Functional parameters for the development of the central nervous system of piglets

Research Project Veterinary Medicine

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Glossary

CNS	Central nervous system
NBW	Normal birth weight. In this study a piglet with a birth weight near the mean birth weight of the litter. The mean weight of the litter was calculated after excluding all LBW piglets.
Low birth weight infant	Infant with a birth weight less than 2.5 kg (Berk 2006; Berk 2008a; Berk 2008b)
LBW	Low birth weight. In this study a piglet with a birth weight 1-2 standard deviations below the mean birth weight of the litter.
Preterm infant	Infant born several weeks before its due date. The birth weight of the infant can be appropriate for the amount of time spent in the womb (Berk 2006; Berk 2008a; Berk 2008b)
Small-for-date infant	Infant with a birth weight below the expected birth weight considering the length of the pregnancy (Berk 2006; Berk 2008a; Berk 2008b)
Very low birth weight infant	Infant with a birth weight less than 1 kg OR an infant born after <30 weeks of pregnancy (Berk 2006; Berk 2008a; Berk 2008b)

Abstract

Because of improved health care the survival rate of preterm infants and low birth weight infants is increased. These infants often have brain abnormalities resulting in a higher incidence of some diseases or disorders in these infants. To limit these brain abnormalities and their consequences, research has to be done. Due to ethical and practical constraints this research cannot be done in humans, so a suitable translational model is needed. Brain growth in human neonates and piglets is comparable concerning the timing of the brain growth spurt and the growth of some brain regions. This suggests that piglets should be a suitable translational model for the growth and development of the central nervous system (CNS) of human neonates. A test battery is needed to measure the relative stage of development of the CNS in neonatal piglets before piglets can be used as animal model. After a literature study 25 tests were conducted on 25 piglets of 0 to 28 days of age to see if the tests were feasible and aversive in piglets. Aversive and non-feasible tests were deleted and the remaining 13 tests were conducted on low birth weight (LBW) piglets and normal birth weight (NBW) piglets for three weeks. The tests were assessed on presence of a turning point (e.g. a test was first absent but after a few days present), normal distribution and differences in the outcomes between the LBW group and NBW group. None of the tests showed a turning point. Not a single test had a normal distribution at all point of time and there were no significant differences in the outcomes between the LBW group and the NBW group. Therefore, the test battery that was developed during this pilot study was not appropriate to measure the functions of piglets before weaning. It can also be concluded that the development of LBW piglets is not by definition retarded concerning the CNS.

Introduction

Because of the improved health care the mortality rate of preterm infants and even of (very) low birth weight infants remarkably decreased over the last decades (Als et al. 2005). For example, in the Netherlands the in-hospital mortality rate was 30% in the 80's and decreased to 11% in the 90's (Stoelhorst et al. 2005). Premature births have as a consequence that 'the immature brain is at increased risk of intraventricular and periventricular haemorrhage, and hypoxic-ischaemic damage'. Especially children born before 33 weeks of gestation have a higher incidence of structural brain abnormalities (Stewart et al. 1999). Examples of these brain abnormalities are white matter damage such as thinning of the corpus callosum, ventricular enlargement, basal ganglia haemorrhage, brainstem abnormalities and several other injuries (Peterson et al. 2000). Due to the increased risk of brain damage of the immature brain premature infants have a higher incidence of attention deficit hyperactivity disorder (ADHD), simple phobias and separation anxiety disorder later in life. Also, these children have an impaired visual-motor integration. Their cognitive function is impaired (Bhutta 2001) which in most cases leads to an IQ score of more than 1 standard deviation below the population mean (Peterson et al. 2000). To limit such brain abnormalities and to minimalize the consequences of these brain abnormalities in the later life of preterm and (very) low birth weight infants research is needed. Of course, due to ethical and practical constraints this research cannot be done in humans and therefore a suitable animal model with similar growth and development of the central nervous system is needed.

