

Emotion and cognition in
low birth weight pigs

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Emotion and cognition in low birth weight pigs

Emotie en cognitie in varkens met een laag
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Unless someone like you
cares a whole awful lot,
nothing is going to get better.
It's not.

- *Dr. Seuss*

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General introduction

The welfare of farm animals is a relevant topic of study because increasing numbers of animals are kept in intensive production systems. While there are many different definitions of (farm) animal welfare, the so-called ‘five freedoms’, as originally formulated by the Brambell committee (1965), remain part of modern frameworks for animal welfare assessment (Mellor, 2017). One such recent modification of the five freedoms states that an animal has good welfare when it has the freedom to adequately react to:

- Hunger, thirst or incorrect food
- Thermal and physical discomfort
- Injuries or diseases
- Fear and chronic stress, and thus,
- The freedom to display normal behavioral patterns “that allow the animal to adapt to the demands of the prevailing environmental circumstances and enable it to reach a state that it perceives as positive” (Ohl and van der Staay, 2012).

This definition reflects how our understanding of animal welfare has evolved. Earlier definitions rely mostly on an animal’s physiological functioning (e.g., nutritional status, body temperature, health), reflecting an emphasis on physical wellbeing as a read-out of animal welfare. However, the importance of psychological constructs such as emotion and cognition are increasingly recognized as essential components of animal welfare assessment (Dawkins, 2008).

EMOTION AND ANIMAL WELFARE

In this thesis, an animal’s emotional or affective state is considered a combination of short-term emotions in response to specific stimuli and longer-term, general moods (Mendl *et al.*, 2010). An emotion is reflected in physiological, behavioral, and cognitive processes (Désiré *et al.*, 2002; Paul *et al.*, 2005). For example, an animal experiencing fear may show an increased heart rate, perform specific postures and vocalizations, and be more inclined to direct its attention to potentially threatening stimuli. In humans, emotions include a conscious subjective feeling (Paul *et al.*, 2005). In animals, this component of emotional state cannot be directly measured (Dawkins, 2008) as it requires self-report (i.e., “I feel fearful”). The presence of this subjective feeling in animals is assumed to be based on the presence of the measurable

objective components of emotion, such as behavior and physiology (Dantzer, 2002).

The concept of emotion has always been closely linked to animal welfare. After all, the presence of negative emotions such as fear was already considered a cause for poor welfare according to the five freedoms. In addition, most animal welfare definitions include (chronic) stress as a cause for poor welfare (e.g., Ohl and van der Staay, 2012). Stress can be defined as a condition where “an environmental demand exceeds the natural regulatory capacity of an organism” (Koolhaas *et al.*, 2011). While this does not necessarily imply an emotional response, chronic stress is likely to be associated with a more negative emotional state as stress is often caused by aversive situations (Paul *et al.*, 2005).

The focus on fear and chronic stress used by most welfare definitions suggests two important points concerning the relationship between emotion and animal welfare. First, it demonstrates a focus on negative emotional states and their potential to reduce welfare. This neglects the potential of positive emotional states to improve animal welfare (Boissy *et al.*, 2007; Yeates and Main, 2008). When assessing animal welfare, both the intensity (e.g., mild fear vs. intense fear) and the valence (i.e., negative vs. positive) of emotions should be taken into consideration.

Second, while negative emotions such as fear can reduce animal welfare, they are also part of an animal’s full emotional range. Both positive and negative emotions are required for an animal’s ability to show normal, species-specific behavior, which is also considered important for animal welfare (Korte *et al.*, 2007). Negative emotions can fulfill an important function for animal welfare. For example, negative social interaction may elicit short-term fear and stress, but are also necessary for the formation of a stable dominance hierarchy, allowing social species to live in groups without unnecessary aggression (Turner and Edwards, 2004). Similarly, acute stressors do not have to be avoided at all costs. As long as an animal can appropriately respond to such stressors, animal welfare does not have to be negatively affected (Ohl and van der Staay, 2012).

COGNITION AND ANIMAL WELFARE

An animal’s cognitive abilities consist of the mechanisms by which the animal processes information about its environment, including acquiring sensory

information, storing this information as memories, recalling this when necessary, and deciding how to act on it (Shettleworth, 2001, 2010). Unlike an animal's emotional state, these cognitive abilities do not provide a direct read-out for whether the animal is experiencing good welfare. However, the ability to perform normal, species-specific behavior is considered an important component of animal welfare. In addition, animals have to be able to successfully interact with their (captive) environment in order to avoid chronic stress (Ohl and van der Staay, 2012). Often, such behavior and interaction rely on an animal's cognitive abilities.

For example, social interaction is a crucial component of the normal, species-specific behavior for most commercially farmed animals (Keeling and Gonyou, 2001). The establishment of a stable social hierarchy, in which animals are aware of their relative dominance position compared to others, relies on learning and memory processes (Held *et al.*, 2002). Animals have to be able to recognize conspecifics as being familiar or unfamiliar, and remember what their position in the dominance hierarchy is, in order to appropriately respond to them (Wechsler and Lea, 2007).

Farm animals also rely on their cognitive abilities to successfully cope with other elements of their captive environment (Held *et al.*, 2002). Spatial memory is required for learning and remembering where specific resources within their housing system can be found (Mendl *et al.*, 2001; Wechsler and Lea, 2007), while operant learning allows farm animals to successfully interact with housing equipment such as automatic feeding stations (Puppe *et al.*, 2007). In addition, cognitive enrichment has been suggested (Meehan and Mench, 2007) and applied (Zebunke *et al.*, 2013) as a means of welfare improvement for farm animals. For this strategy to be successful, the animals need to have the required cognitive skills to interact with such enrichment items.

