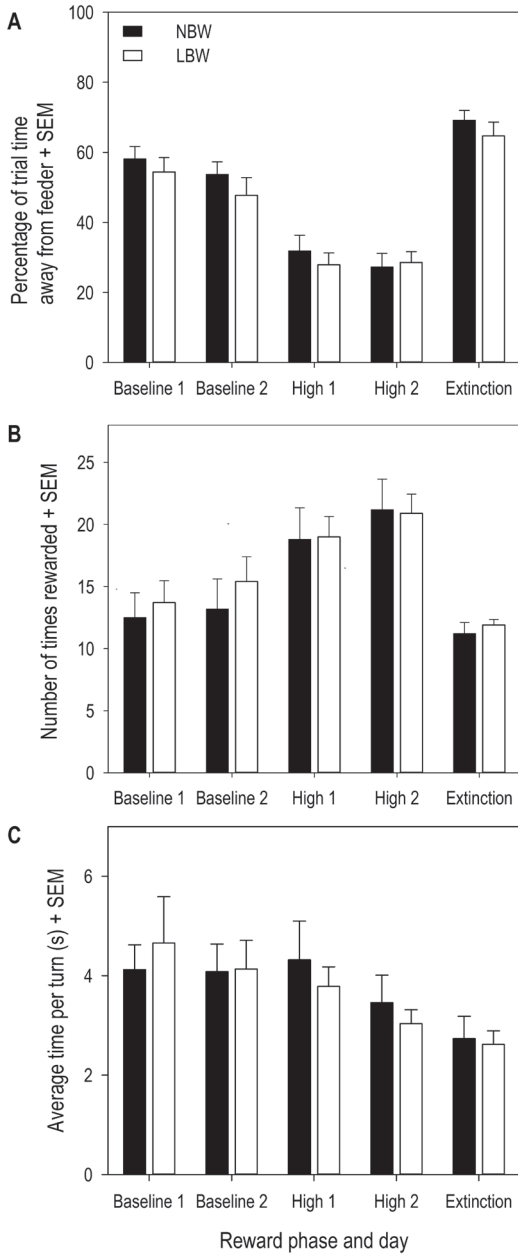
Figure 4. Overview of the results in the nose wheel task for all phases (in order of testing: baseline, high reward and extinction) for the low and normal birth weight pigs. (A) The percentage of time away from the feeder; (B) The number of times rewarded, and (C) The average time per turn.
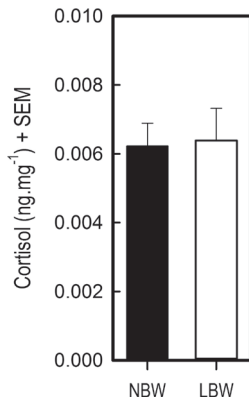
Figure 5. Hair cortisol concentrations in ng.mg-1 in flank hair of normal and low birth weight pigs at 12 weeks of age.

DISCUSSION

The present study aimed to compare the motivation to obtain food rewards between low birth weight (LBW) and normal birth weight (NBW) pigs. To this end, we used varying numbers of rewards in two separate tests; the runway task and the nose wheel task (an operant conditioning task using a progressive ratio schedule). In the runway task, we found that NBW pigs had a shorter leave time in the high reward phase, but no other birth weight effects were found in either test. Both the results of the runway and the nose wheel task showed a phase effect between the baseline and high reward phase (increased motivation) and high reward and extinction phase (decreased motivation). Both tasks are thus sensitive to experimental manipulations which influence the motivation for food rewards in pigs (e.g. size of reward).

Suitability of the two tasks to study motivation

We hypothesized that all pigs would show an increased motivation for a higher number of food rewards and a decrease in motivation during the extinction phase. The phase effects in both the runway and the nose wheel task confirm these hypotheses. This is in accordance with the underlying theory that animals have a higher motivation for resources that are more desired (Kirkden and Pajor, 2006), i.e. in the present study a larger food reward. This indicates

that both the runway and the nose wheel task are appropriate tasks to detect motivational differences for food (rewards) in pigs.

We found differences in motivation within phases; both the run time and the session duration decreased over days during the baseline phase of the runway task, indicating either an increased motivation or that the pigs learned to perform the task more efficiently. In line with these results, in the nose wheel task, the percentage of time away from the feeder decreased over the two baseline days. Moreover, in the high reward phase, the number of times rewarded increased and the average time per turn decreased over the two days. For the high reward phase, this can be explained by a learning effect, as the pigs might remember the high reward from the previous day. They are then likely to adjust their behavior, showing an increased motivation the next day.

It is possible that, despite sufficient training, pigs were still learning during the baseline phase. Alternatively, it may indicate that food motivation increased across successive days. Pigs were growing during the entire research period (Figure 2), which affects their feed intake and thus might influence food motivation. Therefore, another baseline phase after the high reward phase in the nose wheel task would improve our study design, since the high reward effect was now confounded with day effect and body weight. It is important to note that for both tasks the day effects were smaller than the phase effects. In addition, we found no difference in leave or run time in the runway task between the baseline and re-baseline, which were conducted 3 days apart.

In the obstacle phase of the runway, the leave time, run time and (consequently) session duration decreased over the three successive sessions. Since behaviors of the animals were not recorded during these trials, the cause of the decreased leave and run times over sessions remains speculative. Possible explanations are that the pigs became more efficient at passing the objects placed in the runway, that they showed more exploratory behavior in the first session or that their fear or restraint towards the unfamiliar objects decreased. Adding unfamiliar obstacles in a runway task may thus be a useful addition in studies assessing fearfulness or anxiety, and the speed of its decline over sessions. It would then be useful to record behaviors to determine whether the stimulus is actually perceived as fear-inducing.

All pigs showed reduced effort in the extinction phase in both tasks, with an increased leave and run time in the runway task, and a higher percentage of time away from the feeder and lower number of times rewarded in the nose wheel task. This result can be interpreted as pigs being quick to learn that effort was no longer rewarded. In addition, it demonstrates that the pigs were motivated to perform the tasks for the rewards rather than performing them simply to run or to turn the nose wheel.

Birth weight effects on motivation

Previous studies have found that LBW piglets have a faster learning rate than NBW piglets and it was suggested that this could be due to a higher motivation for food (Murphy et al., 2013; Chapter 2, also published in Antonides et al., 2015a). Our current results do not support this hypothesis. In the runway task during the high reward phase, the leave time was shorter in the NBW pigs than in the LBW pigs. If the motivation of NBW pigs was higher than that of LBW pigs in this phase, it would be expected that they would not only leave the start box faster, but also that they would run faster. However, run time was not affected in this phase. Moreover, this was the only significant birth weight effect found on any measure in both tasks. This result alone is not a strong indicator of a motivational difference for food between LBW and NBW pigs.

Previous studies in humans and rats have found that the type of nutrients is important for the altered food preferences in SGA individuals and LBW rats, where especially carbohydrate and fatty diets are preferred (Lussana et al., 2008; Barbieri et al., 2009). Growing pigs have a high need of proteins in their diet (Campbell et al., 1984). In the current study, chocolate M&M's® were used as a food reward. M&M's® are relatively high in carbohydrates, but low in fat and proteins. It would be interesting for future studies to explore the effects of other food rewards with higher fat and protein contents on motivation in pigs.

Motivation tasks: critical notes

Although the present study has indicated that the runway and the nose wheel task can be used to measure motivation, other factors possibly influencing the results must be taken into account. The body weights of the LBW pigs remained lower than the body weights of NBW pigs during the entire study, cor-

roborating previous results (Gondret et al., 2005; Chapter 2, also published in Antonides et al., 2015a). Any physiological difference between the LBW and NBW pigs could influence their ability to perform the tasks. However, Baxter et al. (2008) found that LBW piglets were equally vigorous as their NBW counterparts. In addition, using two different motivation tasks makes it less likely that body size and strength influenced the results.

As an adaptive reaction in the uterus, the brains and heart of growth restricted piglets are sometimes spared and thus receive relatively more nutrients than other organs, resulting in asymmetrical growth (Amdi et al., 2013). It is important to note that the piglets used in this study did not show bodily asymmetries typical for IUGR, as IUGR piglets have reportedly more welfare complications and abnormalities than symmetrical LBW pigs (Rutherford et al., 2013). It is therefore unlikely that IUGR has affected the ability of LBW pigs to perform the tasks.

Pigs showed different rotation techniques in the nosewheel task. Some pigs could rotate the disk for multiple turns with one push of their nose while other pigs needed more, less efficient pushes; one pig even used its teeth to rotate the disk. This could create a difference in the extent to which the pigs experienced the workload. Ferguson et al. (2009) also described individual differences in (mini)pigs that were subjected to an operant task, operating a lever. Because of the variation in applied techniques, it is difficult to design a task without individual variation. Even though different rotation techniques were applied in the nose wheel task, this test was still sensitive enough to measure a difference in motivation between the different phases.
The average time per turn decreased in the extinction phase compared to the high reward phase. This can be explained by the fact that the time at the feeder included the time the pigs needed to consume the food rewards. Since no rewards were given during the extinction phase, compared to eight rewards in the high reward phase, it is logical that the calculated average time per turn decreased. It would have been more accurate to subtract the time needed for consumption from this measure.

A possible drawback of food motivation tasks is that the tested animals can approach satiation during a session (Kirkden and Pajor, 2006). This is especially of importance when a higher amount of food (rewards) is provided. However,

during the nose wheel task, all pigs reached the maximum time of 900 s during the high reward phase, making it unlikely that they became satiated.

Hair cortisol concentration

At 12 weeks of age, flank hair cortisol did not differ between LBW and NBW pigs. In Chapter 2 (also published in Antonides et al. 2015a) we found significantly lower cortisol levels in flank hair of LBW pigs compared to NBW pigs, using the same method as the current study. This implies that the LBW piglets in that study experienced less stress compared to their NBW siblings. When comparing housing conditions, the pigs in that study were housed in much smaller pens than in the current study. As suggested in the discussion of that paper, the difference in cortisol between NBW and LBW animals may have been due to the relatively less space available per pig for the (larger) NBW animals.

In the current study, all pigs had ample space available. This may explain why we did not find differences in cortisol levels between birth weight groups. It is therefore advised to house pigs (and other animals used in research) in large pens or cages, in order to avoid housing conditions to influence test results. This especially holds for experiments in which body size differs between experimental groups.

CONCLUSION

We found that NBW pigs had a shorter leave time in the high reward phase in the runway, but no other birth weight effects were found in either test. Therefore, we conclude that there is no motivational difference between LBW and NBW pigs for food rewards. However, both the results of the runway and the nose wheel task showed a phase effect between the baseline and high reward phase (increased motivation) and high reward and extinction phase (decreased motivation). This is in accordance with the underlying theory that animals have a higher motivation for resources that are more desired. Our results show that both the runway and the nose wheel task are sensitive enough to measure differences in motivation, thus supporting the notion that these tasks are suitable motivation tasks for pigs.

ACKNOWLEDGEMENTS

Chapter 4

Does litter size affect emotionality, spatial learning and memory in piglets?

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ABSTRACT

Average litter size has steadily increased over the past decades in the pig farming industry. Large litters are associated with an increase of piglets born with a lower birth weight and reduced overall piglet viability. The aim of our study was to investigate whether litter size affects emotionality, learning and memory in pigs. Ten piglets from large litters (≥18 piglets) were compared with ten piglets from small litters (≤13 piglets). Piglets from two different suppliers, using different breeds, (hereafter called: Source) were tested. Effects were determined of Litter size and Source on birth weights and growth rates, on emotionality of the piglets in an open field test (OFT) at 5 weeks of age, and on effects of OFT-induced stress as indicated by salivary cortisol. The effects of Litter size and Source on spatial learning and memory in a holeboard task were assessed between 9 and 14 weeks of age. Small litter piglets from Source 1 grew faster than large litter piglets from the same source. This effect of Litter size was not found in piglets from Source 2. In the OFT, no effects of litter size on behaviors were found. However, piglets from Source 1 had lower baseline cortisol levels, made more escape attempts and showed higher locomotor activity during the OFT than piglets from Source 2. During the acquisition phase of the holeboard task, piglets from Source 2 learned the reference memory component faster and reached a higher overall working memory level in the reversal phase than piglets from Source 1. Our results show that source (i.e. supplier and/or breed) influenced performance in behavioral tasks, and that the occurrence of litter size effects was supplier or breed dependent.

Keywords: litter size, pig, open field, cognitive holeboard, breed, supplier

INTRODUCTION

Over the last few decades, high production levels of domesticated pigs with regard to growth and reproduction have been established through genetic selection, the control of reproductive cycles and improvements in husbandry management (Prunier et al., 2010). This has caused a considerable increase in the average litter size of pigs, especially in countries with a large pig industry such as The Netherlands, Denmark, France and Germany (Rutherford et al., 2011). Large litter sizes may affect the welfare of both the sow and her piglets (Rutherford et al., 2013). For example, large litters are associated with increased discomfort for the sow during farrowing and an increase in sow teat damage (Norring et al., 2006; Mainau et al., 2010). Sows have a limited uterine capacity, therefore large litters cause intra-uterine crowding (IUC). The uterine circulation increases with a larger number of fetuses, but not proportionally. The limited extent to which the blood flow of the uterus can be increased, results in a decrease of blood flow, oxygen and nutrient availability per fetus (Père and Etienne, 2000). IUC causes competition for resources between fetuses and has detrimental effects on placental development, resulting in increased pre- and neonatal mortality and reduced overall piglet viability (Wähner and Fisher, 2005).

Litter size correlates negatively with size and weight at birth, and birth weight variability within large litters is greater compared to that in small litters (Quiniou et al., 2002; Beaulieu et al., 2010). Litter size also shows a negative correlation with pre-weaning weight gain (de Passillé and Rushen, 1989). Moreover, the risk of crushing by the sow is higher in piglets from large litters, as they are generally weaker at birth. Due to their smaller average size, they also have poorer thermoregulatory abilities, and possibly a reduced colostrum intake. Thus, large litter piglets have decreased vitality and therefore an increased risk to die before weaning (Herpin et al., 2002; Rutherford et al., 2013).

Human babies that are born at term but small for gestational age (SGA) or have experienced fetal growth restriction, show poorer neurodevelopmental outcomes than babies that are appropriate for gestational age (Arcangeli et al., 2012). A follow-up study of SGA children showed that they had poorer school performance at adolescence than controls (Larroque et al., 2001). The authors

argue that "fetal adaptation to conditions that retard growth during gestation may not be successful in maintaining brain development". Similarly, SGA and fetal growth retardation in rats have negative effects on postnatal growth, metabolism, neurological development and learning ability (Ogata et al., 1985; Saito et al., 2009).

Studies on pigs that are born with a low birth weight (LBW) have been inconclusive. Gieling et al. (2012) found that LBW piglets had higher working memory scores than NBW piglets at the start of the reversal phase of a holeboard task. In a follow-up study, LBW piglets selected with stricter criteria showed improved reference memory performance in both the acquisition and the reversal phase of the same holeboard task (Chapter 2, also published in Antonides et al., 2015a), warranting further studies. It is conceivable that all piglets from large litters suffer from IUC, as they deal with greater competition over oxygen, space and nutrients than piglets from small litters. Therefore, normal birth weight (NBW) piglets from large litters may have undergone more limitations during fetal (cognitive) development than NBW piglets from small litters.

In the present study, we assessed emotionality and learning ability in ten NBW piglets from large litters and ten NBW piglets from small litters. Of each litter size category, five piglets originated from one supplier, and five from another supplier. We exposed all piglets to an open field test (OFT), in which behaviors such as locomotor activity and vocalizations can be used as measures of emotionality (Donald et al., 2011). We then assessed longer-term effects of litter size on cognitive development using the spatial cognitive holeboard task for pigs (Gieling et al., 2012; Chapter 2, also published in Antonides et al., 2015a).

We expected that piglets from large litters would display more emotional reactivity during the OFT, as expressed by more locomotion, vocalizations and defecations, more suspicion towards a novel object, and less time spent in the center of the OFT than piglets from small litters. Additionally, we expected large litter piglets to show a greater surge in cortisol after the OFT than small litter piglets. In the holeboard task, we expected large litter piglets to show lower memory scores and longer trial durations and latencies than piglets from small litters.

MATERIALS AND METHODS

Ethical note

This study was reviewed and approved by the animal ethics committee (DEC) of Utrecht University, The Netherlands. The study was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid their suffering.

Animals

Based on the information of litter sizes per supplier over the 6 months prior to selection, we determined the upper and lower 25th percentile of these data, resulting in a selection criterion of 13 or less piglets for small litters, and 18 or more piglets for large litters. Because in only a few litters less than 13 piglets were born, the animals were ordered as two separate batches from a pig breeding farm, hereafter called Source 1. We obtained 10 piglets (T40 × Pietrain), five from each litter size category (small or large). Unfortunately, due to technical problems it was impossible to obtain the second ordered batch from the same source. Instead, a second batch of 10 piglets (Large White × 426 PIC), five from each litter size category, was supplied by another pig breeding farm, hereafter called Source 2. Note that the effects of source are indistinguishable from effects of breed. The experimental design changed from a simple test of the effects of litter size (small vs. large) to a two-factorial design with the factors Litter size and Source.

However, for answering our main question whether litter size affects emotionality, spatial learning and memory in piglets, we still had 10 piglets from small litters and 10 piglets from large litters at our disposal. If effects of Litter size are robust, then Source should not be relevant. At the same time, this design enables us to assess the effects of the additional factor Source and its interaction with Litter size. It adds a second question, namely whether effects of Litter size (if present) are robust, i.e. whether or not it is affected by Source (see also Festing et al., 1998; Shaw et al., 2002).

One piglet per litter from a total of 20 litters was selected within 24 hours after

birth. All piglets were born to multiparous sows. All piglets of each litter were weighed, including stillborn piglets and piglets that died shortly after birth. The male piglet closest to the average birth weight within its litter was selected and given a different color ear tag. The male piglet second closest to the average weight of the litter was also marked, in case the selected piglet died before weaning. After the selection process, the piglets remained at the pig breeding farm until weaning.

Housing

At weaning, when the piglets were approximately four weeks old, they were transported to the research facilities of the commercial pig breeding farm of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. After arrival at the testing facility, all animals were housed under non-SPF (conventional) conditions. The four groups of five piglets were housed in adjacent pens by litter size category (small or large litter) and supplier (Source 1 or Source 2). The pens (4 m × 5 m) were enriched with straw bedding, rubber balls, a gunnysack and chewing sticks. Ambient temperature in the stable was measured daily and ranged between 4 ℃ to 27 ℃ for the piglets from Source 1 (March – May 2014), and between 8 ℃ and 34 ℃ for the piglets from Source 2 (April – July 2014). Each pen contained a wooden nest box (3 m × 1.5 m) with plastic flaps along the front. Rubber mats, covered with a thick layer of sawdust and straw were placed on the concrete floor of the nest boxes. A heat lamp warmed the nest box until the piglets were 8 weeks of age. The stable in which the pens were located was naturally lighted and ventilated (unheated). Fluorescent lights in the stable were on from 7:30 h to 16:30 h.

To facilitate individual recognition, the animals were marked with a sprayed letter on their back (Porcimark marking spray, Kruuse, Denmark). Food and water were available ad libitum, except during the 5 weeks of holeboard testing, when the animals were fed a quarter of their daily required amount of food in the morning before testing, half of the amount after testing and the remainder in the late afternoon. This feeding schedule ensured that the piglets would not feel saturated during testing and would be motivated to search for food rewards in the holeboard. Starting in the second week after arrival at the research facility, a radio was playing continuously at a moderate volume − slightly louder during daytime than at night − to mask noises that could distract or startle the pigs.

Birth weights and growth

The piglets were weighed on the day they were selected (birth weight), on the day they arrived at the research stables (4 weeks old), and then once per week until behavioral testing ended at the age of 15 weeks.

Open field test

Open field apparatus
The arena used for the open field test (OFT) measured 2.5 m × 2.5 m with synthetic walls of 1.5 m high and was built adjacent to the piglets' home pens by Ossendrijver B.V. (Achterveld, The Netherlands). The arena had one entry door and the concrete floor was covered with a thin layer of sawdust. A camera (JVC Adixxion GC-XA1) recorded continuously during testing hours, and was suspended directly above the arena in such a manner that the entire open field arena was recorded. The arena was divided into nine equal segments on-screen: one center segment and eight outer segments (four corner and four wall segments).

Open field testing
The OFT was conducted 1 week after arrival at the research facility, when the piglets were approximately 5 weeks old. The piglets had never before been socially isolated. They were taken from their home pen and led through a small corridor into the open field arena, where they remained for 10 minutes. After 5 minutes, the experimenter that was standing next to the open field arena manually dropped a novel object (a colorful tambourine toy) in the center segment of the arena. During the OFT, four behavioral measures of activity were registered manually, using a score form by an observer who stood next to the arena: (1) the number of escape attempts, defined by jumping up against the wall with all four legs off the floor; (2) the total number of defecations; (3) the total number of times the piglet looked at the novel object; and (4) the total number of times the piglet touched the novel object with its snout. During the OFT, eye contact with the piglet was avoided. From the video recordings we scored the total number of vocalizations, the total number of line crossings (placing two or more legs in the segment during movement from another segment), and the total time spent in each type of segment (center or outer).

Salivary cortisol

Two saliva samples were collected per piglet: the first 5 minutes before and the second 20 minutes after the OFT. The second sample was drawn around the expected peak of increased cortisol in reaction to the OFT (Kirschbaum and Hellhammer, 2000). Measuring salivary cortisol is preferred over blood sampling, since the method of sampling is much less aversive (Mormède et al., 2007). The piglet was allowed to chew on two cotton swabs (Cotton Swabs 150 × 4 mm WA 2PL, Heinz Herenz, Hamburg, Germany) until they were thoroughly moistened with saliva. The swabs were then immediately stored in tubes on ice. After the samples were centrifuged at 2200 - 3000 g for 10 - 15 minutes at room temperature, the saliva was pipetted in 2 ml Eppendorf tubes and stored at -20 °C (as adapted from (Merlot et al., 2011). All samples were assayed on the same day in duplicate by radioimmunoassay using methods, adapted from the manufacturer's procedure (Coat-a-Count cortisol TKCO, Siemens Solution Diagnostics, Los Angeles, CA, USA).

Baseline cortisol levels could be established for all piglets. The samples of three piglets taken after the open field test did not contain sufficient saliva for analysis.

Holeboard test

The holeboard apparatus

The holeboard apparatus – built by Ossendrijver B.V., Achterveld, The Netherlands – was located next to the home pens. The apparatus measured 530 × 530 cm, with synthetic grey walls that were 80 cm high, and a blue slatted synthetic floor that was elevated 85 cm from the ground. The arena was surrounded by a 40 cm wide corridor, which contained the main entrance to the apparatus. Via a guillotine door in the center of each side wall, operated manually by the experimenter using a rope and pulley system, the pig could enter the holeboard arena. The arena contained a four by four matrix of 'holes' where the pig could search for food rewards (for details, see Figure 2 in Chapter 1 of this thesis). A hole consisted of a plastic food bowl with a false bottom (Road Refresher Large, Prestige Pet Products, Essex, England), covered by a red plastic ball (JollyBall Dog Toy, ø 24 cm, 1400 g, Jolly Pets, Ohio, USA) to prevent the pig from locating the rewards using visual cues.

The pig had to lift the ball using its snout to uncover the food bowl and consume the reward. Chocolate M&M's® were placed as rewards on the false bottom of the bowl. Underneath the false bottom of all holes, 3 inaccessible M&M's® were replaced daily to ensure that pigs could not locate rewards using olfactory cues. Whenever a ball was lifted from the food bowl, the connection between a sensor in the bowl and a magnet in the ball was broken, which generated a signal that was registered by an interface (LabJack), connected to a laptop. This signal was processed by a tailor-made software program (Blinq Systems, Delft, The Netherlands). A steel wire attached to the top of the ball ensured that the ball always fell back into place with the magnet located directly above the sensor. Lifting a ball multiple times during a 10-second-period, without lifting another ball, was recorded as one visit.

Holeboard testing procedure

After the open field test at 5 weeks of age, piglets were habituated to the waiting area and the holeboard apparatus for approximately 15 minutes per day for 4 weeks. The habituation period lasted until all piglets were comfortable staying in the test arena alone, lifted the balls and consumed rewards, which was at approximately 9 weeks of age. From age 9 to 14 weeks, the piglets were tested in the holeboard task in three successive phases: the habituation phase (6 trials), the acquisition phase (40 trials) and the reversal phase (40 trials). Pigs that had reached the pre-set criterion of an average reference memory score ≥ 0.7 at the end of the acquisition phase, over the last trial block (trials 37-40), were switched to the reversal phase. If a pig did not meet the criterion after 40 acquisition trials, its acquisition phase was extended until it did, with a maximum of 60 trials. If a pig did not reach the criterion within 60 acquisition trials, it was excluded from testing in the reversal phase. This was the case for one small litter piglet from Source 1.

During the habituation trials, all 16 holes were baited with M&M's® chocolates. In the acquisition phase, each pig was assigned its own configuration of four baited holes. In total, four different configurations were used, in such a way that, across pigs, every hole was baited equally often (see Figure 2 in Chapter 1 of this thesis). The reversal configuration was the 180° rotated pattern of baited holes used during the acquisition phase.

For each trial, the entry door was randomly determined by the software that

controlled holeboard testing and collected the data. A trial was started when the pig entered the arena with its two front legs, and ended automatically when all rewards had been found, or when the maximum trial duration of 450 s had elapsed, whichever event occurred first. Piglets always received two trials in close succession. The pen order in which the piglets were tested was alternated each testing day. A pig inside the holeboard could smell and hear its pen mates in the waiting pen next to the holeboard apparatus. Fluorescent lights on the sloping ceiling of the stable and the position of the experimenter standing in the corridor of the holeboard arena served as visual extra-maze cues for spatial orientation.

Statistical analyses

All data were analyzed using SAS version 9.4 for Windows (SAS Institute, Cary, North Carolina, USA). Normal distribution of the residuals of all variables was assessed using the Shapiro-Wilk test (SAS PROC UNIVARIATE). All analyses of variance (ANOVA) were performed using SAS PROC GLM, except for the open field data which were analyzed parametrically or as ranked scores (Friedman's Two-way Nonparametric ANOVA for ranked data (Friedman, 1937) using PROC ANOVA. Where appropriate, we performed Sidak post-hoc comparisons between the four groups of piglets.

Birth weights and growth
Birth weights of the piglets were analyzed by a Litter size by Source ANOVA. Growth from 4 to 15 weeks of age was analyzed using an ANOVA with the repeated measures factor Week, and with Litter size and Source as between-subjects factors, and their interactions.

Open field data
Intra- and inter-rater reliabilities were determined using a Spearman's rank correlations test (as described in Martin and Bateson, 1993). Intra-rater reliability was determined for the variables number of escape attempts, number of times the piglet looked at the novel object, and number of times the piglet touched the novel object. These variables were scored twice by the same observer: directly during OFT and again from the video recordings. The number of vocalizations, number of line crossings and percentage of time spent in center or wall segments were scored independently by two observers from the

video recordings. Each observer scored 50% of all video recordings. Additionally, an overlap of 15% of all video recordings was scored by both observers to determine inter-rater reliability. For subsequent analyses, the scores of the two observations (live and from recordings by the same observer, or from the recordings by two observers) were averaged.

As there were eight outer segments and one center segment, the mean percentage of time a piglet spent in an outer segment (i.e. near the walls and in the corners) was determined. The average time spent per outer segment was compared with time spent in the center segment by adding segment (center or outer) as repeated measures factor to determine whether piglets showed wall hugging behavior (thigmotaxis) indicated by spending, on average, more time in an outer than in the center segment.

All other OFT variables were rank-transformed because they did not meet the normality assumption, and were analyzed using the Friedman's Two-way Nonparametric ANOVA for ranked data (Friedman, 1937).

Salivary cortisol data
Baseline salivary cortisol values, cortisol values after the OFT, the absolute increase in cortisol and the proportional increase in cortisol with respect to baseline levels, were analyzed with a two factorial ANOVA with the factors Litter size and Source, and their interaction.

Holeboard data
In the six habituation trials preceding actual holeboard testing, the total number of visits (TV) and number of unique hole visits (UHV) were recorded per trial. Note that UHV corresponds to the number of rewards that were found, as all holes were baited during this phase. TV and UHV scores were analyzed using a repeated measures ANOVA with Litter size and Source as between-subjects factors and Trials as within-subject factor.

During the acquisition and reversal phase, the following variables were recorded or calculated (van der Staay et al., 2012): Working memory (WM): a ratio defined by the number of visits that yield a food reward divided by the number of visits and re-visits to the rewarded set of holes; Reference memory (RM): a ratio that is defined by the number of visits and re-visits to the rewarded set of

holes divided by the number of visits and re-visits to all holes; Trial duration (TD): the time (s) between entering the holeboard and finding all four rewards (when not all rewards were found, the maximum trial duration of 450 s was recorded); and the Inter-visit interval (IVI): the average time (s) between two hole visits.

For all holeboard variables measured in the acquisition and reversal phase, means of 4 successive trials (trial block means) were calculated. Trial block means of variables expressing latencies or durations (TD and IVI) were log10-transformed to meet the normality assumption. Analyses were performed for the acquisition phase (40 trials; blocks 1-10), the reversal phase (40 trials; blocks 11-20) and the transition phase. The transition phase is the switch from the acquisition phase to the reversal phase, i.e. the last trial block mean of the acquisition phase compared to the first trial block mean of the reversal phase (block 10 compared to block 11). This is a measure of the response flexibility of an animal: a large difference may indicate that the animal faced difficulties to adapt to the new configuration of baited holes. Alternatively, it may imply that piglets had learned the assigned configuration in the acquisition phase very well. The extra acquisition trials that a piglet received when it had not yet reached the pre-set criterion of RM ≥ 0.7 after 40 trials were excluded from analyses. Holeboard data were analyzed using a repeated measures ANOVA with Litter size and Source as between-subjects factors, Trial blocks as within-subject factor, and their interactions.

RESULTS

Birth weights and growth

Piglets from large litters had lower average birth weights (1.31 ± 0.05 kg) than piglets from small litters (1.75 ± 0.10 kg) (Litter size: $F_{1,16}$¬ = 12.40, P = 0.003) (Figure 1A). There was no difference in birth weight between piglets from the two different sources (Source: $F_{1,16}$ = 0.00, P = 0.98; Litter size by Source interaction: $F_{1,16}$ = 0.30, P = 0.59).

Source and litter size differentially affected the average body weight (Litter size by Source interaction: $F_{1,16}$ = 7.75, P = 0.01) and the growth rate across weeks 4 to 15 (Week by Litter size by Source interaction: $F_{11,176}$ = 2.70, P = 0.003; Figure 1B). Sidak post-hoc comparisons between the four groups of piglets re-

vealed that body weight of the piglets from small litters of Source 1 exceeded that of the other three groups of piglets, starting in week 6. The body weights of the other three groups of piglets did not differ from one another.