The domestic pig (*Sus scrofa)* shows many similarities with humans concerning anatomy and physiology. Therefore, the domestic pig is used a lot as pre-clinical model in several areas, for example organ transplant surgery and pediatric nutrition (Miller & Ullrey 1987; Dixon & Spinale 2009). Several studies suggest that the development of the CNS of neonatal piglets and human neonates are similar. The brain growth spurt is of special interest because of the enhanced vulnerability of the brain during this period. Research in rats show that only mild restrictions during the brain growth spurt, that occurs during the suckling phase, lead to irreversible changes. These changes have an effect both on physical configuration of the brain and on the behavior of the animal. Also the changes are permanent, even with full attempts at rehabilitation (Dobbing 1974). Dobbing and Sands stated that pigs are the best model for the brain growth spurt of human, better than sheep or rats and even better than the rhesus monkey, a sub-human primate. This is because of the timing of the brain growth spurt of these animals. Sheep, rats and rhesus monkeys all have the biggest proportion of the brain growth spurt prenatally, while pigs have the biggest proportion of their brain growth spurt postnatal, like man. Also the peak in the growth spurt is around birth both in pigs and man (Dobbing & Sands 1979). Another finding of the investigators was that man and pigs have almost the same percentage of adult brain weight at birth, 27% and 25% respectively (Dobbing & Sands 1979). Conrad and coworkers investigated brain growth of piglets from 2 to 24 weeks of age using longitudinal MRI studies to test if piglets are useful as translational model for human neonates. They measured total brain growth and growth of some brain regions, namely the cortex, hippocampus, diencephalon, cerebellum and the brainstem. Total brain growth increased 130% in males and 121% in females from 2 to 24 weeks, with a maximum increase in brain growth at the age of 4 weeks, which is comparable to brain growth of human neonates (Conrad et al. 2012). The brain growth of the neonatal piglets cannot be attributed to neocortical neurogenesis, but mainly to changes in neuropil and myelination, which is also similar to human brain growth (Jelsing et al. 2006). These studies suggest that piglets should be a suitable translational model for the growth and development of the central nervous system of human neonates.

The aim of the study is to develop a complete test battery with which enables to measure the relative stage of development of the central nervous system of neonatal piglets. The tests of the battery should be performable on piglets under field conditions, not aversive and the tests should be sensitive to developmental changes. Another point of interest is the ability of the tests to distinguish piglets with a low birth weight (LBW) from piglets with a normal birth weight (NBW). If the results of the tests differ between these two groups it can be seen as an indication that piglets with a low birth weight do have a lag in the development of the central nervous system. These LBW piglets then could be a suitable translational model for the growth and development of the central nervous system of premature infants.

In this pilot study first a design for a test battery is made by performing tests found in literature. These tests are conducted on piglets of several age categories. Tests that appear to be performable on piglets are then conducted on LBW piglets and NBW piglets twice a week from birth until three weeks of age. The collected data can be plotted to see if there are tests with a turning point. It is possible that a test is present in the first few days of the piglets' life, but after a few days absent, or the other way around. If there is a turning point it is the question if it takes place at another point of time in the LBW piglets compared to the NBW piglets. This turning point is of special interest because it can be seen as a milestone in the development of the central nervous system. By means of statistical analyses it is investigated if the results of the LBW piglets differ from the results of the NBW piglets. With this information the research question can be answered, which is stated as follows: 'are the tests of the test battery appropriate to measure the development of functions in piglets before weaning in an objective manner?'

Materials and methods

Study design

A literature study was done to gain information about tests that are used in humans and other species to determine development of newborns. During phase 1 selected tests were conducted once on piglets of several age categories to determine if these tests are really feasible in piglets. Also the averseness was rated in this phase. Feasible tests that were not aversive to the piglets were conducted for three weeks in a group of low birth weight piglets and a group of normal birth weight piglets (phase 2). With the outcomes of this follow-up statistical calculations were performed to see if LBW piglets significantly differ from NBW piglets in regards to the tests.

General conditions

The 51 piglets that were used were all Topigs 20 x Duroc piglets bred on 'De Tolakker', the breeding farm of the veterinary faculty at the Utrecht University. The animals were housed for 28 days at the farrowing stables (4.32 m^2) together with their mother and siblings. The sow was caged to prevent crushing the piglets. The piglets had free access to water by means of one nipple drinker. From day 0 to day 7 the piglets received ad libitum Miliwean Yoghurt (Trouw Nutrition Hifieed B.V., Boxmeer) as a complement to milk of the sow. From day 8 to day 28 the piglets received ad libitum milk kibbles (Romelko, De Heus Voeders B.V., Ede) to get used to solid food before they were weaned. Also the piglets received a handful of corn cob mix (CCM, De Heus Voeders B.V., Ede) twice a day. The piglets were weaned at 28 days of age by first removing the sow from the farrowing stable. Thereafter the piglets were moved to the weaned piglets section.

Literature study

Literature was searched through Scopus and PubMed. The search terms used are as follows: neonatal assessment, neurobehavioral assessment, neurodevelopmental assessment, gestational age, postnatal, pre-term infants, newborn infants, neurobehavioral maturity. If possible, terms were combined by placing 'and' in between or by adding a search field to enhance specificity of the results. Also the reference-lists of the found articles and reviews were read, to gain new articles. The title of an article was then used as search term. The articles and reviews were read and evaluated for usability. Articles and reviews were marked usable if the tests that were used in an assessment were mentioned or if there was information about the set-up of an assessment.