EMOTION AND COGNITION IN PIGS

As emotion and cognition are increasingly recognized to be of relevance for animal welfare, there is a need for improved understanding of these processes in farm animals. Pigs are a suitable species for such research, as they are commercially reared in large numbers, the majority of which are kept in intensive farming conditions (Park *et al.*, 2017). While pig welfare is known to be influenced by the conditions of their captive environment (Hemsworth,

2018), the potential effects of selective breeding on animal welfare are less well known (Rauw and Gomez-Raya, 2015). In pigs, selection for specific traits which are beneficial for production has been successful, resulting in increased growth rate and sow fecundity (Canario *et al.*, 2014). Therefore, pigs provide us with an opportunity to assess how selective breeding can affect farm animal welfare through emotion and cognition. Such assessments rely on accurate measures of emotional and cognitive functioning in pigs.

Assessing emotional state in pigs

As emotion is reflected by physiological, behavioral and cognitive processes, any of these components can be measured to assess emotional state in pigs (Moors, 2009). Negative emotional states such as fear and stress are often assessed using physiological responses. For example, cortisol is a commonly used measure of stress (Mormède *et al.*, 2007). Pigs show increases in cortisol production after aversive procedures such as tail docking (Numberger *et al.*, 2016), suggesting it could be related to a negative emotional state. However, relying solely on physiological measures of (negative) emotion can be problematic. First, such measures may be confounded by processes that are unrelated to emotional state. For example, cortisol is also increased after physical activity (Otvic, 2014). Second, physiological measures of emotion only provide a measure of emotional intensity, i.e. they can provide an indication of which animal is more stressed. They do not provide information about emotional valence, as an animal displaying an increase in cortisol could be experiencing a negative emotion such as anxiety or a positive emotion such as excitement (Yeates and Main, 2008). Therefore, using a combination of different measures of emotional state is preferred (Leliveld *et al.*, 2016). Such a combination can provide necessary context, i.e. was the animal more active or did it display behavioral signals of emotion alongside the physiological response.

There are many different behavioral tests of emotion in pigs which, when designed correctly, can specifically draw out measures of emotional state (Murphy *et al.*, 2014). So far, such tests have mostly focused on the assessment of negative emotional states. For example, frequently used tests include the open field test, which measures fear of a novel environment (Forkman *et al.*, 2007), and the human approach test, which elicits an approach-avoidance conflict when a pig is confronted with an unfamiliar human (Murphy *et al.*, 2014). Similar to physiological measures of emotion,

such tasks capture differences in emotional intensity. For example, based on the results of an open field test, one group of animals is found to be more or less fearful than another group. Ideally, behavioral tests of emotional state also capture differences in emotional valence (Murphy *et al.*, 2014).

The judgment bias task

Judgment bias has been suggested as a measure of emotional state which can be used to assess differences in emotional intensity and valence. It is a form of cognitive bias, where an individual's emotional state influences cognitive processes such as attention, memory and judgment (Mendl *et al.*, 2009). Judgment bias is based on the notion that a negative emotional state leads to a pessimistic judgment of ambiguous, i.e. emotionally neutral, information, whereas a positive emotional state leads to an optimistic judgment of ambiguous information.

Judgment bias has been assessed in a variety of species, including pigs (Douglas *et al.*, 2012). This can be done by training animals to distinguish between two distinct stimuli. The animal has to learn to associate one of these stimuli with a positive outcome (such as a food reward), and the other with a negative outcome (such as punishment or a lack of reward). Furthermore, the animal is trained to perform a specific behavioral response to each stimulus. Once these behavioral responses are consistently performed, judgment bias can be measured by presenting the animal with stimuli that are intermediate between the positive and negative stimuli (Mendl *et al.*, 2009). These are expected to be ambiguous to the animal, i.e., not associated with a positive or negative outcome. If an animal performs the behavior it has learned to perform in response to the positive stimulus, this is scored as an optimistic response and considered indicative of a positive emotional state. The behavioral response associated with the negative stimulus is scored as pessimistic, being indicative of a negative emotional state.

A study design that has successfully measured judgment bias in pigs relies on discrimination training using auditory cues (Murphy *et al.*, 2013b, 2015). Tones of different frequencies (high vs. low) are used as stimuli, while a large and small food reward are used as positive and negative outcomes, respectively. A pig is presented with either the positive or negative stimulus and has to then approach one of two spatially distinct goal-boxes (**Figure 1.1**), one of which is associated with the positive outcome and the other with the negative outcome. In other words, if the positive stimulus is played, the

pig has to approach the correct goal-box where it can consume the large reward. If the negative stimulus is played, the pig has to approach the other goal-box, where it can consume the small reward. Once pigs consistently approach the correct goal-boxes in response to the different tone cues, they are presented with tones of intermediate frequencies between the reference tones. If a pig approaches the goal-box associated with the large reward after hearing an intermediate, ambiguous tone, this is scored as an optimistic choice, while an approach to the goal-box associated with the small reward is scored as a pessimistic choice.