Open field test

Behaviors in the open field test
Reliabilities: The intra- and inter-rater reliabilities for the OFT observations were ≥ 0.73 and ≥ 0.78, respectively.

Piglets from Source 1 made more escape attempts (Friedman's ANOVA: Source: $F_{1,16} = 5.57$, P = 0.03; Figure 2A) and line crossings (Friedman's ANOVA: Source: $F_{1,16} = 4.81$, P = 0.04; Figure 2B) than piglets from Source 2. Piglets from Source 2 tended to vocalize more during the OFT than piglets from Source 1 (Friedman's ANOVA: Source: $F_{1,16} = 3.48$, P = 0.08). Litter size had no effect on any of these three variables.

The piglets spent a higher percentage of time in the center segment than on average, in each of the eight outer segments (Segment: $F_{1,16} = 12.72$, P = 0.003), i.e. they showed no thigmotaxis in the OFT. Litter size or Source did not affect the percentage of time spent in center or outer segments.
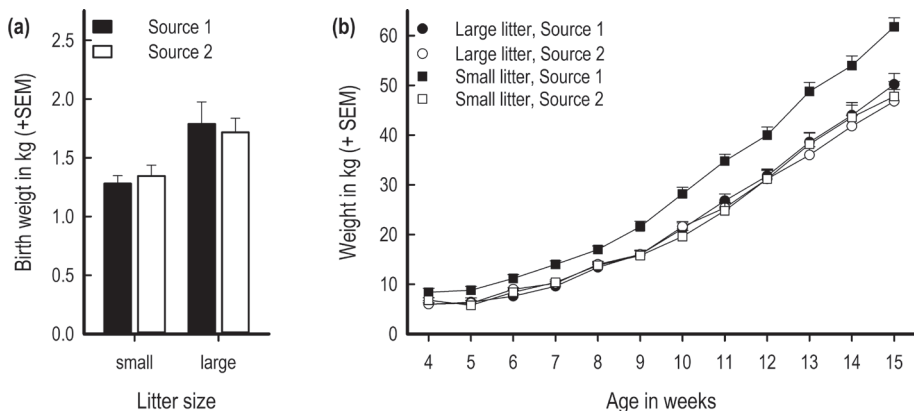

Figure 1. (A) Births weights and (B) Growth of piglets from small and large litters, depicted by source, from weaning at 4 weeks of age until 15 weeks of age.
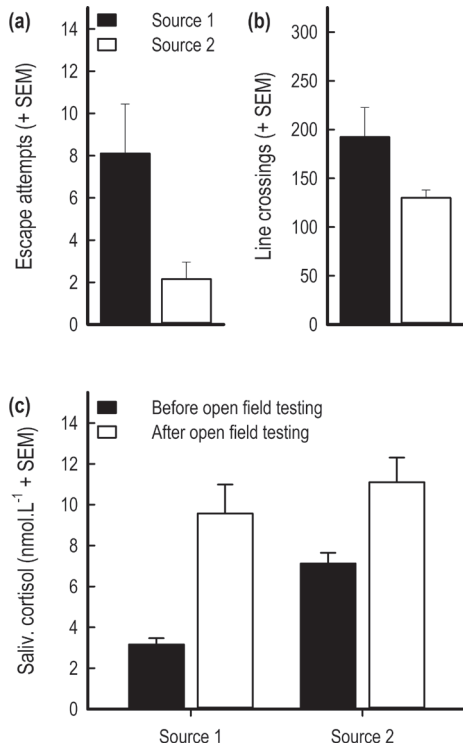
Figure 2. Behaviors in the open field test, depicted by source. The test was conducted individually at the age of five weeks and lasted ten minutes. (A) Number of escape attempts; (B) Number of line crossings; (C) Salivary cortisol values before and after the open field testing, depicted by source.

Cortisol

Sampling of saliva after the OFT yielded insufficient amounts for cortisol determinations in one small litter piglet from Source 1 and two small litter piglets from Source 2.

Repeated measures ANOVA revealed that piglets from Source 2 had higher baseline salivary cortisol values than piglets from Source 1 (Source: $F_{1,13}$ = 4.85, P = 0.046; Figure 2C). All groups showed increased levels of salivary cortisol after the OFT ($F_{1,13}$ = 36.60, P < 0.0001). The absolute increase was similar for suppliers and litter sizes.

However, piglets from Source 1 showed a higher proportional increase in salivary cortisol after the OFT (295 ± 31%) than piglets from Source 2 (151 ± 11%) (Source: $F_{1,13}$ = 15.86, P = 0.002).

Holeboard task performance

Table 1 shows a summary of the effects of source and the differential effects of litter size per source on holeboard performance of the piglets.

Habituation trials
In the six habituation trials preceding actual testing, no effects of litter size or source on the total number of visits (TV) or unique hole visits (UHV) were found. The four groups of piglets showed a constant number of visits (Trials: $F_{5,80} = 0.41$, $P = 0.84$), but a change in UHV over the six habituation trials (Trials: $F_{5,80} = 2.88$, $P = 0.02$). However, the change of UHV was not a systematic increase as might be expected, but an unsystematic fluctuation across the six trials.

The average UHV for all animals over the six trials was 14 ± 0.4 unique hole visits. UHV in this phase corresponds to the number of rewards found, as all 16 holes were baited. Therefore, this average UHV of 14 shows that the animals effectively searched throughout the holeboard for rewards before actual testing started.

Working memory
Working memory (WM; see Figure 3A) performance slightly improved for all animals during the acquisition phase (Trial blocks: $F_{9,144} = 3.96$, $P = 0.0002$) and the reversal phase (Trial blocks: $F_{9,135} = 7.07$, $P < 0.0001$), and decreased for all piglets in the transition phase (Trial blocks: $F_{1,16} = 18.05$, $P = 0.0006$; Figure 3A).

Neither Litter size nor Source affected WM performance during the acquisition phase. In the reversal phase, piglets from Source 2 showed higher average WM scores than piglets from Source 1 (Source: $F_{1,15} = 5.34$, $P = 0.04$). Of the four groups of piglets, small litter piglets from Source 1 had, on average, the lowest WM scores in the reversal phase (Litter size by Source interaction: $F_{1,15} = 4.64$, $P = 0.048$).

Reference memory
Reference memory (RM; see Figure 3B) performance during both the acquisition (Trial blocks: $F_{9,144} = 81.42$, $P < 0.0001$) and reversal phase (Trial blocks:

$F_{9,144} = 51.04$, P < 0.0001) increased in the four groups. They showed a similar drop in RM scores in the transition phase (Trial blocks: $F_{1,16} = 108.59$, P < 0.0001). During the acquisition phase, piglets from Source 2 showed faster RM learning (Trial blocks by Source interaction: $F_{9,144} = 2.44$, P = 0.01) than piglets from Source 1 (Figure 3B).

RM performance of large litter piglets from Source 1 appeared to be least affected by the transition (Litter size by Source interaction: $F_{1,16} = 5.70$, P = 0.03). During the reversal phase, small litter piglets from Source 2 outperformed the other three groups on the average RM performance (Litter size by Source interaction: $F_{1,16} = 7.51$, P = 0.01).

Trial duration

The log10-transformed Trial duration (TD; see Figure 3C) decreased in both the acquisition (Trial blocks: $F_{9,144} = 22.49$, P < 0.0001) and the reversal phase (Trial blocks: $F_{9,144} = 31.09$, P < 0.0001), and increased in the transition phase for all groups (Trial blocks: $F_{1,16} = 99.67$, P < 0.0001).

During the acquisition phase, the piglets from Source 1 showed a different change in TD across trial blocks than piglets from Source 2 (Trial blocks by Source interaction: $F_{9,144} = 2.44$, P = 0.01). The average TD during the transition was different for piglets from small and large litters from the two sources (Litter Size by Source interaction: $F_{1,16} = 4.76$, P = 0.04). The piglets from the large litters of Source 1 appeared to perform, on average, the fastest.

Inter-visit interval

The log10-transformed Inter-visit interval (IVI; see Figure 3D) slightly declined during the acquisition (Trial blocks: $F_{9,144} = 3.38$, P = 0.0009) and reversal phase (Trial blocks: $F_{9,144} = 10.76$, P < 0.0001), and increased during the transition phase for all animals (Trial blocks: $F_{1,16} = 37.25$, P < 0.0001). During the acquisition phase, piglets from Source 1 showed a different change in IVI than piglets from Source 2 (Trial blocks by Source interaction: $F_{9,144} = 2.78$, P = 0.005). Large litter piglets from Source 2 tended to show a steeper decrease of IVI in the reversal phase than the other three groups (Trial blocks by Source by Litter size interaction: $F_{9,144} = 1.71$, P = 0.093).

a) Effect of source on holeboard performance			
Phase	**Measure**	**Difference found between sources**	**See figure**
Acquisition	Reference memory (RM)	Source 2: Piglets show faster increase in RM	3B
	Trial duration (TD)	Different slopes in TD between piglets of the two sources	3C
	Inter-visit interval (IVI)	Different slopes in IVI between piglets of the two sources	3D
Reversal	Working memory (WM)	Source 2: Piglets show better WM performance	3A

b) Differential effect of litter size on holeboard performance per source				
Phase	**Measure**	**Effect in pigs of**	**Litter size groups**	**See figure**
Transition	Reference memory (RM)	Source 1	Large litters: Smaller drop in RM score	3B
	Trial duration (TD)	Source 1	Large litters: Smaller rise in TD score	3C
Reversal	Reference memory (RM)	Source 2	Small litters: Higher RM scores, i.e. better performance	3B
	Inter-visit interval (IVI)	Source 2	Different slope between litter size groups	3D

Table 1. Overview of effects of source and the differential effects of litter size per source on performance in the holeboard task. (A) Effects of source; (B) Differential effects of litter size per source.

DISCUSSION

The aim of the present study was to investigate whether litter size affects emotionality, spatial learning and memory in piglets. We expected that piglets born in large litters would display higher emotionality during the open field test (OFT), show a greater surge in cortisol after the OFT, and perform worse in the holeboard task than piglets from relatively small litters.

Supplier or breed?

Due to technical problems, the design of the study changed from a simple two-groups comparison (small vs. large litter) to a factorial design with as extra factor Source (supplier/breed). In this factorial design, the effects of litter size are determined by comparing all piglets from small litters with all piglets from large litters, i.e. this main effect is tested with the same number of animals for both levels of the factor "litter size" as in the original setup (see also Shaw et al., 2002). If this effect is robust, then Source should not be relevant. At the same time, this design enables to assess the effects of the additional factor Source and its interaction with Litter size.

According to Festing et al. "(...) factorial experimental designs (...), in which
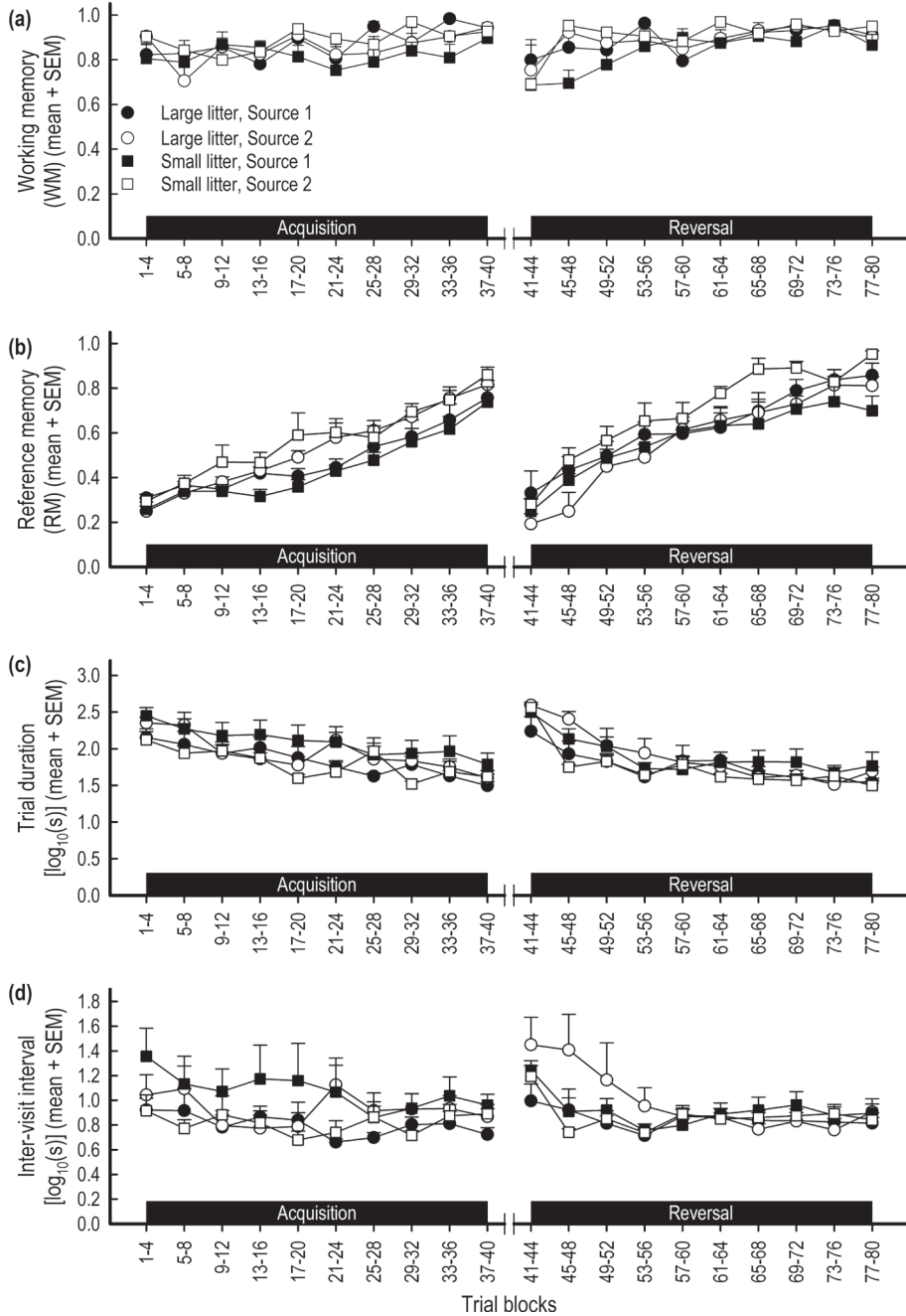
Figure 3. Holeboard performance of all piglets, depicted by litter size per source. (A) Working memory; (B) Reference memory; (C) Trial duration; (D) Inter-visit interval.

two or more factors (for example, treatments, time, sex, strain, age, or diet) are varied simultaneously, usually make more efficient use of resources (including experimental animals) than do designs involving only a single factor (…).” (Festing et al., 1998). In the present design, testing the effects of Litter size can be considered as the confirmatory component, whereas the effects of Source and its interaction with Litter size can be considered as an exploratory component of the study. Experiments with a confirmatory and an exploratory component are not unusual (Festing and Altman, 2002).

This change in design thus does not affect precision (Festing, 1992; Shaw et al., 2002). It does add a second question, namely whether this effect (if present) is robust, i.e. whether or not it is affected by Source (supplier/breed).
We do not know whether the interaction effects between Source and Litter size found on growth rates and several behavioral measures in the open field and the holeboard task point to differential effects of either supplier or breed. These are, however, interesting new findings that raise questions worthy of future investigations.

Surprisingly, source had a profound effect on activity and cortisol increase during the OFT and some effects on memory performance in the holeboard task. Moreover, interactions between litter size and source were found. These interaction effects imply that litter size has differential effects on growth and cognitive performance in piglets of different sources (i.e. suppliers and/or breeds). Both suppliers provided roughly the same environmental conditions in terms of thermal regulation, feed and enrichment. However, they provided different crossbreeds of pigs. It is not possible to disentangle, in case of interaction effects, whether these effects are due to genetics of the piglets or to different management practices. Rather, genetics, management and/or environmental factors may have influenced development and behavior in our pigs differently.

Growth

The birth weights of piglets from large litters were lower than those of piglets from small litters, which is in line with earlier studies (Bergstrom et al., 2009; Beaulieu et al., 2010). Surprisingly, small litter piglets from Source 1 grew faster than the other three groups over the course of the experiment. This implies that litter size had an effect on growth in piglets of Source 1, but not in

piglets of Source 2. It also shows that the growth of piglets from Source 2 was more homogeneous than that of piglets from Source 1.

Source 2 supplied piglets bred and reared under SPF conditions. The growth of pigs from this source may therefore be less affected by differences in litter sizes and birth weight. However, small litter piglets from Source 1 showed faster growth and thus had higher body weights than large litter piglets from the same source at the end of the experiment. In the pig industry, high body weights are preferable as heavy pigs yield more meat.

Open field test

No effects of litter size were found on behavioral measures or cortisol increase in the OFT. Piglets from Source 1 showed higher locomotor activity and tended to vocalize less during the OFT than piglets from Source 2. Piglets in an OFT study treated with a stress reducing drug, and piglets accompanied by another piglet, showed more locomotion and less vocalization than controls (Donald et al., 2011). This would imply that piglets from Source 2 showed higher emotionality in the OFT.

In a study comparing active and passive behavioral response piglets in the OFT, active piglets were found to vocalize less, make more escape attempts, have lower baseline cortisol levels and show a greater increase in cortisol after the OFT than passive piglets (Hessing et al., 1994). Piglets from Source 1 in the present study tended to vocalize less, made more escape attempts, had higher baseline cortisol values and a greater proportional increase in cortisol after the OFT than piglets from Source 2. Thus, when comparing our results to those of the study of Hessing et al. (1994), Source 1 seems to supply active behavioral response pigs, and Source 2 passive behavioral response pigs. It should be noted that the difference in proportional increase in cortisol may be due to a ceiling effect, i.e. that all piglets reached a maximum value of cortisol after the OFT.

Piglets show no wall hugging behavior (thigmotaxis)

It has been reported that pigs show a tendency to spend more time near the walls than in the center segment of an open field. Forkman et al. (2007) argue

that farm animals evolved in open areas, and thus may not show thigmotactic behavior. We found that piglets spent relatively more time in the center segment than in outer segments. This implies that piglets do not show thigmotactic behavior, which is commonly found in rodents (Walsh and Cummins, 1976). In a review, Murphy et al. (2014) also concludes that it is unlikely that pigs show thigmotactic behavior in the OFT. Our results also imply that wall hugging behavior in an OFT is not a useful measure to assess emotionality in young piglets.

In summary, we found strong effects of source on locomotor behavior and cortisol measures during the OFT, yet no effects of litter size. We therefore argue that either supplier or breed has profound effects on emotionality responses in piglets, and that piglets from different suppliers or breeds can express different combinations of response behaviors when measuring emotionality.

Holeboard task

All piglets acquired the holeboard task and improved their performance during both the acquisition and the reversal phase, and were affected by the transition to another set of baited holes from the acquisition to the reversal phase. This corroborates earlier findings of studies using the holeboard task in pigs (Arts et al., 2009; Gieling et al., 2012; Chapter 2, also published in Antonides et al., 2015a).

Strong effects of source on holeboard performance
Piglets from Source 2 showed faster reference memory (RM) learning in the acquisition phase and different changes in trial duration (TD) and inter-visit interval (IVI) than piglets from Source 1. In the reversal phase, piglets from Source 2 had higher WM scores than piglets from Source 1.

Taken together, piglets from Source 2 partially outperformed piglets from Source 1 in the holeboard task, showing faster RM learning in the acquisition phase and higher WM scores in the reversal phase of the task.

Effects of litter size on holeboard performance: different per source
Litter size had a differential effect on holeboard performance of the piglets purchased from the two different sources. In the acquisition phase, no effects

of litter size on holeboard performance were found. In the transition phase, large litter piglets from Source 1 showed a smaller drop in RM performance and a smaller rise in trial duration (TD) than small litter piglets of the same source. This may indicate that large litter piglets from Source 1 were more flexible in re-learning where to find rewards than small litter piglets. Alternatively, this may imply that they had a weaker consolidation of the original configuration of baited holes than small litter piglets.

In the reversal phase, litter size had an effect on RM performance only in piglets of Source 2: small litter piglets showed higher overall RM scores than large litter piglets. Additionally, an effect of litter size in piglets from Source 2 was found on the changes of IVI during the reversal phase. Although these changes are nonlinear and it is thus difficult to interpret this result, it does show that there were some litter size effects within piglets of Source 2 that were not found in piglets of Source 1.

Critical notes

It is possible that the difference in litter size between small (≤ 13 piglets) and large litters (≥ 18 piglets) in our study was not large enough to detect effects of litter size. It may, therefore, be advisable to increase the difference in litter size between groups in future studies. However, this may prove to be challenging, as small litters are expected to become increasingly infrequent in the pig industry (Rutherford et al., 2013).

The set-up of our study included piglets from two different suppliers, supplying different breeds. If litter size would have a strong and robust effect on performance in behavioral tasks, we would expect that litter size effects are robust over different breeds of pigs and (thus) over pigs from different suppliers. However, as we included both litter size and source as factors in our analyses, interaction effects were estimated based on 5 piglets per group. It is plausible that this has reduced the power of our analyses for interaction effects. Although we found indications that source and litter size had a differential effect on several behaviors, we recommend that this is further investigated using more animals.

CONCLUSION

Taken together, our results show that source (i.e. supplier and/or breed) can have a strong effect on performance in behavioral tasks, and that factors such as litter size may affect pigs' performance of one source, while it may not or differently affect pigs from another source. These findings show that it is important to realize that results from behavioral tasks may not be generalizable across different breeds of pigs or across pigs from different suppliers.

Part II

Iron deficiency

Chapter 5

Pre-weaning dietary iron deficiency impairs spatial learning and memory in the cognitive holeboard task in piglets

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ABSTRACT

Iron deficiency is the most common nutritional deficiency in humans, affecting more than two billion people worldwide. Early-life iron deficiency can lead to irreversible deficits in learning and memory. The pig represents a promising model animal for studying such deficits, because of its similarities to humans during early development. We investigated the effects of pre-weaning dietary iron deficiency in piglets on growth, blood parameters, cognitive performance and brain histology later in life. Four to six days after birth, ten male sibling pairs of piglets were taken from ten different sows. One piglet of each pair was given a 200 mg iron dextran injection and fed a control milk diet for 28 days (88 mg Fe/kg), whereas the other sibling was given a saline injection and fed an iron deficient milk diet (21 mg Fe/kg). Due to severely retarded growth of two of the iron deficient (ID) piglets, only eight ID piglets were tested behaviorally. After dietary treatment, all piglets were fed a balanced commercial pig diet (190-240 mg Fe/kg). Starting at 7.5 weeks of age, piglets were tested in a spatial cognitive holeboard task. In this task, 4 of 16 holes contain a hidden food reward, allowing measurement of working (short-term) memory and reference (long-term) memory (RM) simultaneously. All piglets received 40-60 acquisition trials, followed by a 16-trial reversal phase. ID piglets showed permanently retarded growth and a strong decrease in blood iron parameters during dietary treatment. After treatment, ID piglets' blood iron values restored to normal levels. In the holeboard task, ID piglets showed impaired RM learning during acquisition and reversal. Iron staining at necropsy at 12 weeks of age showed that ID piglets had fewer iron-containing cells in hippocampal regions CA1 and dentate gyrus. The number of iron-containing cells in CA3 correlated positively with the average RM score during acquisition across all animals. Our results support the hypothesis that early-life iron deficiency leads to lasting cognitive deficits. The piglet as a model animal, tested in the holeboard, can be useful in future research for assessing long-term cognitive effects of early-life diets or diet-induced deficiencies.

Keywords: iron deficiency, cognition, development, growth, spatial learning, memory, hematology, pigs

INTRODUCTION

The effects of nutrition on development and health has received much attention in the past decades. Good nutrition is essential for proper development and growth, physical health, mental health and well-being. Malnutrition can be defined as any disorder of nutrition, such as over nutrition, under nutrition and unbalanced nutrition, with one or more micronutrient or mineral deficiencies (Allison, 2000). Nearly a third of the world population is suffering from one or more forms of malnutrition, and around half of the deaths among children under the age of five are associated with it (de Onis et al., 1993). Consequences of malnutrition include impaired mental and physical development, disability, and death. The most common nutritional deficiency is iron deficiency, affecting more than two billion people of all ages worldwide (WHO, 2000; Ramakrishnan and Yip, 2002). Iron is an essential micronutrient and is required for many biological functions in the body, such as gene regulation, binding oxygen and serving as a cofactor in many enzymes. Furthermore, iron plays an important role in neural functioning, for example in myelination, neurotransmission and energy metabolism (Youdim et al., 2010). The severity of and specific impairments caused by iron deficiency are dependent on the timing of the deficiency. There are three periods in human development with an elevated risk for iron deficiency: the early neonatal period, toddlerhood, and adolescence in females (Georgieff, 2011). The most common period for iron deficiency in humans is between zero and five years of age (McLean et al., 2009), thus during early development. Studies have shown that children who suffered from early iron deficiency (from the late fetal period through two years of age) perform worse in motor, cognitive, social and emotional tasks compared to controls that were fed a balanced diet (Walter et al., 1989; Lozoff and Georgieff, 2006). In a longitudinal follow-up study, children who had suffered from severe iron deficiency in infancy still showed behavioral and developmental deficits – such as reduced mental and motor functioning, social and attentional problems – ten years after iron treatment (Lozoff et al., 2000).

In order to elucidate the biochemical and neurophysiological mechanisms underlying iron deficiency, the use of rodent iron deficiency models has increased in the recent past. Such studies have shown that iron deficiency causes numerous abnormalities on the morphological, neurochemical and behavioral

level. Deficits resulting from iron deficiency suggest impairments specifically in the prefrontal cortex (PFC) and hippocampus (Rao et al., 2012). The hippocampus is known to be involved in spatial learning and memory (Bird and Burgess, 2008). Indeed, hippocampal dysfunctions impair spatial learning and memory (Olton et al., 1978; Da Silva et al., 1986). Compromised spatial orientation learning has also been found in cognitive and behavioral iron deficiency studies. For example, a study on the effects of maternal iron deficiency on hippocampal development in mice pups found impaired spatial memory function, reduced neurogenesis and reduced volumes of the hippocampus in iron deficient (ID) animals (Ranade et al., 2013). In the Morris water maze test, a spatial learning and memory task, ID rats took longer to find the hidden platform compared to controls (Yehuda et al., 1986). Similar results were found for rats that suffered from perinatal iron deficiency when tested in adulthood after iron repletion, showing that iron repletion after early iron deficiency does not reverse iron deficiency induced impairments (Bourque et al., 2008). Perinatal iron deficiency in rats causes differential regional losses of brain iron content, with the most profound declines found in the hippocampus and prefrontal cortex (Siddappa et al., 2003). Iron deficiency extended to the end of weaning in rat pups caused brain iron concentration to decline and delayed the learning of sensorimotor skills (Beard et al., 2006). Interestingly, most of these abnormalities do not recover after iron repletion (Yehuda and Youdim, 1989; Bourque et al., 2008; Ranade et al., 2013). This suggests that iron deficiency during early development causes irreversible impairments in brain structure and function. These impairments are believed to be due to rapid brain growth during early development and its associated high iron demand (Lozoff and Georgieff, 2006).

Mice and rats are the most commonly used animal model species in neurodevelopmental research (Clancy et al., 2007). However, these rodents have a different timing of early development than humans, both pre- and postnatally. For example, the developing brain of rats is believed to be comparable to that of a newborn human baby, with regard to the degree of maturation, around postnatal day 12-13 (Romijn et al., 1991). Because of developmental differences and differences in brain morphology between rodents and humans, more and better suited animal models are needed to study the long-term cognitive effects of iron deficiency in a controlled manner. Other animal models for studying cognitive effects of iron deficiency have been suggested that more

resemble the early development of humans, including guinea-pigs (Fiset et al., 2015), pigs (Rytych et al., 2012) and primates (Golub et al., 2007).

In comparison to other animals mostly used in neurotranslational research, the brain growth spurt and pattern of brain development in pigs most resembles that of humans (Dobbing and Sands, 1979; Conrad et al., 2012). The pig has a relatively large brain that is, like the human brain, gyrencephalic (with cerebral convolutions), whereas the rodent brain is lissencephalic (smooth surface). Although a difference in size of left and right hippocampi is found in humans but not in pigs, MRI studies have shown that hippocampi of both species show a similar sex-dependent growth trajectory (Conrad et al., 2012). Additionally, the distribution of grey and white matter is comparable between pigs and humans (Lind et al., 2007). Pigs are social and intelligent animals which can be trained in complex cognitive tasks (Mendl et al., 2010). The pig is a precocial species, and thus can be weaned almost immediately after birth. This has the advantage that behavioral tasks can be conducted with piglets at as young as one or two weeks of age (Dilger and Johnson, 2010). Moreover, pigs are easily available with well-documented life history information available. Pigs can thus serve as a promising model animal to examine environmental effects during the neonatal period, when the brain is rapidly developing, on learning and memory later in life.

The resemblance of the pig's digestive system and metabolic processes to those of humans makes the pig a suitable model species for studying effects of nutrition (Puiman and Stoll, 2008). In particular, pigs may be useful to study the effects of iron deficiency, as they have limited iron stores at birth, a high requirement of iron and a low external supply of iron (Starzyński et al., 2013). The ancestor of the domesticated pig, the wild boar, copes with its high iron needs by rooting and thus ingesting iron from soil. Piglets reared in confinement do not have access to this natural source of iron. Moreover, sow's colostrum and milk do not meet the piglets' high iron requirements, even if the sow is fed a high iron lactation diet or administered an injection of iron dextran (Brady et al., 1978). In commercial pig farming, it is therefore common practice to provide piglets with an intramuscular injection of iron dextran on day 3 to 6 after birth (Svoboda and Drabek, 2005).

Studies investigating iron deficiency in pigs have mainly looked at the underlying molecular mechanisms or investigated the prevention and correction of iron deficiency (Lipiński et al., 2013; Starzyński et al., 2013). A recent study that looked at the effects of dietary induced iron deficiency on learning and memory in neonatal piglets, found that severely ID piglets could not acquire a food motivated double T-maze task, whereas mildly ID piglets showed deficits in reversal learning (Rytych et al., 2012). However, the performance of ID pigs was assessed during the period of dietary ID treatment, and not during or after iron repletion. The present study investigated the effects of severe pre-weaning iron deficiency on cognitive performance in piglets later in life. To this end, we used the spatial cognitive holeboard task for piglets (Arts et al., 2009; Gieling et al., 2012). In this task, four hidden rewards can be found in a matrix of 4x4 possibly rewarded sites, allowing for working memory and reference memory to be measured simultaneously. Working memory is a form of short-term memory that is used during a specific trial (van der Staay et al., 2012) and that, once used, should be forgotten (Dudchenko, 2004). This includes, for example, which sites an animal has already visited during a trial, i.e. this information is relevant only within a trial. Reference memory is a form of long-term memory that involves the general rules of a task, such as where to find hidden rewards (van der Staay et al., 2012). Information stored in reference memory is relevant across trials. Additionally, effects of pre-weaning iron deficiency on blood iron parameters during and after treatment, and brain histology after iron repletion was investigated.