The literature study yielded around fifteen articles, whereof five articles tended to be useful. Also one book was found usable. With this selection an overview was made with possible tests that are applied in humans and several other species to determine development of a newborn. Subsequently, the tests in this overview were discussed by the author and a few researchers. With knowledge of previous studies and practical experience all tests that were thought to be not performable were removed from the list. This resulted in 25 possible tests, as listed in Table 1. The list with these tests was the starting point of the study. It is worth mentioning that none of the tests found in literature were especially developed for piglets. One book was used with tests for farm animals in it, the other tests are selected from articles about dogs and men.

Phase 1

All the tests as listed in Table 1 were conducted once on five piglets of five age categories. The following age categories were distinguished: 0 days, 7 days, 14 days, 21 days and 28 days. In this phase the gender and weight of the piglets were not considered relevant. The five piglets per age category were randomly selected. During the performance of the tests a scoring form was completed by the same person for each piglet. The possible outcomes of the tests were 0= absent, 1= partly present and

2= present. The tests that were not feasible in piglets were removed from the test battery. During the performance of the tests a person was present to film the proceedings. The tests were conducted in the aisle of the farrowing stables and the piglets were only fixated or restrained when necessary.

During phase 1 the observers continually looked at how aversive the tests were for the piglets. To determine this the piglets were placed back in the box after the performance of a test and observed and filmed for two minutes. When a piglet showed normal behavior directly the tests was not marked as aversive. When a piglet isolated itself from the other piglets of the litter and/or was shivering (Landa 2012), the test was marked as aversive and was removed from the test battery.

Table 1 - Overview of the 25 possible tests selected after the literature study. This overview contains the name of the test, the action, the expected response and where a test was found.

TEST	ACTION	EXPECTED RESPONSE	REFERENCES
CROSSED EXTENSOR REFLEX	Pinching a hind foot	Flexion of the same foot, extension of the opposing hind limb	(Fox 1964; Robinson 1966; Brazelton & Nugent 1973)
MAGNUS REFLEX	Turning the head to one side	Extension of all the limbs in the direction the head is turned	(Fox 1964; Robinson 1966; Amiel-Tison 2002; Lester et al. 2004)
TONIC NECK REFLEX	Forced upward extension of the head and neck	Extension of the front limbs, flexion of the hind legs	(Fox 1964)
EXTENDED NECK RESPONSE	Raising the animal while holding it only at the mastoid region	Flexion of all limbs and spinal column	(Fox 1964)
LANDAU REFLEX	Holding the animal in horizontal position only by holding it beneath the armpits	Extension of the head, neck, spinal column and hind limbs	(Fox 1964)
RIGHTING RESPONSE	Animal is placed on its side	Animal gets up	(Fox 1964)
PALPEBRAL BLINK REFLEX	Tactile stimulation of the skin between the eyes	Animal blinks	(Fox 1964; Robinson 1966)
LABIAL REFLEX	Stroking the labial area of the animal	Movement of the head and lips towards the region of stimulation	(Fox 1964)
AURICULONASOCEPHAL REFLEX	Stroking the animal behind one ear	Animal turns it head towards the stimulated side	(Fox 1964)
GALANT REFLEX	Touching the flank with a blunt probe	Flexion of the body towards the stimulated side	(Fox 1964; Robinson 1966)
ABDOMINAL REFLEX	Animal in prone position, stroking the abdomen with a blunt probe	Contraction of the abdominal muscles	(Fox 1964; Robinson 1966)
NOCICEPTIVE WITHDRAWAL REFLEX	Pinching the skin between the claws with two fingertips	Withdrawal of the limb, vocalisation and avoidance behaviour	(Fox 1964)
SUPERFICIAL ANAL REFLEX	Tactile stimulation of the external anal sphincter with a blunt probe	Contraction of the external anal sphincter	(Fox 1964)