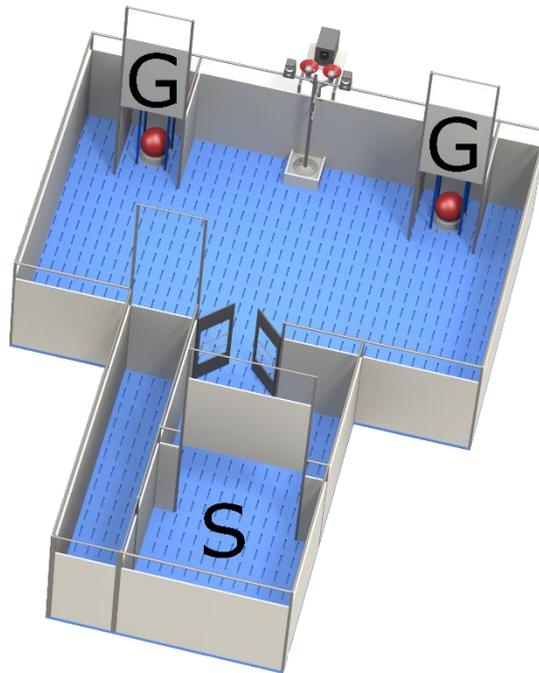


Figure 1.1 Overview of the judgment bias task apparatus, with start box (S) and goal-boxes (G). Each goal-box contains a food bowl, covered by a red ball to mask visual cues (illustration: Yorrit van der Staay).

Assessing cognition in pigs

As cognition consists of many different processes, there are a variety of tasks that can be used to measure the cognitive abilities of pigs. For example, there are multiple types of mazes to assess the different ways in which pigs learn and remember spatial routes and locations (Gieling *et al.*, 2011). As there are so many cognitive domains, it is preferable to use a task which captures multiple measures of learning and memory, for the simple reason that it provides more information. In line with that suggestion, since a pig's performance in a cognitive task can be confounded by processes other than cognition (e.g., motor abilities, motivation; van Eck *et al.*, 2016), it is important that a task provides an assessment of these processes as well (Wainwright and Colombo, 2006). This allows for assessment of the influence of confounding factors on task performance.

A cognitive task for pigs should also be catered to pigs' species-specific behavior and abilities (Koolhaas *et al.*, 2006). First, the cognitive task should be matched with pigs' sensory abilities. For example, tasks using auditory or olfactory cues are likely most suitable for pigs, as these appear to be their strongest sensory capacities (Lind *et al.*, 2007). This also means that such cues can intervene with a study design, e.g. when hiding food rewards in a maze, it must be made sure that the pig cannot locate them based on smell. Second, the cognitive domain that is assessed by a task should be ecologically relevant for pigs, so that its results can be used to predict their behavior in a real-world setting (Gioia and Isquith, 2004), be it in a wild or captive environment. An ecologically relevant task will also ensure that pigs are actually able to learn it, allowing for potential impairments to be measured. As pigs are social animals spending most of their time foraging in a large territory (Graves, 1984), tasks based on spatial or social learning are likely most relevant for pigs.

The spatial holeboard task

A behavioral task which appears suitable to measure cognition in pigs is the spatial holeboard task (Arts *et al.*, 2009; van der Staay *et al.*, 2012). In this task, pigs have to search for the locations of four hidden food rewards in an open arena containing a 4 x 4 matrix of potentially baited holes (food bowls; **Figure 1.2A**). To learn which holes are baited, pigs are free to visit and revisit the holes in any order within a certain time period. As training in the holeboard continues, pigs are expected to become increasingly efficient at

finding the rewards, that is, they are expected to decrease the number of visits to un-baited and/or previously visited holes. The holeboard provides an ecologically relevant task for pigs, as it relies on spatial learning and memory. Furthermore, the design of this task has taken pigs' natural behavior and sensory capacities into account. To obtain a reward, a pig has to lift a large ball covering the food bowl (**Figure 1.2B**), mimicking pigs' rooting behavior (Studnitz *et al.*, 2007). Also, each food bowl is equipped with a false bottom covering inaccessible rewards, ensuring the pigs cannot solve the task based on odor cues.

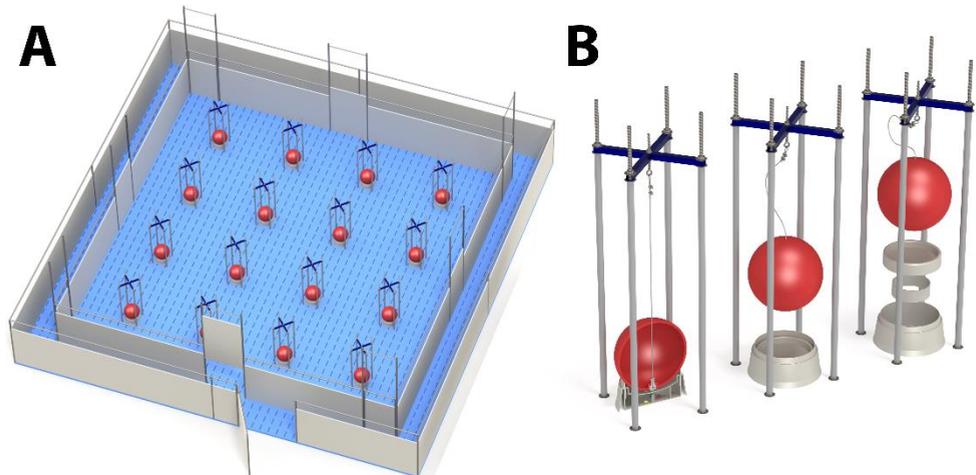


Figure 1.2 **A** Overview of the spatial holeboard task apparatus, with the main arena containing 16 food bowls (holes). **B** Details of the food bowls. Each bowl is covered by a red ball to hide visual cues. The bowls are equipped with a false bottom covering four candies to mask odor cues.

The spatial holeboard collects information on several different measures of cognition (van der Staay *et al.*, 2012). This task simultaneously assesses spatial reference and working memory. Reference memory is a long-term memory, which holds information that remains relevant as training progresses (Olton and Papas, 1979). A pig requires reference memory to store information such

as the number of rewarded holes and where these baited holes are located. In the holeboard, reference memory can be scored as the ratio between visits to rewarded and unrewarded holes, meaning reference memory performance increases as pigs learn to only visit baited holes (van der Staay *et al.*, 2012). Working memory is a short-term memory, which holds information that is only relevant within a single training trial (Dudchenko, 2004). Working memory stores information such as which holes have already been visited during a trial. In the holeboard, working memory is scored as the ratio between first visits and all visits (including revisits) to the baited holes, i.e., it reflects the avoidance of revisiting holes that have already been checked (van der Staay *et al.*, 2012).

