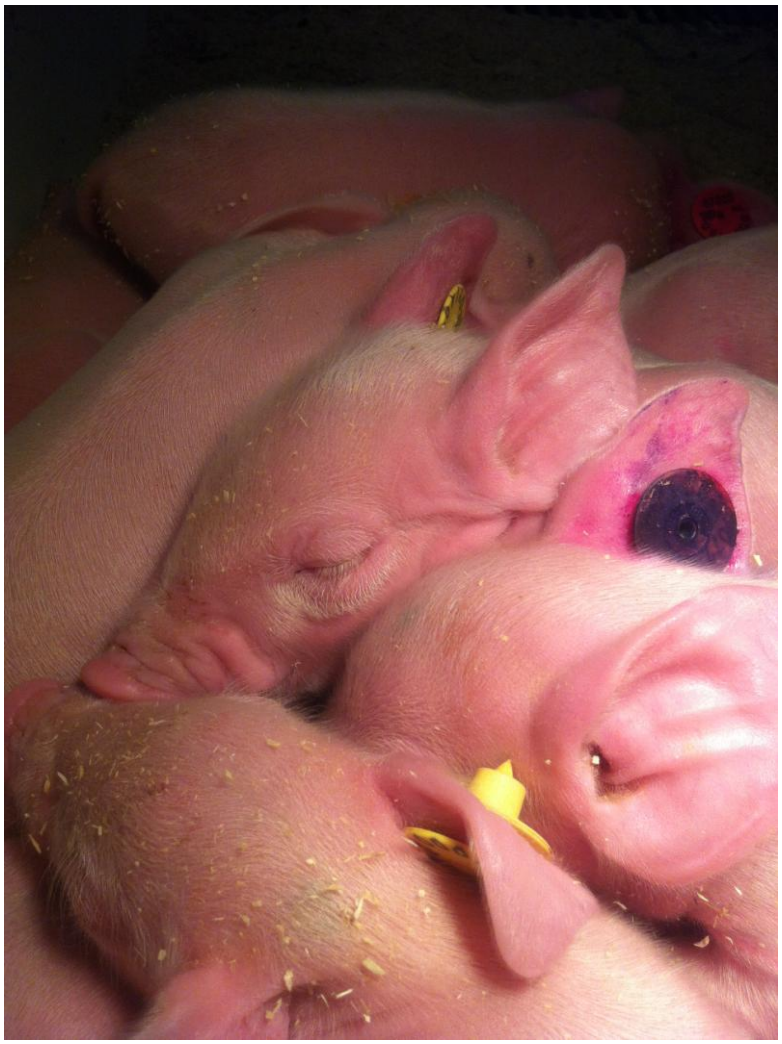
Efficacy and safety of prenatal allopurinol treatment in low birth weight piglets with special emphasis on learning ability



Student: Alexandra Antonides, BSc.
Student number: 3032213

Supervisor: Elise Gieling, MSc., PhD student, Emotion and Cognition program

Department of Farm Animal Health, Faculty of Veterinary Medicine

"I like pigs. Dogs look up to us. Cats look down on us. Pigs treat us as equals."

Sir Winston Churchill

Index

Abstract

Allopurinol is a medicine which is currently studied in a clinical trial in humans to possibly prevent brain damage caused by acute shortage of oxygen around birth. Earlier studies showed that allopurinol can limit brain damage by reducing the formation of free radicals after acute asphyxia/hypoxia. However, no research has yet been conducted on the long-term cognitive effects after chronic, prenatal treatment of prolonged but mild asphyxia during pregnancy. In the present study, pigs were used as model species for studying the long-term effects of allopurinol on cognition. In addition, we investigated possible side effects of this treatment. Six sows were treated with allopurinol during the last trimester of pregnancy. Six untreated sows served as controls. After birth, piglets and placentas were measured, and umbilical cord mixed blood samples were taken. For cognitive tests, piglets with normal birth weight (NBW) and low birth weight (LBW) were selected per sow. It is believed that low birth weight reflects intra-uterine growth restriction, caused by a shortage of oxygen, i.e. mild asphyxia and/or nutrients that reach the fetus. An Open Field test was conducted, and learning ability was assessed using the cognitive Holeboard task. Treatment reduced placenta length and increased piglet length, but did not affect other measures. LBW piglets vocalized more in the Open Field test than NBW piglets, suggesting that they are more easily stressed. No differences were found in Holeboard task performance, although there are indications that allopurinol might reduce learning ability. Further research is needed to elucidate these indications.

Acknowledgements

Hereby I would like to express my gratitude to the people that made it possible for me to do this internship. I want to thank my supervisor Elise Gieling for the endless hours of testing piglets together, which have been challenging, instructive and entertaining; and for all the talks, interesting discussions and laughs. Thanks to Franz Josef van der Staay for helping me find this internship and for his kind help during the process of this research. The farmers Jan, Dirk and Zias have allowed me a glance into the world of extensive farming, which has intrigued me to such an extent that I hope to find a job in farming innovation after graduating. Thanks to Eimear Murphy for our conversations and her animated way of talking about the piglets.

I am grateful that I got to dive into the world of pig cognition. Although not a flattering thought, I have realized that these animals resemble us humans more than we would like to admit. The piglets we trained and tested for this study have moved me, made me laugh, and allowed me to learn about and wonder at their behaviors and personalities. Therefore, I want to thank them in particular for this special and moving experience over the past six months.

Introduction

Complications around birth

Pregnancy and giving birth are complex processes, which are therefore prone to complications. When complications occur, they may have severe consequences for both the mother and the progeny, possibly resulting in physical or neurological deficits and disorders (e.g. learning disabilities: Colletti 1979) or even death in the neonate. Complications during pregnancy include stillbirth, bleedings, premature rupture of the membranes, and pre- and post-term births (Caughey 2007). To illustrate the consequences of such complications: there is an increased risk of premature delivery for women with bleedings during pregnancy, and the mean birth weight of their children is lower than in women who did not have this complication (Lam et al. 2000). Furthermore, early or threatened labor and malposition of the fetus is shown to be related to the development of asthma in the child (Annesi-Maesano et al. 2001). Prenatal exposure to stress increases pregnancy complications (Norbeck & Tilden 1983) as well as the risk for postnatal behavioral disorders such as autism, schizophrenia and depression (Kinney et al. 2008).

Complications around birth include maternal seizures, bleedings and wounds, complications of delivery, breathing delay in the infant, and blood flow or oxygen restrictions to the infant during the process of birth (e.g. Werner et al 1967; Voldner et al 2009). Complications around birth due to shortage of oxygen (hypoxia) and blood flow restriction (ischemia) is a major cause of cognitive disability, developmental delay and mortality in human neonates and children (van Handel et al. 2007). For example, hypoxic-ischemic complications together with low birth weight and chronic shortage of oxygen have been shown to be significantly associated with lower neuropsychological performance in 7-year old children (Seidman et al. 2000). This included perceptual-motor and verbal-conceptual abilities, as well as academic achievement skills. In another study, physical and intellectual development retardation in 2-year old children was especially pronounced in children that suffered from perinatal stress (Werner et al 1967). Thus, perinatal complications can induce serious impairments in cognitive functioning over extensive periods of time.

Brain damage and allopurinol

When any tissue is deprived of oxygen and blood supply for a prolonged period of time, cell death and tissue damage will occur. Although the return of blood flow and oxygen supply (reperfusion) to these tissues is necessary to prevent further damage, the return of blood flow to previously blood-restricted tissue actually causes increased tissue damage (Fellman & Raivio 1997). During reperfusion, there is an excess of oxygen after oxygen deprivation, which causes the production of oxygen-derived free radicals. These free radicals are believed to be generated mainly by the enzyme xanthine oxidase (McCord 1985) and are highly reactive, therefore exerting cytotoxic effects. Numerous studies have shown that therapeutics acting as free radical scavengers can reduce this damage (e.g. in muscular heart tissue: Gardner et al. 1983; in rat kidneys: Baker et al. 1985). Allopurinol is such a free radical scavenger, as well as an inhibitor of xanthine oxidase (Moorhouse 1987). Administration of allopurinol has been shown to reduce the formation of free radicals, thereby limiting tissue damage caused by reperfusion. This has been studied in several animal models, including adult dogs (Allan et al. 1986), fetal lambs (Thiringer 1987) and rat pups (Palmer et al. 1990; Williams et al. 1992). A study in human newborns investigated the effect of high-dose allopurinol administration on severely oxygen deprived neonates (van Bel 1998). Intravenous administration of allopurinol was conducted within 2 hours after birth. Results indicated a beneficial effect of treatment on free radical formation, cerebral blood volume and electrical brain activity, without toxic side effects.

Recently, a clinical trial was conducted in pregnant women at term of whom the fetus was suspected to suffer intra-uterine hypoxia (Kaandorp et al. 2010). Allopurinol was administered maternally before birth, after which short and long-term effects of treatment were measured in the neonates. Results are expected to be published soon. Thus, allopurinol is currently being investigated for its effects on brain damage prevention in human newborns.

In all these cases however, administration of allopurinol was conducted in a single dosage, shortly before or during hypoxia-ischemia and reperfusion damage occurred. Thus, all treatments have been acute and consisted of a single

administration of allopurinol. In the current study, we investigate the safety and efficacy of allopurinol by applying prolonged instead of acute treatment, and investigate the long-term effects of this treatment on cognitive abilities in piglets. Moreover, instead of acute asphyxia, we focus on prolonged, mild asphyxia and investigate whether low birth weight and chronic prenatal allopurinol treatment affects cognitive ability.

Low birth weight piglets as study animal

In the present study, the commercial pig (*Sus scrofa*) was used as model species to investigate the long-term effects of prolonged, prenatal treatment of allopurinol. Pigs have relatively large brains and are physiologically more similar to humans then for example rodents, which are more commonly used to investigate drug effects and behavioral (dys)functioning (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). The pig is therefore believed to be a more appropriate model species for translational research, e.g. for modeling brain disorders and testing potential therapeutics (Lind et al. 2007). Additionally, the use of pigs raises less ethical concerns as opposed to the use of other large animals, such as primates and companion animals (Rollin 2006).