PATELLA REFLEX	Tapping the patella tendon with a reflex hammer	Flexion of the knee	(Fox 1964; Robinson 1966)
RADIALIS REFLEX	Tapping the tendon of the m. extensor carpi radialis with a reflex hammer	Extension of the limb	(Robinson 1966)
PALPEBRAL BLINK REFLEX WITH LIGHT	Shining into the eye with a flashlight	Animal blinks	(Fox 1964)
PUPIL REFLEX	Shining into the eye with a flashlight	Pupil size decreases	(Fox 1964; Robinson 1966)
VISUAL ORIENTATION	Shining in the lateral visual field with a flashlight	Animal turns the head towards the flashlight	(Robinson 1966; Brazelton & Nugent 1973)
VISUAL FIXATION	Moving an object in the visual field of the animal	Animal follows the object	(Brazelton & Nugent 1973; Lester et al. 2004)
STARTLE REFLEX	Scaring the animal with a loud noise	Sudden abduction of the limbs, flexion of the head and blinking with the eyes	(Fox 1964)
AUDITORY ORIENTATION	Sound stimulus (ringing of a bell)	Animal turns the head towards the stimulus	(Fox 1964; Brazelton & Nugent 1973; Lester et al. 2004)
DORSAL PLACEMENT OF THE FEET	Placing the animal on its dorsal side of a foot	Animal draws foot up and places it correctly	(Kuiper & Van Nieuwstadt 2008)
CROSSING OF THE LEGS	Crossing the legs of the animal and let the animal put weight on it	Animal places the legs in correct position	(Kuiper & Van Nieuwstadt 2008)
WHEELBARROW	Lifting the hind limbs of the animal and let it walk	Animal walks on front limbs	(Kuiper & Van Nieuwstadt 2008)
MENACE REFLEX	Moving an object fast in the direction of the eyes	Animal closes the eyes	(Kuiper & Van Nieuwstadt 2008)

Phase 2

The remaining tests (Table 2) on the test battery were performed two times a week (Monday and Thursday) for three weeks instead of four weeks because of limited time(in total seven measuring points). When a litter of piglets was born, they were all weighed and the mean weight and the standard deviation (SD) were calculated. Piglets were selected for the LBW group if their weight was one to two SD under the mean weight of the litter. To select the piglets for the NBW group, the remaining piglets (minus the piglets with a weight >2 SD under the mean weight) were weighed again and a new mean weight was calculated. In both groups 13 piglets were selected. This amount was chosen because the piglet mortality in the postpartum period at 'De Tolakker' over the last twelve months was 12.4% and the results of at least ten piglets per group were needed. The piglets received an ear-tag with a blue backside directly after the selection procedure, so it was possible to quickly distinguish them from the other piglets in the litter. Only the smallest piglets received their ear-tag at the latest at day 2.

Exclusion criteria

Piglets were excluded if they showed clinical signs that were observed by veterinarians, students, caretakers or researchers of the animals and/or if they had any medication other than the standard treatments. The standard treatments on the farm are tail docking, iron injection and injection with Baycox® 50 mg/ml Oral Suspension (Bayer, Diegem, Belgium), all administered on day 4.

Statistics

A t-test was conducted in SPSS 22.0 for Windows to see if the mean birth weight of the LBW group was significantly different from the mean birth weight of the NBW group. The mean weights were significantly different if the p-value was ≤ 0.05.

The mean values of the seven measuring points were calculated for the LBW group and the NBW group and plotted in Microsoft Excel 2013 to see if there were tests with a turning point.

To check the normality of the data a Shapiro-Wilk test was conducted in SAS 9.4 for the untransformed data and the ranked data. First the mean values per litter and per group were calculated. These calculated values were ranked over all measuring points, groups and litters. The data were appointed being Normal if the p-value was ≥ 0.05.

To see if there were differences between the LBW group and the NBW group a Fisher's exact probability test was done per test and per point of time. The tests were conducted at the website vassarstats.net. For performing this test the results of the test battery were divided into two groups: absence (0) = 0 and presence (1 or 2) = 1, were after the table on the website was completed. A difference was marked as significant if the p-value was ≤ 0.05.

Results

Phase 1

At the first test day the tests were conducted on six piglets: four new-born piglets, one piglets of one week of age and one piglet of two weeks of age. After the first test day we decided to remove four tests from the test battery because they were not feasible in piglets. The first removed test was *'extended neck response'*. This test was deleted because it was not possible to hold a piglet only at the mastoid region. The second deleted test was the *'abdominal reflex'*. For this test the piglets had to be in prone position, but they became so upset that conducting the reflex was not possible anymore. The third test that was deleted was the *'radialis reflex'* because it was very difficult to observe any reaction. Last the *'pupil reflex'* was removed from the test battery because when a flashlight shone into the eye the piglet closed the eye so that observing any response was not possible.

Furthermore a few modifications were made in the performance of the test. First the order of the tests was changed. Initially, the order was random, but the animals were most compliant if the tests where fixation or holding was necessary were conducted at the end. The test battery was therefore modified so that the tests without fixation or holding were conducted first, subsequently the tests were performed were fixation of the animal on its side was needed. The tests whereby holding the piglet was needed were performed at the end of the test battery. Further it seemed that conducting the tests on the ground was difficult for the researchers. Therefore, the piglets were placed on a table from that point on. To ensure the piglets did not fall off of the table, they were placed in a Plexiglas crate. An additional benefit of placing the piglets in the Plexiglas crate was that they were able to move less and therefore less fixation was needed.