We expected that ID piglets would show a decline in blood iron values during dietary iron deficiency treatment, and impaired performance in the holeboard task compared to the control group, by making more errors in the task (i.e. showing lower memory scores) and taking more time to complete the task. Furthermore, as brain iron concentration has been shown to be normalized in rodents after two weeks of iron repletion (Erikson et al., 1997; Piñero et al., 2000), we expected to find no effect of treatment on the pigs' brain iron concentration at 12 weeks of age.

MATERIALS AND METHODS

Ethics note

This study was reviewed and approved by the local ethics committee (DEC, DierExperimenten Commissie) and was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid suffering.

Subjects

Ten sibling pairs of male piglets were selected from ten different litters [(Terra × Finnish landrace) × Duroc] at the commercial pig breeding farm of Utrecht University, The Netherlands. This was done in two runs with two weeks in between to ensure that enough male piglets with similar average weights could be selected. During the first days, all piglets were allowed to ingest colostrum from the sow in order to trigger their immunocompetence (Rothkötter et al., 2002). This was believed to have little to no influence on the iron status of the piglets, because of the low iron content in sow colostrum and milk (Brady et al., 1978). During this time, no creep feed was provided. From each litter, two healthy male piglets with birth weights closest to the average birth weight of the entire litter were selected (51.3 ± 3.9 % of the piglets within the ten litters was male). One piglet of each pair was randomly assigned to the ID treatment group, the other to the control group. Four to six days after birth, selected piglets were separated from the sow and transported to the experimental facilities, which was on the same day for all piglets per run in order to reduce mixing stress. In the first week after arrival, the animals were weighed daily to closely monitor weight gain, after which they were weighed weekly. Two ID piglets were euthanized during the first week after arrival, as they did not consume sufficient amounts of milk formula and therefore did not gain weight. Thus, the experiment was conducted with 8 ID piglets and 10 control piglets. The experiment was performed blind, in such a way that neither the experimenters nor the animal caretakers or other people directly involved in this research were aware which piglets underwent the iron deficiency treatment, and which piglets served as controls. The treatment groups were unblinded after the statistical analyses were completed.

Housing

The piglets were housed in a multifunctional room in the clinic of the Department of Farm Animal Health (Utrecht University, The Netherlands) in four adjacent identical pens, each measuring 1.25 m × 2.50 m. They were housed in groups of five pigs (one ID group consisted of three animals after two animals were euthanized due to lack of growth, which was one week after arrival), sorted by treatment group and age. The concrete floor of the pens was covered with a layer of sawdust and straw. Chains, rope and plastic balls were offered as enrichment and were changed regularly. When the animals were approximately ten weeks old, the pens became too small for the piglets according to the guidelines for housing pigs of that age (Forbes et al., 2007). It was thus decided to rehouse the piglets in pens measuring 2.50 × 2.50 m, which required moving the animals to an adjacent, identical room. Rehousing was done in two runs, so that it was performed at the same age in both groups. As the animals were led to the experimental room and back daily for testing, this was expected to have no effect on behavioral performance in the holeboard task. Room temperature was gradually decreased from 26 °C in the first week to 20 °C at the end of the experiment. During the first week, a heating lamp was suspended 1 m above the floor in each pen. This was done to ensure the piglets would not chill, as young piglets' thermoregulatory abilities are low (Herpin et al., 2002). The room had a light-dark regime of 12:12 hours with lights on at 7 a.m. A radio was playing continuously at a moderate volume to mask environmental noise, slightly louder during daytime (7 a.m. to 4 p.m.) than at night.

Treatment

The control group received a needle-free injection of 1 ml iron dextran containing 200 mg iron (MS FerroPig, Schippers Export B.V., The Netherlands) on day 4-6 after birth (directly before transport to the experimental facilities). The treatment group was administered 1 ml of saline (NaCl) in the same manner. As the experiment was performed blind, this was done via a third person, who was informed about the coding of the treatment groups and experimental diets (blinded to the experimenters) and communicated this with the animal caretakers who applied the injections. After arrival in the experimental facilities, the piglets were fed one of two custom diets prepared by the Mead John-

son Pediatric Nutrition Institute Technical Center (Evansville IN, USA). The control group was fed a balanced milk replacer, formulated to achieve an iron content of 100 mg Fe/kg formula. The treatment group received an ID diet formulated to achieve 10 mg Fe/kg, but balanced for all other nutrients. The actual iron content in samples was determined at 88 mg Fe/kg diet for the balanced formula and 21 mg Fe/kg diet for the ID formula.

Experimenters and animal caretakers were blinded to the iron content of the formulas, which were provided with a code on the packaging by the supplier. Milk replacer was freshly prepared five times per day; at 7.30 a.m., 11 a.m., 2 p.m., 4 p.m. and 9 p.m. and fed in two feeding bowls per pen (from two weeks of age in two troughs, to reduce competition). The amount of milk replacer fed was adjusted daily to meet the consumption of the animals and ranged from 1 L to 4 L per feeding. The piglets were fed these diets for 28 days (for a timeline, see Figure 1 and Table 1). Then, all animals received the same commercial piglet feed ad libitum (containing 190-240 mg Fe/kg). The animals were weaned gradually: during the first week of weaning (day 28 - 35 after the start of the experimental diet) in addition to the commercial piglet feed, the assigned milk replacer was still provided (6 L on the first day of weaning, which was gradually decreased to 2 L at the end of weaning). Water was available ad libitum.

Timing of the conducted events during the experiment		
Event	Conducted at (age in days ± 1)	Timing relative to start of experiment (i.e. to transport to experimental facilities)
Birth	0 days	5 (± 1) days
Iron dextran / saline injection	5 days	0 days (0 weeks)
Experimental diet	5 days until 33 days	0-28 days (0-4 weeks)
Gradual transition to regular feed	33 days until 40 days	28-35 days (4-5 weeks)
Blood sampling	5, 19, 33, 47, 85 days	0 days (0 weeks), 14 days (2 weeks), 28 days, (4 weeks), 42 days (6 weeks) and 80 days (approx. 11.5 weeks)
Start of behavioral testing	54 days	49 days (7 weeks)
End of behavioral testing	Between 77 days (min. 40 acq. trials) and 83 days (max. 60 acq. trials)	Between 72 days (10 weeks; min. 40 acq. trials) and 78 days (11 weeks; max. 60 acq. trials)
Euthanasia and brain histology	85 days	80 days (approx.. 11.5 weeks)

Table 1. The timing of events conducted during the experiment, relative to the birth of the piglets (middle column) and relative to weaning and the start of the experimental diets (right column).

Blood sampling

On the day of transport to the experimental facilities (age 4-6 days, see Table 1), blood was collected from the ear veins of the piglets to determine blood hematocrit and hemoglobin levels, using the The epoc® Reader and Host Mobile Computer (Alere Inc., Waltham MA, USA). At 2, 4 and 6 weeks after the start of the experimental diet and at 12 weeks of age (Figure 1), due to difficulties with collecting blood from the ear veins, blood samples were taken from the jugular artery (3.5 ml) to determine hematocrit, hemoglobin and serum iron values. Blood hemoglobin and hematocrit were determined using the Siemens ADVIA® 2120i System with ADVIA Multispecies Testing software. Serum iron was determined using the Beckman Coulter UniCel DxC 600 according to standard procedures.
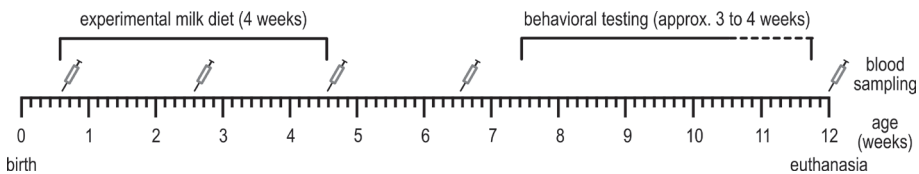

Figure 1. Timeline of the events conducted during the experiment. See Table 1 for more detailed information.

Apparatus

The holeboard apparatus (Ossendrijver BV, Achterveld, The Netherlands) consisted of a square arena of 360 × 360 cm with a 4x4 matrix of food bowls, surrounded by a small corridor (40 cm) with a slatted black synthetic floor (for details, see Figure 2 in Chapter 1 of this thesis). The synthetic walls were 80 cm high and had a steel bar on top (total height: 1 m). The apparatus was elevated 25 cm off the floor. The arena could be entered through four different guillotine doors, one on each side. The entry door was randomly assigned in each trial and was operated from outside the arena using a rope and pulley system. Pigs entered the holeboard through the main entrance and always turned left into the corridor until they found an open door, through which they entered the testing arena. Piglets inside the holeboard arena were able to see the surrounding walls of the experimental room and the ceiling with two

rows of fluorescent tubes, as well as the two experimenters standing in front of the holeboard to the right of the main entrance door. The experimenters avoided eye contact with the piglets during trials. Auditory extra-maze cues were a radio that was playing continuously during testing hours, and the piglet's pen mates in the waiting area in front of the holeboard apparatus, where they were housed during testing.

The sixteen food bowls were covered by red plastic balls which could be lifted by the piglets with their snout (JollyBall Dog Toy, ø 24 cm, 400 g), to prevent the piglets from finding the rewards by sight. To ensure that the rewards were not found by smell, every food bowl contained three rewards (replaced daily) under a false bottom. The apparatus was cleaned with a water hose at the end of each testing day and after a trial if an animal had defecated.

Hole visits were automatically recorded using custom made software (Blinq Systems, Delft, The Netherlands). A visit was scored when a pig lifted the ball and the connection between the magnet in the ball and the sensor in the food bowl was broken. This signal was registered by an interface (LabJack) and sent to a PC. An iron wire attached to the top of the ball ensured that the ball would always fall back into place with the magnet located directly above the sensor. A visit was not counted as a revisit when the same ball was lifted within 10 s and no other holes were visited in between. A trial started when a pig entered the arena with both front legs and ended when a piglet found all four rewards or when the maximum time of 450 s was reached, whichever event occurred first.

Behavioral testing

During the weeks after arrival in the experimental facilities and before testing started, all piglets were gradually habituated to the experimenters, hallway and holeboard apparatus in two sessions of 30 to 60 minutes per day. At the start of holeboard habituation, all balls were lifted and in each of the 16 bowls multiple mini-marshmallows were placed as reward, as these were easy to consume for the young animals. When all animals had been seen consuming rewards from the bowls, all balls were lowered in order for the piglets to learn to lift the balls to find rewards. Habituation was done with all piglets of a pen in the arena, then in groups of two or three and eventually individually. During

this time, pen mates were present in the waiting area in front of the holeboard, thus piglets in the holeboard could still hear and smell their pen mates. Piglets that seemed to have trouble learning to lift the balls in order to find rewards were allowed longer habituation sessions, in order to ensure that all piglets had roughly the same level of performance in finding rewards before testing in the holeboard started. At the end of the habituation period and during holeboard testing, M&M's® chocolates were used instead of mini-marshmallows. Holeboard testing started when all piglets had learned to search for rewards while being alone in the holeboard, which was when the piglets were approximately 7.5 weeks old (Figure 1).

All piglets received six individual habituation trials (two per day) in which all holes were baited. Then, the animals received two trials in close succession per day (massed trials) for the first four testing days, after which they received four massed trails per day, in sets of two trials in close succession each. Each piglet was assigned its own configuration of baited holes, in which 4 of the 16 holes were baited. In total, four different configurations were used, in such a way that every hole was baited equally often. All piglets received 40-60 acquisition trials (12-17 testing days) and 16 reversal trials (4 testing days). A piglet was switched to the reversal phase when its RM score averaged above 0.7 over the last four trials (one day) after at least 40 acquisition trials. After a maximum of 60 acquisition trials, all pigs were switched to the reversal configuration, regardless of their performance. The reversal configuration was the 180° rotated pattern of baited holes used during acquisition, i.e. a change from configuration A to C, B to D, C to A, or D to B. All piglets received 16 reversal trials, thus all piglets received a minimum of 56 trials and a maximum of 76 trials.

Brain histology

Due to the unexpected death of one control animal at 11 weeks of age, brain sections of 9 control animals and 8 ID animals were analyzed. At the end of the experiment (at 12 weeks of age), all animals were euthanized by an intracardial injection with an overdose of pentobarbital (Euthasol®, AST Farma B.V. Oudewater, The Netherlands). Directly afterwards, brains were dissected and weighed. Both hippocampi were then carefully removed and weighed, after which the left hippocampus was cut in half and the dorsal part was stored in 4% phosphate buffered formaldehyde, pH 7.0 (Klinipath, Duiven, The

Netherlands) at 4°C, which was refreshed after two hours and within 24 hours replaced with 70% ethanol. Samples were then stored at 4°C until slicing. Brains were sliced in 0.12 M phosphate buffer saline on ice using a vibratome (VT 1200S Leica Biosystems, Nussloch, Germany). Dorsal hippocampal samples were sliced in such a way that in each container the distance between the sections was 240 μm. Sections were collected in series of six sections and stored in tubes of 0.12 M phosphate buffer saline and 0.1% sodium azide at 4°C. Staining was done using the free-floating method; 40 μm sections were stained in baskets with 8 compartments. PBS was used as a negative control. All steps were performed using a tilt shaker (WS-10, position 10, Edmund Buehler Gmbh, Hechingen, Germany). Sections were washed in 0.05 M tris-buffered saline. Staining took place in the dark to prevent influence by light on immunohistochemical reactions.

Iron staining was done using a commercial kit (Prussian blue iron stain kit, Polysciences Inc., Warrington, USA). Sections were washed with 0.12 M PBS, incubated in a solution of 4% potassium ferrocyanide and 4% hydrochloric acid (1:1) for 45 min at room temperature, then washed with AD. The reaction was intensified with 3,3'-diaminobenzidine tetrahydrochloride DAB catalyzed by H_2O_2 (Sigma-Aldrich, 1 ml DAB stock + 1.5 ml TBS + 2.0 μl H_2O_2) for 5 minutes. Afterwards, sections were mounted on superfrost slides (Thermo Scientific, Braunschweig, Germany) and dried overnight at 37 °C followed by dehydration through graded alcohol series (70% to 100%) and xylene. Slides were then embedded with DePeX (Serva electrophoresis, Heidelberg, Germany) and coverslipped. Four hippocampal areas of interest were chosen: CA3, CA1, dentate gyrus (DG) and subiculum (sb). The CA3 region is associated with spatial pattern recognition and short-term memory; the CA1 region is involved in short- and intermediate memory temporal pattern separation (Kesner et al., 2004). Both CA3 and CA1 are involved in signaling the animal's presence in particular regions of space (i.e. self-location; Barry and Burgess, 2014). The DG is thought to be involved in fine spatial information processing (Kesner et al., 2004; Hunsaker et al., 2008), and the subiculum in memory retrieval and spatial encoding (Stafstrom, 2005).

Visual scoring was performed using a microscope (Olympus B×40) at 10 × 0.25 magnification. An ocular counter was used where a randomized sequence of 20 squares out of 100 squares (unit of 1 square: 1 mm2) was scored. Iron-contain-

ing cells were visible as brown dots, which were counted per mm² and averaged for each hippocampal area over five sections per animal. Scoring was done with coded sections, in such a way that the person that scored the sections did not know which animal of which treatment group was scored (blind procedure).

Statistical analyses

All analyses were performed using the statistical software SAS (version 9.4, SAS Institute, Cary, NC, USA). Normal distribution of all variables was assessed using the Shapiro-Wilk test (SAS PROC UNIVARIATE). All variables expressing latencies or durations were log10-transformed to meet the normality assumption.

Birth weights of control and ID animals used in the experiment were compared using a mixed model ANOVA with litter as random effect. The effects of treatment on the growth curves were analyzed with a mixed model analysis of variance (ANOVA) to account for clustering of piglets within litters and repeated measurements within piglets, with the fixed effects Treatment (ID or control), Week, and the Treatment ¬by Week interaction.

From the holeboard trials, the measures in Table 2 were calculated (van der Staay et al., 2012). These measures were analyzed using the mean of four trials, resulting in trial blocks. The first 40 acquisition trials thus divided into trial blocks 1–10 were analyzed, yet not the extra acquisition trials that a piglet received when it had not yet reached the criterion of RM > 0.7 after 40 trials. The following 16 reversal trials were also analyzed in blocks of 4 trials, thus divided into 4 trial blocks (trial blocks 11–14). The holeboard data analyses were performed for three different phases: acquisition, transition and reversal. The transition phase is the switch from the acquisition phase to the reversal phase, i.e., the last trial block of the acquisition compared to the first trial block of the reversal (trial block 10 compared to trial block 11). This is a measure of the response flexibility of an animal: a large difference means that the animal faced difficulties to adapt to the new situation.

Effects of treatment on performance in the habituation trials in the holeboard (six successive trials preceding testing in which all holes were baited), on the learning curves of the acquisition phase (10 successive trial block means of

4 trials each) and reversal phase (4 successive trial block means of 4 trials each), and on the transition between the acquisition and reversal phase, were analyzed using mixed model ANOVAs. For holeboard habituation trials, fixed effects were Treatment, Trial and the Treatment by Trial interaction. For the holeboard acquisition, transition and reversal phase, fixed effects were Treatment, Trial blocks and the Treatment by Trial blocks interaction.

Treatment effects on blood hematocrit, hemoglobin and serum iron were analyzed for five time points (0, 2, 4 and 6 weeks after the start of treatment and at 12 weeks of age, see Figure 1) using mixed model ANOVAs. Fixed effects were Treatment, Week and the Treatment by Week interaction. (Note that the number of observations differed per blood collection moment and per variable due to technical difficulties during either the blood collection or the analyses; for the number of observations per time point and per variable see Supplementary Table 1.) In case of significant interaction effects of Treatment by Week on the blood values, we additionally performed analyses on the separate time points to assess at which time points the differences occurred. For these individual analyses, a Bonferroni correction was applied, to correct for multiple comparisons.

The effects of treatment on brain weight, relative brain weight (% of body weight at euthanasia), average hippocampal weight (mean of the weight of left and right hippocampus), relative hippocampal weight (% of total brain weight) and iron-containing cell count after iron staining of dorsal hippocampal sections were analyzed using a mixed model ANOVA with the fixed effect Treatment. Hippocampal weights and relative hippocampal weights were log10 transformed to meet the normality assumption. In all mixed model analyses, a random effect for litter was added, and the correlation of repeated measures within piglets was addressed using an autoregressive heterogeneous (1) structure for the residuals (SAS PROC MIXED).

Correlations between the linear trend component and general mean of reference memory scores in the acquisition phase and the iron-containing cell count after staining of each hippocampal area (CA3, CA1, DG, sb) were calculated using Pearsons product-moment correlation coefficient.

Holeboard task measures and their definition

Measure	Definition
Working memory (WM)	A ratio defined by the number of visits that yield a food reward divided by the number of visits and re-visits to the rewarded set of holes
Reference memory (RM)	A ratio that is defined by the number of visits and re-visits to the rewarded set of holes divided by the number of visits and re-visits to all holes
Trial duration (TD)	The time between entering the holeboard and finding all four rewards (when not all rewards were found the maximum trial duration of 450 s was recorded)
Inter-visit interval (IVI)	The average time between two hole visits during a trial
Latency to the first visit (LFV)	The latency until the first visit during a trial
Latency to the first reward (LFR)	The latency until the first rewarded visit during a trial
Total visits (TV)	The total number of hole visits made during a trial
Unrewarded visits (URV)	The total number of unrewarded hole visits made during a trial
Rewarded visits (RV)	The total number of rewarded hole visits made during a trial
Number of visits until 1st reward (Vfirst)	The number of hole visits until the first reward was found
Number of visits until 2nd reward (Vsecond)	The number of hole visits between the first and second reward were found
Number of visits until 3rd reward (Vthird)	The number of hole visits between the second and third reward were found
Number of visits until 4th reward (Vfourth)	The number of hole visits between the third and fourth reward were found

Table 2. Measures recorded or calculated in the holeboard task. For an explanation of the 'number of errors per reward' measures, see Gieling, 2013, pp. 173–176. In short, it is the number of errors that were made before the next reward was found.

RESULTS

Weights and growth

Figure 2 shows the body weights of the animals from birth to 12 weeks of age. The birth weights of the selected piglets did not differ between siblings ($t10 = -0.27$; $p = 0.79$). However, over the course of the experiment, the body weights of the control piglets was higher than that of the ID piglets (Treatment: $F_{1,203} = 22.06$; $p < 0.0001$) and control animals grew faster than ID animals (Treatment by Week interaction; $F_{12,203} = 3.13$; $p = 0.0004$). This difference in body weights between treatment groups was present from week 3 onward (Week 3: $F_{1,203} = 5.00$; $p = 0.03$, and all subsequent weeks with associated $p < 0.05$).

Holeboard performance

All results of the statistical analyses of the holeboard performance in all phases (habituation, acquisition, transition and reversal) are shown in Supplementary

Table 2.

Habituation trials
In the habituation phase (six successive trials in which all holes were baited, which preceded formal testing), ID piglets made fewer total visits (Treatment: $F_{1,87}$ = 5.70; p = 0.02) and found fewer rewards ($F_{1,87}$ = 7.72; p = 0.01) than control animals. No trial effect or interaction effect was found (Supplementary Table 2A). In the last habituation trial, the number of total visits (Trial: $F_{5,87}$ = 2.23; p = 0.06, Treatment by Trial interaction: $F_{1,87}$ = 0.01; p = 0.92) and rewards found (Trial: $F_{5,87}$ = 1.44; p = 0.22, Treatment by Trial interaction: $F_{1,87}$ = 0.01; p = 0.91) did not differ between treatment groups (see Supplementary Figure 1). Thus, all piglets searched throughout the entire holeboard for rewards equally effective at the end of the habituation trials.

Working memory
Acquisition phase: Both groups showed a similar increase in working memory (WM) performance over the successive acquisition trial blocks (Trial blocks: $F_{9,149}$ = 2.17; p = 0.03; Figure 3A). No difference was found in WM in the average performance level between treatment groups, nor an interaction between Treatment and Trial blocks.

Transition: When switching from the acquisition to the reversal phase, WM performance dropped similarly in both groups (Trial blocks effect: $F_{1,23}$ = 46.25; p < 0.0001). The average WM performance level during transition was unaffected by treatment.

Reversal phase: WM performance of both groups increased similarly during the reversal phase (Trial blocks: $F_{3,55}$ = 11.41; p < 0.0001). There was a trend that control animals had, on average, a higher WM performance in the reversal phase than the ID animals ($F_{1,55}$ = 3.27; p = 0.08). No interaction between Treatment and Trial blocks was found (Supplementary Table 2B).

Reference memory
Acquisition: Both groups of piglets showed the same increase in reference memory (RM) performance during acquisition (Trial blocks: $F_{9,151}$ = 22.13; p < 0.0001). The control piglets had, on average, higher RM scores than the ID piglets (Treatment: $F_{1,151}$ = 17.56; p < 0.0001; Figure 3B). No interaction

between Treatment and Trial blocks on RM performance was found.

Transition: RM scores dropped in the transition from acquisition to reversal (Trial blocks: $F_{1,23} = 66.75$; $p < 0.0001$). Although inspection of Figure 3A suggests that the drop in performance of the control group was larger than that of the ID group, this impression was not confirmed statistically (Treatment by Trial blocks interaction: $F_{1,23} = 2.18$; $p = 0.15$). On average, the RM scores of the control animals during transition were higher than those of the ID piglets (Treatment: $F_{1,23} = 5.88$; $p = 0.02$).

Reversal: RM scores increased in the reversal phase for all animals (Trial blocks: $F_{3,55} = 23.98$; $p < 0.0001$). Control animals had higher average RM scores during this phase than ID animals (Treatment: $F_{1,55} = 5.14$ $p = 0.03$). No interaction between Treatment and Trial blocks was found (Supplementary Table 2B).

Durations and latencies
In both groups, the trial duration (TD), the inter-visit interval (IVI) and the latency until the first reward was found (LFR) decreased over time in both the acquisition phase and reversal phase (Trial blocks effect, Supplementary Table 2B), whereas the latency to the first visit (LFV) only declined in the reversal phase. All duration and latency measures increased in the transition from acquisition to reversal, except for LFV. Treatment had no effect on TD (Figure 3B), IVI, LFV or LFR in any phase, nor were any interactions found between Treatment and Trial blocks for these measures (Supplementary Table 2B).

Number of hole visits
The total number of visits (TV), the number of unrewarded visits (URV) and the number of rewarded visits (RV) did not differ between the groups in any phase (Supplementary Table 2B). For all animals, TV and URV decreased during the acquisition and reversal phase, and increased in the transition from acquisition to reversal. RV only increased in the acquisition phase (Trial blocks, Supplementary Table 2B). No interaction effects were found for these measures.

Number of visits before finding the 1st, 2nd, 3rd and 4th reward
The ID group made more visits in the acquisition phase before finding the first (Treatment: $F_{1,149} = 8.33$; $p = 0.004$) and second (Treatment: $F_{1,148} = 5.35$; p
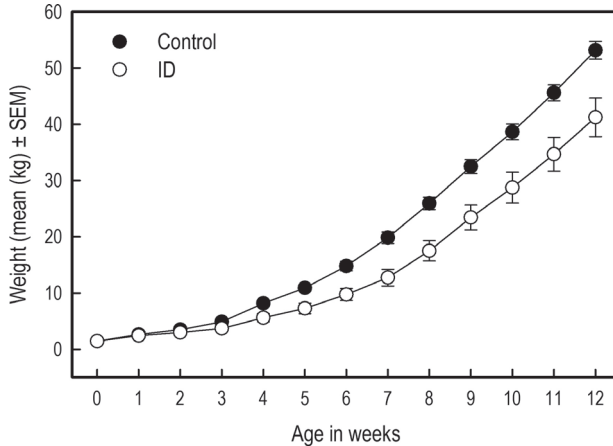
Figure 2. The body weights of ID (n = 8) and control (n = 10) piglets in kilograms over the course of the experiment. The difference in body weights between treatment groups was significant from week 3 onward (week 3: p = 0.03). Note: The absence of error bars indicates that the SEM is smaller than the plot symbol.

= 0.02) reward than the control group did. Additionally, the ID pigs needed more visits to find the fourth reward in the transition to a different set of rewarded holes (Treatment: $F_{1,19}$ = 12.75; p = 0.002) and during the reversal phase (Treatment: $F_{1,51}$ = 15.15; p = 0.0003). The increase in number of visits before finding the fourth reward from the last acquisition to the first reversal trial block was larger in ID piglets than in control animals (Treatment by Trial blocks interaction: $F_{1,19}$ = 4.41; p = 0.049).

Blood iron values

For all three blood parameters hematocrit (Hct) (Treatment by Week interaction: $F_{4,58}$ = 14.11; p < 0.0001) hemoglobin (Hb) ($F_{4,58}$ = 35.90; p < 0.0001) and serum iron ($F_{3,55}$ = 16.54; p < 0.0001), values showed a different curve for the two treatment groups (Supplementary Table 3). In order to investigate at which time points there were treatment effects on these measures, we additionally looked at the effects of treatment per sampling time point (Supplementary Table 4). Because a Bonferroni correction was thus applied, differences with an associated p-value of < 0.01 were considered significant in these analyses.

Before the start of the experimental diet, blood hematocrit ($F_{1,58}$ = 0.30; p =

0.59) and hemoglobin ($F_{1,58}$ = 0.39; p = 0.54) values did not differ between siblings. After 2 weeks of dietary treatment, Hct ($F_{1,58}$ = 23.24; p < 0.0001) and Hb values ($F_{1,58}$ = 44.90; p < 0.0001) but not serum iron ($F_{1,55}$ = 3.42; p = 0.07) were lower in the ID group than in the control group. After 4 weeks and thus at the end of treatment, Hct ($F_{1,58}$ = 30.79; p < 0.0001), Hb ($F_{1,58}$ = 134.28; p < 0.0001) and serum iron ($F_{1,55}$ = 48.29; p < 0.0001) were all lower in ID animals than in control animals (Figure 4). Six weeks after the start of treatment, which was two weeks after the transition to regular feed, Hct ($F_{1,58}$ = 8.82; p = 0.004) and Hb ($F_{1,58}$ = 17.87; p < 0.0001) values were still higher in control animals than in ID animals. Serum iron did not differ between treatment groups at this stage ($F_{1,55}$ = 0.87; p = 0.35). At 12 weeks of age, thus 7.5 weeks after treatment and transition to regular feed, ID animals tended to show higher Hct values than control animals ($F_{1,58}$ = 5.79; p = 0.02), but no differences in Hb ($F_{1,58}$ = 3.86; p = 0.05) or serum iron ($F_{1,55}$ = 0.14; p = 0.70) were found between treatment groups (Figure 4; Supplementary Table 4).

Brain weights

Treatment did not affect the absolute brain weight at 12 weeks of age ($F_{1,6}$ = 1.58; p = 0.26). The ID animals had, however, higher brain weights relative to their total body weights (0.20 ± 0.017 %) than the control animals (0.16 ± 0.005 %) ($F_{1,6}$ = 11.88; p = 0.014). Treatment had no effect on absolute hippocampus weight ($F_{1,6}$ = 0.64; p = 0.45) nor on the hippocampal weight relative to the total brain weight ($F_{1,6}$ = 0.13; p = 0.73).

Brain iron concentrations

Iron-containing cell count following iron staining showed a difference in iron-containing cells between treatment groups in two hippocampal regions (Figure 5 and 6). In the CA3 region, there was no significant difference between control group and ID group ($F_{1,6}$ = 3.47; p = 0.11). In the CA1 region, the sections of ID animals contained fewer iron-containing cells compared to sections of control animals ($F_{1,6}$ = 10.67; p = 0.02). Similarly, in the dentate gyrus (DG) there were fewer iron-containing cells in ID pigs' sections compared to control pigs' sections ($F_{1,6}$ = 25.18; p = 0.002). There was no significant difference in
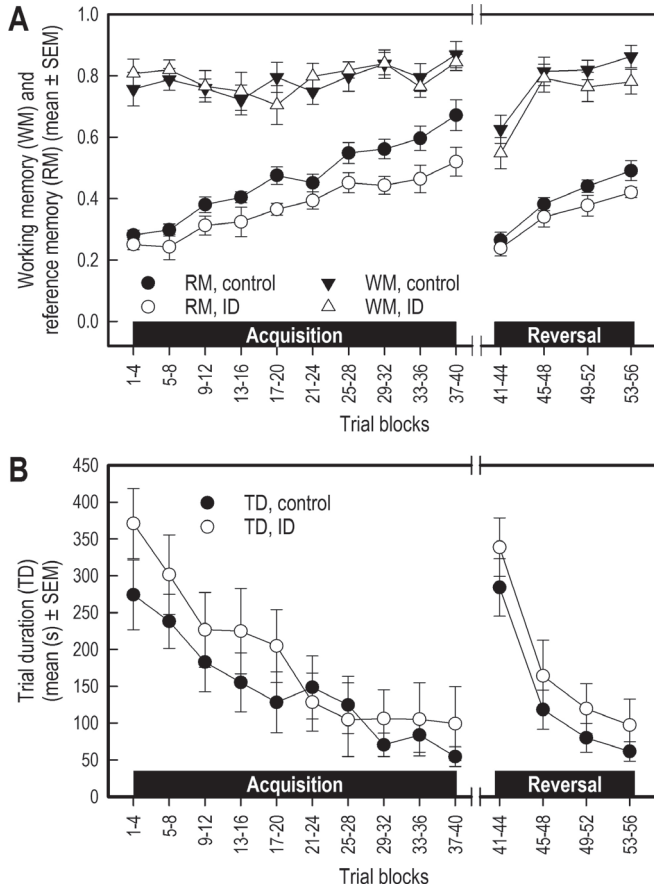
Figure 3. Performance of ID (n = 8) and control (n = 10) piglets in the spatial cognitive hole-board task during the acquisition phase (trials 1-40) and the reversal phase (trials 41-56). (A) Working memory (WM) and reference memory (RM) performance. ID animals showed impaired RM performance in the acquisition, transition and reversal phase; see Supplementary Table 2 for results of the statistical analyses. (B) Trial duration (TD). Note that TD was analyzed statistically after log10 transformation whereas the untransformed means and SEMs are depicted here.

iron-containing cells in the subiculum between the treatment groups.