In stead of acute asphyxia, we focus on prolonged, mild asphyxia and the effect of chronic treatment with allopurinol on this mild asphyxia. Low birth weight (LBW) is believed to reflect intra-uterine growth restriction, caused by a chronic shortage of oxygen and nutrients supply to the fetus (Biri et al. 2007; Cox & Martin 2009). As opposed to humans, it is practically impossible to individually monitor piglets and determine intra-uterine growth restriction (IUGR) during pregnancy using ultra-sound. Birth weight has however been shown to be an important read-out parameter of IUGR (Cox & Martin 2009). In humans, low and very low birth weight children have a higher risk of cognitive deficits and behavioral problems later in life (McCormick et al. 1990; Hack et al. 1994; Grunau et al. 2002). LBW may reflect chronic and mild instead of acute and severe hypoxia-ischemia, of which the resulting brain damage might be prevented by chronic, prenatal administration of allopurinol. LBW is common in commercial pigs, especially when litter size increases

(Quiniou et al. 2002). Recently, Gieling et al. (2011b) showed that LBW in piglets is related to (mild) cognitive impairments.

To investigate the effects of prenatal sow treatment with allopurinol on LBW piglet cognition, we have looked at different parameters: umbilical cord blood gas values, placenta measures, piglet measures, performance in the Open Field test for emotionality, and performance in the cognitive Holeboard test for pigs.

Umbilical cord blood gas measurements

Fetal oxygenation and intrapartum asphyxia (shortage of oxygen) is believed to be reflected in umbilical cord blood parameters (Thorp & Rushing 1999) such as blood pH, pO_2, pCO_2, concentrations of electrolytes and hematocrit value. Umbilical cord blood pH lower than 7.0 in humans has been associated with neonatal death and is a reliable indicator of birth asphyxia (Thorp et al. 1989; van den Berg 1996). Furthermore, low pH and low oxygen tension have been associated with developmental retardation (Soothill et al. 1995). In newborn piglets, low pH (< 7.0) and high pCO_2 (> 100 mm Hg) are indicators of mortality and reduced viability, which can be attributed to intra-uterine asphyxia (Randall 1971). Research focusing on blood gas parameters from the umbilical cord has shown that LBW infants show a high concentration of potassium, lower protein and glucose concentration, and no difference with NBW infants for CO_2, sodium, chloride and pH (Pincus et al. 1956).

In order to rule out the possibility that acute asphyxia has occurred during birth in the piglets we used, a condition that may mask effects of mild, chronic hypoxia, we collected and analyzed umbilical cord mixed blood samples.

Open Field test for emotionality

The novel environment or Open Field test is widely used to assess emotionality in animals. In this test, an animal is placed or led into a novel open space and kept there for a certain amount of time, surrounded by walls which prevent the animal from escaping. During this time, behavior of the animal is scored. Although the test was initially designed to assess emotionality in rodents (Hall & Ballachey 1932; Archer 1973), it has also been adjusted for use in bigger animals, such as cows (de Passilé et al. 1995) and pigs (Fraser 1974). In general, measures consist of number and type of vocalizations, activity measures, and number of defecations. In a factor analysis,

Passilé et al. (1995) showed with an open field test in calves that defecation and vocalizations, along with walking, may represent fearful responses to novelty and social separation. In a validation test of the open field test in pigs, Donald et al. (2011) showed that pigs treated with the stress-reducing drug Azaperone were more active and vocalized less than untreated pigs. The same tendencies were shown by pigs that entered the arena with a familiar companion instead of alone; although to a lesser degree. Similarly, Rutherford et al. (2012) showed that pre-treatment with Azaperone resulted in higher scores on positive behaviors, including activity, and lower negative scores, including vocalisations, than untreated piglets in an open field test.

These results suggest that low activity and a high number of vocalizations are indicators of a stressful response to a novel environment and the social isolation. The number of defecations has also been shown to measure emotionality, or stressful response, in rats (Pare 1964). In the current study, we therefore scored activity measures, number of vocalizations and number of defecations in the Open Field test. Halfway through the Open Field test, we presented a novel object and measured responses towards the novel object.

Spatial cognitive Holeboard

Apart from single measures in determining any differences between LBW and NBW piglets and treated and untreated piglets, we especially wanted to look at long-term cognitive effects of prolonged treatment with allopurinol. Because of a growing interest for pigs as a model species for biomedical and bio-behavioral research (Gieling et al. 2011a), cognitive tasks adapted from primate and rodent research have been adjusted to suit the physical and cognitive abilities of pigs. Behavioral tests should be adapted to pig anatomy and ability in order for the pigs to be able to execute the task. For example, continuous lever pressing with a hoof for at least 10 seconds proved to be difficult to train in miniature pigs (Ferguson et al. 2009), indicating that this is not a suitable set-up for a behavioral test in pigs. Furthermore, cognitive tests should be complex enough to be able to detect differences in performance (Kornum & Knudsen 2011).

In this study, we looked at learning and memory measures to determine cognitive functioning over time. To this end, we used the spatial cognitive Holeboard

for pigs (Arts et al. 2009; Gieling et al. 2011b). This is a free choice maze, meaning that the animal is free to walk around and visit or revisit any site in the arena, in order to find multiple hidden rewards. In the Holeboard, the arena consists of 16 sites equally distanced from each other in a 4 x 4 matrix, of which four sites contain a food reward (van der Staay et al. 2012). After a reward is found and consumed, the site remains empty for the rest of the trial. The best strategy for finding all four rewards is thus for the animal to remember during a trial which sites are visited, which sites were empty, and which rewarded sites have already been visited. 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). Working memory is a form of short-term memory that the animal uses within a testing session, but not between sessions (Olton & Samuelson 1976; Dudchenko 2004), such as which sites have been visited and which have not. Reference memory is a form of long-term memory, used for the general rules of a task (Olton & Samuelson 1976); for example, remembering which sites always contain a reward. This kind of memory takes several trials to form, and is maintained over long periods of time. As proposed by Arts et al. (2009), instead of using one starting position, we used four starting positions. This was done in order to prevent the pigs from developing a fixed pattern of visiting the rewarded sites, or search strategy, thereby reducing working memory load (van der Staay et al. 1990) and thus making the task easier.

Hypotheses

- ✓ For placenta and piglet measurements, we expect to find no difference between treated and non-treated animals in any of the measures.
- ✓ For umbilical cord blood gas values, we expect no deviating values, as we do not expect acute asphyxia to have occurred.
- ✓ In the Open Field test, we expect:
    - No effect of treatment on emotionality measures;
    - Higher emotionality in LBW compared to NBW piglets.
- ✓ In the spatial holeboard task, we expect:
    - Untreated LBW piglets to adapt slower in the reversal phase than untreated NBW piglets (as shown in Gieling et al. 2011b)
    - Treated NBW piglets to perform equally to untreated NBW piglets
    - Treated LBW piglets to perform better than untreated LBW piglets
    - Better performance includes a steeper curve in RM and/or WM performance and a steeper decline in trial duration

Methods

Ethical note

The study was reviewed and approved by the local ethics committee (DEC, **d**ier**e**xperimenten**c**ommissie), 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.

Treatment and selection procedures

In this study, pigs of the Utrecht university farm "De Tolakker" (The Netherlands) were used. This farm is a breeding facility, where sows are kept to produce piglets which are sold to a fattening farm around ten weeks of age. Twelve sows were used for this study. For practical reasons such as time constrictions and housing conditions, six sows were used in order to form a first batch of piglets in October 2011, and a second batch was formed in December 2011 using six different sows. In this way, we were able to train and test more animals. The same treatment schedule was used for both batches. In each batch, six sows that had at least given birth to one litter of piglets before and had an expected farrowing date within the same week, were selected for this study. Three of these sows were treated with allopurinol (Ratiopharm Nederland bv., Haarlem, The Netherlands) during the last trimester of their pregnancy, starting 32 days before the expected farrowing date (the gestation period in pigs is around 115 days). Treatment consisted of adding powdered allopurinol (15 mg/kg) mixed with some water and honey to 1 kg of standard pellets for pregnant sows. The dose was adjusted on Wednesdays after weekly weighing of the sows on Mondays during the entire treatment period. Each morning, the sows were given treatment individually, containing the right amount of medicine for their weight. This feeding procedure was repeated each morning during the treatment period. If no piglets were born in the morning, treatment continued for that day. Three untreated sows served as control.

In the week in which all six sows were expected to give birth and therefore were already moved to the farrowing stable, the sows were kept under constant surveillance throughout the day and night. As soon as farrowing started, the following actions were conducted. A piglet was intercepted with clean towels at the moment of delivery. In order to link the placenta to the piglet, a suture string containing a node code was sprayed with alcohol and knotted to the umbilical cord (if the cord was still attached to the piglet after delivery) before clamping and cutting it at around 10 cm away from the piglet. This node code correlated to the piglet's ear tag which it received directly after delivery, making it possible to later link the placenta to the piglet.

Umbilical cord blood gas samples

After the umbilical cord was cut between the clamp and the suture string, the clamp was removed and a mixed venous blood sample was taken – if blood flow was sufficient. Blood was collected in an Eppendorf tube, after which the blood was extracted using a syringe. This syringe was put on ice. Using the blood-gas analyzer Radiometer ABL80 FLEX, the following blood gas parameters were determined: pH, pO_2, pCO_2 and Hematocrit.

We were able to collect blood samples from piglets of each sow (see Appendix Table 1 for the amount of samples collected per sow), resulting in 26 blood samples from piglets of treated sows and 19 blood samples from piglets of untreated sows.

Placenta measurements

For placenta measurements, we added measures of placentas collected and measured in an earlier experiment (ter Haar 2012, Master thesis) in which sows were treated with allopurinol in the same manner. In these sows however, piglets were delivered using a caesarean section. In this group of sows, consisting of two treated and three control sows, 15 placentas were measured in total. In the first batch of piglets born naturally in October 2011, 62 placentas were measured, and in the second batch of December 2011, 48 placentas were measured. Thus, a total of 125 placentas were used (74 of treated and 51 of untreated sows; see Appendix Table 1 for number of placentas per sow).

After all piglets were born, we collected the placentas which the sow excreted during or after farrowing. These placentas were put on ice and later stored at 4 degree Celsius. In the following week, after all sows had farrowed and placentas were collected, the placentas were measured. In order to do this, placentas that were mainly undamaged and could be unfolded into their natural shape, were measured using a rope that was later held against a ruler to decide the measures in centimetres. Measures included: placenta length (measured along the inside of the placenta from one end to the other); placenta width (measured along the base of the placenta at the broadest point); and placenta circumference (measured by placing the rope exactly around the placenta). Additionally, each placenta was weighed on a weighing scale (10 g accuracy, Breuer Weegtechniek JB-800). Using pictures taken directly from above with a ruler alongside the placenta for scale, surface area was calculated with PDF-Xchange Viewer 2.5.