At the second and third test day the remaining tests were conducted on nineteen piglets. After the completion of all test animals it was decided to remove another eight tests from the test battery because of the feasibility of the test. The first deleted test was the *'magnus reflex'* because this test was scored a 0 in 24 animals and only a 2 in one animal. Another reason to remove this test was that performing the test was difficult in the piglets of three and four weeks because of their weight. The *'landau reflex'* was also deleted, because a reaction was absent in 23 animals and present in only two animals. This test was difficult to perform in piglets of three and four weeks of age. The third deleted test was the *'auriculonasocephalic reflex'* because a reaction to this reflex was only present in one piglet and absent in the other 24 piglets. Also the *'galant reflex'* was removed because this reflex was only present in one piglet and absent in 24 piglets. The 'palpebral blink reflex with light' was deleted as well because 23 piglets were not responding to this test. Also *'visual orientation'* was deleted because a reaction to this behaviour was absent in 21 piglets, partly present in two piglets and present in two piglets. Furthermore this test was difficult to interpret correctly. *'Visual fixation'* was deleted as well, because this behaviour was absent in 18 animals, partly present in four animals, present in two animals and not conducted in one animal. The last deleted test was the *'auditory orientation'*, for 22 piglets did not react to the sound of the bell. The *'menace reflex'* was not deleted from the test battery despite this reflex was only partly present in two piglets. The reason this test was not deleted is because of personal interest of the researchers.

Phase 2

The remaining 13 tests are listed in Table 2. These tests were conducted on 13 low birth weight piglets in the LBW group. The piglets in this group had an average weight of 0.80 kg (± 0.20). Five piglets of the LBW group died during the period of data collection. Three piglets died at the age of one day because of weakness (one piglet) and because they were crushed by their mother (two piglets). One piglet died at the age of two days for the same reason. The fifth piglet died at day eight because of chronic wasting. The dead piglets were removed from the database and therefore not included in any calculation. The 13 piglets of the NBW group had an average weight of 1.26 kg (± 0.11). None of the piglets of the NBW group died during the test period. The conducted t-test had a p-value of 0.14 which means that the mean birth weight of the LBW group and the NBW group do not significantly differ.

Table 2 - Overview of the tests conducted in phase 2. The tests were conducted in which they are listed in the overview.

TEST	ACTION	EXPECTED RESPONSE	REFERENCES
LABIAL REFLEX	Stroking the labial area of the animal	Movement of the head and lips towards the region of stimulation	(Fox 1964)
PALPEBRAL BLINK REFLEX	Tactile stimulation of the skin between the eyes	Animal blinks	(Fox 1964; Robinson 1966)
MENACE REFLEX	Moving an object fast in the direction of the eyes	Animal closes the eyes	(Kuiper & Van Nieuwstadt 2008)
DORSAL PLACEMENT OF THE FEET	Placing the animal on its dorsal side of a foot	Animal draws foot up and places it correct	(Kuiper & Van Nieuwstadt 2008)
CROSSING OF THE LEGS	Crossing the legs of the animal and let the animal put weight on it	Animal places the legs in correct position	(Kuiper & Van Nieuwstadt 2008)
STARTLE REFLEX	Scaring the animal with a loud noise, while animal walks on the aisle	Sudden abduction of the limbs, flexion of the head and blinking with the eyes	(Fox 1964)
WHEELBARROW	Lifting the hind limbs of the animal and let it walk	Animal walks on front limbs	(Kuiper & Van Nieuwstadt 2008)
PATELLA REFLEX	Tapping the patella tendon with a reflex hammer	Flexion of the knee	(Fox 1964; Robinson 1966)
CROSSED EXTENSOR REFLEX	Pinching a hind foot	Flexion of the same foot, extension of the opposing hind limb	(Fox 1964; Robinson 1966; Brazelton & Nugent 1973)
NOCICEPTIVE WITHDRAWAL REFLEX	Pinching the skin between the claws	Withdrawal of the limb, vocalisation and avoidance behaviour	(Fox 1964)
SUPERFICIAL ANAL REFLEX	Tactile stimulation of the external anal sphincter with a blunt probe	Contraction of the external anal sphincter	(Fox 1964)
RIGHTING RESPONSE	Animal is placed on its side	Animal gets up	(Fox 1964)
TONIC NECK REFLEX	Forced upward extension of the head and neck	Extension of the front limbs, flexion of the hind legs	(Fox 1964)