Correlations between brain iron and memory performance

The mean RM scores in the acquisition phase of all animals correlated positively with the number of iron-containing cells in CA3 sections (rpm = 0.520; p = 0.03; see Supplementary Table 5). This exploratory correlation analysis suggests a moderate relationship between the mean RM performance during acquisition and iron content in the hippocampal CA3 region (Figure 7).

DISCUSSION

This study investigated the effects of early-life iron deficiency on growth, cognitive performance, blood parameters and brain histology in piglets later in life. Our results show that severe pre-weaning iron deficiency in piglets causes impairments in physical and mental development, and has long-lasting effects on brain histology.

Iron deficiency impairs growth

At the start of the experiment, siblings did not differ in birth weight. However, over the course of the experiment, the body weights of the control animals were higher than those of the early iron deficient (ID) animals and the control animals had a higher growth rate. The weight difference became apparent starting from the third week of age and lasted until the end of the experiment, although iron deficiency treatment had ended at approximately 5 weeks of age. As we chose to group-house our piglets, we could not measure individual food intake, which ideally should be taken into account in nutritional studies that include growth performance as a read-oud parameter (e.g. Fiset et al., 2015). However, iron deficiency is well-known to impair growth and cause reduced appetite in both rats (Beard et al., 1995) and piglets (Ishaya, 2012). A study investigating the effects of iron deficiency on T-maze performance in piglets found no difference in body weights between ID and control animals (Rytych et al., 2012). However, that study lasted until the age of 28 days, whereas we measured body weights until 12 weeks of age and did not find a difference in weights until 3 weeks of age.

In an iron deficiency study in rats, it was found that impaired growth due to iron deficiency was caused by negative effects on the cell division in growing organs (Canale and Lanzkowsky, 1970). In human infants, iron deficiency also causes poor growth and reduced weight gain (Ryan, 1997). Growth is an important indicator of health and nutritional status of children, as disturbances in health and nutrition invariably affect child growth (de Onis et al., 2006). Our data support the notion that iron is an essential micronutrient for neonatal growth and development.
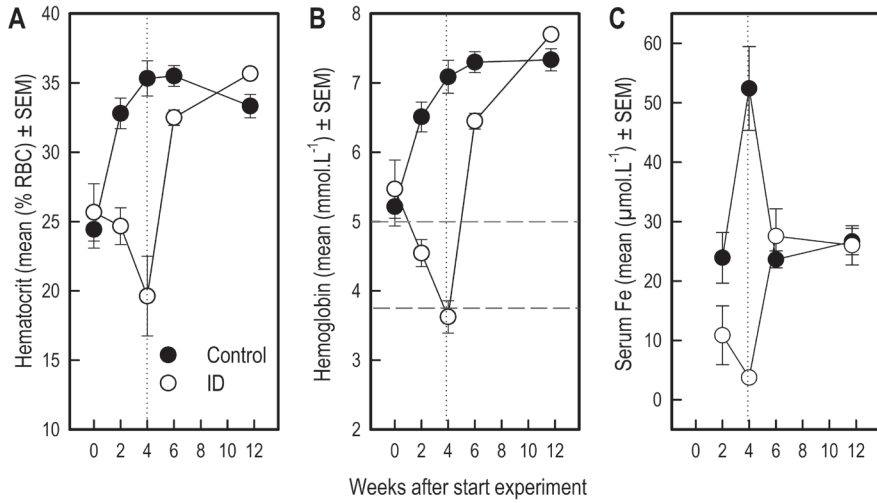
Figure 4. Blood iron values in ID and control animals from 0 to 12 weeks after the start of the experiment (4-6 days of age). Dietary ID treatment lasted for 28 days (dotted vertical line). (A) Hematocrit values (% red blood cells); (B) Hemoglobin values. The upper dashed horizontal line indicates a hemoglobin value of 5, below which piglets are considered anemic, the lower dashed line indicates a value of 3.75, below which piglets are considered severely anemic (Ishaya, 2012); (C) Serum iron values. Note that the number of observations varied per measure and sampling time point, as reported in Supplementary Table 1. See Supplementary Table 3 and 4 for results of the statistical analyses.
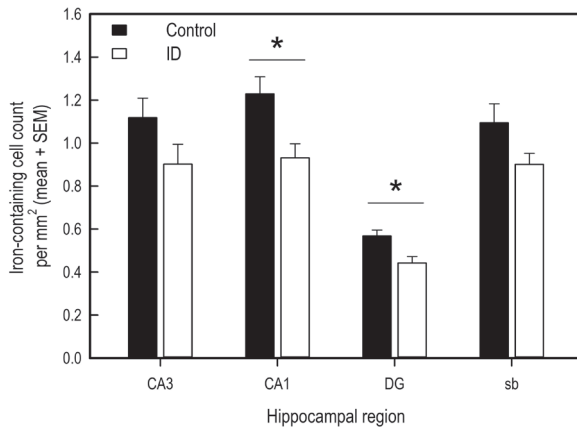

Figure 5. Number of iron-containing cells per mm2 in iron stained hippocampal sections of ID (n = 8) and control (n = 9) animals in hippocampal regions CA3, CA1, dentate gyrus (DG) and subiculum (sb) at 12 weeks of age.
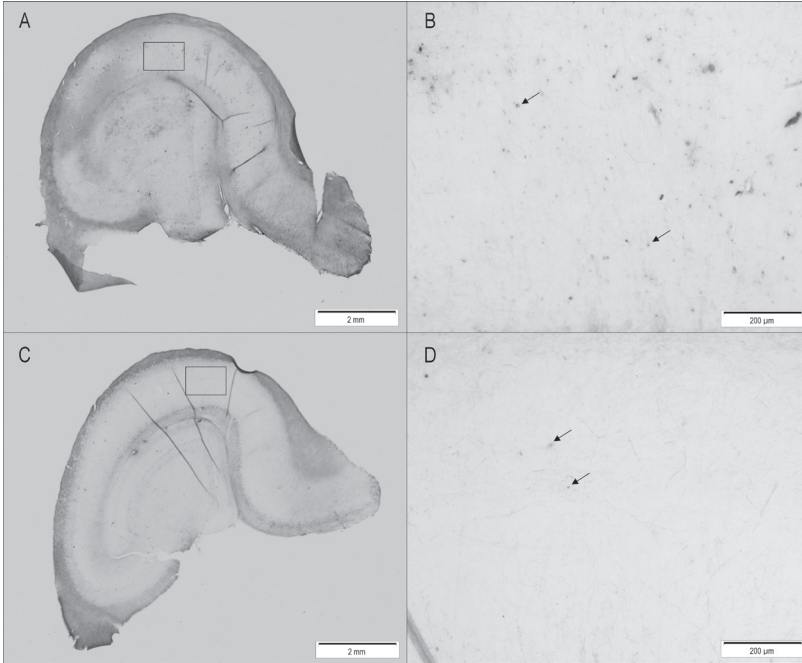
Figure 6. Representative pictures of iron stained hippocampal sections of control animals (A and B) and ID animals (C and D) at 12 weeks of age. Sections of ID animals contained significantly fewer iron-containing cells in CA1 (p = 0.02) and dentate gyrus (p = 0.002) regions than sections of control animals (A and C: scale bar = 2 mm, magnification 1.26×; B and D: scale bar = 200 μm, magnification 12.6×).

Figure 7. Correlation between average reference memory scores in the acquisition phase of the holeboard task and iron-containing cell count in hippocampal sections after iron staining of ID (n = 8) and control (n = 9) animals at 12 weeks of age.

Impaired memory performance due to iron deficiency treatment

Our study confirms earlier findings that (young) piglets are able to acquire the holeboard task (Arts et al., 2009; Gieling et al., 2012; Bolhuis et 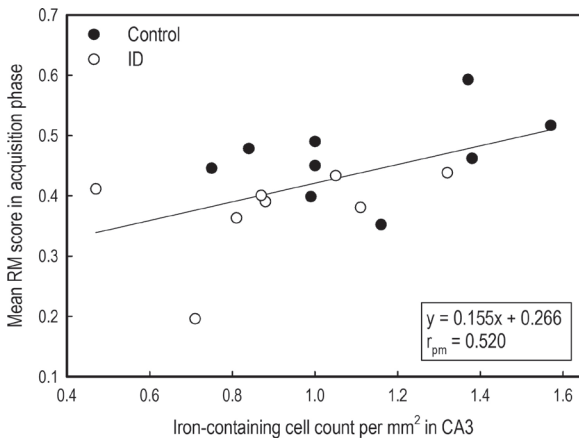al., 2013; Haagensen et al., 2013). All piglets acquired the task: their memory scores improved over time and trial durations and latencies declined over the course of the experiment. During the six habituation trials preceding testing in which all holes were baited, ID piglets made fewer total visits and found fewer rewards than control piglets, i.e. showed less exploration. However, this effect had disappeared by the end of the habituation trials.

This reduced exploration or motivation may be due to the main behavioral symptoms of iron deficiency, such as lethargy, lack of concentration and attentional problems (Lozoff and Brittenham, 1986; Pollitt, 1993). Although we did not record this behavior, ID piglets already seemed to need more time to learn to search for food rewards than control piglets during habituation to the testing environment, i.e preceding the habituation trials. In an iron deficiency study in rats, it was found that ID rats showed decreased locomotion and a slower rate of habituation (Beard et al., 2002). In future behavioral studies looking at the effects of iron deficiency in pigs, it may therefore be interesting to record behaviors such as time needed for habituation prior to testing.

During the acquisition and reversal phase, ID pigs reached lower reference memory (RM) scores than the control pigs. These results confirm findings from human studies that a major symptom of iron deficiency is a decline in cognitive performance (Yehuda and Youdim, 1989). The reduced exploration or motivation found in the habituation trials as expressed in fewer total visits and fewer rewards found, was not found in the acquisition or reversal trials. As RM is calculated as a ratio, both components of the ratio would be equally affected by a reduction in exploration or motivation, and thus the outcome would not change. Measures that can serve as indices of motivation or speed, such as trial duration, inter-visit interval and latency to the first visit, did not differ between the groups. Moreover, RM performance at the start of both the acquisition and reversal phase did not differ between groups, indicating that both groups started out at the same level of performance in both phases. Similarly, the number of rewarded visits did not differ between groups in any phase. This supports the notion that the reduced performance of the ID animals was a cognitive effect,

and was not caused by low motivation to perform the task.

The effects of iron deficiency on several types of cognitive performance have been tested in humans, such as selective attention, working memory, executive function tasks and spatial memory (Lozoff and Georgieff, 2006). With the present study we confirm in particular the finding that iron deficiency leads to impairments in reference memory performance. This corroborates earlier findings in rats, in which declines in RM scores were found in the Morris water maze up to four weeks after iron repletion (Yehuda et al., 1986). Comparably, RM performance was affected in radial arm maze performance of rat pups born to ID mothers (Ranade et al., 2013). In a study investigating the effects of iron deficiency on spatial memory in a double T-maze task in pigs, severely ID piglets could not acquire a food motivated double T-maze task, whereas mildly ID piglets showed deficits in reversal learning (Rytych et al., 2012). However, the diets in that study were fed for the total duration of the study, thus piglet performance in the study by Rytych et al. does not provide information about long-term effects of early-life iron deficiency. The piglets in the current study were tested approximately 3 weeks after treatment ended (Figure 1).

The hematocrit and hemoglobin values of ID pigs two weeks after treatment ended indicate that they may not have been fully iron repleted at the start of behavioral testing, which was one week later. However, it may be argued that the slow repletion progress indicates that treatment caused at least some physiological changes to occur, such as a reduced iron uptake ability of ID pigs. Our finding that brain iron was still reduced at 12 weeks of age, which was around 7.5 weeks after treatment ended, is also an indicator that ID pigs were not able to fully recover their ID status within the duration of our study. In an MRI study in pigs, it was found that rapid brain growth in piglets lasts until around 12 weeks of age (Conrad et al., 2012). Thus, although the pig brain is not yet fully grown by that age, most of the growth spurt and brain development has occurred by that time.

To assess whether ID treatment affects cognitive development after this period of rapid brain growth, it may be interesting to monitor and assess physical and cognitive development for a longer period of – or at a later – time. For example, an iron deficiency study in guinea-pigs found decreased locomotor activity at 42 days as compared to 24 days of age (Fiset et al., 2015), suggesting

that some detrimental effects of iron deficiency may not show until later in life. Due to a lack of space to house the rapidly growing pigs in our experimental facilities, we could not conduct our study for a prolonged period of time.

Our findings, in combination with previous findings of iron deficiency studies, suggest that early-life iron deficiency causes developmental deficits in the brain. It is suggested that the detrimental effects of iron deficiency on cognition involves the reduction of brain iron, receptor and transporter densities, and changes in serotonin and dopamine pathways (McCann and Ames, 2007; Youdim et al., 2010).

Additional to and in line with the negative effects of iron deficiency on memory scores, ID piglets made more visits before finding the first and second reward in the acquisition phase, and more visits before finding the fourth reward in the transition and reversal phase. When making more errors during a task, memory load is increased, as more information has to be stored for a longer period of time then when fewer errors are made. This increase in errors can therefore be an indication that memory load was increased for ID piglets, or that the ID piglets' executive attention to complete the task was reduced (Gieling et al., 2014). This is interesting with regard to iron deficiency, as studies in humans have shown that children that suffered early-life iron deficiency display attentional problems (Lozoff and Georgieff, 2006).

Working memory (WM) and trial duration (TD) scores did not differ between the treatment groups in any phase. Our results thus support the notion that WM and RM calculated in the manner of this study are independent measures that measure different forms of memory (van der Staay et al., 2012). However, visual inspection of the graphical representations of WM and TD results (Figure 3) suggests that the control group had higher WM scores than the ID group in the reversal phase and lower TD over the entire course of the experiment. These impressions, however, were not statistically confirmed.

Anemia in ID animals, iron overload in control animals?

During treatment, all blood iron values decreased in ID animals and increased in control animals. At 12 weeks of age, 7.5 weeks after dietary treatment and transition to regular feed, blood iron values of both treatment groups recovered to similar values. These results show that piglets have some iron stores

at birth, which deplete within several weeks after birth if not given iron treatment, either dietary or by means of an iron injection.

In a similar study investigating the effects of early-life iron deficiency on blood parameters in piglets, three groups of piglets were formed: a control group, a mildly ID and a severely ID group (Rytych et al., 2012). As in our study, control animals received a 200 mg iron dextran injection. Piglets were separated from the sow 48 hours after farrowing, housed individually and fed the assigned milk formula diet. The control group was fed a 100 mg iron/kg diet and the severely ID group 10 mg iron/kg diet, which are similar values to those in the current study. Hb values of their ID animals dropped to lower levels as our ID animals after 4 weeks of treatment (2.7 mmol/L compared to 3.7 mmol/L). Hematocrit values followed a comparable decline in their (severely) ID animals and increase in control animals as in the current study.

Hematocrit

In a study investigating different administration methods and injection sites of iron on hematocrit (Hct) values in piglets, Hct values varied from 29% at 4 days of age (before iron injection) to 40% at 28 days of age (Koch and Hines, 1969). Different methods and injection sites did not significantly affect Hct values. Buzzard et al. (2013) found average Hct values of 29% in healthy piglets between 3 and 4 weeks of age. Although the values found in the control animals in our study are slightly higher at these ages, their Hct values do fall within the range that Koch and Hines found during and after iron treatment, and the Hct values of our ID animals dropped far below this range to 19% at the end of treatment. After transition to regular feed, Hct values of ID animals recovered to values similar to those of control animals.

Hemoglobin

The most common parameter to indicate iron deficient anemia is hemoglobin (Hb) (McLean et al., 2009). Hb levels of 6.25 mmol/L or above are assumed normal in piglets, with pigs considered anemic below value of 5 mmol/L. Growth is affected below 4.375 mmol/L and a value below 3.75 mmol/L indicates severe anemia (as converted from g/dL values in Ishaya, 2012). Our results thus imply that all piglets were nearly anemic before treatment, Hb values of control animals rose to normal levels due to iron treatment, and ID animals became severely anemic (3.63 ± 0.23 mmol/L) at the end of treatment.

Indeed, as Ishaya (2012) argues happens below a Hb value of 4.375 mmol/L, growth of ID animals was impaired, even after treatment had ended. After transition to regular feed, ID animals' Hb values recovered to normal values, similar to those of control animals.

Serum iron

In our control animals, we found surprisingly high values of serum iron at the end of treatment (52.41 ± 7.06 μmol/L). High levels of blood serum iron may occur due to iron overload (Cornelius and Kaneko, 1963), which may have been the case in our control animals, as they received both an iron injection and an iron-sufficient diet during treatment. Serum iron of ID animals dropped severely during treatment (3.74 ± 0.21 μmol/L), showing that iron reserves were nearly depleted by the end of treatment. Large variation in piglet serum iron values have been reported (Bernát, 1983). In a study investigating factors that influence serum iron in pigs, control animals showed values within the range of 18 to 35 μmol/L from 0 to 15 weeks of age (Braham et al., 1967), which is in line with the values found in all animals in our study two weeks after treatment had ended.

Our results show that piglets are born with low iron stores at birth, which deplete quickly if not given iron treatment, and that an iron administration in common husbandry practice may cause iron overload. An excess of iron can be toxic and cause oxidative damage (Lipiński et al., 2010). In their study, Lipiński et al. showed that by administrating iron to piglets in two doses at day 3 and 10 instead of in one dose, toxicity can be reduced and iron uptake is improved. Moreover, Yu et al. (2002) argue that a 200 mg iron dextran injection does not contribute to overall performance of piglets at 15 days of age when creep feed is provided from day 7 onward, and consequently may not be a necessity.

Brain sparing in iron deficient animals

The absolute weights of the brains at 12 weeks of age did not differ between the groups. However, as the ID animals were lighter than the control animals, the relative brain weight compared to total body weight was higher in the ID group than in the control group. This suggests that brain sparing has occurred in the ID animals, meaning that proportionally more energy or nutrients were used

for brain development compared to the rest of the body due to their restricted growth (Dobbing, 1971; Hall, 2011) (considering the lower brain iron content in ID as opposed to control animals, this does not include the distribution of iron). For the weights of hippocampi, no differences were found between the groups. A study looking at a rat iron deficiency model found reduced volumes of the hippocampus in ID rats (Ranade et al., 2013). It is possible that we did not find these effects because we measured weight instead of volume.

Early-life iron deficiency reduces hippocampal iron content

Brain histology after scheduled necropsy at 12 weeks of age showed that in hippocampal regions CA1 and the dentate gyrus (DG), fewer iron-containing cells were present in ID animals than in control animals. During early development, iron is prioritized to red blood cells at the expense of the brain and other organs when iron supply does not meet the demand (Lozoff and Georgieff, 2006). This may explain why regional brain iron content, but not blood iron values, were lower in ID pigs than in control animals at 12 weeks of age.

In an iron deficiency study in rat pups, in which a repletion group received an iron-adequate diet after two weeks of ID dietary treatment, brain iron and ferritin were normalized after 14 days, whereas brain transferrin was higher than in control animals (Erikson et al., 1997). In their ID group, which received the ID diet for the entire duration of the study, regional brain iron was decreased in cortex and hippocampus. Transferrin concentrations increased drastically in the hippocampi of these ID animals. Transferrin is involved in iron transport and distribution in all tissues, and elevated levels are associated with iron deficiency anemia (Macedo and Sousa, 2008). In another iron deficiency study with rats that looked at the effects of timing and duration of iron deficiency on brain iron metabolism, two weeks of iron repletion after an ID diet from postnatal day 10 to 21 was sufficient to restore (both overall and regional) brain iron concentration (Piñero et al., 2000). It is in comparison to these studies thus surprising that in our piglets we found iron levels to still be lower in ID animals compared to control animals after 7.5 weeks of iron repletion; even when blood iron values had restored to normal. It may be that as the dietary treatment in our study was longer than in the rat studies repletion groups (four weeks vs. two weeks or eleven days, respectively), and at a relatively later developmental stage as rat brains show a different degree of maturation (Romijn et

al., 1991), the induced iron deficiency in our ID piglets had stronger negative effects on brain iron concentrations.

The CA1 region plays an important role in short- and intermediate-term memory and self-location (Kesner et al., 2004; Barry and Burgess, 2014). The dentate gyrus (DG) has been suggested to play an important role in learning and memory by processing and representing spatial information (Kesner et al., 2004). Specific destruction of DG cells in rats caused a decrease in performance in a previously acquired radial arm maze task (Tilson et al., 1988; Walsh et al., 1986). In an iron deficiency study that investigated the effects of maternal iron deficiency on rat pup performance, reduced neurogenesis and reduced hippocampal pyramidal and granule cells correlated to impaired radial arm maze performance, in which especially reference memory (RM) scores were affected (Ranade et al., 2013). This correlation between detrimental effects on hippocampal histology and a reduction in memory performance due to iron deficiency is in line with our finding that RM scores correlated positively to iron-containing cells in the CA3 region of the hippocampus.

Our findings that early-life iron deficiency causes both a decrease in memory performance later in life and in brain iron in hippocampal regions that play a role in learning and memory, strongly suggest that iron is essential for these structures to develop and function normally. Although these results may in part be attributed to ID pigs not having fully recovered their iron status at the time of testing, our results do suggest that early-life iron deficiency affects early development of pigs at least for an extended period of time after ID treatment has ended, which has not been shown before in pigs. These findings corroborate previous findings of early-life iron deficiency studies in both humans and rodents.

CONCLUSION

Our results show that severe pre-weaning iron deficiency in piglets leads to impaired physical and cognitive development later in life. Iron deficient (ID) animals showed retarded growth and impaired reference memory performance in the holeboard task weeks after ID treatment had ended. Our results suggest that early-life iron deficiency affects early development of pigs for an extended period of time. During treatment, blood parameters showed that ID animals

became severely anemic, yet their blood values recovered to normal values after transition to iron-sufficient feed after weaning. Serum iron values of control animals showed an indication that iron overload occurred, which may be due to the possibly excessive and toxic administration of 200 mg iron per piglet, which is common in current pig husbandry practice. Brain histology at 12 weeks of age (7.5 weeks after transition to iron-sufficient feed) showed that hippocampal regions CA1 and dentate gyrus of ID animals had fewer iron-containing cells than those of control animals. The number of iron-containing cells in the CA3 region correlated positively to reference memory performance in the acquisition phase of all animals. Our findings strongly suggest that iron is essential for brain structures involved in memory and learning to develop and function normally. We conclude that piglets can be used as a model animal to investigate the long-term effects of early-life diets and dietary-induced deficits, and that the holeboard is a sensitive enough task to detect these effects. This task can be used in future research to unravel the pathway through which iron deficiency affects cognition and to develop therapies for treating iron deficiency in both the pig industry as well as in humans.

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIAL

Supplementary Figure 1. The total number of visits (TV) and number of rewards found (REW) in ID (n = 8) and control (n = 10) animals in the six separate habituation trials in the holeboard task, in which all sixteen holes were baited with a reward.

Number of samples per time point of blood collection						
		Age in weeks				
Measure	**Treatment**	**0**	**2**	**4**	**6**	**12**
Hematocrit (Hct)	ID	6	8	8	6	6
	control	7	10	9	10	6
Hemoglobin (Hb)	ID	6	8	9	6	6
	control	7	10	9	10	6
Serum iron	ID	ND	8	9	8	8
	control	ND	10	10	10	9

Supplementary Table 1. Number of observations per blood collection for blood value analysis.

A) Holeboard habituation (Hab)										
		Treatment			Trials			Treatment x Trials		
Measure	Phase	F	df	P≤	F	Df	P≤	F	df	P≤
Total number of visits (TV)	Hab	5.70	1,87	**0.0191**	2.23	5,87	0.0579	0.45	5,87	0.8154
Rewards found (REW)	Hab	7.72	1,87	**0.0067**	1.44	5,87	0.2192	0.44	5,87	0.8181

B) Holeboard acquisition (Acq), transition (Trans), reversal (Rev)										
		Treatment			Trial blocks			Treatment x Trial blocks		
Measure	Phase	F	df	P≤	F	Df	P≤	F	df	P≤
Working memory (WM)	Acq	0.00	1,149	0.9992	2.17	9,149	**0.0273**	0.66	9,149	0.7460
	Trans	1.22	1,23	0.2799	46.25	1,23	**<0.0001**	0.44	1,23	0.5119
	Rev	3.27	1,55	0.0761	11.41	3,55	**<0.0001**	0.33	3,55	0.8018
Reference memory (RM)	Acq	17.56	1,151	**<0.0001**	22.13	9,151	**<0.0001**	1.15	9,151	0.3285
	Trans	5.88	1,23	**0.0235**	66.75	1,23	**<0.0001**	2.18	1,23	0.1530
	Rev	5.14	1,55	**0.0273**	23.98	3,55	**<0.0001**	0.30	3,55	0.8264
Trial duration (TD)	Acq	0.97	1,151	0.3258	9.66	9,151	**<0.0001**	1.32	9,151	0.2290
	Trans	1.63	1,23	0.2144	94.09	1,23	**<0.0001**	0.13	1,23	0.7199
	Rev	1.52	1,55	0.2225	42.51	3,55	**<0.0001**	0.03	3,55	0.9915
Inter-visit-interval (IVI)	Acq	0.39	1,151	0.5320	2.19	9,151	**0.0257**	1.25	9,151	0.2716
	Trans	0.74	1,23	0.3994	24.55	1,23	**<0.0001**	0.09	1,23	0.7613
	Rev	0.58	1,55	0.4484	18.53	3,55	**<0.0001**	0.46	3,55	0.7088
Latency first visit (LFV)	Acq	0.60	1,151	0.4399	0.79	9,151	0.6279	0.30	9,151	0.9744
	Trans	0.15	1,23	0.6976	0.30	1,23	0.5866	0.04	1,23	0.8476
	Rev	0.53	1,55	0.4697	3.17	3,55	**0.0313**	0.24	3,55	0.8711
Latency first rewarded visit (LFR)	Acq	2.70	1,151	0.1022	4.32	9,151	**<0.0001**	0.52	9,151	0.8558
	Trans	0.67	1,23	0.4215	34.30	1,23	**<0.0001**	0.20	1,23	0.6561
	Rev	0.73	1,55	0.3967	12.96	3,55	**<0.0001**	0.11	3,55	0.9546
Total number of visits (TV)	Acq	0.12	1,151	0.7297	5.33	9,151	**<0.0001**	1.18	9,151	0.3124
	Trans	1.29	1,23	0.2679	100.92	1,23	**<0.0001**	0.01	1,23	0.9057
	Rev	1.74	1,55	0.1931	15.12	3,55	**<0.0001**	1.22	3,55	0.3109
Unrewarded visits (URV)	Acq	0.71	1,151	0.3991	7.11	9,151	**<0.0001**	1.16	9,151	0.3268
	Trans	1.27	1,23	0.2715	118.57	1,23	**<0.0001**	0.00	1,23	0.9670
	Rev	2.48	1,55	0.1213	21.72	3,55	**<0.0001**	1.16	3,55	0.3324
Rewarded visits (RV)	Acq	1.33	1,151	0.2510	2.11	9,151	**0.0322**	0.83	9,151	0.5867
	Trans	0.02	1,23	0.8826	2.63	1,23	0.1182	0.28	1,23	0.6025
	Rev	0.12	1,55	0.7309	0.64	3,55	0.5948	1.02	3,55	0.3895
Rewards found (REW)	Acq	0.63	1,151	0.4280	1.57	9,151	0.1296	0.96	9,151	0.4765
	Trans	1.01	1,23	0.3261	24.92	1,23	**<0.0001**	0.11	1,23	0.7423
	Rev	0.70	1,55	0.4067	9.90	3,55	**<0.0001**	0.32	3,55	0.8133
Visits before 1st reward (Vfirst)*	Acq	8.33	1,149	**0.0045**	6.16	9,149	**<0.0001**	0.41	9,149	0.9302
	Trans	0.47	1,23	0.5004	15.81	1,23	**0.0006**	0.85	1,23	0.3664
	Rev	0.09	1,55	0.7706	4.62	3,55	**0.0059**	0.48	3,55	0.6968
Visits before 2nd reward (Vsecond)*	Acq	5.35	1,148	**0.0221**	7.79	9,148	**<0.0001**	0.45	9,148	0.9061
	Trans	1.26	1,23	0.2725	37.13	1,23	**<0.0001**	2.71	1,23	0.1132
	Rev	1.09	1,55	0.3014	9.52	3,55	**<0.0001**	1.06	3,55	0.3740
Visits before 3rd reward (Vthird)*	Acq	2.08	1,144	0.1511	8.11	9,144	**<0.0001**	0.65	9,144	0.7532
	Trans	0.38	1,21	0.5439	40.46	1,21	**<0.0001**	0.00	1,21	0.9713
	Rev	0.56	1,52	0.4595	9.26	3,52	**<0.0001**	0.11	3,52	0.9548
Visits before 4th reward (Vfourth)*	Acq	1.27	1,131	0.2612	10.07	9,131	**<0.0001**	1.76	9,131	0.0816
	Trans	12.75	1,19	**0.0020**	39.14	1,19	**<0.0001**	4.41	1,19	**0.0492**
	Rev	15.15	1,51	**0.0003**	10.31	3,51	**<0.0001**	1.86	3,51	0.1486

Supplementary Table 2. Performance of ID and control animals in the spatial cognitive holeboard task during habituation (hab) (A), and the acquisition (Acq), transition (Trans), and reversal (Rev) phases (B). *: For further information about the operational definitions of these variables, see Gieling, 2013, 173–176

Treatment effects on blood parameters

Blood parameter	Treatment			Week			Treatment x Week		
	F	df	P≤	F	df	P≤	F	df	P≤
Hematocrit	21.75	1,58	**<0.0001**	19.63	4,58	**<0.0001**	14.11	4,58	**<0.0001**
Hemoglobin	38.01	1,58	**<0.0001**	51.96	4,58	**<0.0001**	35.90	4,58	**<0.0001**
Serum iron	25.91	1,55	**<0.0001**	2.39	3,55	0.0790	16.54	3,55	**<0.0001**

Supplementary Table 3. Treatment effects on hematocrit, hemoglobin and serum iron of ID and control pigs in a mixed models analysis with Treatment effect, Week effect and their interaction. See Supplementary Table 4 for effects per sampling time point.