Piglet measurements and rearing conditions

All piglets were weighed and measured directly after birth. Measurements consisted of snout to end of skull length and snout to tail base length. Weighing was conducted in a crate on a weighing scale (10 g accuracy, Breuer Weegtechniek JB-800). Each piglet's ponderal index (weight/length^3) was calculated, which has been shown to be an important parameter for assessment of fetal condition in newborn infants (e.g. Fay et al. 1991). After all piglets were born and measured, we calculated the average birth weight of the litter in order to select piglets of low birth weight (LBW). This was defined as all animals weighing at least one standard deviation below litter average. To select normal birth weight (NBW) piglets, LBW piglets were excluded and average weight was recalculated using the weights of the remaining piglets. The piglet that had a weight closest to this new average and had the same sex as the selected LBW animal was selected as a NBW piglet. As many NBW as LBW piglets were selected within a litter. As not every litter consisted of more than 1 LBW piglet and not all LBW piglets survived, the number of piglets per sow differed from 1 – 3 piglets per birth weight class (see Appendix Table 2). Piglets of each litter selected for cognitive testing received a different colour ear tag from the rest of the litter.

All piglets were reared under standard conditions (no tail docking or castration) for the first four weeks after birth, staying with the sow and their littermates during this time. If piglets selected for our study were suspected to receive too little colostrum during the first hours after birth, we milked the sow to hand feed the animal. This was done to increase chances of survival to weaning age. After a period of four weeks, as common procedure, the piglets were weaned by removing the sow from the piglets. One week after weaning, we moved the selected piglets for our study to the experimental unit, and divided all littermates into two groups. These groups therefore contained both treated and untreated piglets, of both normal and low birth weight. The first batch of October '11 consisted of two groups of 13 animals; the second batch of December '11 contained 8 and 9 animals. Since two sows (one untreated sow of each batch) had produced litters with a very high average birth weight and no piglets below 1250 g, we decided to exclude these sows and thus their piglets from further analysis. Table 1 shows the total size of the tested groups. All procedures followed were similar for both batches of piglets.

Table 1: Size of groups tested in Open Field and Holeboard task.

Birth weight	Treatment	Piglets
LBW	Allopurinol	10
	Control	8
NBW	Allopurinol	10
	Control	9

LBW = low birth weight; NBW = normal birth weight

The two groups of piglets were housed in adjacent, enriched pens of 4 x 5 m, in a naturally lighted and ventilated stable. Temperature was measured daily and ranged from -3 to 25 degrees. Each pen contained a piglet nest where a heating mat (20 – 30 degrees Celsius) was placed and a heat lamp was hung in the corner, out of reach from the piglets. Isolation in the nest was ensured by plastic flaps hanging along the side, a layer of straw on the roof, and rubber mats covered by a thick layer of sawdust in the nest. Both the nest and the concrete floor of the pen were covered with straw. Until the age of 7 weeks, food was provided *ad libitum*, after which 100% of required intake was provided, divided in two portions (¼ in the morning, ¾ in the

afternoon) to control motivation in the behavioral tests. A radio played 24/7, though slightly louder during testing hours to mask sudden background noises.

After transfer to the new pens, the piglets were allowed to get accustomed to their new surroundings for one week. To habituate the animals to humans, we visited the piglets for a minimum duration of ten minutes each day during this week. These visits consisted of sitting in the pen, walking around, making noises and touching the animals when they approached. M&M chocolates and some corn cob mix were fed to attract their attention.

Open Field test

One week after arrival (at 5 weeks of age), we conducted the Open Field test with each piglet individually. A novel environment to the piglets with high walls was used for this test. The concrete floor was covered with sawdust. In the first batch of piglets, this was an area of 250 x 205 cm, with two wooden and two plastic walls of at least 150 cm high. In the second batch of piglets, as the arena used for the first batch was no longer available, the novel arena was 250 x 150 cm, with plastic walls of 120 cm high.

Piglets were tested per pen in a random order and were returned to their pen mates directly after testing. A piglet was allowed access into the testing arena alone, after which the door was closed and the observations started. The test lasted for ten minutes. After five minutes, a novel object (a colourful tambourine) was presented by slowly letting it descend on a string in the centre of the arena, 80 cm from the back wall. By letting the tambourine gently hit the ground and thus make a sound, we made sure the piglet noticed the object. During the entire ten minutes, we scored activity measures by watching live camera images on a screen with a grid. The camera was placed directly above the Open Field in the centre, so we could see the entire area on screen. The grid divided the screen into sixteen equal segments, which we divided into three components: centre segments, wall segments and corner segments (Figure 1). By using Observe6 (software written for CNS Research, Bayer AG, Germany, 2002), we scored the number of line crossings within and between segments, the number of entries into the different segments, and the time spent in each segment. Furthermore, we scored total line crossings and total entries into each

segment. These were used as measures of activity. Additionally, we counted the total number of vocalizations, the number of escape attempts (defined as jumping against the wall), the number of defecations during the trial, the number of times the piglet looked at the novel object and the number of times it touched the novel object.

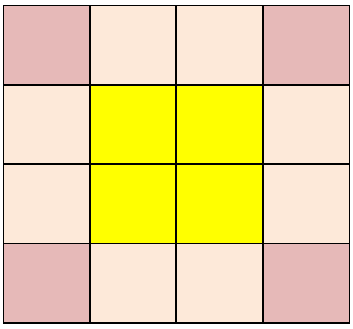


Figure 1: Segmentation of the Open Field. The Open Field was divided into segments in order to assess activity measures. Yellow = centre segment, dark pink = corner segment, pink= wall segment.

Holeboard test

The testing arena containing the spatial Holeboard for pigs was situated in the same stable as the housing pens. All pigs housed in the same pen were led through a corridor leading to the straw-bedded waiting area adjacent to the Holeboard arena before testing. Above and around the testing area, extra-maze cues were present. Piglets were tested individually in a randomized order, during which they could still hear and smell their pen mates. A radio was playing in order to minimize the effects of sudden background noises. The testing apparatus (manufactured by Ossendrijver BV, Achterveld, The Netherlands) was a cognitive Holeboard, consisting of a square arena containing a matrix of 4x4 plastic food bowls (Figure 2). The floor consisted of slatted blue plastic; the 80 cm high walls were synthetic and had a steel bar across the top (1 m). The four sliding entry doors were positioned in the centre of each wall and were operated from outside the apparatus via a string and pully system. The entry site was randomly assigned per trial. Pigs voluntarily walked through a small corridor surrounding the arena, until they found an open door through which they could enter the testing arena. Upon entry, the trial started. Both the corridor and the arena were elevated above the floor.

The animals were able to move freely through the test apparatus and could visit all sites easily. In order to prevent the animals from locating the rewards by smell, three rewards (M&M's, replaced daily) were placed under the false bottom of the food bowls. In order to prevent the pigs from finding rewards by sight, the food bowls were covered by red plastic balls (JollyBall Dog Toy, 24 cm in diameter, 400g) that could be lifted with the snout in order to access the reward, and fell back onto the food bowl once the piglet retracted its snout. The apparatus was rinsed with water daily, and between trials if a piglet had defecated during a trial.

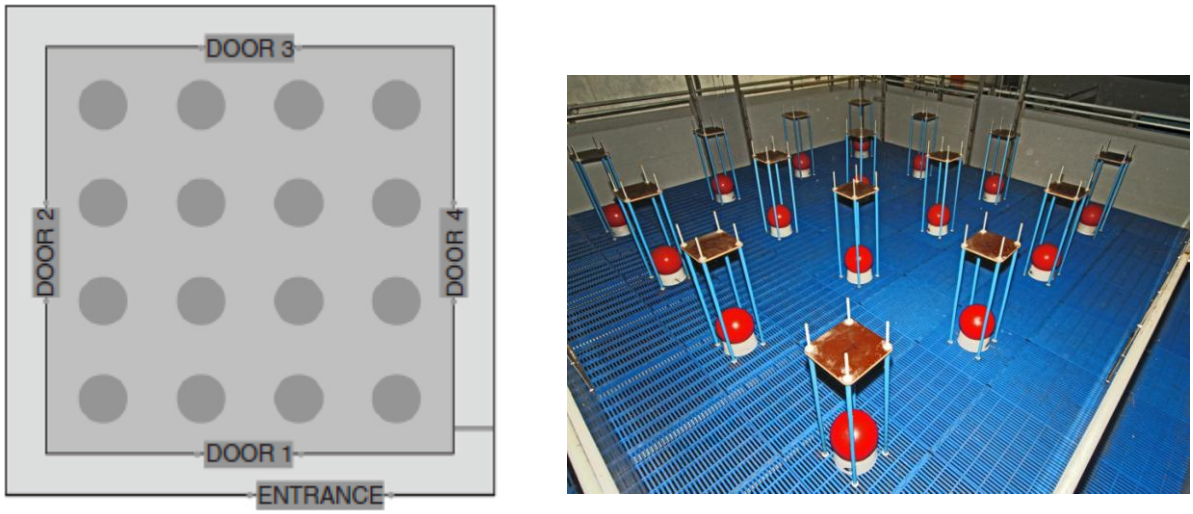


Figure 2: The cognitive Holeboard for pigs. A schematic presentation (left panel) and photo (right panel) of the cognitive Holeboard used to assess spatial memory in piglets. Through the main entrance, the piglets would enter the corridor (width: 40 cm), through which they could only walk in a clockwise manner (left). The door that allowed access to the Holeboard arena (530 x 530 cm) was randomly assigned each trial. The 16 food bowls inside the arena were symmetrically arranged (space between bowls: 95 cm; space between wall and bowls: 73 cm; wall height: 80 cm).

Habituation

A habituation period of 2 weeks was applied to allow the pigs to get accustomed to walking through the corridor from their pen to the waiting area adjacent to the Holeboard, staying in the waiting area and spending time in the Holeboard arena. During habituation in the Holeboard, all food bowls contained one M&M. During habituation sessions, bowls were refilled directly after baits had been consumed, in order for the piglets to learn how to lift a ball and find a reward. Piglets were

habituated in the Holeboard arena in increasingly smaller groups, first with all pen mates, and eventually in pairs. When in pairs, once a piglet was seen lifting a ball and consuming the reward underneath it multiple times, it was let into the Holeboard alone. Once all piglets could enter the Holeboard alone and had learned how to find rewards, habituation was finished and testing could start.