When the results were plotted, none of the tests had a clear turning point. The labial reflex, the crossed extensor reflex, the menace reflex and the patella reflex showed a floor effect. This means that the outcomes of the test at all measuring points is (approximately) zero. The palpebral blink reflex, the wheelbarrow and the dorsal placement of the feet showed a ceiling effect. This means that the outcomes of the test at all measuring points is (approximately) two. Therefore these tests were not subjected to further analyses. The superficial anal reflex as well as the righting response partly showed a ceiling effect. The superficial anal reflex showed this effect from measuring point five till seven in both groups, the righting response showed the effect in the LBW group from measuring point two until point seven. These two tests were therefore subjected to further analyses, along with the startle reflex, the crossing of the legs, the nociceptive withdrawal reflex and the tonic neck reflex. Figure 1 shows the plots of the tests that were analyzed further. The Shapiro-Wilk test for normality was performed on these tests but none of the tests were normal distributed at all points of time, as seen in Table 3 and 4. The outcomes of the Fisher's exact test (Table 5-10) show that none of the outcomes of the tests differ at any point of time between the LBW group and the NBW group.

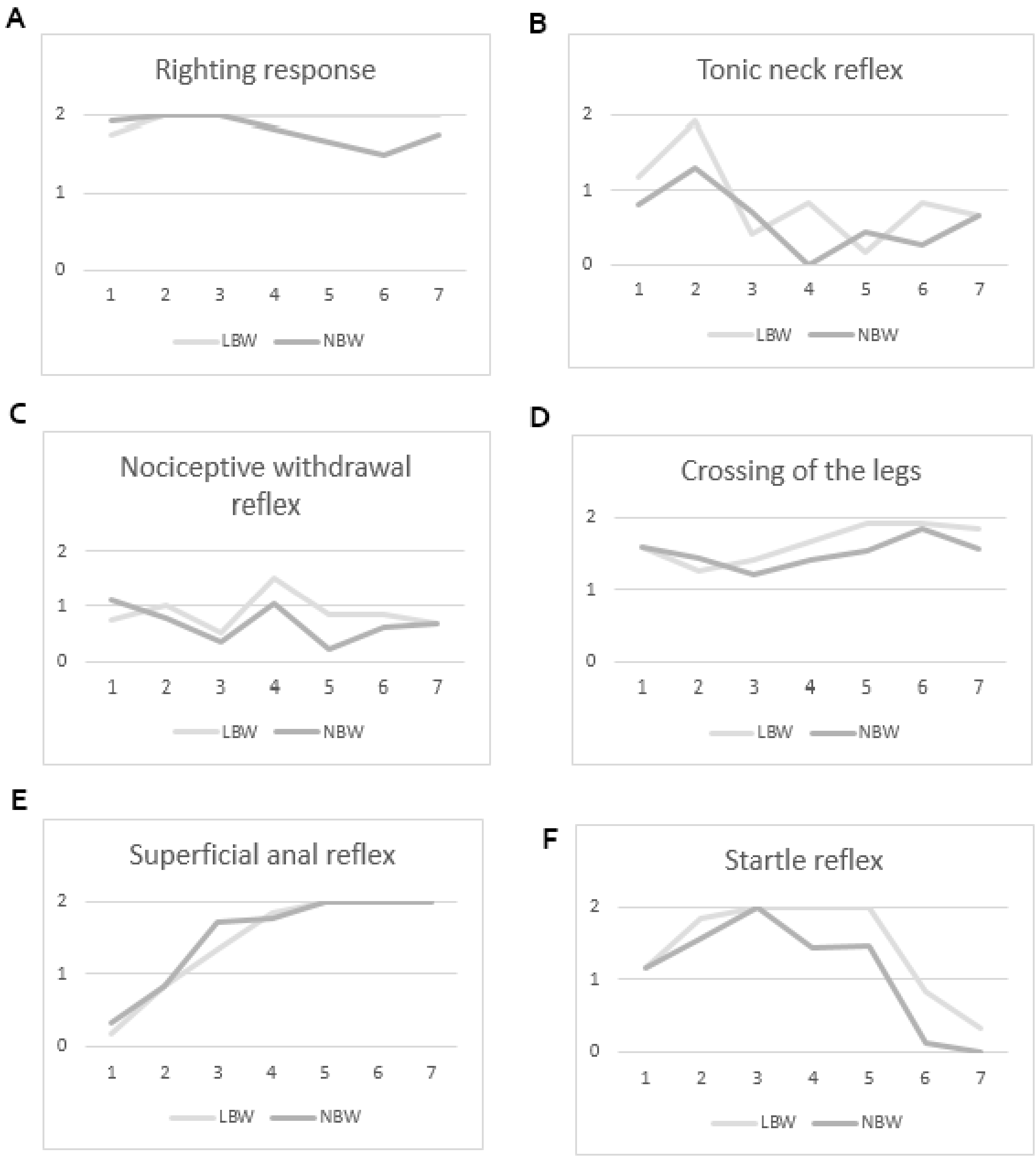

Figure 1- Graphs of the mean outcomes of the 7 measuring points plotted for the LBW group and the NBW group for the righting response (A), the tonic neck reflex (B), the nociceptive withdrawal reflex (C), crossing of the legs (D), the superficial anal reflex (E) and the startle reflex (F).