Effects of treatment per sampling time point

	Hematocrit			Hemoglobin			Serum iron		
wk	F	df	P≤	F	df	P≤	F	df	P≤
0	0.30	1,58	0.5888	0.39	1,58	0.5362	-	-	-
2	23.24	1,58	**<0.0001**	44.90	1,58	**<0.0001**	3.42	1,55	0.0697
4	30.79	1,58	**<0.0001**	134.28	1,58	**<0.0001**	48.29	1,55	**<0.0001**
6	8.82	1,58	**0.0043**	17.87	1,58	**<0.0001**	0.87	1,55	0.3538
12	5.79	1,58	0.0193	3.86	1,58	0.0543	0.14	1,55	0.7050

Supplementary Table 4. Treatment effects on blood values of ID and control pigs per sampling time point during dietary treatment (which lasted for 28 days from age 4-6 days) and after transition to regular feed (dotted line). Note that a Bonferroni correction was applied, and that consequently effects of the treatment are considered statistically significant if $p < 0.01$.

Correlations between iron-containing cell count in hippocampus and RM scores

All animals (n=17)		CA3	CA1	DG	sb
RM acquisition linear trend	r_{pm}	0.390	0.120	0.246	0.214
	p<	0.122	0.647	0.341	0.409
RM acquistion mean	r_{pm}	0.520	0.160	0.193	0.239
	p<	**0.032**	0.540	0.457	0.355
RM reversal linear trend	r_{pm}	0.386	0.061	0.108	-0.001
	p<	0.126	0.816	0.680	0.997
RM reversal mean	r_{pm}	0.258	0.069	0.075	0.147
	p<	0.317	0.793	0.775	0.573
RM difference score	r_{pm}	0.464	0.162	0.118	0.244
	p<	0.061	0.535	0.652	0.345

Supplementary Table 5. Correlations between iron-containing cell count in hippocampal areas after iron staining and RM scores during the acquisition phase of the holeboard task.

Chapter 6

Non-anemic iron deficiency from birth to weaning does not impair growth or memory in piglets

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Abstract

Early iron deficiency is associated with impaired (cognitive) development, the severity of which depends on the timing and duration of the under-supply of iron. To design effective treatment and prevention strategies for iron deficiency in humans, suited animal models are needed. In an earlier study (Chapter 5, also published in Antonides et al., 2015b) we separated 10 pairs of piglets from their mothers within a few days after birth and reared one sibling with artificial iron-deficient (ID) and the other with balanced control milk until weaning. ID piglets grew slower and showed poorer reference memory performance than their controls in a spatial holeboard task, even weeks after iron repletion. One putative intervening factor in that study was pre-weaning maternal deprivation. In an attempt to refine the piglet iron-deficiency model, we assessed whether piglets reared by sows without receiving iron supplementation, can serve as an animal model of iron deficiency. As sow milk is inherently iron-deficient, piglets normally receive a prophylactic iron injection. Ten pairs of piglets were housed with foster sows until weaning (4 weeks). One sibling per pair was randomly assigned to the control group (receiving iron dextran injections: 40 mg iron per kg body mass on days 3 and 10), the other to the ID group. From weaning, all pigs were fed a balanced commercial diet. Blood samples were taken in week 1, 3.5, 6 and 12. Pre-weaning blood iron values of ID piglets were lower than those of controls, but recovered to normal values after weaning. Hemoglobin of ID piglets did not reach anemic values. Hematocrit and hemoglobin of ID animals did not decrease, and serum iron even increased pre-weaning, suggesting that the piglets had access to an external source of iron, e.g. spilled feed or feces of the foster sows. Growth, and spatial memory, assessed in the holeboard from 10 to 16 weeks of age, was unaffected in ID pigs. We conclude that sow-raised piglets are not a suitable model for iron-deficiency induced cognitive deficits in humans. Based on our previous and the present study, we conclude that growth and memory are only impaired in piglets that suffered from pre-weaning anemia.

Keywords: iron deficiency, cognition, development, spatial learning, memory, pigs, anemia

INTRODUCTION

Iron deficiency is a form of malnutrition that is caused by a nutritional short-age of the micronutrient iron. The need for iron increases considerably during growth, pregnancy and lactation (McLean et al., 2009; Gambling et al., 2011). If iron shortage during early development is not restored quickly enough, neo-nates are at risk of developing iron deficiency and eventually anemia. Suck-ling neonates run a great risk of developing iron deficiency because of the low iron supplies in maternal milk (Fransson and Lönnerdal, 1980; Bates and Prentice, 1994). Worldwide, approximately two billion people suffer from iron deficiency, of which the highest prevalence is among children under the age of five (McLean et al., 2009). Early-life iron deficiency in humans is known to cause retarded growth and irreversible deficits in the development of motor and cognitive skills and memory functions (Beard, 2003; Lozoff and Georgieff, 2006). The timing and duration of the under-supply of iron is crucial for the severity of these adverse effects. Rodent models have elucidated some impor-tant mechanisms involved in the development of iron deficiency, on both the (neuro)physiological and behavioral level. Results from both human and ro-dent studies suggest that early-life iron deficiency causes irreversible deficits in brain structure and function (Yehuda and Youdim, 1989; Lozoff and Geor-gieff, 2006).

In addition to rodents, pigs have recently received increasing attention as ani-mal model species in translational research (e.g. Lind et al., 2007; Kobayashi et al., 2012). Although widely used, rodent models for human conditions have recently raised concerns. The reliability of these small animal models is ques-tioned, as successful pre-clinical studies in rodents scarcely translate to effec-tive clinical use in humans (van der Worp et al., 2010; Macleod, 2011). Large animal models may show less discrepancies in study outcomes with humans. Animals that more closely resemble humans are likely to yield more reliable and thus more relevant study results (Festing and Altman, 2002). In this light, the pig is thought to be a more suited and promising animal model species for translational research than rodents (Gieling et al., 2011b). In addition, pigs have a (neuro)anatomy, physiology and developmental pattern that more closely resemble those of humans (Conrad et al., 2012). As in humans, the pig's brain growth spurt occurs perinatally, whereas in rodents it occurs post-

natally (Dobbing and Sands, 1979).

The pig in particular seems to be a suited animal model to study the effects of early-life iron deficiency on development (Miller and Ullrey, 1987). Piglets are born with low iron supplies of approximately 50 mg, which is mostly found in hemoglobin (Venn et al., 1947). Their rapid growth causes a need for 7-10 mg iron daily in the first weeks of life (Venn et al., 1947; Svoboda and Drabek, 2005). Sow milk provides piglets with only around 1 mg of iron per day, which does not suffice to provide the piglets with the amount of iron needed (Csapó et al., 1996). It is believed that piglets of the wild boar, the ancestor of the pig, ingest iron through rooting in soil. In the pig farming industry, however, the barren environment in which piglets are reared provides no such external source of iron. Moreover, for decades pig breeders have been selecting pigs for increasingly larger litter sizes and fast growth (Rauw et al., 1998). This may increase the need for iron and cause more severe iron deficiency in neonatal piglets (Furugouri and Kawabata, 1975).

In pig husbandry, it is therefore common practice to provide new-born piglets with an iron dextran injection, to prevent development of iron deficiency anemia. Recently, we conducted a study to investigate the effects of dietary induced pre-weaning iron deficiency in piglets on spatial learning and memory (Chapter 5, also published in Antonides et al., 2015b). We used 10 pairs of piglets from ten different litters. One piglet from each pair was randomly assigned to the iron deficiency group, the other served as control. The control piglets were administered an iron injection; the iron deficient (ID) treatment animals received a saline injection. Animals were then separated from the sow at 4 to 6 days of age, and fed artificial milk diets for 28 days. Control animals received a balanced milk diet, whereas ID animals received an iron deficient milk diet. After treatment, all piglets were weaned and fed a balanced piglet diet. ID piglets showed impaired reference memory learning capability in a spatial holeboard task (measured after iron repletion) as well as impaired growth (permanent), lower blood iron values (during treatment) and lower brain iron concentrations (measured 8 weeks after iron repletion). ID animals were clinically anemic at the end of treatment, as assessed by their hemoglobin values.

In a similar study by Rytych and colleagues (2012), piglets were assigned to a control, mildly ID or severely ID diet from 2 to 28 days of age, during which

their growth, blood parameters and cognitive performance was assessed. Control animals received an iron injection. Severely ID piglets showed impaired learning in a double T-maze task, whereas mildly ID piglets showed deficits in reversal learning. All ID animals (mild and severe) became anemic. Treatment did not affect growth of the piglets. This lack of effect may have been due to the short duration of the study (28 days).

However, the piglets in both studies (Rytych et al., 2012; Chapter 5, also published in Antonides et al., 2015b) were separated from the sow within a few days after birth, in order to feed them the controlled experimental diets. This is very early compared to separation from the sow in industrial pig husbandry, where piglets are usually weaned at around four weeks of age. Early-life maternal deprivation is known to increase long-term stress responses in rats (Suchecki and Tufik, 1997) and to cause intellectual damage in human infants (Yarrow, 1961). Rat pups that experienced 24 hours of maternal separation at postnatal day 9 showed reduced hippocampal plasticity at adulthood (Roceri et al., 2002). This shows that such a stressful and emotional event during early brain development may cause permanent deficits in brain function. The hippocampus is involved in spatial learning and memory (Bird and Burgess, 2008) and hippocampal dysfunctions are known to impair spatial learning and memory (Olton et al., 1978; Da Silva et al., 1986).

Complementing this finding, maternally deprived rats showed delayed acquisition and a higher degree of persistent behavior in the Morris water maze task compared to control animals (Oitzl et al., 2000). As the holeboard is also a spatial learning and memory task, these results raise the concern whether the results from our previous iron deficiency study (Chapter 5, Antonides et al., 2015b) may have been influenced by maternal deprivation. Oitzl and collegues showed that maternal deprivation in their rats amplified individual behavioral differences at senescence. The lasting detrimental effects of iron deficiency on memory performance found in our previous study might also have been influenced or amplified by early maternal deprivation. In order to refine the piglet iron deficiency model, early maternal deprivation should ideally be prevented in the experimental set-up. In addition, this would increase the welfare of the piglets, as they are spared the experience of separation stress at an extremely young age.

In the present study, we investigated whether piglets that stayed with the sow until weaning without receiving iron supplementation might serve as a suited, refined animal model for iron deficiency in humans. To this end, the piglets were allowed to consume only sow milk (neither iron supplementation nor additional feed were provided) until weaning at four weeks of age.

We measured hematology, growth, and spatial memory performance in ID and control piglets. Based on previous findings of pre-weaning, dietary induced iron deficiency in piglets (Rytych et al., 2012; Chapter 5, Antonides et al., 2015b), we expected that ID piglets would develop anemia as indicated by their hemoglobin values. Also, pre-weaning hemoglobin, hematocrit and serum iron values were expected to be lower in ID animals than in control animals. We predicted that ID piglets would show retarded growth and impaired spatial orientation performance, indicated by lower spatial memory scores in the cognitive holeboard task. The results of this study may yield a refined, more natural and less invasive piglet model of iron deficiency.

MATERIALS AND METHODS

Ethics note

This study was reviewed and approved by the local animal ethics committee of Utrecht University (DEC, DierExperimenten Commissie) and was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid suffering.

Animals and housing (pre-weaning)

Pairs of piglets [(Terra × Finnish landrace) × Duroc] from 12 different litters, all born within the same week, were selected from the commercial pig breeding farm of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands (see Supplementary Table 1 for an overview of the data of selected piglets and foster sows). All piglets of each litter were weighed within 24 hours after birth, including stillborn piglets. The pair of piglets of the same sex that had a weight closest to the average weight of the litter was selected and marked with a differently colored ear tag. For two sibling pairs, due to

poor growth and reduced health of one of the siblings, a sibling of a different gender was selected, resulting in two male-female sibling pairs. One of the piglets of each selected pair of piglets was assigned randomly to the control group, the other to the ID group.

All piglets remained with their maternal sow during the first two days after birth, to allow them to ingest colostrum. Then, the selected piglets were transferred to one of three foster sows at two days of age and were exchanged for piglets of the foster sow. We ensured that these foster sows had more teats available than the number of piglets to nourish, to avoid teat competition and increase survival chances. The three foster sows fed 9, 11 and 12 piglets. The use of foster sows was chosen in order to only withhold additional feed from the piglets selected for our study, leaving all other piglets of the piggery unaffected by our experiment. Otherwise, we would have had to withhold all piglets of the 12 selected litters additional feed. This would have reduced their growth performance and thus have caused substantial financial losses for the piggery. Cross-fostering piglets at a young age is done on a regular basis in conventional practice, for example to increase survival chances of low birth weight piglets (Deen and Bilkei, 2004). Heim et al. (2012) showed that early postnatal cross-fostering does not affect behavior, survival or growth performance of adopted piglets.

All selected piglets remained with the foster sows until weaning at four weeks of age. During this period, they did not receive any additional milk replacer or creep feed and thus only had access to sow milk, which is low in iron content (Csapó et al., 1996). Water was available through a drinking nipple. During the first four weeks (pre-weaning) all selected piglets were weighed once every two days in order to closely monitor their growth. One pair of piglets was excluded from the experiment before weaning due to poor growth, which necessitated our interference with extra care and additional feed. Thus, the experiment was conducted with 11 sibling pairs, consisting of 12 male and 10 female piglets. After weaning, all piglets were weighed once per week until they had reached the age of 17 weeks, which was when the experiment ended.

Iron treatment and blood collection

On day 3 and day 10 after birth, control animals received an iron dextran

injection of 0.2 ml/kg body weight, containing 200 mg Fe/ml (MS Ferro-Pig, Schippers Export B.V., The Netherlands), as adapted from Lipiński et al. (2010). This corresponded to 40 mg Fe/kg body weight, based on the weight on the day of injection. We decided to adjust the injections to body weight and not to birth weight (as in Lipiński et al., 2010) as this, we argue, is more accurate. The ID piglets received two saline injections of 0.2 ml/kg body weight. Lipiński and colleagues argue that the amount of iron normally administered in conventional practice (100 – 200 mg per animal) is excessive and may even be toxic. They showed that reducing and spreading the iron administration reduces iron toxicity and allows the body to use the iron more effectively. In the present study, we therefore used this administration scheme.

At the age of 1, 3.5, 6 and 12 weeks, blood samples were taken from the jugular vein. Piglets were fixated by hand during all blood sampling moments except at 12 weeks of age, when a pig snare was used to briefly restrain the animals. Blood hemoglobin and hematocrit were determined using the Siemens ADVIA® 2120i System with ADVIA Multispecies Testing software. Serum iron was determined using the Beckman Coulter UniCel DxC 600 according to standard procedure.

Experimental housing (post-weaning)

At approximately four weeks of age, all selected piglets were weaned and transported to the experimental facility (located next to the farm they originated from), where they were housed in two adjacent pens (both 4 m × 5 m) from November 2014 until February 2015. Sibling pairs were separated and housed in different pens. Growth of the piglets was expected to be more balanced if piglets in a pen were of a more uniform weight (Francis et al., 1996), therefore in forming the groups, pigs of similar weights were housed together. Both groups contained a balanced number of ID and control animals (see Supplementary Table 1). Each piglet received a sprayed number on its back to allow fast identification. At the experimental facility, all piglets received commercial pig feed ad libitum (Prevent piglet feed, De Heus Voeders BV, Ede, The Netherlands). Feed was provided in a large feeding trough in order to reduce feeding competition (4 m × 0.30 m). Water was available ad libitum through a drinking nipple.

The pens had a concrete floor covered with straw as bedding material. Each pen contained a wooden nest box (3 m × 1.5 m) with plastic flaps along the front for easy access and heat preservation. Inside the nest box, two heat lamps were suspended approximately 1 m above the floor, which were removed when the piglets were 12 weeks old. Nest box flooring consisted of rubber mats and a thick layer of sawdust and straw as bedding. Different sized plastic balls and pig sticks were provided as enrichment. The stable in which the pens were located was naturally lighted and ventilated (not heated). Lights were on from 7:30 a.m. until 4:30 p.m. Temperature ranged from -1 °C to 14 °C and was recorded daily. After the end of the study, all pigs were fattened to slaughter weight and transported to and slaughtered in a commercial slaughter house.

Holeboard apparatus

The spatial cognitive holeboard for pigs (built by Ossendrijver BV, Achterveld, The Netherlands) was a square arena measuring 5.30 × 5.30 m, with grey synthetic 80 cm high walls with a steel bar on top. The square testing arena was surrounded by a 40 cm wide corridor and contained a guillotine door on each side through which piglets could enter the arena (for details, see Figure 2 of Chapter 1 of this thesis). The apparatus had a blue plastic slatted floor. The arena contained 16 possibly rewarded sites in a four by four matrix, consisting of plastic food bowls (Road Refresher Large, Prestige Pet Products, Essex, England). These bowls contained false bottoms; in the actual bottoms of all bowls, three M&M's® were placed daily to assure that the piglets could not use olfactory cues to find the rewards. To prevent piglets from using visual cues to find the rewards, each food bowl was covered with a red plastic ball (JollyBall Dog Toy, Ø 24 cm, 1400 g, Jolly Pets, Ohio, USA). The piglets always entered the corridor clockwise and were allowed to leave the arena through the door closest to the main entrance of the apparatus. During testing, pen mates were housed in the waiting pen in front of the holeboard apparatus. This allowed the piglet inside the arena to smell and hear, but not see its pen mates during testing. It also served as an auditory extra-maze cue for the piglet during testing. The fluorescent lights on the sloping ceiling of the stable and the position of the experimenter standing in the corridor of the holeboard arena served as visual extra-maze cues for spatial orientation within the arena. During testing, eye contact with the animal that was tested was avoided.

The experiment was controlled through a laptop which collected the data. A sensor in the food bowl sent a signal whenever the connection with the magnet in the ball was lost, which was the case when the ball was lifted by the piglet with its snout. This signal was registered via an interface (LabJack), transferred to the laptop and processed by a custom-made software program (Blinq Systems, Delft, The Netherlands). If a ball was lifted multiple times within a 10-second period and no other food bowl was visited in between, this series of events was counted as one single visit. The entry door was randomly determined by the software before each trial and was opened manually by the experimenter before a piglet entered the corridor, using a rope and pulley system. It has been demonstrated in a study using rats as subjects that randomization of the start position forestalled the development of a preferred pattern of visiting the holes (van der Staay et al., 1990). This effect of randomizing start positions has since then been corroborated in a number of holeboard studies (reviewed in van der Staay et al., 2012).

Habituation and holeboard training

During the first week after arrival, piglets were allowed to get accustomed to their new pen and pen mates. During this week, they were habituated to humans for 30 to 60 minutes per day. Afterwards, all pigs were gradually habituated to the hallway leading to the holeboard apparatus and its waiting area. Piglets were habituated to the holeboard arena from six to ten weeks of age. At the start of these holeboard habituation sessions, all piglets of a pen were allowed to enter the holeboard arena and corridor together, with all arena doors left open. During these sessions, all food bowls contained multiple food rewards. The balls were lifted to facilitate the learning process of finding food rewards, and bowls were refilled as soon as the rewards in a bowl were consumed. The group size of pigs let into the holeboard together was gradually decreased over the habituation sessions, until piglets were comfortable to enter the arena alone. The balls were gradually lowered to allow piglets to learn to lift the balls to uncover the food bowls and find the rewards.

Holeboard testing

When all piglets were physically able to lift the balls and were comfortable entering the arena alone, holeboard testing started. This was the case at 10

weeks of age. Each piglet received six habituation trials in which all 16 holes contained a reward; two trials per day on three successive days. After the habituation trials, the acquisition trials started, in which each piglet was assigned to its own configuration of four baited holes. In total, four different configurations were used in such a way that, across all piglets, every hole was baited equally often. Piglets received two daily trials in close succession (massed trials) on working days. If after at least 40 acquisition trials a predetermined learning criterion was reached (an average reference memory score of > 0.7 over the last four trials), the pigs were switched to the reversal configuration. The reversal configuration was the 180° rotated pattern of baited holes used during the acquisition phase. After maximally 60 acquisition trials, all piglets were switched to the reversal phase, regardless of their performance. All piglets received a total of 20 reversal trials.

Holeboard variables

A trial was started manually when a piglet entered the arena with both front legs. A trial was ended automatically by the software when all rewards were found, or after 300 s, whichever event occurred first. For the six habituation trials preceding the acquisition phase (all holes baited), the total number of visits (TV) and the total number of rewards found (REW) were automatically recorded by the software. For the acquisition and reversal trials, the following variables (van der Staay et al., 2012) were either recorded or calculated automatically by the software:

- Working memory (WM), a ratio defined by the number of visits that yield a food reward divided by the number of visits and re-visits to the rewarded set of holes;
- Reference memory (RM), a ratio defined by the number of visits and re-visits to the rewarded set of holes divided by the number of visits and re-visits to all holes;
- Trial duration (TD) in seconds, the time between entering the holeboard and finding all four rewards, or the maximum trial duration of 300 s if the pig did not find all rewards;
- Inter-visit interval (IVI) in seconds, the average time between successive hole visits;
- Latency to the first visit (LFV) and Latency to the first rewarded visit

(LFR) in seconds;
- Total visits (TV), unrewarded visits (URV) and rewarded visits (RV);
- Number of visits until the 1st (Vfirst), 2nd (Vsecond), 3rd (Vthird) and 4th (Vfourth) reward were found (for a detailed explanation see Gieling (2013), pp. 173-176).

Statistical analyses

Data were analyzed with the statistical software SAS (version 9.4, SAS Institute, Cary, NC, USA). Normal distribution of the residuals of all variables was assessed using the Shapiro-Wilk test (SAS PROC UNIVARIATE). Birth weights of animals used in the experiment were compared using a mixed model ANOVA with Litter as random effect. The effects of treatment on the growth curves from 4 to 17 weeks of age and on blood iron values were analyzed with a mixed model ANOVA to account for clustering of piglets within litters and repeated measurements within piglets, with the fixed effects Treatment (control or ID), Week, and their interaction, with a random effect for Litter. In case of significant interaction effects of Treatment by Week on the blood iron values, we additionally performed analyses on the separate blood sampling time points, in order to assess at which time points differences were found. For these individual analyses, a Bonferroni correction for multiple comparisons was applied. Note that the number of observations differed per blood collection moment and per variable due to technical difficulties during either blood collection or the analyses of the samples. For the number of observations per time point and per variable see Supplementary Table 2.

The six successive habituation trials were analyzed separately with a mixed model ANOVA with the fixed effects Treatment (control or ID), Trials and their interaction, with a random effect for Litter. For the acquisition and reversal phase, means of trial blocks (four successive trials each) were calculated for all variables. The first ten trial blocks of the acquisition phase (block 1-10) were analyzed; thus excluding the extra acquisition trials that a piglet received when it had not yet reached the preset criterion of RM > 0.7 after 40 trials. The reversal phase consisted of five trial blocks (block 11-15). All variables expressing latencies or durations were log10-transformed to meet the normality assumption. Two outliers in the data of the variable LFV were detected using the online outlier detector QuickCalcs (GraphPad Software, Inc., La Jolla CA,

USA) and replaced by missing values. The effects of treatment on holeboard performance were analyzed with a mixed model ANOVA with the fixed effects Treatment (control or ID), Trial blocks and their interaction, with a random effect for Litter.

The holeboard data analyses were performed for three different phases: acquisition, transition and reversal. The transition phase is the switch from the acquisition phase to the reversal phase, i.e. the last trial block of the acquisition phase compared to the first trial block of the reversal phase (block 10 compared to block 11). This is a measure of the response flexibility of an animal: a large difference means that the animal faced difficulties to adapt to the new situation.

RESULTS

Blood iron values

Hematocrit (Hct) and hemoglobin (Hb) values of ID animals were lower than those of control animals over the different sampling time points from 1 to 12 weeks of age (Treatment: both $p < 0.0001$, see Figure 1 and Table 1). All blood iron values increased over time for all animals (Week: Hct and Hb: $p < 0.0001$; serum iron: $p = 0.007$). Both Hct and Hb showed a steeper increase in ID animals than in control animals (Treatment by Week interaction: Hct: $p = 0.007$; Hb: $p = 0.001$). Serum iron values of ID animals tended to show a steeper increase than those of control animals (Treatment by Week interaction: $p = 0.05$; Figure 1C).

Blood iron levels in ID animals during treatment
Visual inspection of Figure 1 suggests that blood iron values of ID animals rose between week 1 and week 3.5. Therefore, we compared blood iron values within ID animals between these sampling time points using one-sample t-statistics on the difference scores. These tests revealed that there was no difference in ID piglets' Hct ($t9 = 0.95$; $p = 0.37$) and Hb ($t9 = 0.99$; $p = 0.35$) values between week 1 and 3.5, whereas their serum iron values were higher in week 3.5 than in week 1 ($t9 = 3.29$; $p = 0.01$). Note that serum iron values were log10 transformed for this analysis to meet the normality assumption.

In order to investigate at which sampling time points treatment effects were present, we additionally analyzed differences between treatment groups at each separate sampling time point (Table 2). Because a Bonferroni correction was applied, differences with an associated p-value of < 0.01 were considered significant in these analyses.

Treatment effects on blood parameters

Blood parameter	Treatment			Week			Treatment x Week		
	F	Df	P≤	F	df	P≤	F	df	P≤
Hematocrit	16.50	1,64	**<0.0001**	10.89	3,64	**<0.0001**	4.39	3,64	**0.0072**
Hemoglobin	20.87	1,64	**<0.0001**	26.88	3,64	**<0.0001**	6.04	3,64	**0.0011**
Serum iron	2.96	1,66	0.0900	4.37	3,66	**0.0072**	2.74	3,66	0.0503

Table 1. Effects of feeding piglets iron-deficient sow milk as only nourishment until weaning on blood iron values. Blood parameters of ID and control animals are compared over the course of the experiment (0 to 12 weeks). Effects per sampling time point are listed in Table 2. Results that are considered significant (p < 0.05) are depicted in bold.

Hematocrit

At 1 week of age, a measuring time point that fell between the two iron or saline injections, Hct values tended to be lower in ID animals than in control animals (p = 0.01, after a Bonferroni correction, this result was a trend). At the end of treatment and before weaning at 3.5 weeks, Hct values were lower in ID animals than in control animals (p < 0.0001). At 6 and 12 weeks of age, no differences in Hct values were found between treatment groups (Figure 1A; Table 2).

Hemoglobin

Hb values were lower in ID animals than in control animals at 1 week of age (p = 0.002) and at the end of treatment before weaning at 3.5 weeks of age (p < 0.0001). At 6 weeks of age, Hb values still tended to be lower in ID animals than in control animals (p = 0.01, after a Bonferroni correction, this result was a trend). No difference in Hb values between treatment groups were found at 12 weeks of age (Figure 1B; Table 2).

Serum iron

Serum iron values were lower in ID animals than in control animals at 1 week of age (p = 0.006). Near the end of treatment at 3.5 weeks and at 6 and 12

weeks of age, no differences in serum iron values between treatment groups were found (Figure 1C; Table 2).

Weights and growth

There was no difference in birth weight between siblings ($F_{1,10}$ = 2.22; p = 0.17). Over the course of the experiment, there was no difference in average weight between treatment groups (Treatment: $F_{1,270}$ = 1.39; p = 0.24; Figure 2). Weight gain in the two groups was similar (Week: $F_{13,270}$ = 395.79; p < 0.0001; Treatment by Week interaction: $F_{13,270}$ = 0.43; p = 0.96).

Holeboard performance

The results of the statistical analyses of the holeboard data are listed in Table 3. In the six habituation trials in which all sixteen holes contained rewards, there was no treatment effect on the total number of visits made (TV) nor on the
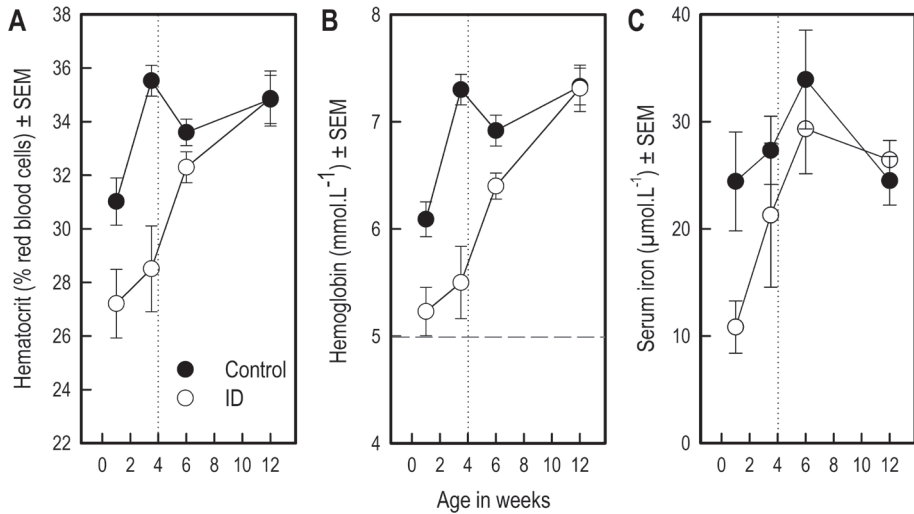
Figure 1. Blood hemoglobin, hematocrit and serum iron values of ID and control animals from 1 to 12 weeks of age. Weaning and transition to regular feed was at 4 weeks of age (dotted vertical line). (A) Hematocrit values; (B) Hemoglobin values. The dashed horizontal line indicates a hemoglobin value of 5, below which piglets are considered anemic (Ishaya, 2012); (C) Serum iron values. The number of successfully collected and analyzed samples varied per measure and sampling time point; for the number of successful observations per time point and measure, see Supplementary Table 2. Mean values and standard error of the mean (SEM) are depicted per treatment group and time point.

	Hematocrit			Hemoglobin			Serum iron		
Effects of treatment per sampling time point									
Age (weeks)k	F	df	P≤	F	df	P≤	F	df	P≤
1	6.33	1,64	0.0144	10.58	1,64	**0.0018**	7.96	1,66	**0.0063**
3.5	17.38	1,64	**<0.0001**	25.16	1,64	**<0.0001**	0.62	1,66	0.4333
6	2.78	1,64	0.1002	6.95	1,64	0.0105	0.55	1,66	0.4590
12	0.00	1,64	0.9742	0.00	1,64	0.9863	0.47	1,66	0.4938

Table 2. Effects of feeding piglets iron-deficient sow milk as only nourishment until weaning on blood iron values per sampling time point in ID and control animals during the pre-weaning period (0-4 weeks) and after transition to regular feed (at 4 weeks of age; dotted line) are listed. Note that a Bonferroni correction was applied: effects of the treatment are considered statistically significant if $p \leq 0.01$ (depicted in bold).

number of rewards found (REW), which in this phase is also an indication of the number of different holes visited. For all animals, TV did not change throughout these six trials. However, REW increased for all animals during this phase (Trials: $p = 0.02$), i.e. the piglets learned to visit more of the 16 food bowls during a trial. There was no Treatment by Trial interaction for TV or REW (Table 3A).