Testing and reversal phase

All animals were tested in two consecutive trials each working day (Monday through Friday), starting at 7 weeks of age. Each piglet was assigned to a reward configuration, in which 4 out of 16 bowls contained a reward. Four configurations were used in total (A-D, see Figure 3). Each piglet was trained on its own configuration for at least 40 trials (20 testing days) and a maximum of 60 trials (30 testing days), depending on their reference memory (RM) performance. If the piglet had reached the RM performance criterion of > 0.7 for at least two consecutive days (4 trials, see "Measures" below for calculation of this variable) after 40 training trials, it could start the next phase of reversal training. If the RM criterion was not reached, the training phase was extended until criterion was reached. After a maximum of 60 trials, independent of RM performance, all remaining piglets started in reversal training. The reversal phase consisted of turning their old configuration 180 degrees (A to C; B to D, and vice versa), which was the configuration that least resembled the training configuration. Testing including the reversal phase ended when a piglet had received a total of 84 trials (42 testing days), independent of which performance it had reached on the task.

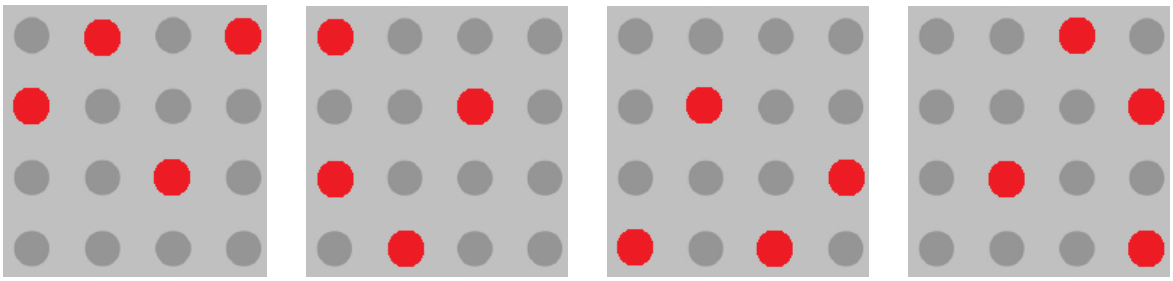


Figure 3: Configurations A, B, C and D (left to right) used for the Holeboard task

Measures

Measures included the number of visits to rewarded bowls (correct), the visits to unrewarded or previously rewarded bowls (errors), and total trial duration. Visits, latencies and trial duration were recorded in real time using Experiment Control for Utrecht University. A trial was started manually when the piglet had entered the arena with both front legs, and ended automatically when all four rewards had been found or 600 seconds had elapsed, whichever event occurred first. A visit was scored when the piglet lifted the ball with its snout, which was automatically registrated with a magnet inside the plastic ball and a magnet sensor in the false bottom. When the signal between magnet and magnet sensor was interrupted by a piglet lifting the ball, this was registered by an interface (LabJack) and sent to the computer programme (Experiment Control), which automatically calculated the measures reference memory and working memory. A revisit to a previously visited bowl was only scored when another bowl was visited in between, or 10 seconds had elapsed between visits.

Working memory ratio was calculated as (number of rewarded visits) / (number of visits and revisits to the rewarded set of bowls), reflecting the ability of the animals to avoid revisiting baited bowls (Brinks et al. 2007; van der Staay et al. 2006). Reference memory ratio was calculated as (number of visits and revisits to the rewarded set of bowls) / (number of visits and revisits to all bowls), which is an index for the ability of animals to discriminate between baited and unbaited holes. Ratio measures were used as these are less biased by incomplete trials, in which the animal does not collect all rewards (van der Staay et al. 2011).

Trial duration was the time elapsed between entering the Holeboard and finding the last reward. If the piglet did not find all rewards, this measure was assigned the maximum value (600 s). For each measure, block mean values of four trials (two days) were calculated (methods adapted from Arts et al. 2009; Gieling et al. 2011b).

Data analyses

Using SPSS 16.0 for Windows, the data were analysed using a linear mixed model for the umbilical cord blood gas values, placenta measures, piglet measures and Open Field test data. For umbilical cord blood gas values, piglet and placenta measures, 'treatment' was a fixed factor and 'sow' a random factor. 'Litter size' was used as covariate, because the litter size is a major determinant of the weight of the piglet in a litter, with larger litters having smaller piglets. As we did not have sufficient blood gas samples of LBW and NBW piglets and could not link most placenta's to a piglet, we could only compare treated with untreated samples. For the piglet measures we also left out LBW and NBW as a factor, since the measures we took – length and weight – obviously correlate with a low or normal birth weight. Therefore, we only looked at the effect of treatment on these measures. For the Open Field test, we added birth weight class (low or normal) and the interaction term 'treatment * birth weight class' as fixed factors. Again, 'sow' was a random factor and 'litter size' the covariate. Each variable was checked for normality by plotting parameter estimates against parameter residuals in a Q-Q plot (in which the residuals should show the same trendline as the estimates) and a scatter plot (in which residuals should be evenly distributed around 0).

To analyse the data of the Holeboard test, we used SAS 9.2. We averaged NBW and LBW piglet data per sow, and used these data as repeated measures of the sow. Therefore, for each variable (trial duration, working memory and reference memory) we had two measures per trial block for each sow, which we tested in a General Linear Model for Repeated Measures with trial blocks as second repeated measures factor.

Results

Umbilical cord blood gas values

The pH values of all sampled piglets did not include any values below 7.0. The distribution of the pH values was normal (Appendix Figure 1). The pCO_2 values were all below 100 mm Hg, although we did have two samples with a very low pCO_2 (< 25 mm Hg). Appendix Table 1 shows how many umbilical cord blood samples were collected per sow. Treatment did not have an effect on any blood gas parameter (Table 2).

Table 2: Effect of treatment on umbilical cord blood gas measures.

Blood gas measure	df	F	p-value
pH	1	1,603	0,238
pCO_2	1	0,008	0,930
pO_2	1	1,217	0,339
Hct	1	0,315	0,588

Placenta measures

The outcomes of the analyses of placenta measure data are shown in Table 3. Appendix Table 1 shows how many placentas were collected per sow. Allopurinol treatment had an effect on placenta length: placentas of treated sows were shorter than placenta's of untreated sows (linear mixed model; df = 1; F = 4,886; p = 0,044; see Figure 4). All other measures were not affected by treatment (Table 3).

Table 3: Effect of treatment on placenta measures

Placenta measure	df	F	p-value
Length	1	4,886	**0,044**
Width	1	0,249	0,626
Cicrumference	1	2,557	0,130
Surface area	1	0,320	0,581
Weight	1	0,200	0,663

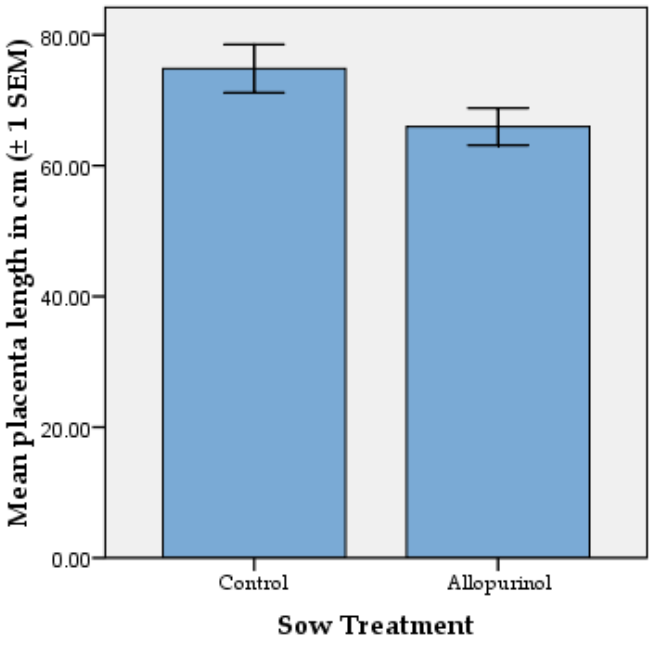


Figure 4: Effect of prenatal sow treatment on placenta length. Mean length of placentas of sows treated with allopurinol and placentas of control sows. Placenta length was lower in sows treated with allopurinol (linear mixed model; n treated = 74; n untreated = 51; df = 1; F = 4,886; p = 0,044. See Appendix Table 1 for number of placentas collected per sow).

Piglet measures

Table 4 shows the outcome of the analyses on piglet measures, showing that allopurinol treated piglets had a higher full length than the control piglets (linear mixed model; df = 1; F = 6,347; p = 0,026; Figure 5). No treatment effects were found for snout length, birth weight and ponderal index (Table 4).

Table 4: Effects of treatment on piglet measures

Piglet measure	df	F	p-value
Snout length	1	0,801	0,387
Full length	1	6,347	**0,026**
Birth weight	1	3,044	0,106
Ponderal index	1	1,479	0,247

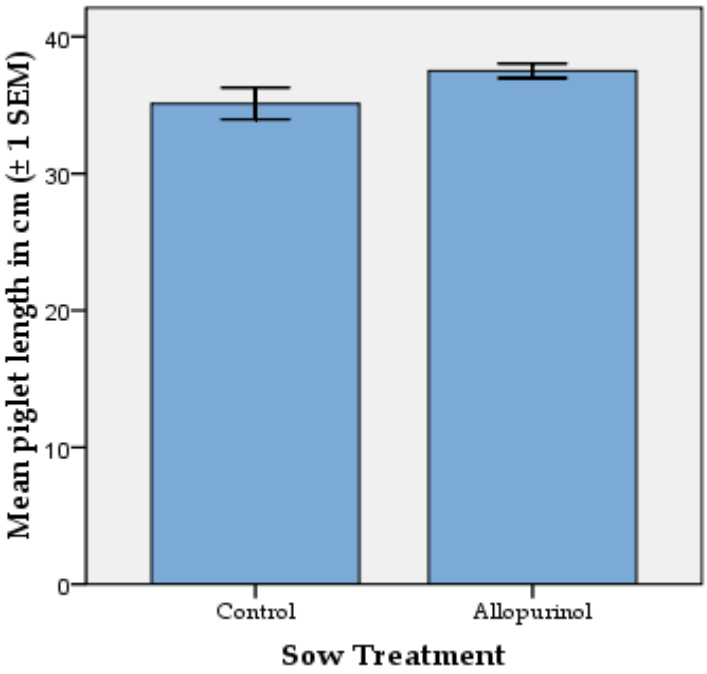


Figure 5: Effect of sow treatment on piglet length. Mean length of newborn piglets was higher in piglets of treated sows (linear mixed model; df = 1; F = 0,6347; p = 0,026. Appendix Table 4 shows litter size per sow).