In addition to spatial memory, the holeboard also allows for assessment of cognitive-behavioral flexibility when a reversal of the task is applied (Bolhuis *et al.*, 2004). This is done by switching a pig to a new configuration of baited holes, forcing it to switch strategies (i.e., instead of only visiting the previously baited holes, explore the holeboard to find the new configuration; **Figure 1.3**). Finally, the holeboard captures measures of motivation (e.g., the latency to visit a hole) and exploration (e.g., the number of holes visited in a trial; van der Staay *et al.*, 2012).

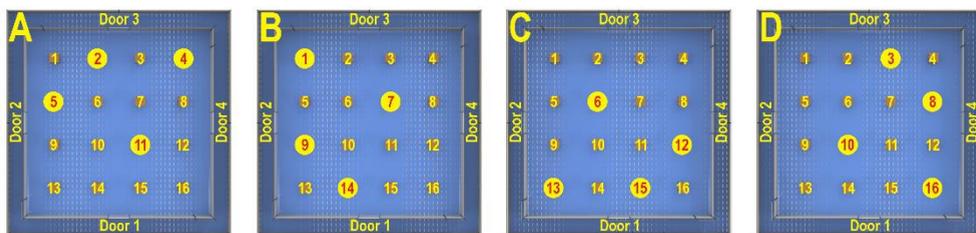


Figure 1.3 The different reward configurations (A-D) used during holeboard training. Baited holes are highlighted (illustrations: Yorrit van der Staay).

LOW BIRTH WEIGHT IN PIGS

On commercial pig farms, there has been selection for increased sow fecundity to increase production. This selection has been successful, with average litter sizes having increased. Where the ancestor of commercial pigs, the wild boar, produces litters of four to six piglets, litters on commercial farms have increased to an average of 16 piglets born per litter (Rutherford *et al.*, 2011; Rangstrup-Christensen *et al.*, 2018). Unfortunately, there appears to be a trade-off between litter size and the sow's ability to provide sufficient oxygen and nutrients for optimal development of all fetuses (Père and Etienne, 2000; Wähner and Fischer, 2005). In larger litters, not all fetuses receive sufficient blood supply and space, resulting in intra-uterine growth restriction (IUGR; Prunier *et al.*, 2010). As these larger litters are increasingly common on commercial farms, the occurrence of low birth weight (LBW) piglets (**Figure 1.4**) has also increased (Quiniou *et al.*, 2002; Rutherford *et al.*, 2013).

LBW piglets have been the subject of numerous studies assessing their growth and survival, mainly due to an interest in their commercial performance. This has resulted in considerable knowledge of certain aspects of LBW piglets' welfare. For example, LBW piglets are more likely to suffer from injuries and disease (Rutherford *et al.*, 2013; Calderón Díaz *et al.*, 2017), are less likely to have sufficient food intake (Scheel *et al.*, 1977; Le Dividich *et al.*, 2017) and have impaired thermoregulation (Tuchscherer *et al.*, 2000; Herpin *et al.*, 2002). Whether the welfare of LBW pigs is also influenced by impaired emotional and cognitive development has received far less scientific attention. This is of interest, as in spite of their increased mortality (Quiniou *et al.*, 2002; Fix *et al.*, 2010), a considerable proportion of LBW pigs survive to slaughter age (Calderón Díaz *et al.*, 2017).

Effects of LBW on emotion and cognition have been reported in other species, such as humans, sheep and rats (Hunter *et al.*, 2016). The findings of human studies are most relevant as a comparison to pigs, as the IUGR leading to LBW is comparable between humans and pigs. In both species, IUGR occurs naturally (Hunter *et al.*, 2016), due to an impaired supply of oxygen and/or nutrients to the fetus (Cox and Marton, 2009). Another similarity between humans and pigs is that they are perinatal brain developers (Pond *et al.*, 2000; Lind *et al.*, 2007). In such species, the majority of brain development, including HPA axis maturation, lasts from the late prenatal until the early postnatal period (Conrad *et al.*, 2012). This means that in both species, IUGR

can affect (early) brain development. Therefore, it is reasonable to assume that the adverse effects of LBW on emotion and cognition previously reported in humans could be found in pigs as well.