Working memory and reference memory
Working memory and reference memory were not affected by treatment in any phase (Table 3B, Figure 3). All piglets showed an increase in WM and RM performance in both the acquisition and reversal phase, and a decrease in these measures in the transition to a different set of baited holes.

Latency first visit and trial duration
There was no treatment effect on LFV in the acquisition and transition phase (Table 3B, Figure 4A). However, control animals showed a steeper decline in LFV in the reversal phase than ID animals (Treatment by Trial blocks interaction: $p = 0.046$).

Trial duration (TD) was not affected by treatment in any phase. TD decreased in all animals in the acquisition and reversal phase, and increased in the transition phase (Table 3B, Figure 3B).

Visits before fourth reward
All animals showed a decrease in the number of visits before the fourth reward was found (Vfourth) during the acquisition and reversal phase, and an increase in Vfourth in the transition phase (Table 3B, Figure 4B). There was

no effect of treatment or Treatment by Trial blocks interaction on Vfourth in the acquisition phase.

In the transition to a different set of baited holes, control animals showed a larger increase in Vfourth than ID animals (Treatment by Trial blocks interaction: $p = 0.02$). This in turn may explain the observation that, in the reversal phase, control animals showed a steeper decrease in Vfourth than ID animals (Treatment by Trial blocks interaction: $p = 0.002$; Figure 4B).
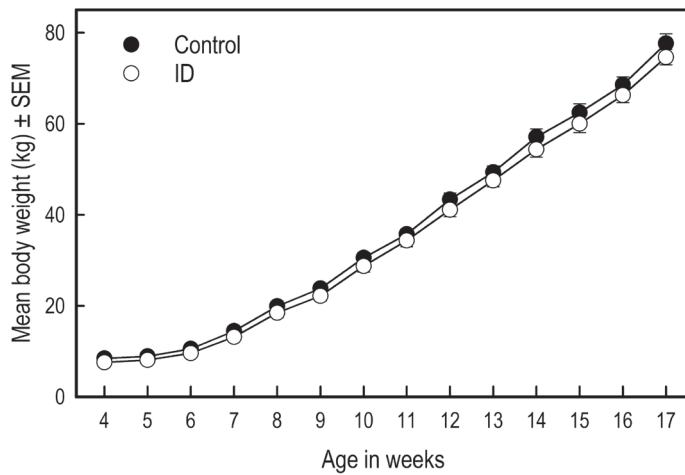
Figure 2. Growth of ID and control piglets from weaning at 4 weeks of age to 17 weeks of age. Mean values and standard error of the mean (SEM) are depicted per treatment group.
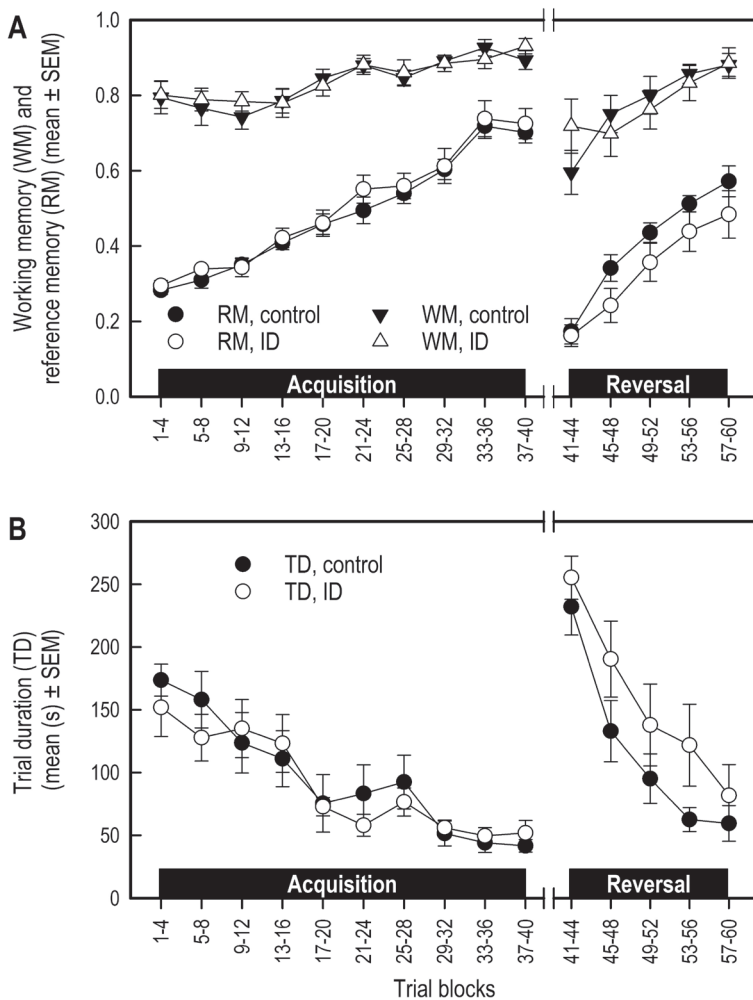
Figure 3. Performance of ID and control piglets in the spatial cognitive holeboard task during the acquisition phase (trials 1-40) and the reversal phase (trials 41-60). (A) Working memory (WM) and reference memory (RM) performance; (B) Trial duration (TD). Note that TD was analyzed statistically after log10 transformation of the block means whereas the untransformed block means and SEMs are depicted here. For the results of the statistical analyses, see Table 3. Mean values and standard error of the mean (SEM) are depicted per treatment group.
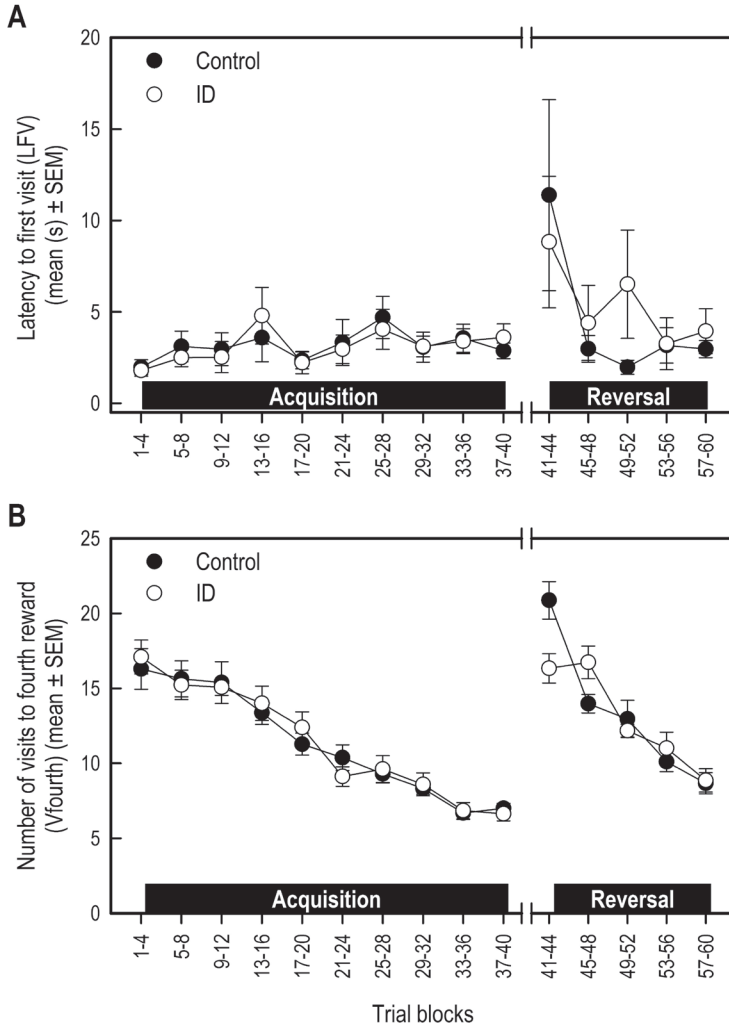
Figure 4. Performance of ID and control piglets in the spatial cognitive holeboard task during the acquisition phase (trials 1-40) and the reversal phase (trials 41-60). (A) The latency to the first visit (LFV); (B) The number of hole visits until the fourth reward was found (Vfourth). For the results of the statistical analyses, see Table 3. Mean values and standard error of the mean (SEM) are depicted per treatment group.

A) Holeboard habituation (Hab)

Measure	Phase	Treatment			Trials			Treatment x Trials		
		F	df	P≤	F	Df	P≤	F	df	P≤
Total number of visits (TV)	Hab	1.46	1,110	0.2302	0.56	5,110	0.7273	1.16	5,110	0.3349
Rwwards found (REW)	Hab	1.28	1,110	0.2607	2.78	5,110	**0.0212**	0.72	5,110	0.6121

B) Holeboard acquisition (Acq), transition (Trans), reversal (Rev)

Measure	Phase	Treatment			Trial blocks			Treatment x Trial blocks		
		F	df	P≤	F	Df	P≤	F	df	P≤
Working memory (WM)	Acq	0.22	1,190	0.6379	8.63	9,190	**<0.0001**	0.45	9,190	0.9034
	Trans	2.97	1,25	0.0969	25.32	1,25	**<0.0001**	0.75	1,25	0.3949
	Rev	0.01	1,74	0.9356	5.57	4,74	**0.0006**	0.77	4,74	0.5486
Reference memory (RM)	Acq	0.92	1,190	0.3390	50.60	9,190	**<0.0001**	0.42	9,190	0.9225
	Trans	0.00	1,27	0.9725	201.94	1,27	**<0.0001**	0.41	1,27	0.5269
	Rev	1.24	1,76	0.2687	38.19	4,76	**<0.0001**	1.22	4,76	0.3074
Trial duration (TD)	Acq	0.00	1,190	0.9904	15.54	9,190	**<0.0001**	0.79	9,190	0.6299
	Trans	1.06	1,27	0.3124	270.76	1,27	**<0.0001**	0.03	1,27	0.8531
	Rev	2.78	1,76	0.0998	27.61	4,76	**<0.0001**	0.88	4,76	0.4805
Inter-visit-interval (IVI)	Acq	0.00	1,190	0.9513	2.89	9,190	**0.0031**	0.66	9,190	0.7471
	Trans	0.53	1,27	0.7192	44.46	1,27	**<0.0001**	0.13	1,27	0.4728
	Rev	2.00	1,76	0.1617	10.01	4,76	**<0.0001**	0.88	4,76	0.4792
Latency first visit (LFV)	Acq	0.03	1,188	0.8584	4.11	9,188	**<0.0001**	0.91	9,188	0.5211
	Trans	0.04	1,27	0.8482	4.66	1,27	**0.0399**	0.75	1,27	0.3928
	Rev	0.18	1,76	0.6705	2.90	4,76	**0.0275**	2.54	4,76	**0.0464**
Latency first rewarded visit (LFR)	Acq	0.02	1,190	0.8796	6.66	9,190	**<0.0001**	1.12	9,190	0.3519
	Trans	1.08	1,25	0.3082	29.15	1,25	**<0.0001**	0.00	1,25	0.9611
	Rev	4.70	1,74	0.0334	9.50	4,74	**<0.0001**	0.05	4,74	0.9944
Total number of visits (TV)	Acq	0.03	1,190	0.8703	32.43	9,190	**<0.0001**	0.65	9,190	0.7548
	Trans	1.70	1,27	0.2028	52.36	1,27	**<0.0001**	1.36	1,27	0.2532
	Rev	0.17	1,76	0.6796	9.35	4,76	**<0.0001**	1.49	4,76	0.2133
Unrewarded visits (URV)	Acq	0.01	1,190	0.9063	42.87	9,190	**<0.0001**	0.70	9,190	0.7082
	Trans	1.23	1,27	0.2769	130.11	1,27	**<0.0001**	1.40	1,27	0.2464
	Rev	0.00	1,76	0.9829	17.15	4,76	**<0.0001**	1.71	4,76	0.1558
Rewarded visits (RV)	Acq	0.70	1,190	0.4050	1.73	9,190	0.0844	0.49	9,190	0.8791
	Trans	3.43	1,27	0.0750	7.16	1,27	**0.0125**	1.25	1,27	0.2735
	Rev	2.13	1,76	0.1488	4.21	4,76	**0.0039**	1.00	4,76	0.4139
Visits before 1st reward (Vfirst)[*]	Acq	0.60	1,190	0.4385	27.04	9,190	**<0.0001**	1.37	9,190	0.2046
	Trans	0.27	1,26	0.6080	34.85	1,26	**<0.0001**	0.18	1,26	0.6743
	Rev	0.28	1,75	0.6000	12.35	4,75	**<0.0001**	1.95	4,75	0.1103
Visits before 2nd reward (Vsecond)[*]	Acq	0.12	1,190	0.7342	28.03	9,190	**<0.0001**	1.13	9,190	0.3431
	Trans	0.09	1,24	0.7701	74.79	1,24	**<0.0001**	0.14	1,24	0.7072
	Rev	0.02	1,72	0.8774	18.09	4,72	**<0.0001**	1.24	4,72	0.3034
Visits before 3rd reward (Vthird)[*]	Acq	0.70	1,188	0.4048	39.33	9,188	**<0.0001**	0.67	9,188	0.7372
	Trans	0.00	1,22	0.9908	90.91	1,22	**<0.0001**	0.02	1,22	0.8940
	Rev	0.02	1,68	0.9026	18.61	4,68	**<0.0001**	0.57	4,68	0.6869
Visits before 4th reward (Vfourth)[*]	Acq	0.03	1,184	0.8525	34.04	9,184	**<0.0001**	0.68	9,184	0.7225
	Trans	7.99	1,19	**0.0108**	232.98	1,19	**<0.0001**	6.82	1,19	**0.0171**
	Rev	0.07	1,61	0.7934	30.92	4,61	**<0.0001**	4.81	4,61	**0.0019**

[*]: For further information about the operational definitions of these variables, see Gieling, 2013, pp. 173–176

Table 3. Effects of feeding piglets iron-deficient sow milk as only nourishment until weaning on performance in the spatial cognitive holeboard task during (A) The habituation phase (Hab) in which all sixteen holes were baited, and (B) The acquisition phase (Acq), transition phase (Trans) and reversal phase (Rev). The transition phase is the switch from the acquisition phase to the reversal phase, i.e. the last trial block of the acquisition phase compared to the first trial block of the reversal phase.

DISCUSSION

The present study aimed to investigate whether piglets that remained with the sow until weaning, without providing them with additional feed or iron supplementation, could serve as a refined, less labor-intensive animal model for iron deficiency in humans. In this refined model, early maternal deprivation as a putative intervening factor was eliminated. Leaving the piglets with a sow until weaning also spares them the welfare-compromising stress of early removal from the sow.

Hematology, growth and cognitive performance was assessed in ID and control piglets. Our results show that piglets reared by a sow without iron supplementation until weaning at four weeks of age did not become clinically anemic. Moreover, their long-term growth and cognitive performance was unaffected. In comparison with previous studies, early-life iron deficiency-induced clinical anemia seems essential in retarding growth and developing long-term impairments of memory performance.

Blood iron values

Blood iron values were lower in ID animals than in control animals during treatment. After weaning and transition to iron-sufficient feed, blood iron values of ID piglets recovered to normal, comparable to those of control animals. This finding confirms that sow milk is low in iron content (Csapó et al., 1996).

No clinical anemia in ID animals

Surprisingly, pre-weaning hematocrit (Hct) and hemoglobin (Hb) values of ID animals did not decrease, and their serum iron values even rose between 1 and 3.5 weeks of age. In contrast, in our previous study assessing the effects of a more severe dietary ID treatment in young piglets (Chapter 5, Antonides et al., 2015b), there was a steep decline in Hct, Hb and serum iron during treatment in the ID animals. In that study, control animals received a 200 mg iron injection on day 3, ID animals received a saline injection. All piglets were separated from the sow at four to six days of age. Then, control animals were fed an iron sufficient diet (88 mg iron/kg diet) and ID animals an iron deficient diet (22 mg iron/kg) for 28 days. Similarly, piglets in the study by Rytych et al. (2012)

were separated from the sow 48 hours after farrowing and received a control (100 mg iron/kg), mildly (25 mg iron/kg) or severely (10 mg iron/kg) ID milk diet for four weeks. Similar to our previous study (Chapter 5, Antonides et al., 2015b), the mildly and severely ID piglets in the study by Rytych et al. (2012) showed a decline in Hct, Hb and serum iron values during treatment. In both studies, all ID animals (mildly and severely) became clinically anemic, whereas the ID animals in the current study did not.

We argue that, comparing the results of Chapter 5 (Antonides et al. 2015b) and Rytych et al. (2012) with the findings of the present study, it is highly likely that piglets had access to an external source of iron while they were housed with the foster sows. A study by Sansom and Gleed (1981) investigated consumption of sow feces by suckling piglets by radioactive labeling the sows' feed and, consequently, their feces. They showed that piglets ingest around 20 g of sow feces per day (ranging from 5 to 85 g). This, they argue, may prevent piglets from becoming anemic, provided that one gram of fresh feces contains approximately 2 mg iron. Another possibility is that the piglets in our study consumed feed that was spilled by the foster sows. This could explain why, contrary to expectations, the blood iron values of our ID piglets did not decrease during treatment. We thus assume that these two external oral sources (feces and/or spilled feed) could have provided sufficient iron to prevent the development of anemia.

Control animals: iron administration and serum iron values
In two recent iron deficiency studies in piglets, control animals receiving an iron supplementation of 200 mg iron (Rytych et al., 2012; Chapter 5, Antonides et al., 2015b) showed serum iron values at four weeks of age that were nearly twice as high as that of the control animals in the present study, which received two injections containing lower doses of iron. Additionally, the serum iron values of the control animals in Chapter 5 (Antonides et al. 2015b) were much higher during than after treatment (Rytych et al. did not measure after treatment). This may indicate that the amount of iron that was administered in those studies was indeed excessive, as argued by Lipiński et al. (2010).

Growth unaffected in ID animals

The most frequently used read-out parameter of performance and health of

animals is growth (de Onis et al., 2006). In our study, withholding piglets from iron supplementation and additional feed before weaning did not affect their growth. Figure 2 suggests that control animals had slightly higher weights, yet this finding was not confirmed statistically. A study by Yu et al. (2002) also showed no effects of an iron injection on growth performance in piglets. In their study, by offering creep feed, piglets did not become clinically anemic. In our previous iron deficiency study in piglets, ID piglets did become clinically anemic and showed impaired growth (Chapter 5, Antonides et al., 2015b). Our study results complement the findings of Yu et al. (2002) that early-life iron deficiency that does not lead to anemia does not affect growth.

No effects of ID treatment on memory performance

As in previous studies using the holeboard task for pigs (Arts et al., 2009; Gieling et al., 2012), all piglets in our study acquired the holeboard task: their memory scores improved and trial durations and latencies declined during the course of training.

In the habituation trials, in which all 16 holes were baited, no differences were found for the total number of visits or total number of rewards found between treatment groups. In contrast, in our previous iron deficiency study inducing more severe dietary ID in young piglets leading to anemia (Chapter 5, Antonides et al., 2015b), ID piglets made less total visits and found less rewards than control animals in the habituation trials. This indicates that cognitive deficits were already apparent in ID animals before formal training in the holeboard started in that study.

The most important indicators of memory performance in the holeboard task are working memory (WM) and reference memory (RM), which are forms of short- and long-term memory, respectively. We compared the RM scores during both the acquisition and the reversal phase of the control animals of the present study with those of the control animals of our previous iron deficiency study (Chapter 5, Antonides et al., 2015b, see supplementary Figure 1). Visual inspection of the RM learning curves corroborates the notion that the control groups of both studies showed a very similar RM performance. If maternal deprivation would have had an effect on the piglets, then the learning curves of these two studies would have been different. This supports the premise that

maternal deprivation did not affect the performance of control pigs in the previous study. Nonetheless, we cannot completely exclude the possibility that the interaction of maternal deprivation and iron deficiency caused the cognitive deficits found in the ID pigs of our previous study. To test this possibility, the effects of iron deficiency must be compared between sow-reared and sow-deprived pigs in the same study. However, it will be challenging to match the degree of iron deficiency in such a study.

Visual inspection of Figure 3 suggests that the control group had higher RM scores and shorter trial durations in the reversal phase than the ID group. These impressions, however, were not confirmed statistically. We found that control pigs showed a steeper decline in latency to the first visit (LFV) and in errors before finding the fourth reward (Vfourth) in the reversal phase than ID pigs. This is an indication that control animals learned faster in the reversal phase. However, LFV showed strong variation over the trial blocks, making it an unreliable measure on which to base conclusions.

Vfourth was higher in control animals at the start of the reversal phase, explaining the steeper decline in control animals during the reversal phase. The higher score for this measure in the first trial block of the reversal may indicate that control animals had a stronger recollection of their original set of baited holes than ID animals. This may be due to better memory. However, considering the lack of any effects on other memory scores, these findings are not a strong indicator of treatment effects on memory performance.

In our previous study (Chapter 5, Antonides et al., 2015b), RM performance was impaired in ID piglets in both the acquisition and reversal phase. Comparably, piglets fed a severely ID diet for four weeks in the study by Rytych et al. (2012) were unable to acquire a T-maze task; piglets fed a mildly ID diet showed impaired reversal learning in the task compared to control animals.

In a review on iron deficiency studies in both humans and animals, McCann and Ames (2007) report that induced iron deficiency that does not lead to anemia in general does not lead to impaired growth or reduced performance in behavioral tasks. Our findings corroborate this notion in pigs. We consider the proposed refined and less invasive piglet model that we assessed in this study unsuited to serve as an animal model for iron deficiency in humans, as growth

and memory of ID animals was unaffected. However, the results of our study do indicate that the severity of the induced early-life iron deficiency crucially determines the impact on long-term development and cognition in piglets. The development of anemia seems a reliable indicator and predictor of long-term deficits caused by iron deficiency.

CONCLUSION

The aim of the present study was to assess whether piglets left at the sow until weaning without iron supplementation or additional feed could serve as a refined, less labor-intensive piglet model for iron deficiency in humans. Pre-weaning hematocrit, hemoglobin and serum iron were lower in ID animals than in control animals. These values recovered to normal after weaning, when all piglets were fed the same iron-sufficient pig feed. Surprisingly, pre-weaning hemoglobin and hematocrit values did not decrease in ID animals, and their serum iron values even increased. Importantly, ID piglets did not become clinically anemic, as indicated by their hemoglobin values. This suggests that piglets had access to an external source of iron. As no additional feed was provided until weaning and sow milk is low in iron content, we argue that piglets probably consumed feces or spilled feed from the sow, preventing them from becoming anemic. Growth and memory performance in the holeboard task were unaffected in ID animals. Our results suggest that, as ID animals did not become clinically anemic, the imposed iron deficiency was not severe enough to cause long-term developmental or cognitive deficits. Our proposed animal model is thus not suited as a refined animal model for iron deficiency in humans. However, we did find that early-life iron deficiency that does not result in anemia does not have irreversible effects on long-term development and memory performance in piglets. Thus, the development of anemia in early-life iron deficiency seems to crucially determine whether long-term detrimental effects on physical and mental development arise.

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SUPPLEMENTARY MATERIAL

Information of piglets used in this study

	Pen 1				Pen 2			
Litter	Pig ID	Treatment	Gender	Foster Sow	Pig ID	Treatment	Gender	Foster Sow
1	1	ID	**M**	**3**	12	Control	**F**	**1**
2	2	ID	F	**3**	13	Control	F	**1**
3	3	ID	M	1	14	Control	M	1
4	4	Control	F	1	15	ID	F	1
5	5	Control	F	1	16	ID	F	1
6	6	ID	M	2	17	Control	M	2
7	7	ID	M	2	18	Control	M	2
8	8	Control	M	2	19	ID	M	2
9	9	ID	F	2	20	Control	F	2
10	10	Control	F	*	21	ID	F	**3**
11	11	Control	**F**	3	22	ID	**M**	3

*: This piglet was not housed with a foster sow, but did receive iron injections according to the adjusted schema used in this study.

Supplementary Table 1. Overview of the composition of the experimental groups housed per pen after weaning at 4 weeks of age, and information of the piglets used in this study. Siblings are depicted next to each other, one of them was housed in pen one, the other in pen two. Data marked in bold show litter pairs that deviated from our protocol (according to which same sex siblings should be assigned to the two conditions and pens; also, of all piglets in the study, one piglet was not reared by a foster sow).

Number of samples per time point of blood collection

Measure	Treatment	Age in weeks			
		1	3.5	6	12
Hemoglobin (Hb)	ID	10	11	11	8
	control	10	11	11	10
Hematocrit (Hct)	ID	10	11	11	8
	control	10	11	11	10
Serum Fe	ID	10	11	11	11
	control	8	11	11	11

Supplementary Table 2. Number of samples per blood collection time point for the analysis of hemoglobin, hematocrit and serum iron values.
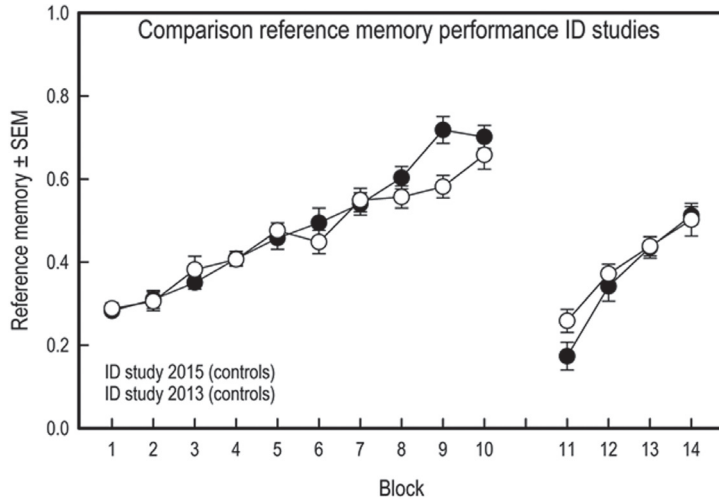
Supplementary Figure 1. Comparison between reference memory scores of the control animals of our previous iron deficiency study (Chapter 5, also published in Antonides et al., 2015b), conducted in 2013, and of the control animals of the current study, conducted in 2015.

Chapter 7

General discussion

Early adverse events can have long-lasting or permanent effects later in life. Especially adverse events during early and rapid development can cause irreversible deficits (e.g. Lozoff et al., 2006). Such deficits may be cognitive, physical or functional, or a combination of these. In this thesis, we studied the effects of low birth weight (LBW) and early-life iron deficiency on development and cognition. We used pigs to study these complications, a) as animal model for elucidating the mechanisms and causes behind these complications in humans, and b) to gain knowledge about and improve the welfare of commercial pigs. We expected that a (very) low birth weight, being born in a large litter, and pre-weaning iron deficiency would all have lasting detrimental effects on physical and cognitive development, such as impaired growth and reduced memory performance. In addition, we expected that LBW pigs would show a higher motivation for food rewards than NBW pigs.

Chapter 2, which addressed the effects of LBW on cognitive development, and Chapter 5, 6 and Appendix I, which addressed cognitive functioning of early-life iron deficient (ID) pigs and its effects on synaptic plasticity in the brain, were mainly focused on developing a large animal model for these conditions in humans.

In Chapter 3, we studied food motivation of LBW and NBW pigs. In Chapter 4, we investigated the effects of litter size and supplier/breed on emotionality and cognitive performance. The piglets from large-sized litters are on average born with a lower birth weight than piglets born in small litters. The study in Chapter 4 may therefore be considered as an extension of the effects of birth weight reported in Chapter 2. Both Chapter 3 and 4 focused on the effects of LBW and litter size on cognition and development in pigs and their results may contribute to improving the welfare of pigs, for example through adjustments of housing and management practices in the pig farming industry.

Summarized, the findings reported in this thesis are:

Chapter 2:   LBW piglets outperformed their normal birth weight (NBW) siblings in cognitive performance in the holeboard task, and had lower hair cortisol values. We speculated that this difference reflected a higher food motivation in LBW piglets and tested this hypothesis in Chapter 3.

Chapter 3:   Contrary to our expectation, LBW and NBW pigs showed no

substantial difference in motivation for food rewards in either of two motivation tasks.

Chapter 4: Supplier/breed, but not litter size, strongly affected emotionality of the piglets. We found a supplier/breed by litter size interaction effect on both growth and cognitive performance in the holeboard task. Pigs born in large and small litters of one supplier did not differ in growth, whereas small litter pigs grew faster than large litter pigs purchased from a second supplier. Pigs from the second supplier outperformed pigs from the first supplier in the acquisition phase of the holeboard task. Small litter pigs from the second supplier outperformed pigs from large litters in the reversal phase of the task, which was not found in pigs from the first supplier.

Chapter 5: Early-life iron deficiency induced by withholding piglets iron supplementation and feeding them iron deficient milk replacer until weaning resulted in development of anemia during treatment. The iron deficient (ID) pigs showed long-lasting impaired growth and memory performance compared to controls. Hippocampal brain iron content was lower in ID pigs at 12 weeks of age, and correlated positively with (reference) memory performance. In addition, plasticity markers were analyzed in brain samples of the ID and control pigs (see **Appendix I**).

Chapter 6: Pigs that were reared with a sow until weaning, without receiving additional (creep) feed or iron supplementation, showed lower blood iron values during treatment than controls, but did not develop anemia. ID pigs did not show impaired growth or cognitive deficits after treatment.

Appendix I: ID pigs showed reduced synaptic plasticity in both the ventral and dorsal hippocampus, which could potentially have led to the impaired cognitive functioning and therefore lower memory performance found in Chapter 5.

Animals in translational research

For understanding both human and animal functioning, and for developing treatments for diseases or other forms of malfunctioning, animal studies are

needed. We can investigate humans that suffer from a specific complication or disease and compare them to healthy controls under similar conditions. However, the effects of confounding factors cannot stringently be controlled in such an approach. Animal research offers the possibility to conduct research under more controlled conditions, and by doing so, may yield more reliable results. Nonetheless, there will always be differences and discrepancies between an animal model and humans. In translational research, we aim to look at processes and functions that are very similar in the model animal and humans. By studying these processes and functions closely in animals in a controlled way, we expect to gain knowledge about those processes in humans as well (van der Staay, 2006; van der Staay et al., 2009).

Rodents have been widely used as an animal model in translational research, due to their quick reproduction rate, small size and low costs. However, concerns have been raised about the 'translational failure' of rodent studies: promising therapeutic interventions in animals do not always translate to the same effects in clinical trials in humans (Perel et al., 2007; van der Worp et al., 2010). Pigs have recently received increasing attention as an animal model for translational research, especially in the area of biomedicine (Lind et al., 2007; Kobayashi et al., 2012). Pigs closely resemble humans in their early development, physiology, and (neuro)anatomy, making them an attractive large animal model for translational research (Dilger and Johnson, 2010; Conrad et al., 2012). Still, the use of pigs in (translational) research, especially behavioral studies with pigs, is started relatively recently. Therefore, in conducting such studies – including those covered in this thesis – we are still searching to find useful, reliable methods and approaches that are suitable for testing pigs and useful in translational research.