Open Field test

Table 5 shows the results of the analyses on the Open Field test for every measure we scored. The table shows the effects of treatment, birth weight class (low or normal) and the effect of the interaction between these two factors on the measures. Only the number of vocalizations during the test was significantly higher in LBW piglets, compared to NBW piglets (linear mixed model; df = 1; F = 4,895; p = 0,036; Figure 6). All other measures were not influenced by either treatment or birth weight class. Appendix Table 2 shows how many piglets of each birth weight class were used per sow.

Table 5: Open Field results

Measure	Treatment	Birth Weight Class	Treatment*BWC
Time spent in wall segments	0,235	0,225	0,756
Time spent in corner segments	0,292	0,354	0,715
Time spent in center segments	0,522	0,809	0,822
Entries wall segments	0,727	0,683	0,442
Entries center segments	0,830	0,405	0,801
Entries corner segments	0,593	0,942	0,449
Crossings in wall segments	0,689	0,629	0,294
Crossings in corner segments	0,590	0,921	0,515
Crossings in center segments	0,979	0,542	0,861
Total number of enties	0,670	0,824	0,418
Total number of crossings	0,718	0,690	0,493
Total time looking at object	0,446	0,189	0,510
Total times touching object	0,473	0,556	0,348
Number of escape attempts	0,465	0,523	0,286
Number of vocalizations	0,563	**0,036**	0,268
Number of defecations	0,912	0,672	0,376

Of each sow, 1 – 3 piglets of each birth weight class were tested (Appendix Table 2).

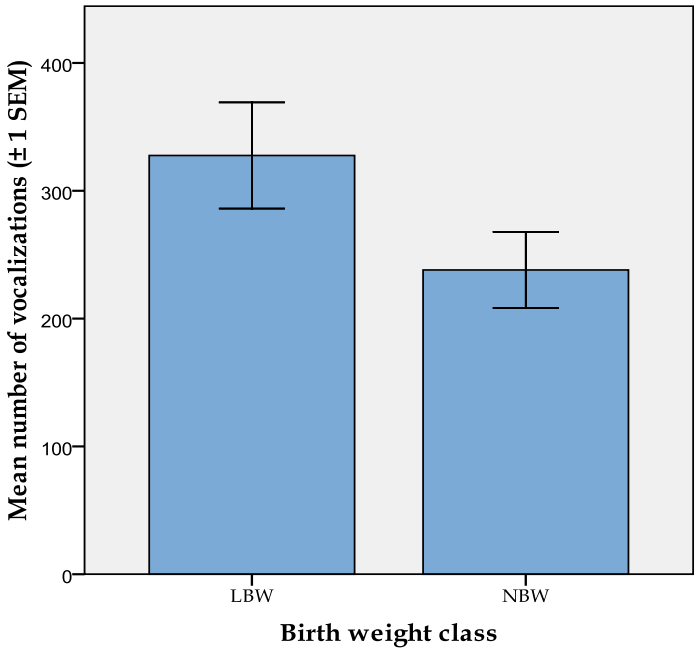


Figure 6: Vocalizations in the Open Field test. The number of vocalizations during the Open Field test for emotionality was significantly lower in LBW piglets than in NBW piglets (linear mixed model; df = 1; F = 4,895; p = 0,036. LBW = low birth weight; NBW = normal birth weight).

Holeboard task

Table 6 shows the outcomes of statistical analyses of piglet performance in the Holeboard task. Treatment, birth weight and their interaction had no effect on TD, WM or RM, in either the training or the reversal phase (Table 6, Figure 7). There was an effect of trial blocks (Bs) on all three measures for both the training and reversal phase (all $p < 0{,}0001$).

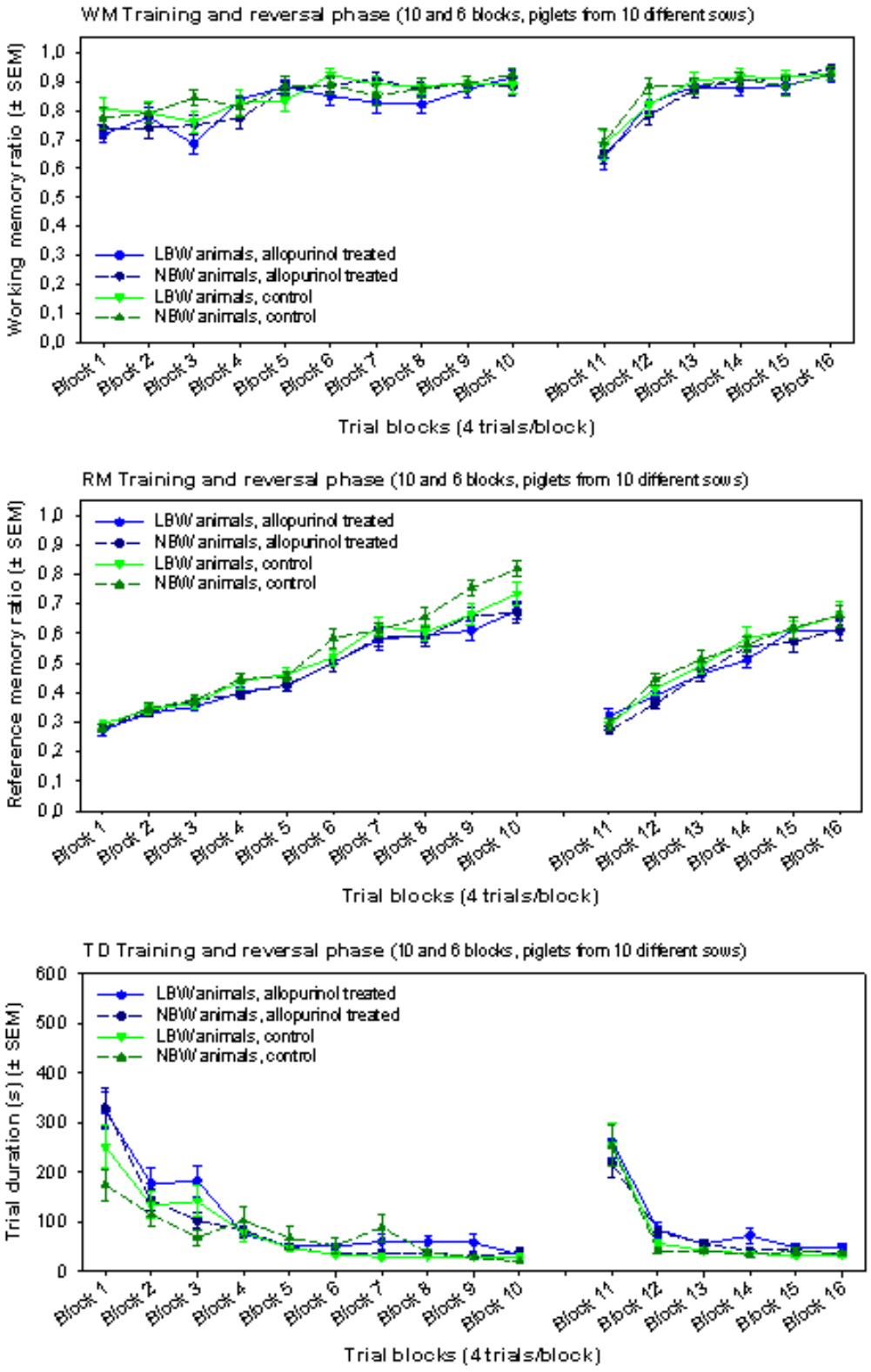


Figure 7: Piglet performance in the Holeboard task. Performance for all groups tested; low and normal birth weight, either treated or untreated. Block 1 – 10 = Training phase; Block 11 – 16 = Reversal phase. WM = Working Memory; RM = Reference Memory; TD = Trial Duration (all ns).

Table 7: Results for the Holeboard task.

		Between subjects			**Within subjects**								
		Treatment (TM)			Blocks (Bs)			Birth weight (BW)			BW x TM interaction		
Measure	Phase	F	df	P <	F	df	P <	F	df	P <	F	df	P <
Trial duration	training	0.08	1,8	0.7877	18.59	9,72	**<0.0001**	0.04	1,8	0.8551	0.62	1,8	0.4536
	reversal	0.20	1,8	0.6698	90.13	5,40	**<0.0001**	0.07	1,8	0.8029	1.23	1,8	0.3129
Working memory	training	0.04	1,8	0.8409	8.94	9,72	**<0.0001**	0.39	1,8	0.5503	1.10	1,8	0.3248
	reversal	0.32	1,8	0.5843	26.63	5,40	**<0.0001**	0.09	1,8	0.7725	0.04	1,8	0.8469
Reference memory	training	0.42	1,8	0.5372	63.21	9,72	**<0.0001**	1.03	1,8	0.3392	0.35	1,8	0.5686
	reversal	0.46	1,8	0.5188	61.16	5,40	**<0.0001**	0.02	1,8	0.8859	0.27	1,8	0.6183
Trials to criterion	training	0.301	1,8	0.5981	*n/a*	*n/a*	*n/a*	2.90	1,8	0.1273	1.10	1,8	0.3245

		Within subjects								
		BW x Bs			Bs x TM			BW x Bs x TM		
Measure	Phase	F	df	P <	F	df	P <	F	df	P <
Trial duration	training	1.62	9,72	0.1263	0.9	9,72	0.5300	1.16	9,72	0.3340
	reversal	0.18	5,40	0.9669	1.23	5,40	0.3129	0.27	5	0.9248
Working memory	training	0.74	9,72	0.6666	1.03	9,72	0.4232	1.55	9,72	0.1469
	reversal	0.58	5,40	0.7165	0.09	5,40	0.9923	1.11	5	0.3682
Reference memory	training	0.67	9,72	0.7364	0.33	9,72	0.9608	0.52	9,72	0.8552
	reversal	0.27	5,40	0.9259	0.54	5,40	0.7412	1.74	5	0.2226
Trials to criterion	training	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*

Discussion

The aim of this study was to investigate the effect of chronic prenatal allopurinol treatment through the sow on postnatal learning ability in low birth weight piglets, using the cognitive spatial Holeboard task for pigs. Additionally, we looked at the effect of treatment on other measures, namely umbilical cord blood cord gases, piglet and placenta measures, and behavior in the Open Field test. By investigating these additional measures, we tried to determine 1) the efficacy of prenatal allopurinol treatment on cognitive development, 2) whether any of the piglets had undergone acute asphyxia during birth, and 3) whether treatment with allopurinol had side-effects on piglet measures and emotionality.