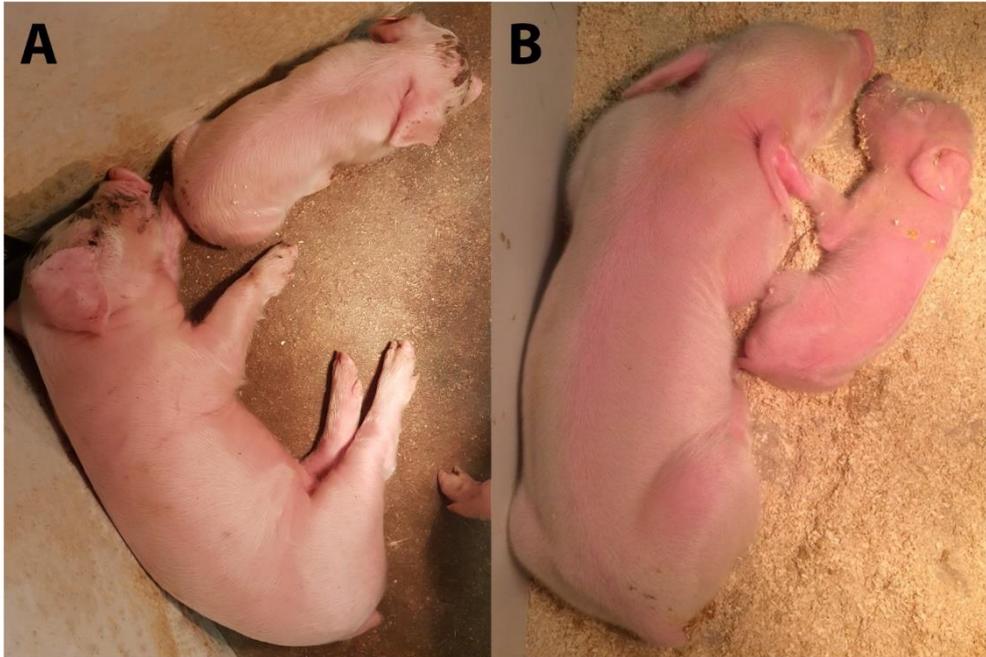


Figure 1.4 Examples of LBW and NBW piglets, photographed within one week after birth. **A** LBW piglet of ~500 grams at birth (right) next to NBW sibling of ~1500 grams at birth. **B** LBW piglet of ~700 grams at birth (right) next to NBW sibling of ~1800 grams at birth.

Low birth weight and emotion

In humans, LBW is considered a risk factor for developing emotional disorders such as anxiety and depression (Räikkönen *et al.*, 2008; Lahti *et al.*, 2010). Apparently, LBW in humans can result in a long-term vulnerability to more negative emotional states. This relationship between LBW and emotion could be sex-specific. In general, the incidence of developing emotional disorders such as depression is much higher in females (Kessler, 2003). The

effects of LBW on emotion in particular also appear to be increased for females, with a higher risk of depression found for LBW females, but not LBW males, in several studies (Costello *et al.*, 2007; Van Lieshout and Boylan, 2010).

The potential effects of LBW on the emotional development of pigs have almost exclusively been studied using physiological measures of stress (Rutherford *et al.*, 2013). Specifically, these studies have mostly relied on (changes in) plasma cortisol concentration. LBW piglets have increased circulating plasma cortisol compared to NBW piglets at different time points during the first week after birth (Wise *et al.*, 1991; Klemcke *et al.*, 1993). Furthermore, LBW piglets show an exaggerated increase in plasma cortisol concentration after an acute stressor at three months old (Poore and Fowden, 2003). However, these findings provide limited evidence of lower welfare due to long-term increased stress in LBW pigs. Only two studies so far have assessed behavioral measures of emotion in LBW pigs. In an open field test, LBW piglets vocalized more than their NBW siblings (Gieling *et al.*, 2014). Studying male piglets, Murphy *et al.* (2015) found that LBW pigs showed a more negative emotional state based on judgment bias than NBW pigs. Potential differences in emotional development between LBW females and males have not yet been assessed in pigs.

Low birth weight and cognition

In humans, LBW is associated with an increased risk of cognitive impairments. These effects of LBW on cognition are long-term, with reports of learning difficulties throughout adolescence (O’Keeffe *et al.*, 2003; Lindström *et al.*, 2017; Yu and Garcy, 2018) and lower academic performance as adults (Strauss, 2000; Larroque *et al.*, 2001). Whereas these findings are quite general, i.e., lower academic performance could be due to poor performance on any number of cognitive domains, there are also more specific reports of learning difficulties in LBW children. For example, they have long-lasting attention deficits (Suffren *et al.*, 2017) and impaired spatial learning abilities (Leitner *et al.*, 2005).

Multiple studies have already assessed the effects of LBW on cognition in pigs. However, they have produced contradictory results. There have been reports of impaired spatial learning in LBW pigs (Gieling *et al.*, 2012; Radlowski *et al.*, 2014), comparable cognition based on both spatial learning (Gieling *et al.*, 2014) and discrimination learning (Murphy *et al.*, 2013a, 2015)

and one study reporting improved spatial learning in LBW pigs (Antonides *et al.*, 2015a).

It is of interest that possible effects of stress on cognitive performance in LBW pigs are often not accounted for in such studies. Both chronic and acute stress are known to influence learning and memory (Mendl, 1999; Conrad, 2010; Sandi, 2013). Furthermore, such effects of stress can be sex-specific (Bowman *et al.*, 2003; Healy *et al.*, 2009). These findings are of importance for studies assessing the effects of LBW on cognition. As LBW pigs have been shown to have altered stress responses (Rutherford *et al.*, 2013), this could influence their cognitive performance. As this influence could be sex-specific, extending our knowledge of cognition in LBW pigs by accounting for potential sex-dependent effects of stress would be beneficial.

AIM AND OUTLINE OF THIS THESIS

LBW could be a welfare problem for commercially housed pigs as it may impair their cognitive and emotional development. The aim of this thesis is to evaluate if and how LBW influences emotion and cognition in pigs.

Part I: Pre-weaning effects of low birth weight

In **Part I**, the effects of LBW on pre-weaning development are examined. To do this, **Chapter 2** provides an assessment of HPA axis development in NBW and LBW piglets of both sexes. Neonatal hair cortisol concentration was measured as a marker for intra-uterine stress. In addition, markers of acute and chronic stress in sows were measured to assess potential effects of maternal stress on LBW and NBW offspring. In **Chapter 3**, the neurological and emotional development of LBW and NBW piglets is compared. Piglets were subjected to a standard examination of neurological functioning at the first day after birth. At the age of three weeks, they were assessed in a human approach test, where behavior indicative of fear was scored.