Table 3- Outcomes of the Shapiro-Wilk test for the LBW group for the normal data and the ranked data (R) whereby an 'x' means that the results of a test on a certain measuring point were normal distributed.

	STARTLE REFLEX	SUPERFICIAL ANAL REFLEX	CROSSING OF THE LEGS	NOCICEPTIVE WITHDRAWAL REFLEX	TONIC NECK REFLEX	RIGHTING RESPONSE
1						
1R						x
2		x		x		
2R				x		
3						
3R						
4						
4R						
5						
5R						
6						
6R						
7					x	
7R					x	

Table 4- Outcomes of the Shapiro-Wilk test for the NBW group for the normal data and the ranked data (R) whereby an 'x' means that the results of a test on a certain measuring point were normal distributed.

	STARTLE REFLEX	SUPERFICIAL ANAL REFLEX	CROSSING OF THE LEGS	NOCICEPTIVE WITHDRAWAL REFLEX	TONIC NECK REFLEX	RIGHTING RESPONSE
1		x		x	x	
1R		x		x	x	
2		x	x	x	x	
2R			x	x	x	
3			x	x	x	
3R			x	x	x	
4			x			
4R			x			
5					x	x
5R					x	x
6				x		x
6R				x		x
7			x	x	x	
7R			x		x	x

Table 5 - Outcomes of the Fisher's exact probability test for the startle reflex. The LBW group and the NBW group are compared for all 7 measuring points.

	1	2	3	4	5	6	7
LBW							
0	3	1	0	0	0	5	7
1 or 2	5	7	8	8	8	3	1
NBW							
0	6	3	0	3	3	11	13
1 or 2	7	10	13	10	10	2	0
Fisher's exact probability	1	1	1	0.26	0.26	0.33	0.38

Table 6 - Outcomes of the Fisher's exact probability test for the superficial anal reflex. The LBW group and NBW group are compared for all 7 measuring points.

	1	2	3	4	5	6	7
LBW							
0	7	5	3	1	0	0	0
1 or 2	1	3	5	7	8	8	8
NBW							
0	9	7	2	1	0	0	0
1 or 2	4	6	11	12	13	13	13
Fisher's exact probability	0.61	1	0.33	1	1	1	1

Table 7 - Outcomes of the Fisher's exact probability test for the crossing of the legs. The LBW group and NBW group are compared for all 7 measuring points.

	1	2	3	4	5	6	7
LBW							
0	0	0	0	0	0	0	0
1 or 2	8	8	8	8	8	8	8
NBW							
0	0	0	0	0	0	0	0
1 or 2	13	13	13	13	13	13	13
Fisher's exact probability	1	1	1	1	1	1	1

Table 8- Outcomes of the Fisher's exact probability test for the nociceptive withdrawal reflex. The LBW group and NBW group are compared for all 7 measuring points.

	1	2	3	4	5	6	7
LBW							
0	4	4	6	2	5	4	6
1 or 2	4	4	2	6	3	4	2
NBW							
0	6	8	9	5	11	9	8
1 or 2	7	5	4	8	2	4	5
Fisher's exact probability	1	0.67	1	0.66	0.33	0.65	0.66

Table 9 - Outcomes of the Fisher's exact probability test for the righting response. The LBW group and NBW group are compared for all 7 measuring points.

	1	2	3	4	5	6	7
LBW							
0	0	0	0	0	0	0	0
1 or 2	8	8	8	8	8	8	8
NBW							
0	0	0	0	0	0	0	0
1 or 2	13	13	13	13	13	13	13
Fisher's exact probability	**1**	**1**	**1**	**1**	**1**	**1**	**1**

Table 10 - Outcomes of the Fisher's exact probability test for the tonic neck reflex. The LBW group and NBW group are compared for all 7 measuring points.