As we did not find any pH below 7.0 nor pCO_2 values above 100 mm Hg, we can assume that acute asphyxia did not occur during birth in the piglets we sampled (Randall 1971; van den Berg 1996). One sample in which we found a low value of pCO_2 included very deviating values for all other measures, strongly indicating that analysis of this sample had failed. The other sample with a low pCO_2 (19 mm Hg) showed a high pH (7.67, which was the highest value we found) but otherwise normal values for all other measures. Randall (1971) showed that a low pH and high pCO_2 is an indication of reduced viability. However, the reverse combination was not found nor mentioned in their study sample. The combination of a high pH and low pCO_2 has been linked to low alveolar and arterial oxygen concentrations in research on Mount Everest climbers (West et al. 1983; Grocot et al. 2009), but the pO_2 value for the piglet with these values was normal (46 mm Hg) in comparison the low pO_2 found in such studies (below 30 mm Hg). In an *in vivo* study in rats, respiratory activity increased at high pH and decreased at low pCO_2 (Harada et al. 1985), but the combination of both is not mentioned. Therefore, we may discard this measure as an artifact or error of sampling, as the measures of this sample taken together do not give a conclusive result compared to available literature on this matter.

In sum, we conclude that none of the piglets from which we sampled umbilical cord blood had suffered from acute birth asphyxia. This included 13 of 37 animals we tested in the Open Field and Holeboard task (Appendix Table 4), which we argue is a indicative sample size to exclude the possibility of acute asphyxia in

the piglets tested. It would have been more reliable if all piglets were sampled, but lack of umbilical cord blood flow after clamping it in some piglets did not allow for this. In future research, it may be advisable to use arterial instead of umbilical cord blood of piglets for this analysis, so that all piglets can be sampled. Alternatively, other methods may be used, e.g. evaluating (anti-)oxidative parameters of placental tissue (Biri et al. 2007).

For placenta measures, we found that allopurinol treatment reduced the length of placentas, but not other placenta measures such as circumference or surface area. We did not have sufficient data to say anything about the differences between placentas of LBW or NBW piglets, as in some sows many umbilical cords would break before we could tie a node code to the cord. Therefore we were able to link only a few placentas to the piglets that they had contained, and we had to exclude birth weight effects. As placenta measurements were conducted fairly roughly, namely with a rope which we later held against a ruler, measures may not have been accurate enough. Additionally, placentas are flexible, allowing for manual stretching and shrinking, depending on how they are laid out. Therefore, we cannot be certain that the significant outcome is solid and relevant. Still, as all placentas were measured using consistent measuring and handling methods, and many placentas were used, the significant outcome may be valid. If the effect we found is in fact a result of treatment, the question remains what the biological relevance of this effect may be. In other words, what consequences does a shorter (but not smaller) placenta have for the piglets? In the piglet measures, we found that treated piglets were longer in their full length but not snout length, although they were not significantly heavier than untreated piglets. The fact that their snout and thus head was not significantly longer, indicates that the head was relatively smaller compared to the body in treated piglets. Reduced head growth, or asymmetric growth, may be an indicator of poor neurological outcome (Lin et al. 1991). Although we did not find differences in performance in the Holeboard task (Figure 7), there are indications that LBW piglets had reduced learning abilities when treated (see Discussion on the Holeboard task later in this chapter), which would support this finding.

Thus, treatment resulted in shorter placentas and longer piglets. This seems to be contradictory, as one would expect these measures to be positively correlated. Perhaps, allopurinol has a mild effect on intra-uterine growth processes, which would then have a negative effect in placentas and a positive effect in the piglets. Further research focused on this exact mechanism is needed. However, as in both placentas and piglets treatment has no effect on weight or size but only on length, we assume there is little biological relevance of this effect. It should be mentioned that we were not able to extract all placentas but did measure all piglets of each sow (totals included 125 placentas and 227 piglets). Therefore, there is a discrepancy in the number of placentas and the number of piglets measured, which can influence outcomes due to differences in accuracy.

In the Open Field test, none of the measures were affected by treatment. Thus, prenatal treatment with allopurinol did not affect emotionality or stress responses in piglets according to our data, although our sample size was rather small (6 treated and 4 control sows). We did however find an effect of birth weight class on the number of vocalizations during the test. Vocalization has been shown to correlate with unpleasant and painful events, such as unanaesthetized castration in pigs, branding in cows, and exposure to a novel environment in many animal species (Rushen 2000). Studies by Donald et al. (2011) and Rutherford et al. (2012) showed that the stress-reducing drug Azaperone decreased vocalizations. Therefore, we can assume that high frequencies of vocalization are an indicator of stress in the animal. Studies by Fraser (1974) and Donald et al. (2011) both showed that vocalization is a response to social isolation in pigs, since pair-wise testing especially reduced vocalization scores in their study as compared to individual testing. In an open field test for calves, vocalization was also argued to be a response to novelty and social isolation (de Passille et al. 1995). Our results indicate that LBW piglets are more easily stressed than NBW piglets in situations as social isolation and exposure to novelty, confirming our hypothesis. Social isolation may be more stressful for smaller piglets than for larger litter mates, as they are more vulnerable. In the open field test for calves that De Pastille et al. (1995) conducted, older calves vocalized less. Thus, age has been shown to influence vocalization in the open field test.

Being small and in a sense relatively underdeveloped, it seems logical from an evolutionary point of view to vocalize more than larger litter mates in stressful situations. Smaller piglets will need more attention, protection and nutrition in order to survive than their larger littermates. If vocalizing more results in more attention from the sow, this could be an evolved survival mechanism for LBW piglets. In an experiment on vocalizations of piglets in need and the sow's response to piglet vocalizations, it was shown that (1) isolated unfed piglets vocalized more than isolated fed piglets; (2) isolated low birth weight piglets vocalized more than isolated normal birth weight piglets; and (3) sows vocalized and approached a loudspeaker playing these piglet calls more often than when white noise was played (Weary & Fraser 1995). Another study on piglet and sow vocalizations indicated that both sows and piglets vocalize when they are isolated from each other, and that isolated piglets in a colder environment (14 compared to 30 degrees Celsius) vocalized more, indicating that the piglet signals the need for supplemental heat from the sow (Weary et al. 1997). These studies support the assumption that higher frequencies of vocalizations are due to stronger needs in the LBW animals for protection and nutrition.

The results from the Holeboard task showed no significant differences for either NBW or LBW piglets, or for piglets from treated and untreated sows. However, when looking at the graphs in Figure 7, it can be seen that the LBW allopurinol treated animals were slowest in acquiring the task (all graphs, Training phase, blue line), overall showing the lowest scores for WM and RM, and longest trial durations (TD). Although these differences are not significant, our sample size was small (6 treated and 4 untreated sows), and it is a worrying indication that treatment with allopurinol might reduce learning ability. Therefore, repeating the experiment with a larger sample size is advised, at least before applying this treatment to humans.

To increase sample size, laborious actions such as umbilical blood tapping and placenta measures may be excluded, through which a simpler experimental set up can be acchieved. In that manner, more sows can be included in the experiment. With a larger sample size, these differences in learning abilities may become significant.

Another option is to use other model species, such as rats or mice, which are easier to handle and treat, making it easier to increase the sample size.

There seemed to be a more pronounced difference between LBW and NBW performance in Batch I compared to both batches taken together (e.g. RM performance of the Training phase, Appendix Figure 2). Batch I and II were trained in winter and spring, respectively. Climate conditions such as temperature might have had an influence on piglet performance in the task, for example by increased stress due to cold conditions (e.g. Weary et al. 1997), possibly influencing results. It may therefore be better to conduct all experiments in the same season or at least at similar temperatures.

In an earlier study conducted with LBW and NBW piglets, it was found that LBW piglets showed a retarded acquisition of the first reversal phase after training (Gieling 2011b). This supports the general finding that LBW is related to cognitive impairment (e.g. Hack et al. 1994), which is why we expected to find similar results for LBW compared to NBW piglet performance on the Holeboard task. The fact that we did not find this may be due to the small sample size we used (6 treated vs. 4 untreated sows). Another possibly important factor is that we were not able to provide extra care to the LBW piglets that had the lowest birth weights and therefore had a greater risk of not surviving to weaning age. During the selection procedure, a few LBW piglets that we had selected for our study died, probably due to competition with litter mates. As these weaker LBW piglets are the most interesting ones for this study and not many LBW piglets are produced per litter, it may be advised that special care, comparable to incubator care in human neonates, is provided to the weakest and lightest LBW piglets. When these piglets can be used for such a study, results may be more prominent, and significant differences might be found.

As for efficacy, we did not find that allopurinol enhances cognitive performance, as no differences were found for treated or untreated groups in learning ability. Larger sample sizes of treated and untreated sows, and perhaps complementary studies in other animal species are however needed to corroborate these findings.

Conclusions

Prenatal sow treatment with allopurinol reduced placenta length and increased piglet length. As no other measures of placenta or piglet were affected, we argue that there is little biological relevance to these findings. As full length but not snout length was increased with treatment, treatment may have induced asymmetric growth in the fetuses, which can induce poor neurological outcome. In the Open Field test, we found no effect of treatment on activity or emotionality measures. LBW piglets were found to vocalize more during the test than NBW piglets, which indicates that LBW piglets may be more easily stressed when socially isolated and exposed to novelty. In the Holeboard task, no differences were found for LBW, NBW, treated and untreated piglets in learning ability. We may have been unable to find differences in LBW and NBW performance, which has been found in previous research, due to a small sample size of sows used. However, there is a worrying indication that treatment with allopurinol might reduce learning abilty. Larger sample sizes in further research is therefore needed. We conclude that the safety of chronic allopurinol treatment in the sow for piglet conginition is unclear, and its efficacy concerning reducing brain damage remains undetermined. Due to a small sample size and some indications that allopurinol might cause asymmetric growth and reduce learning ability, further and extended research is needed.

References

Allan G., Cambridge D., Lee-Tsang-Tan L., Van Way C.W., Whiting M.V. 1986. The protective action of allopurinol in an experimental model of haemorrhagic shock and reperfusion. *British Journal of Pharmacology* **89**: 149 – 155.