Part II: Post-weaning effects of low birth weight

In **Part II**, the effects of LBW on post-weaning development are examined. As sex may influence both emotion and cognition in pigs, **Chapter 4** assesses baseline emotion and cognition of female and male pigs. The spatial holeboard task is used to measure cognitive performance, while the judgment

bias task is used to measure emotional state. In **Chapter 5** and **Chapter 6**, these tasks are applied to LBW and NBW pigs at approximately four months of age. These studies also included markers of acute and chronic stress, as stress is closely linked to both cognition and emotion.

Part III: Judgment bias as a measure of emotional state

In **Part III**, the validity of judgment bias as a measure of emotional state in pigs is assessed. **Chapter 7** provides a literature review of the use of judgment bias tasks to measure emotional state in animals. We discuss potential confounding influences on results of judgment bias tasks and provide suggestions for further validation. In **Chapter 8**, Bayesian analyses are used to establish whether judgment bias in pigs is truly an independent measure of emotion. This was done by assessing potential correlations with measures of cognitive performance.

In **Chapter 9**, the results of these studies are combined to discuss (1) the effects of emotion and cognition on the welfare of LBW pigs, and (2) the suitability of our judgment bias task to assess emotional state in pigs.

Part I

Pre-weaning effects of low birth weight

2

Acute and chronic stress in commercially housed sows and its effects on low and normal birth weight offspring

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Under review

ABSTRACT

Commercial pig farms are the source of a variety of stressors for both breeding sows and their offspring. Breeding sows are regularly exposed to on-farm stressors throughout the duration of their production period. Their piglets' HPA axis development can be affected by maternal stress, or by intra-uterine growth restriction as often occurs in larger litters. In the current study, we aimed (1) to assess acute and chronic stress in breeding sows. Specifically, stress responses of primi- and multiparous sows were compared to determine whether sows adapt to on-farm stressors, and (2) to assess whether maternal stress and intra-uterine growth restriction impact HPA axis development of piglets. To do this, salivary cortisol concentrations of 16 primiparous and 16 multiparous sows were assessed as a measure of acute stress. This was done at three time points: 1) while sows were group housed, 2) after sows were separated from the group and 3) after handling procedures. In addition, sows' hair cortisol concentrations were determined as a measure of chronic stress. Hair cortisol concentrations of sows' low birth weight ($n = 64$) and normal birth weight ($n = 54$) offspring were determined on day 3 after birth, to assess *in utero* cortisol production as a measure of HPA axis development. It was expected that if sows adapt to on-farm stressors, the more experienced multiparous sows would show decreased stress responses in comparison to primiparous sows. However, we found a comparable acute stress response of primi- and multiparous sows to separation from the herd. Handling procedures did not influence sows' salivary cortisol concentrations. Both groups of sows showed comparable levels of chronic stress, as measured by hair cortisol concentration. Sows' chronic stress did not influence their offspring's HPA axis development. Birth weight did have an effect on piglets' hair cortisol concentration. This effect was sex-dependent, with female LBW piglets having higher hair cortisol concentrations than male LBW piglets. Overall, our results indicate that breeding sows do not adapt to all on-farm stressors. In addition, low birth weight piglets can be vulnerable to disturbed HPA axis development.

INTRODUCTION

Sows housed on commercial breeding farms are regularly exposed to external stressors during the multiple years of their production period. Common examples are individual housing in crates around farrowing and lactation, which limits the expression of natural behavior (Lawrence *et al.*, 1994; Jarvis *et al.*, 1997, 2002; Damm *et al.*, 2003), and unstable social hierarchies due to regular mixing of unfamiliar sows in group housing (Spoolder *et al.*, 2009; Greenwood *et al.*, 2014; Verdon *et al.*, 2015; Martínez-Miró *et al.*, 2016). As (the absence of) stress and the ability to adequately respond to stressors are components of most definitions of animal welfare (Fraser, 1995; Botreau *et al.*, 2007; Ohl and van der Staay, 2012), increasing our understanding of how sows cope with stressors is important.

The impact of on-farm stressors may differ for primiparous sows (those that are on their first reproduction cycle) and older, multiparous sows, as primiparous sows are unfamiliar with many of the common husbandry procedures surrounding pig production. For example, young sows' salivary cortisol concentration is higher after a first confrontation with unfamiliar conspecifics, compared to subsequent mixing events (van Putten and Buré, 1997; van der Staay *et al.*, 2008; Couret *et al.*, 2009; Ison *et al.*, 2014). As these sows gain experience with mixing events, they receive fewer skin lesions and perform less agonistic behavior, indicating they learned to avoid unnecessary aggression while establishing a dominance hierarchy. Sows also show signs of adaptation to the behavioral restriction imposed by farrowing crates. Higher parity sows perform fewer posture changes and show decreased alertness, as well as having decreased plasma cortisol concentrations compared to primiparous sows (Jarvis *et al.*, 1997, 2001). Together, these studies show that sows appear able to learn how to cope with certain stressors as they gain experience with them. However, only a limited number of on-farm stressors have been assessed in relation to sows' acute stress response.

Additionally, it is unclear how sows' increased experience with stressors relates to chronic stress. Some indications have been found that in spite of sows' potential adaptation to stressors, prolonged exposure to them still results in chronic stress. For example, older sows perform at least as much stereotypic behaviors (Von Borell and Hurnik, 1991; Zhang *et al.*, 2017) and have higher adrenal gland weight (van der Staay *et al.*, 2010) than younger sows, both indicators of chronic stress (Wiepkema and Schouten, 1992; Ulrich-Lai *et al.*, 2006). However, studies of long-term cortisol levels are

lacking. Where plasma and salivary cortisol concentrations provide a measure of acute stress by reflecting instantaneous cortisol concentrations, hair cortisol concentration can provide a measure of chronic stress as it reflects cortisol accumulation over a period of weeks (Davenport *et al.*, 2006). A study assessing hair cortisol in sows over two successive reproductive cycles found no difference in chronic stress from one cycle to the next (Bacci *et al.*, 2014). However, no comparison of primi- and multiparous sows was made, with most sows in that study already being older during the first assessed reproductive cycle (average parity >4). Combining assessments of acute and chronic stress responses in primi- and multiparous sows would be valuable, as this would provide additional knowledge of whether sows learn to cope with on-farm stressors.