PART I

Improved cognition of LBW pigs

In some animal studies, we may find very useful and reliable results which help us to treat complications in humans. In other studies, however, we may find that the animal model is not as suitable as expected for translation to human conditions. The findings in Chapter 2 implicate that LBW has differential effects on cognitive development in humans and pigs. In humans, it is known

that LBW impairs cognitive development (Arcangeli et al., 2012; Larroque et al., 2001). In rats, this finding was confirmed (Ogata et al., 1985; Saito et al., 2009).

Surprisingly, in Chapter 2 we found that LBW pigs outperformed their NBW siblings in a memory task, and that they learned faster. From these results, it may be interpreted that LBW in pigs results in improved cognition, as opposed to humans and rats. It is possible that in utero growth restriction in LBW pigs instigates brain preconditioning, i.e. perinatal sub-lethal stressors may induce protection against future stressors (Stetler et al., 2014). However, this raises the question why LBW would have this effect in pigs and not in humans and rodents. More research on cognitive functioning of LBW pigs is needed to answer this question, as from these study results it is unclear what caused the improved cognition of the LBW animals.

Cortisol levels
In Chapter 2, pigs were housed in pens of 1.25 m × 2.50 m until 10 weeks of age. LBW animals had relatively more space per pig than their NBW siblings, due to their size difference and the loss of one LBW piglet. Having only 0.6 m² available per pig until 10 weeks of age might have caused distress in the larger NBW animals, as they had slightly less space at their disposal, if considered relative to their larger body. This could explain the difference in hair cortisol values between LBW and NBW pigs. In Chapter 3, in which all pigs had ample space available (4 m2 per pig) we did not find effects on cortisol concentrations. In a study assessing the effects of pen size on behavioral and physiological responses in group housed pigs, less space per pig also resulted in higher cortisol concentrations (Barnett et al., 1992). This is interesting for current pig housing guidelines (Forbes et al., 2007), which may prove inadequate to satisfy the pigs' needs.

Selection of LBW animals
Results of previous studies conducted by Gieling et al. (2012) and Murphy et al. (2013) and the studies described in Chapter 2 and 3 of the present thesis on the effects of LBW in pigs on long-term cognitive development, are inconclusive. In Chapter 2 and 3, stricter selection criteria for LBW animals were applied than in previous studies by Gieling et al. (2012) and Murphy et al. (2013). We required lower absolute birth weights of LBW animals, with an

upper limit of 1 kg. This criterion was also used in a study on birth weight and growth performance in pigs by Quiniou et al. (2002), who define 'small piglets' as weighing less than 1 kg. It is important to note that the LBW piglets in our studies did not show asymmetrical growth typical for severe intra-uterine growth restriction (IUGR). IUGR piglets reportedly experience more negative impact on their welfare than symmetrical LBW pigs (Rutherford et al., 2013).

Food motivation, growth rate and feed intake

The study in Chapter 3 focused on the motivation for food (rewards) of NBW and LBW pigs. We argued that LBW piglets have to fight harder for resources in order to beat their low survival chances, and therefore might have an increased motivation to obtain food. We did not find substantial motivational differences between NBW and LBW pigs. This may have been due to the food reward (chocolate M&M's®) used. It is worthwhile to test whether food rewards rich in fats or carbohydrates, tested after a longer period of food deprivation, would yield other results in motivation studies in pigs.

As LBW and NBW pigs differ in size and growth rate, it is difficult to investigate food motivation, controlling the putative confounding factors size, growth rate, and feed intake. Therefore, it may be useful to measure individual feed intake during future studies and correct for size or weight, thus assessing relative feed or energy intake. In our study, we aimed to reduce the possible influence of body size or strength by assessing motivation in two different types of tasks: if size was of influence in the runway task, it was less likely to be in the nose wheel task, where persistence rather than physical effort was needed to gain a high number of rewards.

Effects of breed or supplier

In Chapter 4, due to technical problems after purchasing the first batch of piglets, the remaining batch was purchased from another supplier, supplying a different breed of pigs. Although we did not set out to perform the study in this manner, it did generate some very interesting results. The fact that breed or supplier – we cannot disentangle the two in this set up – had a strong effect on both emotionality and cognitive performance, shows that results of behavioral studies cannot be generalized across suppliers or breeds. In rats, it is also known that performance in behavioral tasks can differ substantially between

strains (e.g. Glowa and Hansen, 1994; Andrews et al., 1995). On the one hand this is a nuisance, as we want study results from scientific research to be as reliable and replicable as possible. On the other hand, such differences can be used for selective breeding purposes in both research and husbandry.

Nonetheless, it is an important reminder that results from studies using a specific type of breed, may not be generalizable to other breeds, and that additional experiments (extended replications) are needed to confirm the findings (van der Staay et al., 2010). That is an important reason why human, rodent and large animal models are all useful and necessary.

It is possible that stronger effects of litter size on cognitive performance can be found using pigs from one supplier or breed only, as litter size did show differential effects per supplier/breed. These interaction effects were, however, estimated based on five piglets per group. It is plausible that this has reduced the power of our analyses for interaction effects. We recommend that effects of litter size on cognitive performance is further investigated using more animals from the same suppliers or breeds.

PART II

Iron deficiency and development

In the second part of this thesis, we investigated the long-term effects of pre-weaning iron deficiency on cognition and development. In Chapter 5, piglets that did not receive iron supplementation and were fed an iron deficient (ID) milk diet for four weeks became anemic during treatment. ID animals showed retarded growth, impaired memory performance and reduced brain iron levels weeks after treatment and iron repletion. Our findings suggest that iron is essential for the developent and normal functioning of brain structures involved in memory and learning. These lasting effects of severe early-life iron deficiency are in line with findings in human and rodent iron deficiency research (Yehuda and Youdim, 1989; Lozoff et al., 2006; Bourque et al., 2008). Our findings thus support the notion that iron is an essential micronutrient for neonatal growth and cognitive development.

It is suggested that the detrimental effects of iron deficiency on cognition

involve the reduction of brain iron, receptor and transporter densities, and changes in serotonin and dopamine pathways (McCann and Ames, 2007; Youdim et al., 2010). Findings in Chapter 5 confirm that brain iron content is reduced due to early-life iron deficiency in pigs. In Appendix I, we show that early-life iron deficiency also reduces hippocampal synaptic plasticity.

The most common parameter to indicate iron deficiency anemia is hemo-globin (McLean et al., 2009). Using the hemoglobin values for the assessment of anemia in pigs (Ishaya, 2012), ID piglets in Chapter 6 did not become ane-mic during treatment. After treatment, ID piglets did not show impairments in growth nor cognitive performance. It has been reported that iron deficiency that does not lead to anemia in general does not lead to impaired growth or reduced performance in behavioral tasks (McCann and Ames, 2007). When comparing results of Chapter 5 and 6, we can confirm in pigs that develop-ment of anemia is essential in retarding growth and developing long-term impairments of memory performance due to iron deficiency.

Palatability of iron deficient milk formula

Ideally, we would have measured individual feed intake and weight gain in Chapter 5, (e.g. Fiset et al., 2015). However, then housing all piglets individu-ally would have been inevitable. Pigs are social animals, and individual hous-ing would not only reduce their welfare, but may also have interfered with the behavioral tests (Fone and Porkess, 2008; McLean et al., 2010). We therefore chose to feed the piglets as close to ad libitum as possible, by feeding them five times per day and adjusting the amount of milk replacer daily to their needs. At the beginning of the study reported in Chapter 5, the first week after re-moval from the sow, ID animals were having trouble to consume the milk and to gain weight. Two animals were consequently euthanized due to poor growth and health performance. ID animals may have had a reduced appetite due to iron deficiency, which has been shown in ID rats (Beard et al., 1995) and ID piglets (Ishaya, 2012). However, as we have deliberated about with veterinarians, iron deficiency was unlikely to already have a strong effect – if any – at such an early stage of the study. Instead, it is possible that the ID milk replacer was less palatable than the control iron-sufficient milk replacer. It would be interesting to further look into palatability of milk replacer that is low in iron content, by measuring individual feed intake and, for example, conduct a preference study in which the animals can choose between iron suf-ficient and iron deficient milk.

IMPLICATIONS OF THIS THESIS' FINDINGS

Selection for very large litters

Selective breeding in the pig farming industry is causing average litter sizes to increase rapidly (Kondrup, 2013; Rutherford et al., 2013). With an increase in litter size, piglets have less space, oxygen and nutrients to share in utero, causing an increase in weight variability within litters and a decline in average birth weight (Quiniou et al., 2002; Rutherford et al., 2011). As a result, the incidence of piglets born with a LBW is increasing.

Although we find an improved cognitive performance of LBW pigs, this is only one aspect of their behavior and performance. More research on the performance, welfare and behavior of LBW piglets can benefit both the welfare of pigs and the production success of farms. It is well-known that LBW reduces life-long weight gain (Douglas et al., 2013), which our findings in Chapter 2 and 3 corroborate. LBW in piglets poses numerous other welfare problems, as they are weaker and slower than their larger litter mates. Survival chances of LBW piglets are low, due to high risks of hypothermia, crushing by the sow and starvation (Rutherford et al., 2013).

With increasing farm sizes and decreasing personal care for individual piglets, weaker piglets are increasingly often left without supervision or help in their struggle to survive. This is not only gravely detrimental to the welfare of these LBW piglets, it also reduces the productivity of the piggery when the mortality rate of LBW piglets is high. Selection for large litter sizes thus seems to have surpassed its peak of efficiency and profitability, both for the animal and the farmer. It is advisable to adjust the selection of litter sizes to more natural and effective proportions. This would cause increased physiological maturity in piglets, promoting better health and survival chances (Tuchscherer et al., 2000).

Necessity of iron dextran injections

In common pig husbandry practice, piglets receive an iron dextran injection in order to prevent development of iron deficiency (Svoboda and Drabek, 2005). However, there has been increasing concern about these iron supplementations. For example, Lipiński et al. (2010) argue that the administration of 100 to 200 mg of iron per piglet is excessive and possibly toxic. When comparing

serum iron values at the end of treatment in control piglets of the studies reported in Chapters 5 and 6, piglets in Chapter 5 receiving a 200 mg iron injection show signs of iron overload (Cornelius and Kaneko, 1963). These high serum iron values are not found in control animals in Chapter 6, which received two spread iron injections of lower doses. Lipiński et al. (2010) argue that reducing and spreading iron administration reduces iron toxicity and allows the body to use the iron more effectively, a notion which our findings seem to confirm.

Not only the high dose of iron injections has been questioned, the necessity of the iron supplementation all together is increasingly being doubted. Yu et al. (2002) showed that a 200 mg iron dextran injection does not contribute to overall performance of piglets at 15 days of age when creep feed is provided from day 7 onward. Offering creep feed has become common practice in commercial husbandry systems, because it improves growth and feed intake after weaning (Okai et al., 1976). In addition, piglets use creep feed to supplement their milk diet (English et al., 1988). This raises the question whether iron injections are still necessary. As we show in both iron deficiency studies in this thesis, oral iron ingestion (i.e. consuming balanced commercial pig feed after treatment ended) recovered blood iron values in ID animals quickly.

Even without offering creep feed, Sansom and Gleed (1981) argue that consumption of sow feces may prevent piglets from becoming anemic. Piglets that remained with the sow (Chapter 6) without receiving iron supplementation or creep feed, showed no decline in blood iron values during treatment and did not become anemic. Consequently, their performance was not impaired. We argue that piglets presumably ingested sow feces and spilled feed, thereby ingesting sufficient iron to prevent development of anemia. It deserves strong recommendation to reevaluate the dose and application of iron injections in husbandry practices. If iron supplementation proves to be unnecessary, this will both benefit the welfare of piglets, which then do not have to undergo an injection at a young age, and reduce workload and costs for the farmer. However, as iron injections have become standard in pig husbandry, it will presumably prove difficult to change this tradition in farming practices.

THESIS CONCLUSIONS

- Although LBW causes numerous welfare problems in young piglets, we find an improved memory performance compared to NBW siblings, though no difference in food motivation;

- Litter size affects growth and cognitive performance in pigs, although we found that this effect is supplier or breed dependent;

- Supplier or breed has a strong effect on emotionality responses of piglets;

- The piglet is a suitable animal model for early-life iron deficiency in humans, provided that the induced iron deficiency leads to anemia.

Appendix I

Early-postnatal iron deficiency reduces synaptic plasticity in the dorsal and ventral hippocampus in pigs

Manuscript in preparation

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ABSTRACT

Iron deficiency (ID) in neonates has been linked to impaired cognitive functioning in both rodent models and humans. The hypothesized mechanisms have so far been mostly limited to iron being a cofactor for several important enzymes. A few studies have shown a possible link between reduced BNDF protein levels and ID but there have not been many links between iron deficiency and reduced synaptic plasticity as a cause of impaired cognitive functioning yet. Pigs could provide an excellent model for early postnatal ID, since both the pig's gyral pattern and the timing of brain development are very similar to humans. By looking into the effects of ID on protein levels of synaptic plasticity markers like synaptophysin, BDNF (mature BDNF, 18kDa proBDNF and 24kDa proBDNF), p75NTR and TrkB in the ventral hippocampus, dorsal hippocampus and prefrontal cortex of newborn piglets, we hope to provide a link between ID and synaptic plasticity as a cause of impaired cognitive functioning. Newborn piglets were either fed a low-iron diet or a normal-iron diet for 4 weeks. After the pigs were 12 weeks of age, brains were harvested and dissected. Western blotting showed decreased synaptophysin and TrkB in the dorsal hippocampus, and decreased BDNF and TrkB in the ventral hippocampus as a consequence of ID. Both a shift towards the 18kDa proBDNF form and a shift towards the p75NTR apoptotic pathway were found in the ventral hippocampus and the same shift towards p75NTR was found in the dorsal hippocampus. Epigenetic changes and changes in proteases that cleave proBDNF could be potential underlying mechanisms leading to alterations in protein levels and a reduction in synaptic plasticity.

Keywords: BDNF, neuroplasticity, hippocampus, neurotrophin, piglets

INTRODUCTION

Nutritional iron deficiency (ID) in neonates and toddlers negatively affects brain development leading to prolonged functional and structural central nervous system (CNS) deficits (Lozoff et al., 2000; Grantham-McGregor and Ani, 2001; Beard, 2003; Beard and Connor, 2003; Walter, 2003). Iron is not only an essential micronutrient required for oxygen transport, but is also a required cofactor for key enzymes in neurotransmitter production (Wang et al., 2002; Beard, 2003; Dunkley et al., 2004). From late prenatal to early postnatal life, the brain undergoes the so-called brain growth spurt. This critical time frame is featured by high demands of iron needed to orchestrate neuronal connectivity and myelination (Dobbing and Sands, 1979; Connor and Menzies, 1996). ID particularly leads to deficiencies in prefrontal cortex and hippocampal signaling (Rao et al., 2012). Interestingly, many of the ID-induced CNS deficits do not recover after iron repletion and eventually lead to sustained impairments (Yehuda and Youdim, 1989; Bourque et al., 2008; Ranade et al., 2013). In a longitudinal follow-up study, it was shown that children who suffered from severe ID during infancy maintained mental and motor impairment, even ten years after the onset of iron treatment (Lozoff et al., 2000). Although ID is often studied in rodents, the brain development of piglets resembles human brain development much more than for instance rats, in part due to the similar timing of the brain growth spurt (Dobbing and Sands, 1979; Conrad et al., 2012). Moreover, both piglets and infants are prone to develop CNS deficits due to restrained perinatal iron availability, underscoring the necessity of iron-containing nutrition (Whiteker, 1965; Lipiński et al., 2010). In Chapter 5, we showed in piglets that pre-weaning dietary ID impairs later spatial learning and memory even after restoring nutritional iron levels (Antonides et al., 2015b). Moreover, we confirmed lower iron content in the CA3 and dentate gyrus regions of the hippocampus. The hippocampus plays an essential role in spatial learning and memory and hippocampal-cortical interactions produce very strong memory traces (Eichenbaum et al., 1996). Connections between the hippocampus and prefrontal cortex (PFC) contribute to memory consolidation (Preston and Eichenbaum, 2013). Rodent models for ID have already shown that iron is required for apical dendrite formation in the CA1 region of the hippocampus (Jorgenson et al., 2004; Brunette et al., 2010). Hippocampal brain-derived neurotrophic factor (BDNF) signaling is strongly associated

with synaptic plasticity and memory formation (Gómez-Palacio and Escobar-Rodríguez, 2006). In this study we investigate whether the lower hippocampal iron content and the coinciding impaired memory performance, are linked to impaired BDNF signaling in the hippocampus and prefrontal cortex. Protein levels of the plasticity markers BDNF, TrkB, p75NTR, and synaptophysin in the hippocampus and prefrontal cortex were measured with western blot.

Furthermore, the neonatal porcine and human brain structures are similar; sharing the same gyral pattern, and the same distribution of gray and white matter (Pond et al., 2000; Lind et al., 2007).

MATERIALS AND METHODS

Brain material was kindly provided by the Faculty of Veterinary Medicine at the University of Utrecht after finishing their own experiments with the piglets.

Animals

Pigs ((Terra × Finnish landrace) × Duroc mix) from the Faculty of Veterinary Medicine at the University of Utrecht were used. Two male sibling piglets with a normal body weight were selected from ten different litters. For each pair, one piglet was randomly assigned to the treatment group (n=10) and the other piglet to the control group (n=10). The piglets were separated from the sow after 4-6 days.

Housing
The piglets were housed in the Clinic of the Department of Farm Animal Health, University Utrecht. They were housed in four adjacent pens with five piglets per pen, sorted by group and age. The pens measured 1.25 m × 2.5 m (from 10 to 12 weeks of age they were housed in pens measuring 2.5 m × 5 m) and the piglets were provided with playing material such as chains and balls. A radio played continuously and room temperature was adjusted to the needs of the piglets ranging from 26°C during the first week to 21°C during the 12th week. There was a 12h light/dark cycle with the lights on 7:00 AM. Water was provided ad libitum via an automated drinking nipple. The piglets were fed four times a day at 7:00 AM, 10:30 AM, 2:00 PM, and 8:00 PM.

Treatment

The experiment was performed double blind, by means of a third person that knew the identity of the treatment groups. Within 24h after birth, the control group received an injection of iron dextran (Gleptosil, 200mg as gleptoferron MS Schippers, Lommel, Belgium), as is common in the farming industry to avoid iron deficiency. The treatment group did not receive this injection. After 4-6 days, the treatment group was fed an iron deficient diet containing 10mg/kg iron whereas the control group was fed an iron rich diet containing 100mg/kg iron. Both diets were provided by Mead Johnson Nutrition and were freshly prepared before each feeding. After 4 weeks the experimental feeding ended and all piglets were fed an iron rich commercially available diet at libitum. At 12 weeks of age, the piglets were euthanized. During the experiment, one piglet died and two had to be put down due to excessive weight loss, resulting in a total of 17 piglets used (control n=9, ID n=8).

Brains

Ventral hippocampus, dorsal hippocampus and prefrontal cortex (PFC) were dissected and provided by the Faculty of Veterinary Medicine, University of Utrecht. The material was packed in aluminum foil and stored at -80°C. One ventral hippocampus sample was lost during storage.

Homogenization

Brain samples were homogenized in 1ml lysis buffer (100mM Tris-HCl, 200mM NaCl, 1mM EDTA, 2mM DTT, 0.05% triton, 1 tablet complete protease inhibitor mix/20ml buffer (Roche, Vilvoorde, Belgium), 1 tablet PhosSTOP phosphatase inhibitor cocktail/10ml buffer (Roche)) using a mini-Bead-Beater (BioSpec products, Bartlesville, OK, USA). Samples were homogenized 3x for 30s with 5min cooling on ice between runs. After 30min cooling on ice, samples were centrifuged at 16,000G for 20min. (4°C), and the supernatant was divided into aliquots and stored at -80°C until further use.

Western Blot

Brain homogenates were separated with SDS-PAGE. Gel percentages and protein load for TrkB, p75NTR, synaptophysin, and BDNF were 7.5% (50μg protein), 10% (25μg protein), 10% (10μg protein), and 14% (40μg protein), respectively. Proteins were transferred onto a PVDF membrane, which was

subsequently blocked (50% Odyssey blocking buffer in PBS, Li-Cor, Lincoln, NE) for 1h at room temperature. The membranes were incubated with primary antibody overnight at 4°C. Antibodies used were 1:250 rabbit anti-TrkB (#9104, Cell signaling technologies Beverly, MA), 1:1000 rabbit anti-p75NTR (#07-476, Millipore Billerica, MA), 1:1000 mouse anti-synaptophysin (#MAB5258, Millipore), and 1:600 rabbit anti-BDNF (#20981, Santa Cruz Biotechnologies Santa Cruz, CA). For normalization, either GAPDH or β-actin was used: 1:2,000,000 mouse anti-GAPDH (#10R-G109A, Fitzgerald Huissen, NL), and 1:2000 mouse anti-β-actin (#F0110, Santa Cruz Biotechnologies). The membranes were washed with PBS and PBS-Tween, and subsequently incubated with secondary antibody for 1h at room temperature: 1:5000 goat anti-rabbit IRDye 800 (#926-32211, Li-Cor), and 1:10,000 donkey anti-mouse IRDye 680 (#926-32222, Li-Cor). Membranes were washed in PBS and PBS-Tween and fluorescent protein bands were visualized using the Odyssey Infrared Imaging System (Li-Cor). ImageJ (http://imagej.nih.gov/ij/) was used to quantify the fluorescent protein bands.

Statistical analysis

For analysis of the data, a two-tailed student's t-test was performed. Statistical significance was set at P=0.05. Dixon's Q test was performed to identify outliers.

RESULTS

Effects of iron deficiency on synaptophysin protein levels

Analysis of the synaptophysin western blots showed that iron deficiency reduced the synaptophysin protein levels both in the dorsal hippocampus (p<0.05, Figure 1) and prefrontal cortex (p<0.05, Figure 1) compared to the control group. No effects were found in the ventral hippocampus (p=0.36, Figure 1).
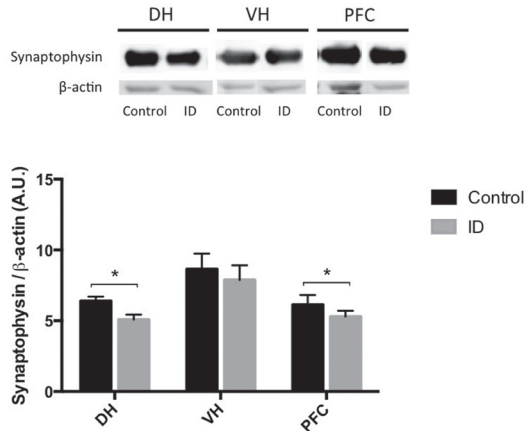
Figure 1. The effect of iron deficiency (ID) on synaptophysin protein levels in the dorsal hippocampus (DH), ventral hippocampus (VH), and prefrontal cortex (PFC). Data are presented as mean + SEM. For the ID groups of the DH and PFC: n=8, VH: n=7. For the control groups: n=9. * P<0.05

Effects of iron deficiency on BDNF protein levels

Analysis of the BDNF western blots showed that ID increased mature BDNF (mBDNF) protein levels in the dorsal hippocampus (p<0.05, Figure 2A). Contrary to the dorsal hippocampus, ID reduced mBDNF protein levels in the ventral hippocampus (p<0.001, Figure 1A), while no effect was found in the prefrontal cortex (p=0.90, Figure 1A).

These same results were found for the 18kDa proBDNF protein levels, with ID increasing 18kDa proBDNF protein levels in the dorsal hippocampus (p<0.05, Figure 2B), while reducing 18kDa proBDNF protein levels in the ventral hippocampus (p<0.001, Figure 2B). Again, no effects were found in the prefrontal cortex (p=0.72, Figure 2B).

A similar pattern emerged for the 24kDa proBDNF isoform. ID had no effect on 24kDa proBDNF protein levels in the dorsal hippocampus (p=0.11, Figure 2C), while ID reduced the 24kDa proBDNF levels in the ventral hippocampus (p<0.001, Figure 2C) and no effects were found for 24kDa proBDNF levels in the prefrontal cortex (p=0.35, Figure 2C).

We also analyzed ratios of the different BDNF isoforms. ID did not affect the 18kDa proBDNF / mBDNF ratio in the dorsal hippocampus (p=0.94,

Figure 2D), but increased the 18kDa proBDNF / mBDNF ratio in the ventral hippocampus (p<0.05, Figure 2D). ID did not affect the 18kDa proBDNF / mBDNF ratio in the prefrontal cortex (p=0.36, Figure 2D).

Results of the 24kDa proBDNF / mBDNF ratio indicated that ID did not affect the 24kDa proBDNF / mBDNF ratio in the dorsal hippocampus (p=0.54, Figure 2E), but decreased the 24kDa proBDNF / mBDNF ratio in the ventral hippocampus (p<0.001, Figure 2E). ID increased the 24kDa proBDNF / mBDNF ratio in the prefrontal cortex (p<0.01, Figure 2E).
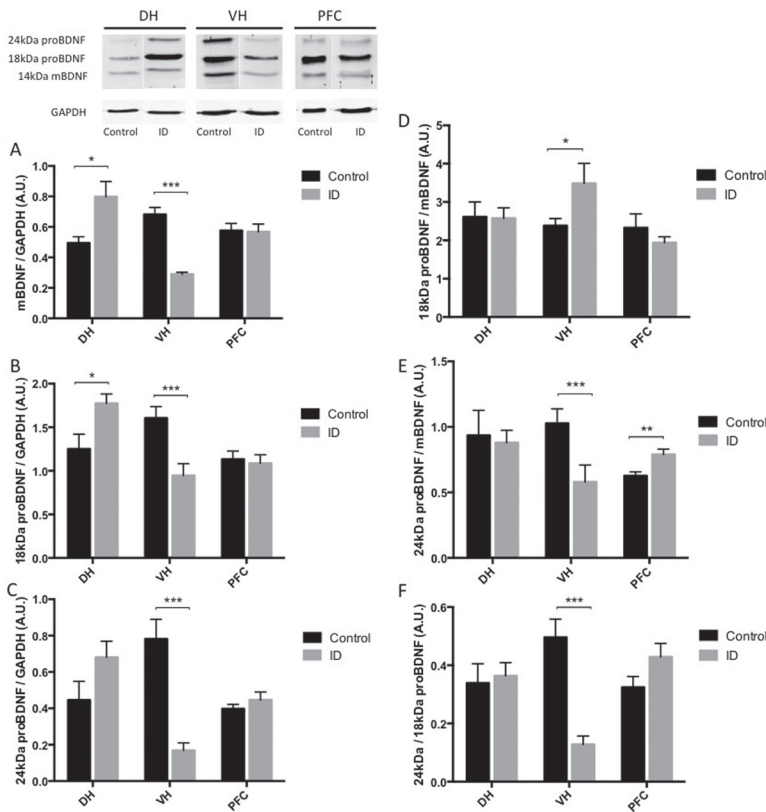
Figure 2. The effect of iron deficiency (ID) on mBDNF (A), 18kDa proBDNF (B), and 24kDA proBDNF (C) protein levels, and 18kDa proBDNF/mBDNF (D), 24kDa proBDNF/mBDNF (E), and 24kDa/18kDa proBDNF ratios in the dorsal hippocampus (DH), ventral hippocampus (VH), and prefrontal cortex (PFC). All data are presented as mean + SEM. For all DH and PFC ID groups: n=8, for the VH ID group: n=7. For all control groups: n=9. * P<0.05 ** P<0.01 *** P<0.001

Furthermore, ID did not affect the 24kDa proBDNF / 18kDa proBDNF ratio in the dorsal hippocampus (p=0.77, Figure 2F), but decreased the 24kDa proBDNF / 18kDa proBDNF ratio in the ventral hippocampus (p<0.001, Figure 2F). ID showed a tendency to increase the 24kDa proBDNF / 18kDa proBDNF ratio in the prefrontal cortex, (p=0.097, Figure 2F).

Effects of iron deficiency on the BDNF receptors

Analysis of the p75NTR western blots showed that ID did not affect the p75NTR protein levels in the dorsal hippocampus, ventral hippocampus and prefrontal cortex (p=0.72, p=0.74, and p=0.24 respectively, Figure 3A). Analysis of the TrkB western blots showed that ID reduced TrkB protein levels in both the dorsal and ventral hippocampus (p<0.01 for both, Figure 3B), while increased TrkB protein levels were found in the prefrontal cortex (p<0.001, Figure 3B).
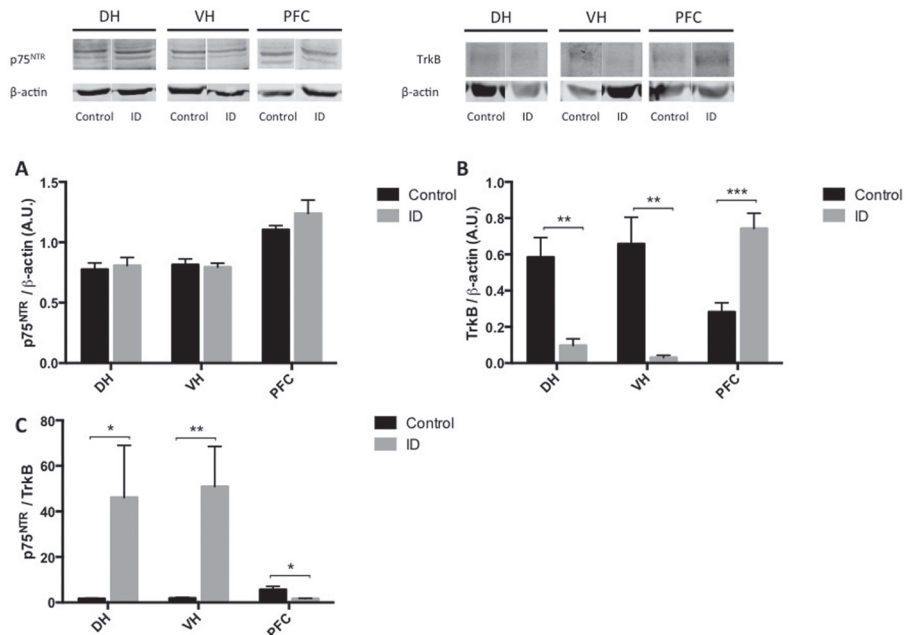

Figure 3. The effect of iron deficiency (ID) on p75NTR protein levels (A), TrkB protein levels (B), and the p75NTR/ TrkB ratio in the dorsal hippocampus (DH), ventral hippocampus (VH) and prefrontal cortex (PFC). All data are presented as mean + SEM. For all DH and PFC ID groups: n=8, for the VH ID group: n=7. For all control groups: n=9. * P<0.05 ** P<0.01 *** P<0.001

Calculation of the p75NTR / TrkB ratio revealed that ID increased this ratio in both the dorsal and ventral hippocampus (p<0.05 and p<0.01 respectively, Figure 3C), while a decreased p75NTR / TrkB ratio was found in the prefrontal cortex (p<0.05, Figure 3C).