Annesi-Maesano I., Moreau D., Strachan D. 2001. *In utero* and perinatal complications preceding asthma. *Allergy* **56**: 491 – 297.

Archer J. 1973. Tests for emotionality in rats and mice: a review. *Animal Behavior* **21**: 205 – 235.

Arts J.W.M., van der Staay F.J., Ekkel E.D. 2009. Working and reference memory of pigs in the spatial holeboard discrimination task. *Behavioural Brain Research* **205**: 303 – 306.

Baker G.L., Corry R.J., Autor A.P. 1985. Oxygen free radical induced damage in kidneys subjected to warm ischemia and reperfusion. *Annals of Surgery* **202**: 628 – 641.

Biri A., Bozkurt N., Turp A., Kavutcu M., Hummetoglu Ö., Durak I. 2007. Role of oxidative stress in intrauterine growth restriction. *Gynecologic and Obstetric Investigation* **64**: 187 – 192.

Brinks V., van der Mark M., de Kloet R., Oitzl M. 2007. Emotion and cognition in high and low stress sensitive mouse strains: a combined neuroendocrine and behavioral study in BALB/c and C57BL/6J mice. *Frontiers in Behavioral Neuroscience* **1**: 8.

Caughey A.B. 2007. Measuring perinatal complications: methodologic issues related to gestational age. *BMC Pregnancy and Childbirth* **7**: 18.

Colletti L.F. 1979. Relationship between pregnancy and birth complications and the later development of learning disabilities. *Journal of Learning Disabilities* **12**: 25 – 29.

Cox P., Martin T. 2009. Pathological assessment of intrauterine growth restriction. *Best Practice & Research Clinical Obstetrics and Gynaecology* **23:** 751 – 764.

de Passillé A.M., Rushen J., Martin F. 1995. Interpreting the behaviour of calves in an open-field test: a factor analysis. *Applied Animal Behaviour Science* **45**: 201 – 213.

Donald R.D., Healy S.D., Lawrence A.B., Rutherford K.M.D. 2011. Emotionality in growing pigs: Is the open field a valid test? *Physiology & Behavior* **104**: 906 – 913.

Dudchenko P.A. 2004. An overview of the tasks used to test working memory in rodents. *Neuroscience and Biobehavoiral Reviews* **28**: 699 – 709.

Fay R.A., Dey P.L., Saadie C.M.J., Buhl J.A., Gebski V.J. 1991. Ponderal index: a better definition of the 'at risk' group with intrauterine growth problems than birth-weight for gestational age in term infants. *Australian and New Zealand Journal of Obstetrics and Gynaecology* **31**: 17 – 19.

Fellman V., Raivio K.O. 1997. Reperfusion Injury as the Mechanism of Brain Damage after Perinatal Asphyxia. *Pediatric Research* **41**: 599 – 606.

Ferguson S.A., Gopee N.V., Paule M.G., Howard P.C. 2009. Female mini-pig performance of temporal response differentiation, incremental repeated acquisition, and progressive ratio operant tasks. *Behavioural Processes* **80**: 28 – 34.

Fraser D. 1974. The vocalizations and other behaviour of growing pigs in an "open field" test. *Applied Animal Ethology* **1**: 3 – 16.

Gardner T.J., Stewart J.R., Casale A.S., Downey J.M., Chambers D.E. 1983. Reduction ofmyocardial ischaemic injury with oxygen-derived free radical scavengers. *Surgery* **94**: 423 – 427.

Gieling E.T., Nordquist R.E., van der Staay F.J. 2011a. Assessing learning and memory in pigs. *Animal Cognition* **14**: 151 – 173.

Gieling E.T., Park S.Y., Nordquist R.E., van der Staay F.J. 2011b. Cognitive performance of low- and normal-birth-weight piglets in a spatial hole-board discrimination task. Pediatric Research **71**: 71 – 76.

Grocot M.P.W., Martin D.S., Levett D.Z.H. 2009. Arterial blood gases and oxygen content in climbers on Mount Everest. *New England Journal of Medicine* **360**: 140 – 149.

Grunau R.E., Whitfield M.F., Davis C. 2002. Pattern of learning disabilities in children with extremely low birth weight and broadly average intelligence. *Archives of Pediatrics & Adolescent Medicine* **156**: 615 – 620.

Hack M, Taylor G., Klein N., Eiben R., Schatschneider C., Mercuri-Minich N. 1994. School-age outcomes in children with birth weights under 750 g. *The New England Journal of Medicine* **12**: 753 – 759.

Hall C., Ballachey E. L. 1932. A study of the rat's behavior in a field. A contribution to method in comparative psychology. *University of California Publications in Psychology* **6**: 1 – 12.

Handel M., Swaab H., de Vries L.S., Jongmans M.J. 2007. Long-term cognitive and behavioral consequences of neonatal encephalopathy following perinatal asphyxia: a review. European Journal of Pediatrics **166**: 645 – 654

Harada Y., Kuno M., Wang Y.Z. 1985. Differential effects of carbon dioxide and pH on central chemoreceptors in the rat in vitro. *The Journal of Physiology* **368:** 679 - 693.

Kaandorp J.J. *et al.* 2010. Antenatal allopurinol for reduction of birth asphyxia induced brain damage (ALLO-Trial); a randomized double blind placebo controlled multicenter study. *BMC Pregnancy and Childbirth* **10:** 8

Kinney D.K., Munir K.M., Crowley D.J., Miller A.M. 2008. Prenatal stress and risk for autism. *Neuroscience and Biobehavioral Reviews* **32**: 1519 – 1532.

Kornum B.R., Knudsen G.M. 2011. Cognitive testing of pigs (*Sus scrofa*) in translational biobehavioral research. *Neuroscience and Biobehavioral Reviews* **35**: 437 – 451.

Lam C.M., Wong S.F., Chow K.M., Ho L.C. 2000. Women with placenta praevia and antepartum haemorrhage have a worse outcome than those who do not bleed before delivery. *Journal of Obstetrics and Gynaecology* **20**: 27 – 31.

Lin C., Su S., River P. 1991. Comparison of associated high-risk factors and perinatal outcome between symmetric and asymmetric fetal intrauterine growth retardation. *American Journal of Obstetrics and Gynecology* **164**:153 – 1542.

Lind N.M., Moustgaard A., Jelsing J., Vajita G., Cumming P., Hansen A.K. 2007. The use of pigs in neuroscience: modeling brain disorders. *Neuroscience and Biobehavioral Reviews* **31**: 728 – 751.

McCord J. M. 1985. Oxygen-derived free radicals in postischemic tissue injury. *The New England Journal of Medicine* **312**: 159 – 63.

McCormick M.C., Gortmaker S.L., Sobol A.M. 1990. Very low birth weight children: Behavior problems and school difficulty in a national sample. *Journal of Pediatrics* **117**: 687 – 693.

Mendl M., Held S., Byrne R.W. 2010. Pig cognition. *Current Biology* **20**: 796 – 798.

Moorhouse P.C., Grootveld M., Halliwell B., Quinlan J.G., Gutteridge J.M.C. Allopurinol and oxypurinol are hydroxyl radical scavengers. *Federation of European Biochemical Societies* **213**: 23 – 28.

Norbeck J.S., Tilden V.P. 1983. Life stress, social support, and emotional disequilibrium in complications of pregnancy: a prospective, multivariate study. *Journal of Health and Social Behavior* **24**: 30 – 46.

Oades R.D., Isaacson R.L. 1978. The development of food search behavior by rats: the effects of hippocampal damage and haloperidol. *Behavioral Biology* **24**: 327 – 337.

Olton D.S., Samuelson R.J. 1976. Remembrance of places passed: spatial memory in rats. Journal of Experimental Psychology **2**: 97 – 116.

Palmer C., Vannucci R.C., Towfighi J. 1990. Reduction of perinatal hypoxic-ischemic brain damage with allopurinol. *Pediatric Research* **27**: 332 – 336.

Pare W.P. 1964. Relationship of various behaviors in the open-field test of emotionality.

Psychological Reports **14**: 19 – 22.

Parks D.A., Granger D.N. 1983. Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals *American Journal of Physiology – Gastrointestinal and Liver Physiology.* **245:** G285 – G289.

Pincus J.B., Gittleman I.F, Saito M., Sobel A.E. 1956. A study of plasma values of sodium, potassium, chloride, carbon dioxide, carbon dioxide tension, sugar, urea and the protein basebinding power, pH and hematocrit in prematures on the first day of life. *Pediatrics* **18**: 39 – 49.

Quiniou N., Dagorn J., Gaudré D. 2002. Variation of piglets' birth weight and consequences on subsequent performance. *Livestock Production Science* **78**: 63 – 70.

Randall G.C.B. 1971. The relationship of arterial blood pH and pCO_2 to the viability of the newborn piglet. *Canadian Journal of Comparative Medicine* **35**: 141 – 146.

Rollin B.E. 2006. The regulation of animal research and the emergence of animal ethics: a conceptual history. *Theoretical Medicine and Bioethics* **27**: 265 – 285.

Rushen, J. 2000. Some issues in the interpretation of behavioural responses to stress. *In: The Biology of Animal Stress* by G.P. Moberg & J.A. Mench, New York: CABI.

Rutherford K.M.D., Donald R.D., Lawrence A.B., Wemelsfelder F. 2012. Qualitative Behavioural Assessment of emotionality in pigs. *Applied Animal Behaviour Science*, in press.

Seidman L.J., Buka S.L., Goldstein J.M., Horton N.J., Rieder R.O., Tsuang M.T. 2000. The relationship of prenatal and perinatal complications to cognitive funcioning at age 7 in the New England Cohorts of the national collaborative perinatal project. *Schizophrenia Bulletin* **26**: 309 – 321.

Soothill P.W., Ajayi R.A., Campbell S., Ross E.M., Nicolaides K.H. 1995. Fetal oxygenation at cordocentesis, maternal smoking and childhood neuro-development. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **59**: 21 – 24.

ter Haar, K. 2012. Differences in placental features of piglets after a prenatal treatment period with allopurinol. Master thesis, Wageningen University, The Netherlands.

Thiringer K., Hrbek A., Kalsson K., Rosén K.G., Kjellmer I. 1987. Postasphyxial cerebral survival in newborn sheep after treatment with oxygen free radical scavengers and a calcium antagonist. *Pediatric research* **22**: 62 – 66.

Thorp J.A., Rushing R.S. 1999. Umbilical cord blood gas analysis. *Obstetrics and Gynecology Clinics of North America* **26**: 695 – 709.