Stress in sows is not only of interest for their own welfare, but also that of their offspring. Prenatal stress has been associated with altered development and functioning of the HPA axis in a variety of species, including pigs (Moisiadis and Matthews, 2014; Otten *et al.*, 2015; McGowan and Matthews, 2018). Piglets from chronically stressed sows show an exaggerated HPA axis response to various common stressors compared to piglets from undisturbed sows, such as mixing with unfamiliar animals (Hausmann *et al.*, 2000; Jarvis *et al.*, 2006b), restraint (Ison *et al.*, 2010) and tail docking (Rutherford *et al.*, 2009). These findings are supported by alterations in behavior observed in prenatally stressed piglets, such as increased escape attempts during an open field test (Otten *et al.*, 2007). Overall, these studies show that prenatal stress could compromise piglets' ability to cope with stressors.

In addition to prenatal maternal stress, piglets' HPA axis development can also be influenced by the increasingly common occurrence of low birth weight (LBW), which is a result of selection for increased litter size (Rutherford *et al.*, 2013). In larger litters, not all fetuses receive sufficient nutrients for optimal development (Wähler and Fischer, 2005). The resulting intra-uterine growth restriction may be associated with increased fetal stress, which has been shown to influence HPA axis functioning. For example, LBW piglets have higher plasma cortisol concentrations than normal birth weight (NBW) piglets throughout the first week of life (Klemcke *et al.*, 1993), as well as an increase in adrenal size and HPA axis activity post-weaning (Poore and Fowden, 2003). To better understand prenatal stress in piglets, either due to maternal stress or suboptimal fetal development, hair cortisol concentration could be a useful additional measure. Neonatal hair cortisol concentration would reflect *in utero* cortisol accumulation, allowing for

assessment of whether prenatal stress impacts HPA axis development (Kapoor *et al.*, 2016). As both maternal stress and litter size may vary with sow parity (Bono *et al.*, 2012), piglets of primi- and multiparous sows may be differently affected.

The aim of this study was to assess on-farm stress responses in sows and their offspring. Specifically, our aims were: (1) to assess acute stress in primi- and multiparous sows. Acute stress responses were measured as increase in salivary cortisol concentration after exposure to multiple on-farm stressors, specifically separation from the herd and handling procedures prior to farrowing, (2) to assess chronic stress in primi- and multiparous sows, using hair cortisol concentrations as a measure of chronic stress, and (3) to assess whether prenatal stress affects HPA axis development differentially in low and normal birth weight piglets by measuring neonatal hair cortisol concentration. We expected primiparous sows to show an exaggerated acute stress response compared to multiparous sows, due to their lack of experience with on-farm stressors. Furthermore, we expected any adaptation to stressors of multiparous sows to be accompanied by a difference in long-term cortisol accumulation, i.e. if adaptation occurs this should result in decreased long-term cortisol accumulation. Concerning prenatal stress in piglets, we expected that maternal stress would be positively correlated with piglet hair cortisol concentration and that LBW piglets would have elevated hair cortisol concentrations compared to NBW piglets.

METHODS

Ethical note

All methods that demanded the handling of live animals were reviewed and approved by the local animal welfare body (Animal Welfare Body Utrecht) and were conducted in accordance with the recommendations of the EU directive 2010/63/EU.

Animals

Sows (Yorkshire x Dutch landrace) were selected from the commercial pig breeding farm of Utrecht University. In total, hair and saliva samples were collected from 16 primiparous sows (first parity) and 16 multiparous sows (third parity and higher, average parity = 4-5). Samples were collected over a

period of 5 weeks (July-August), due to limited availability of primiparous sows.

After the sows farrowed, up to four piglets were selected from each litter based on birth weight. From each litter, all piglets were weighed within 24 hours after birth. One female and one male piglet with a birth weight closest to the litter average were selected as normal birth weight (NBW) piglets. The piglets with the lowest birth weight were selected as low birth weight (LBW) piglets if their birth weight was a minimum of 1 SD below the litter average. Piglet selection resulted in 64 NBW piglets (32 females, 32 males) and 54 LBW piglets (31 females, 23 males).

To assess whether LBW piglets are more common in larger litters, 60 additional litters (from sows that were not selected for the study) were weighed within 24 hours after birth. To allow comparisons with previous studies (which sometimes define LBW as $\leq 1\text{kg}$), a selection criterion based on absolute birth weight was used. Occurrence of LBW piglets was measured as proportion of piglets within a litter weighing less than 1kg at birth.

Housing and handling

During gestation, sows were group-housed in a barn measuring approximately 263 m². It contained two straw-bedded lying areas (87m² each, separated by a 1.5m high wall), a dunging area (71 m²) and a feeding area (18 m²) containing three walk-through electronic feeding stations (Intellitek ESF, Fancom B.V., Panningen, the Netherlands) set on a 24h feeding cycle. Water was available *ad libitum* from five drinkers. Sows had access to a 53m² outside area with concrete floor and *ad libitum* provision of grass silage. The herd consisted of a dynamic group with an average of 160 sows present, ranging in parity from gilts to 9th parity sows. Weekly transfers of animals consisted of six to 10 sows being removed from the herd approximately one week before their expected farrowing date and a similar number being (re-) introduced to the herd approximately four days after insemination (see **Figure 2.1** for a timeline of a sow's reproductive cycle).