DISCUSSION

The data shows that iron deficiency reduces synaptophysin levels in the dorsal hippocampus and prefrontal cortex As previously mentioned, synaptophysin is a protein located in synaptic vesicles and it interacts with several other synaptic proteins, thereby regulating processes such as synapse formation, endocytosis, exocytosis and biogenesis of synaptic vesicles (Valtorta et al., 2004; Mallozzi et al., 2013). Reduced synaptophysin protein levels due to iron deficiency could therefore lead to reduced synaptic plasticity. This could be one potential mechanism leading to impaired cognitive functioning, which has been reported to be an effect of iron deficiency (Beard and Connor, 2003; Lozoff et al., 2006). To our knowledge, this is the first time that iron deficiency is directly linked to reduced synaptophysin levels in the hippocampus and prefrontal cortex. A potential mechanism through which iron deficiency reduces synaptophysin has yet to be discovered. A possible mechanism could lie in the reduction of mBDNF and shift toward 18kDa proBDNF (which will be discussed in more detail later), resulting in reduced synaptogenesis leading to a decrease in synaptic vesicles and thereby a reduction in synaptophysin.

The data also show increased mBDNF and 18kDa proBDNF in the dorsal hippocampus. These findings are surprising since iron deficiency has been reported to reduce BDNF levels in the hippocampus, though no specific details on the different BDNF forms were given (Tran et al., 2008; Estrada et al., 2014). The ventral hippocampus did show a reduction in mBDNF, 18kDa proBDNF and 24kDa proBDNF. One potential mechanism leading to reduced BDNF levels was recently proposed by Tran et al. and focuses on epigenetic chromatin remodeling at the Bdnf locus (Tran et al., 2015). Tran et al. found that early-life iron deficiency induced stable histone modifications (H4 deacetylation, increased H3 K27me3 enrichment and decreased H3 K4me3 enrichment) at the Bdnf-IV promoter. This could possibly lead to chromatin remodeling and thereby reduce Bdnf-IV transcription. One interesting aspect of our data is the increase of BDNF forms in the dorsal hippocampus ver-

sus the decrease of these BDNF forms in the ventral hippocampus. As previously mentioned, the dorsal hippocampus is more related to memory whereas the ventral hippocampus is more related to emotional behavior (Moser and Moser, 1998; Fanselow and Dong, 2010). Still, studies have shown that ID is linked to impaired cognition, whereas the increase of BDNF forms in the dorsal hippocampus would suggest otherwise. These findings, together with the recently proposed mechanism by Tran et al. mentioned above, raise interest in the specific effects of ID on the expression patterns of the different BDNF isoforms in the dorsal and ventral hippocampus and especially the differences in BDNF-IV expression between the dorsal and ventral hippocampus.

Looking at the ratios between the different BDNF forms mBDNF, 18kDa proBDNF and 24kDa proBDNF, the 18kDa proBDNF / mBDNF ratio was found to be increased in the ventral hippocampus of iron deficient piglets, whereas the 24kDa proBDNF / mBDNF ratio was found to be decreased. This indicates an increased amount of 18kDa proBDNF and reduced amount of 24kDa proBDNF compared to mBDNF protein levels. These findings suggest a shift towards the 18kDa proBDNF form in the ventral hippocampus of iron deficient piglets. This is further supported by the decreased 24kDa proBDNF / 18kDa proBDNF ratio. To our knowledge, these findings have not been reported before, but they could potentially provide a new insight into the mechanisms leading to cognitive impairment linked to iron deficiency. ProBDNF is known to exist in different sizes due to cleavage, glycosylation and dimerization. Matrix metalloproteinase 7 (MMP-7) is known to cleave proBDNF into 18kDa proBDNF but not into 24kDa proBDNF (Lee et al., 2001). Looking into the effects of ID on the activity and expression of MMP-7 might provide further insight into the shift towards 18kDa proBDNF. Interestingly, iron deficiency increased the 24kDa proBDNF / mBDNF ratio in the prefrontal cortex, but no other statistically significant differences were found for the BDNF data in the prefrontal cortex. This suggests that the effects of iron deficiency are very brain area specific.

To further analyze the potential importance of the BDNF data provided by the BDNF western blots, BDNF receptor levels were analyzed. No statistically significant effects of iron deficiency on p75NTR receptor levels were found in any of the three brain areas. This finding was supported by Tran et al. who reported no difference in p75NTR mRNA expression between iron deficiency

and control group (Tran et al., 2009).

ID decreased TrkB protein levels in both the dorsal and ventral hippocampus, while an increase was found in the prefrontal cortex. As previously mentioned, binding of mBDNF to TrkB leads to autophosphorylation of TrkB and subsequent activation of the MAPK, PI3K and PLCγ1 pathways, eventually resulting in cell survival and neurite outgrowth (Huang and Reichardt, 2003; Longo and Massa, 2013). Reduced TrkB levels would therefore lead to decreased cell survival and decreased neurite outgrowth, eventually leading to reduced plasticity, which can be linked to impaired cognitive functioning. For this reason, the increased TrkB protein levels are very unexpected and a possible explanation cannot been given at this point, though looking into the effects of ID on TrkB expression via qPCR might be necessary to confirm these results.

The p75NTR / TrkB ratio was increased in the dorsal and ventral hippocampus in ID piglets, while this ratio was decreased in the prefrontal cortex. This increase in the dorsal and ventral hippocampus further supports the idea of reduced plasticity, due to the shift towards the apoptotic effects of p75NTR instead of the proliferative effects of TrkB.

As previously mentioned, preliminary data from Chapter 5 have shown that early-life ID decreases growth, memory scores and iron content in neurons from the hippocampal formation and prefrontal cortex in newborn piglets, together with lower iron content in the CA3 and dentate gyrus regions of the hippocampus, which directly correlated with lower memory performance (Antonides et al., 2015b). The data of our research supports these findings by showing that ID reduces synaptic plasticity in both the ventral and dorsal hippocampus, which could potentially lead to the impaired cognitive functioning and therefore lower memory performance that was found by Antonides et al.

CONCLUSION AND FUTURE PERSPECTIVES

Reduced synaptophysin protein levels, TrkB protein levels and an increased p75NTR /TrkB ratio in the dorsal hippocampus, together with reduced BDNF protein levels, TrkB protein levels and an increased p75NTR / TrkB ratio in the ventral hippocampus suggest that ID decreases synaptic plasticity, thereby leading to impaired cognitive functioning. The unexpected increase

of BDNF protein levels in the dorsal hippocampus and the interesting shift towards 18kDa proBDNF in the ventral hippocampus provide some interesting ideas for the future. Looking into the effects of ID on the expression patterns of specific BDNF isoforms in the dorsal and ventral hippocampus with a special interest in BDNF-IV, and looking into the effects of ID on MMP-7 protein levels or MMP-7 activity could potentially provide some new mechanistic insights into these unexpected, yet interesting findings, for which we provide a scheme explaining the potential mechanisms (Figure 4).
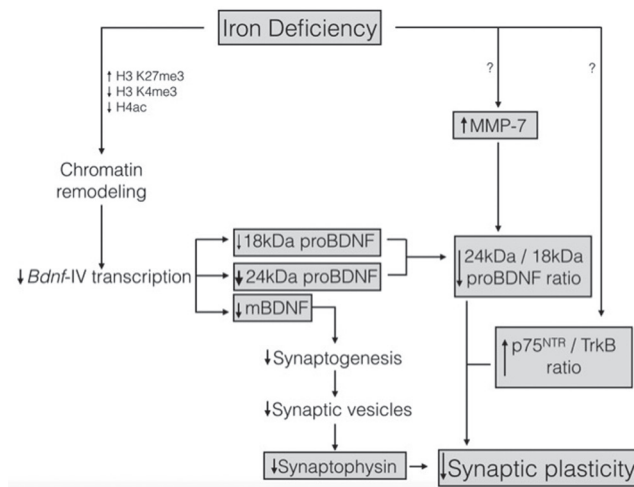
Figure 4: A potential scheme showing the effects of iron deficiency on BDNF and synaptophysin via epigenetic changes and potential changes in MMP-7 activity. Iron deficiency induces epigenetic changes resulting in reduced Bdnf-IV transcription, leading to reduced proBDNF (18 and 24kDa) and reduced mBDNF. Reduced mBDNF in its turn leads to reduced synaptophysin levels due to reduced synaptogenesis. Iron deficiency also increases MMP-7 activity, which leads to a shift towards 18kDa proBDNF. This shift together with an increased p75NTR / TrkB ratio, and the decreased mBDNF and synaptophysin, all lead to reduced synaptic plasticity.

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Nederlandse samenvatting

Jonge dieren zijn kwetsbaar, omdat ingewikkelde processen betrokken zijn bij hun groei en ontwikkeling. Worden deze processen in een bepaalde periode verstoord, dan kan dit een blijvend effect op de ontwikkeling hebben. Een voorbeeld is de ontwikkeling van spraak bij mensen en zang bij vogels. Als een baby of een jonge vogel tijdens een bepaalde gevoelige ontwikkelingsperiode niet de juiste geluiden van spraak of zang te horen krijgt, zullen zij nooit normale spraak of zang ontwikkelen. Een normale mentale en lichamelijke ontwikkeling hangt dus af van de juiste afstemming van deze processen en de factoren die ze beïnvloeden.

Tijdens de vroege ontwikkeling laten dieren over het algemeen een groeispurt zien, waarin organen zoals de hersenen snel ontwikkelen. Het verschilt per diersoort wanneer in de ontwikkeling deze groeispurt plaatsvindt. In knaagdieren ontwikkelen de hersenen voornamelijk na de geboorte, terwijl dit bij mensen vooral rondom de geboorte plaatsvindt. Het rattenbrein is daarom, wat ontwikkeling betreft, op de leeftijd van 12 tot 13 dagen pas het meest gelijk aan het brein van een pasgeboren mensenbaby. Er zijn recentelijk zorgen geuit over het gebruik van knaagdieren als diermodellen voor mensen in onderzoek, zoals onderzoek naar de effecten van een verstoorde ontwikkeling voor de geboorte. De resultaten van knaagdieronderzoek blijken namelijk niet altijd te kunnen worden gegeneraliseerd naar de mens. Dieren die meer op mensen lijken, leveren naar verwachting relevantere en beter bruikbare studieresultaten op. In onderzoek met het doel de resultaten te vertalen naar de mens krijgen grote modeldiersoorten daarom steeds meer aandacht..

Varken als diermodel voor de mens

Varkens vertonen veel meer overeenkomsten met mensen dan knaagdieren. De groeispurt en hersenontwikkeling in varkens vindt net als bij mensen rondom de geboorte plaats en beide hebben relatief grote hersenen. Verder zijn varkens intelligente, sociale dieren en kunnen zij op jonge leeftijd getraind worden voor gedragstaken. Biggen zijn makkelijk verkrijgbaar bij varkenshouderijen. Bovendien kan de fokker uitgebreide achtergrondinformatie over deze biggen verstrekken, wat ze interessante dieren maakt voor gebruik in onderzoek. Het varken is daarom een veelbelovend diermodel om het onderzoek in mensen en knaagdieren te ondersteunen en aan te vullen. Varkens kunnen in het bijzonder goed gebruikt worden om de effecten van bepaalde externe invloeden

kort na de geboorte op mentale en fysieke ontwikkeling te onderzoeken.

Laag geboortegewicht

Bij mensenkinderen worden problemen rondom de geboorte veelvuldig in verband gebracht met een tragere ontwikkeling, (neuro)psychologische achterstand en slechte schoolprestaties op latere leeftijd. In het bijzonder blijkt een laag geboortegewicht (LG) een sterk negatief effect te hebben. Uit onderzoek blijkt dat LG ook bij ratten een negatief effect op ontwikkeling en leervermogen heeft. LG in varkens komt veel voor, door de selectie op grote tomen (nesten). Hoe groter de toom, des te lager is het gemiddelde geboortegewicht van de biggen in een toom, waarbij meer biggen met een LG worden geboren. Dit komt doordat de biggen de beperkte ruimte, voedingsstoffen en zuurstof moeten delen in de baarmoeder. Dit heeft een negatieve invloed op hun ontwikkeling. Europese landen met een grote varkensindustrie hebben een gemiddelde toomgrootte van 14 biggen. Sterke selectie voor hoge productie en almaar toenemende toomgrootten zorgen ervoor dat dit gemiddelde snel stijgt, waarbij tomen van 20 biggen of meer steeds vaker voorkomen. Ter vergelijking: het wilde zwijn, de voorouder van het varken, heeft tomen van gemiddeld 4 tot 6 biggen. LG biggen zijn minder sterk en groot, waardoor zij een grotere kans hebben om door honger, kou of verdrukking door de zeug vroegtijdig te overlijden. Of LG een effect heeft op mentale ontwikkeling en leervermogen van varkens, is nog niet overtuigend bewezen.

IJzertekort

Goede voeding is essentieel voor een goede ontwikkeling, groei en gezondheid. Bijna een derde van de menselijke wereldbevolking lijdt aan een vorm van ondervoeding of een tekort aan essentiële voedingsstoffen, waarvan de meest voorkomende ijzertekort is. IJzer is belangrijk voor veel processen in het lichaam, in het bijzonder voor de werking van de hersenen. Een tekort aan ijzer op jonge leeftijd kan voor een vertraagde of verstoorde ontwikkeling van verschillende vaardigheden en gedrag zorgen. De gradatie van deze beperkingen hangt af van de duur en timing van het ijzertekort. Een ijzertekort op jonge leeftijd zorgt zowel bij ratten als bij mensen voor een verslechterd geheugen en leervermogen, zelfs lang nadat het ijzertekort is verholpen door juiste voeding met voldoende ijzer. Dit suggereert dat ijzertekort op jonge leeftijd perma-

nente beperkingen in het functioneren van de hersenen veroorzaakt. Er wordt vermoed dat dit komt door de snelle groei op jonge leeftijd, waarbij de behoefte aan ijzer groot is.

Biggen worden geboren met weinig ijzerreserves. Door hun snelle groei (zeker in de bio-industrie, waar een sterke selectie plaatsvindt op snel groeiende varkens) hebben zij een grote behoefte aan ijzer. Zeugenmelk bevat echter te weinig ijzer om aan deze behoefte te voldoen. Om die reden krijgen biggen in de varkenshouderij kort na de geboorte standaard een ijzerinjectie toegediend. Door deze injectie niet te geven, is het mogelijk om de effecten van ijzertekort op fysieke en mentale ontwikkeling te bepalen. Daarom is het varken een uitermate geschikt diermodel om de lange-termijn effecten van een vroeg ijzertekort op mentale en fysieke ontwikkeling te onderzoeken.

Leervermogen en geheugen onderzoeken

Wij hebben ons in dit proefschrift vooral gericht op de mentale ontwikkeling. Deze kan door leer- en geheugentaken bij het varken worden onderzocht. De effecten van LG en ijzertekort op leervermogen en geheugen in varkens hebben wij onderzocht met de holeboard taak. Dit is een grote arena met zestien (4x4) locaties waar vier verborgen beloningen (chocolade M&M's®) gevonden kunnen worden (zie Figuur 2 van Hoofdstuk 1 van dit proefschrift). Door de vier beloningen telkens op dezelfde locatie te leggen en de test dagelijks uit te voeren, kunnen de prestaties van de varkens worden bijgehouden over de tijd, zoals het aantal fouten dat het dier maakt en de tijd die hij ervoor nodig heeft om alle beloningen te vinden. Deze leerfase noemen wij de acquisitie. Door de beloningen daarna op andere locaties te leggen, kan het vermogen om opnieuw te leren bepaald worden. Deze leerfase noemen wij de reversal. De studies in dit proefschrift zijn zowel bedoeld als model voor de mens, als ook om de kennis over het welzijn van het varken te verbeteren. De uitkomsten van deze studies kunnen van belang zijn voor de kennis over deze complicaties in mensen en dieren. Zij kunnen ook informatie opleveren die bruikbaar is voor verbeteringen in de veehouderij en voor verbetering van het welzijn van varkens.

Hoofdstukomschrijving en bevindingen

Deel I: Laag geboortegewicht en toomgrootte

In **Hoofdstuk 2** hebben wij de ontwikkeling en leerprestaties van LG biggen vergeleken met normaal geboortegewicht (NG) biggen. LG biggen hadden een blijvende groeiachterstand, wat eerdere onderzoeksresultaten ondersteunt. Verrassend genoeg lieten LG biggen echter een beter leervermogen zien dan NG biggen: zij hadden een beter lange-termijn geheugen tijdens zowel de leerfase (acquisitie) als de her-leerfase (reversal), waarbij de beloningen op een andere plek waren gelegd. Wij vermoedden dat deze verbeterde leerprestatie te maken had met een sterkere motivatie voor voedsel van de LG biggen. Dit zou te verklaren zijn door een grotere behoefte aan voedsel en de grote concurrentie voor voedsel (c.q. melk) op jonge leeftijd, zeker in grote tomen.

In **Hoofdstuk 3** onderzochten wij daarom de motivatie voor een voerbeloning van LG en NG biggen in twee motivatietaken: een runway taak en een nosewheel taak. In de runway taak moesten de dieren 7 m afleggen van een start- naar een eindbox, waar beloningen lagen. Wij onderzochten hoe snel de dieren de startbox verlieten en hoe snel zij naar de eindbox renden voor een kleine beloning (2 M&M's®), een grote beloning (8 M&M's®), wanneer er obstakels in de runway werden geplaatst, en wanneer er geen beloningen werden gegeven.

In de nosewheel taak moesten de dieren een wiel ronddraaien met hun neus om een beloning (wederom 2, 8 of 0 M&M's®) te krijgen. Voor elke volgende beloning moesten zij het wiel één keer meer ronddraaien. Zij moesten dus voor elke volgende beloning meer moeite doen. Door bij te houden hoeveel tijd zij aan de taak besteedden en hoeveel rotaties zij bereid waren te maken voor de beloning, kon de motivatie bepaald worden. Er werd in beide taken een verschil in motivatie gevonden voor de hoogte van de beloning (verhoogde motivatie voor meer beloningen), maar geen verschil in motivatie tussen LG en NG biggen. Motivatieverschillen tussen LG en NG biggen verklaren dus niet de betere leerprestaties van LG biggen die we in Hoofdstuk 2 hebben gevonden.

In **Hoofdstuk 4** onderzochten wij de effecten van toomgrootte op emotionaliteit, ontwikkeling en leervermogen in NG biggen van grote tomen (met meer dan 18 biggen) en kleine tomen (met minder dan 13 biggen). Emotionaliteit werd gemeten in een open field test, waarin de biggen op jonge leeftijd geïsoleerd worden in een onbekende ruimte gedurende 5 minuten. Door gedragingen zoals vocalisaties, mate van activiteit, locatie in het open veld en aantal ontsnappingspogingen te registreren, kan de emotionaliteit van een big worden bepaald. Ook hebben wij de concentratie van het stresshormoon cortisol vóór en na de test in het speeksel van de biggen gemeten. Wij verwachtten een hogere emotionaliteit en verminderd leervermogen in biggen van grote tomen, omdat die meer concurrentie om zuurstof en voedingsstoffen hebben gehad in de baarmoeder. Vermoedelijk hebben zij daardoor een verminderde (mentale) ontwikkeling gehad.

Door technische problemen hebben wij de helft van de dieren van één varkenshouderij afgenomen, en de andere helft van een andere houderij. De twee houderijen leverden verschillende varkensrassen. Interessant genoeg vonden wij sterke effecten van houderij of ras (niet te onderscheiden in deze opzet) op emotionaliteit, groeisnelheid en leervermogen in de holeboard taak. Varkens van houderij 1 reageerden actief emotioneel (bijvoorbeeld door veel onstnappingspogingen te laten zien en weinig te vocaliseren), terwijl varkens van houderij 2 passief emotioneel reageerden. Varkens uit grote en kleine tomen van houderij 1 lieten geen verschil in groei zien; varkens van houderij 2 afkomstig uit kleine tomen groeiden sneller dan de varkens uit grote tomen. Varkens van houderij 2 presteerden beter in de acquisitie fase van de holeboard taak dan varkens van houderij 1. In de reversal fase presteerden biggen uit kleine tomen van houderij 2 beter dan uit grote tomen; dit verschil werd niet gevonden in varkens van houderij 1. Deze resultaten laten zien dat houderij en/of ras een aanzienlijk effect kan hebben op de prestaties van varkens in gedragstaken.

Deel II: IJzertekort
In **Hoofstuk 5** onderzochten wij de effecten van het weglaten van een ijzerinjectie en het geven van een ijzerarm kunstmelkdieet gedurende de eerste 4 weken na de geboorte op de lange-termijn fysieke ontwikkeling en het leervermogen van biggen. De controlegroep kreeg wel een ijzerinjectie en een gebalanceerd kunstmelkdieet. Na het melkdieet kregen alle biggen hetzelfde commerciële biggenvoer, gebalanceerd voor alle nutriënten (waaronder ijzer).

Wij bepaalden ijzerwaarden in het bloed (hemoglobine, hematocriet en serum ijzer), groei, en leervermogen in de holeboard taak. Ook onderzochten wij na het experiment het ijzergehalte in de hippocampus (een hersenstructuur betrokken bij leren en geheugen), en de synaptische plasticiteit van de hippocampus. Synaptische plasticiteit is het vermogen van de verbinding tussen twee cellen – de synaps – om van sterkte te veranderen. De resultaten hiervan hebben we in **Appendix I** beschreven.

Varkens die de vroege ijzertekort behandeling hadden gekregen ontwikkelden anemie (bloedarmoede), lieten een permanente groei-achterstand zien, en hadden een sterk verminderd leervermogen in zowel de acquisitie als de reversal fase van de holeboard taak. Zij hadden minder ijzer in twee gebieden van de hippocampus, en de hoeveelheid ijzer in de hersenen correleerde positief met het leervermogen. De synaptische plasticiteit in de hippocampus was verminderd in hersenen van de dieren met ijzertekort in de eerste weken van hun leven. Dit pleit ervoor dat de hersenen ijzer nodig hebben tijdens de groei om goed te ontwikkelen en functioneren, en dat de hippocampus betrokken is bij leren en geheugen. Ook laten wij met deze resultaten zien dat een vroeg ijzertekort kan leiden tot permanente achterstand in zowel fysieke als mentale ontwikkeling bij varkens.

In **Hoofdstuk 6** herhalen wij dit experiment in een andere opzet, waarbij de biggen bij de zeug worden gelaten om het diermodel te verbeteren. Een vroege scheiding van de moeder is niet alleen nadelig voor het welzijn van jonge dieren, het kan ook de resultaten hebben beïnvloed van Hoofdstuk 5. Aangezien zeugenmelk van nature ijzerarm is, wilden wij onderzoeken of biggen die tot 4 weken leeftijd bij de zeug bleven en in die tijd uitsluitend zeugenmelk kregen, en daarnaast geen ijzerinjectie kregen, soortgelijke resultaten zouden opleveren als beschreven in Hoofdstuk 5. Wij bepaalden opnieuw ijzerwaarden in het bloed, groei, en leervermogen in de holeboard taak.

De varkens die de ijzertekort behandeling hadden ondergaan ontwikkelden tegen de verwachting in geen anemie, lieten geen groeiachterstand zien en hadden een leervermogen vergelijkbaar met dat van de controledieren. Deze biggen hadden blijkbaar toch voldoende ijzer binnengekregen. Aangezien zeugenmelk weinig ijzer bevat, moeten de biggen het ijzer uit een andere bron hebben verkregen. Hierbij valt te denken aan voer dat door de zeug werd

gemorst. Ook zouden de biggen ijzer binnen hebben gekregen door het eten van de mest van de zeug. Met deze studie, in combinatie met de resultaten van Hoofdstuk 5, laten wij zien dat de ontwikkeling van anemie bij een vroeg ijzertekort vermoedelijk essentieel is om permanente nadelige effecten op de ontwikkeling te veroorzaken.

Discussiepunten en aanbevelingen

Laag geboortegewicht en toomgrootte
Hoewel in ratten en mensen gevonden is dat een LG de mentale ontwikkeling negatief beïnvloedt, vonden wij het tegenovergestelde bij varkens in Hoofdstuk 2. De verklaring hiervoor zal door meer onderzoek uitgezocht moeten worden. Hoewel door dit resultaat het varken als modeldier voor LG in mensen niet geschikt lijkt, is het wel een interessante bevinding met betrekking tot varkens zelf.

Echter, LG in varkens levert grote problemen op voor het welzijn en de overlevingskansen van jonge biggen. Met almaar groter wordende varkenshouderijen, waarbij er steeds minder persoonlijke aandacht per dier mogelijk is, is het de vraag of de selectie op grote tomen niet zijn piek van efficiëntie heeft gepasseerd. Lagere overlevingskansen, meer welzijnsproblemen en een vertraagde groei hebben immers niet alleen een nadelig effect op de varkens zelf, maar ook op de efficiëntie van de varkenshouderij. Het is daarom aan te bevelen om de selectie op grote tomen enigszins terug te brengen naar meer natuurlijke en effectieve proporties. Dit zal de overlevingskans, gezondheid en prestaties van de biggen aanzienlijk verbeteren.

Effecten van ras of houderij
Hoewel wij niet hadden verwacht dat ras of houderij een effect zou hebben op prestaties in gedragstaken in Hoofdstuk 4, heeft het wel interessante resultaten opgeleverd. Bij ratten is ook bekend dat rassen verschillend kunnen reageren in gedragstaken. Deze verschillen kunnen worden gebruikt bij het gericht fokken en selecteren op bepaalde eigenschappen, in zowel de varkenshouderij als in wetenschappelijk onderzoek.
Het is echter een belangrijke bevinding dat de gedragsprestaties van varkens kennelijk niet te generaliseren zijn over varkens van verschillende rassen en/of afkomstig van verschillende houderijen. Dit is dan ook de reden dat vervol-

gonderzoek, herhaald onderzoek en onderzoek in verschillende diersoorten zo belangrijk zijn om elkaar aan te vullen.

IJzertekort en anemie

Uit onze ijzerstudies in Hoofdstuk 5 en 6 kunnen wij voorzichtig concluderen dat de ontwikkeling van anemie essentieel is in het veroorzaken van permanente ontwikkelingsproblemen in varkens, zowel fysiek als mentaal. Dieren die geen anemie ontwikkelden tijdens de ijzertekort behandeling in Hoofdstuk 6 lieten geen beperkingen in groei of leervermogen zien, in tegenstelling tot de dieren in Hoofdstuk 5 die wel anemisch werden door de ijzertekort behandeling.

Een belangrijke vraag die de uitkomst van Hoofdstuk 6 oproept, is of de standaard ijzerinjectie bij pasgeboren biggen wel nodig is. Bij de meeste varkenshouderijen worden biggen al kort na de geboorte bijgevoerd met kunstmelk en papjes waar voldoende ijzer in zit. Ondanks dat wij de biggen niet hebben bijgevoerd in Hoofdstuk 6, kregen de biggen toch voldoende ijzer binnen om anemie te voorkomen, vermoedelijk door het eten van mest en gemorst voer van de zeug. In een eerder onderzoek werd al gevonden dat bijvoeren vanaf 7 dagen na de geboorte ervoor zorgt dat biggen geen verminderde prestatie laten zien in groei en ontwikkeling op 15 dagen leeftijd. Het is daarom sterk de vraag of ijzerinjecties nog wel nodig zijn in de varkenshouderij, en het verdient aanbeveling om dit verder te onderzoeken. Als ijzerinjecties overbodig blijken te zijn, zal dit niet alleen voordeling zijn voor biggen, die dan geen injectie op jonge leeftijd hoeven te ondergaan, maar ook handelingen en kosten voor de varkenshouder verminderen. Omdat ijzerinjecties zo standaard zijn geworden in de varkenshouderij, zal het echter lastig zijn om deze traditie binnen varkensbedrijven te veranderen.

Conclusies

- Hoewel LG veel welzijnsproblemen veroorzaakt bij jonge biggen, vonden wij een verbeterd leervermogen ten opzichte van NG biggen, maar geen verschil in motivatie voor een voerbeloning;

- Toomgrootte beïnvloedt de groei en het leervermogen van varkens, hoewel wij vonden dat dit effect ras of houderij afhankelijk is;

- Ras of houderij heeft een sterk effect op emotionaliteit van biggen;

- Het varken lijkt een geschikt diermodel voor vroeg ijzertekort bij mensen te zijn, onder de voorwaarde dat het ijzertekort leidt tot anemie.

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> The same Force formed the sparrow
> That fashioned man, the king
> The God of the Whole gave a spark of soul
> To furred and to feathered thing
>
> And I am my brother's keeper
> And I will fight his fight
> And speak the word for beast and bird
> Till the world shall set things right
>
> ~ *The voice of the voiceless, Poems of experience.* Ella Wheeler Wilcox.

About the author

Alexandra Antonides, or Sanja, was born on May 1st 1986 in Amsterdam, where she has lived ever since. One quality that always stood out was that she was fond of animals. Growing up, she wanted to become a veterinarian. Unfortunately, severe cat and horse allergies steered her away from that career choice. In high school, she was gripped by biology, and in particular animal physiology and behavior. In her senior year, she did a small study on her dog for a biology assignment, testing whether the dog would conduct tricks better when either using only gestures, or only spoken commands (for which she blindfolded her dog, who willingly underwent the challenge).

She then continued to study Biology at the Free University in Amsterdam. For her bachelor internship in her third year, she joined a social sciences research group to conduct a research on group behavior in humans. She was told during her bachelor that if she wanted to study animal behavior, she would have to go to Utrecht University for her master's degree. In particular, by then, she was interested in the animal mind: how an animal perceives its environment, what it thinks and feels. She decided to apply for the prestige master's program Neuroscience and Cognition, track Behavioral Neuroscience. In this master's program, she could combine her interest for the human brain with her interest for animal cognition.

She conducted her first master's internship at the Department of Behavioral Biology, where she joined a study on chimpanzee personalities by offering them several cognitive tests and challenging situations. Her second internship was at the Emotion and Cognition group (now Behavior and Welfare group) of the Department of Farm Animal Health, Faculty of Veterinary Science, where she helped to conduct a study on low birth weight piglets. She then finished her master's program with a literature thesis on improving farm animal welfare. The thesis was almost rejected by the study advisor as "it had little to do with neuroscience". She convinced the exam committee that welfare, and especially personally perceived welfare, has everything to do with neuroscience, especially behavioral neuroscience.

After she received her master's degree, she was approached to conduct two studies at the Emotion and Cognition group as a research assistant, where she had conducted her internship with piglets. She accepted the position which lasted nine months, after which she was offered to continue the research as

a PhD student. Ironically, she thus ended up working at the Faculty of Veterinary Science and has, in the end, managed to fulfill her childhood wish of working with animals and studying their behavior.

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