Thorp J.A., Sampson J.E., Parisi V.M., Creasy R.K. 1989. Routine umbilical cord bloos gas determinations? *American Journal of Obstetrics and Gynecology* **161**: 600 – 605.

van Bel F., Shadid M., Moison R.M.W., Dorrepaal C.A., Fontijn J., Monteiro L., van de Bor M., Berger H.M. 1998. Effect of allopurinol on postasphyxial free radical formation, cerebral hemodynamics, and electral brain activity. *Pediatrics* **101**: 185 – 193.

van den Berg P.P., W.L.D.M. Nelen, Jongsma H.W., Nijland R., Kollée L.A.A., Nijhuis J.G., Eskes T.K.A.B. 1996. Neonatal complications in newborns with an umbilical artery pH <7.00. *American Journal of Obstetrics and Gynecology* **175**: 1152 – 1157.

van der Staay F. J. 2006. Animal models of behavioral dysfunctions: Basic concepts and classifications, and an evaluation strategy. *Brain research reviews* **52**: 131 – 159.

van der Staay F.J., Gieling E.T., Pinzon N.E., Nordquist R.E., Ohl F. 2011. The appetitively motivated "cognitive" holeboard: A family of complex spatial discrimination tasks for assessing learning and memory. *Neuroscience and Biobehavioral Reviews* **36**: 379 – 403.

van der Staay F.J., Bouger P., Lehmann O., Lazarus C., Cosquer B., Koenig J., Stump V., Cassel J.-C. 2006. Long-term effects of immunotoxic cholinergic lesions in the septum on acquisition of the cone-field task and non-cognitive measures in rats. *Hippocampus* **16**: 1061 – 1079.

van der Staay F.J., van Nies J., Raaijmakers W. 1990. The effects of aging in rats on working and reference memory performance in a spatial holeboard discrimination task. *Behavioral and neural biology* **53**: 356 – 370.

van Handel M., Swaab H., De Vries L.S., Jongmans M.J. 2007. Long-term cognitive and behavioral consequences of neonatal encephalopathy following perinatal asphyxia: a review. *European Journal of Pediatrics* **166**: 645 – 654.

Voldner N., Frøslie K.F., Haakstad L.A.H., Bø K., Henriksen T. 2009. Birth complications, overweight, and physical inactivity. *Acta Obstetricia et Gynecologica* **88**: 550 – 555.

Weary D.M., Ross S., Fraser D. 1997. Vocalizations by isolated piglets: a reliable indicator of piglet need directed towards the sow. *Applied Animal Behaviour Science* **53:** 249 – 257.

Werner E., Simonian K., Bierman J.M., French F.E. 1967. Cumulative effect of perinatal complications and deprived environment on physical, intellectual and social development of preschool children. *Pediatrics* **39**: 490 – 505.

West J.B., Hackett P.H., Maret K.H., Milledge J.S., Peters Jr. R.M., Pizzo C.J., Winslow R.M. 1983. Pulmonary gas exchange on the summit of Mount Everest. *Journal of Applied Physiology* **55**: 678 – 687.

Williams G.D., Palmer C., Heitjan D.F., Smith M.B. 1992. Allopurinol preserves cerebral energy metabolism during perinatal hypoxia-ischemia: a ^{31}P NMR study in unanesthetized immature rats. *Neuroscience Letters* **144**: 103 – 106.

Appendix

Appendix Table 1: Number of placentas and umbilical cord blood samples collected per sow

Sow Number	Placentas collected	Umbilical cord blood samples
1	**2**	**-**
2	**4**	**-**
3	3	-
4	2	-
5	4	-
6	**12**	**2**
7	**12**	**3**
8	**13**	**2**
9	9	4
10	6	2
11	10	2
12	**4**	**5**
13	**15**	**10**
14	**12**	**4**
15	5	4
16	5	2
17	7	5

Bold = treated sow. Sow 1 – 5 = first collection, September 2011 (C-section, no blood samples taken); Sow 6 – 11 = second collection, October 2011; Sow 12 – 17 = third collection, December 2011.

Appendix Table 2: Piglets selected per sow for the Open Field test and Holeboard task

Sow number	Birth Weight Class	Piglets
6	**Low**	**2**
6	**Normal**	**2**
7	**Low**	**2**
7	**Normal**	**2**
8	**Low**	**1**
8	**Normal**	**1**
9	Low	3
9	Normal	3
10	Low	3
10	Normal	3
12	**Low**	**1**
12	**Normal**	**1**
13	**Low**	**2**
13	**Normal**	**2**
14	**Low**	**2**
14	**Normal**	**2**
15	Low	1
15	Normal	2
17	Low	1
17	Normal	1

Bold = treated with Allopurinol. Sow 6 – 10 = second collection, October 2011; Sow 12 – 17 = third collection, December 2011.

Appendix Table 3: Blood sample count per group selected for Open Field & Holeboard task

Group	**Umbilical cord blood samples**
Treated LBW	5
Control LBW	1
Treated NBW	3
Control NBW	4

Appendix Table 4: Litter size per sow

Sow Number	Litter Size
1	**13**
2	**15**
3	15
4	14
5	16
6	**16**
7	**13**
8	**9**
9	16
10	16
12	**9**
13	**18**
14	**12**
15	8
17	18

Bold = treated with Allopurinol. Sow 1 – 5 = first collection, September 2011; Sow 6 – 10 = second collection, October 2011; Sow 12 – 17 = third collection, December 2011.

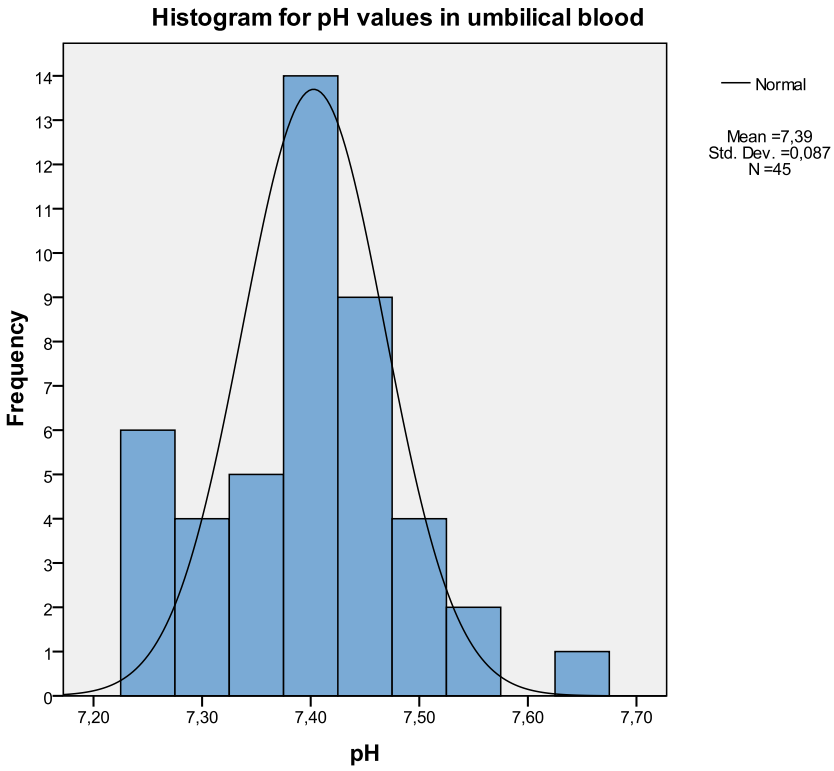


Figure 1: Histogram for pH values of piglet umbilical cord blood samples in this study.

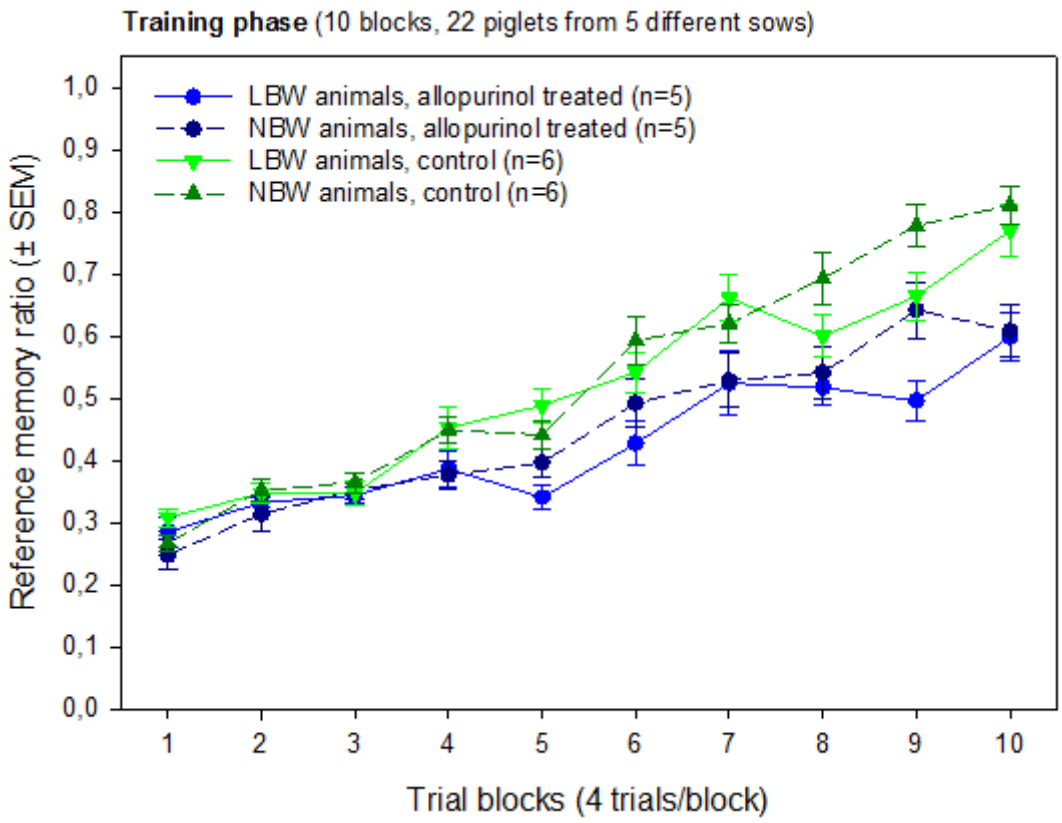


Figure 2: Reference memory scores for Batch I in the Holeboard task.