	1	2	3	4	5	6	7
LBW							
0	3	0	6	6	7	5	4
1 or 2	5	8	2	2	1	3	4
NBW							
0	5	1	7	13	7	10	8
1 or 2	8	12	6	0	6	3	5
Fisher's exact probability	**1**	**1**	**0.40**	**0.13**	**0.17**	**0.63**	**0.67**

Discussion

It was remarkable that the mean birth weight of the LBW group and the NBW group was not significantly different. This may be because of the dead of five piglets, which had a birth weight varying between 540 and 740 grams. Another possible explanation for the absence of the significant difference is that there was no absolute upper limit to the birth weight of the LBW group, only a relative upper limit. For investigation done in the future it is recommended to determine an absolute upper limit.

The outcomes of the righting response of the NBW group show a downward trend from test point 3 until test point 6. This decrease may be caused by the huge weight gain these animals showed. It is possible that the legs were not strong enough to carry the weight of the body. This explanation is plausible because the animals of the LBW group, with a considerable lower weight gain did not show a downward trend.

In both groups the superficial anal reflex showed a ceiling effect starting at test point 5. This reflex is a spinal reflex, which means that the reflex runs directly via reflex arcs in the spinal cord. However, the handbook for clinical examination for the horse and farm animals used at the faculty of veterinary science at the Utrecht University pays special attention to the fact that spinal reflexes only can be conducted in pigs when the animal is very quiet (Kuiper & Van Nieuwstadt 2008). Because the piglets were agitated when they were lifted to conduct the superficial anal reflex, this agitation possibly suppresses the reflex.

The startle reflex shows a downward trend both in the NBW group and the LBW group respectively from test point 3 till test point 7 and from test point 5 till test point 7. The startle reflex was tested in the aisle of the farrowing stables and this aisle was sometimes soiled with water, kibbles of the sows or dead flies. It is possible that the piglets were very interested in these novel objects that they did not pay any attention to sounds anymore. It is also a possibility that the handclap was not loud enough to startle the animals because with increasing age of the piglets the noise in the farrowing stable was also increasing. A third possible explanation is the habituation of the animals to the sound of a handclap. For further investigation it is recommended to use a standardized loud auditory stimulus.

Despite the patella reflex is not further analyzed, the outcomes of this reflex are remarkable. None of the piglets showed at any measuring point a patella reflex. A study of Ochs in cats shows that inhibition of the patella reflex by stimulation of the motor cortex is possible. In Ochs' study the patella reflex was inhibited if parts of the motor cortex, for example the bulbar part of the reticular formation was stimulated using electrodes (Ochs 1955). This reticular formation plays a crucial role in modulating pain perception, arousal and consciousness and also in spinal reflexes and movement (Klein & Cunningham 2013). Because the piglets were very agitated while being fixated on their side during the performance of the test, this could be an explanation of the floor effect of the patella reflex in this study.

Another theory concerning the inhibition of the patella reflex is that the reflex is inhibited by adrenaline. Schweitzer and Wright injected cats with adrenaline and observed an extinction of the patella reflex with an increasing concentration of adrenaline. When a concentration of 0.05 mg adrenaline was injected there was no effect, but when 0.2 mg was injected they saw a slight depression of the reflex and with 0.4 mg adrenaline there was a complete inhibition of the reflex (Schweitzer & Wright 1937). Adrenaline is released during periods of stress, which is in all probability the case in the handled piglets. An inhibition of the reflex by adrenaline is therefore also a possible explanation.

In further studies, a larger number of animals is recommended, because of the significant piglet mortality of 38.5% in piglets of the LBW group in this study. With a larger number of animals more reliable analyses can be done.

The tests found in the literature were tests conducted in human neonates and puppies. These species are both altricial, while pigs are a precocial species. Precocial species have offspring that is fully mobile but depends on their parents for thermoregulation, food and protection from predators, while offspring of altricial species require brooding and feeding because of their incapability to move and to thermoregulate themselves (Mills & Daniel 2010). The differences in maturity that can be distinguished between altricial and precocial species can explain why piglets show other results to the same tests as human neonates and puppies. Other tests that are more suitable for precocial species are needed to test on piglets.

As mentioned in the introduction, a longitudinal MRI study was done in piglets that showed piglets and human neonates do have a comparable brain growth (Conrad et al. 2012). MRI also can be useful to detect brain differences in LBW piglets compared to NBW piglets. The restriction of MRI in a study like this is the fact that MRI only shows structural changes or differences and no functional changes or differences.

Although the developed test battery did not show any results in healthy piglets of different birth weights, the test battery may be useful in piglets with neurological deficits, for example piglets with meningitis. Because these piglets often show epilepsy behavior they can be useful as translational animal model for infants with epilepsy.

Conclusion

It can be concluded that the test battery that was developed during this study is not appropriate to measure the functions of piglets before weaning. Also it can be stated that LBW piglets are not by definition premature concerning the CNS. It is possible that the LBW piglets used in this study were only small for gestational age.

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