Sows were separated from the main herd on the day before they were moved to the farrowing unit. Separation occurred after feeding, with the electronic feeding station giving selected sows access to a pen adjacent to the main herd's facilities. This separation pen measured 35 m² and contained a concrete floor without bedding. Water was available from one drinker.

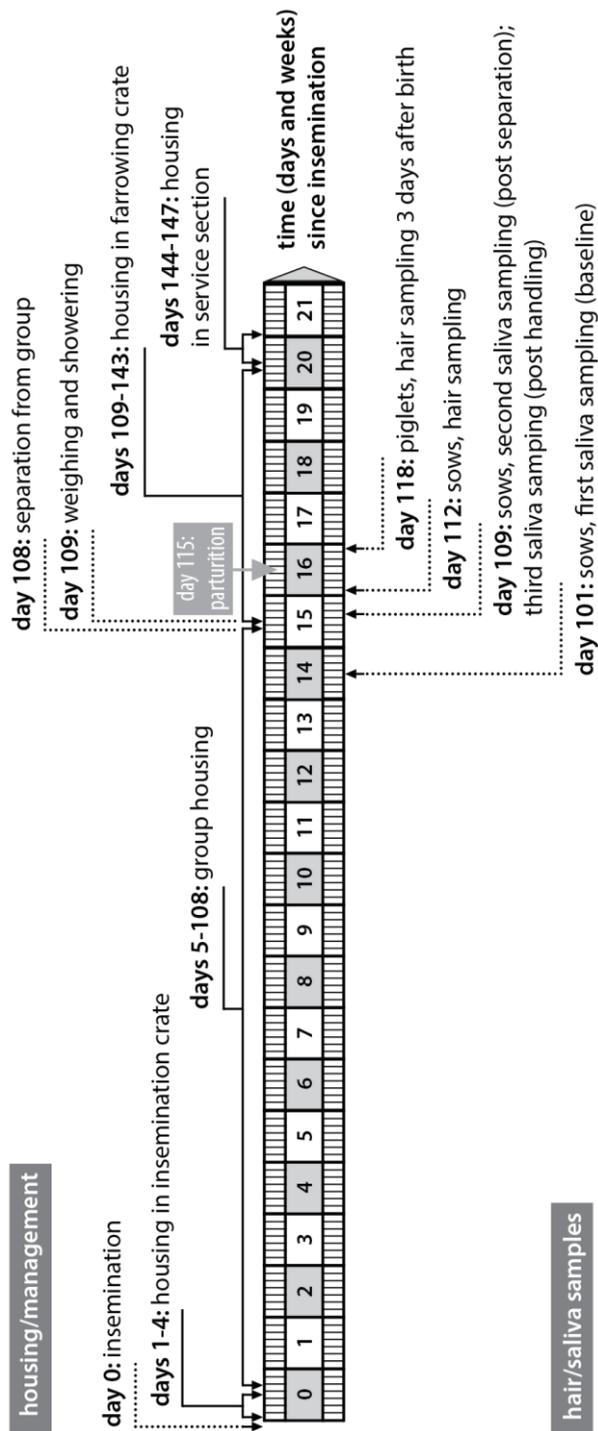


Figure 2.1 Schematic overview of a reproductive cycle of commercially housed sows, as applied during the experiment. Time points of handling procedures (housing/management) and sample collection (hair/saliva samples) are included.

Separation from the herd usually occurred approximately 12-16 hours prior to moving to the farrowing unit. On the day of relocation, the sows were showered in groups of three to seven animals (shower room measured 7.3 m²) and individually weighed. Sows were then escorted to the farrowing unit.

The farrowing unit consisted of a mechanically ventilated, thermostatically controlled room containing 10 farrowing pens. Temperature inside the farrowing unit was maintained at 24°C until one week post-farrowing, after which it was maintained at 20°C. Each pen (2.4 x 1.8 m) was fitted with a centrally positioned farrowing crate (1.8 x 0.6 m). Pens had partially slatted floors with floor heating for the piglets. Sows were fed twice-daily, with water available *ad libitum*. Each sow was provided with a length of rope as chewing substrate.

Saliva samples

Saliva samples were collected from each sow at three time points: (1) a baseline sample collected while sows were group-housed with the main herd, two weeks prior to expected parturition, (2) a post-separation sample, collected while sows were housed in the separation pen prior to relocation to the farrowing unit, and (3) a post-handling sample, collected after sows were showered and weighed on their way to the farrowing unit (**Figure 2.1**).

Baseline and post-separation samples were collected between 8:30 and 9:30 in the morning. Post-handling samples were collected approximately 20 minutes after sows were showered and weighed, to allow for the peak in salivary cortisol response to develop (e.g. Merlot *et al.*, 2011). Saliva was collected by letting each sow chew on two cotton swabs (Cotton Swabs 150 mm × 4 mm WA 2PL; Heinz Herenz, Hamburg, Germany) until they were sufficiently moistened. Swabs were centrifuged using saliva collection tubes (Salivette, Sarstedt, Germany) at around 3524 g for 10 minutes at 10°C. Saliva samples were stored at -20°C until salivary cortisol concentration was determined *in duplo* using a Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics BV, The Hague, the Netherlands).

Hair samples

Sow hair samples were collected three days before expected parturition (**Figure 2.1**). Hair was taken from the left dorsal flank region of each sow

with a disposable razor (using a new razor for each sample). Piglet hair samples were collected three days after birth (**Figure 2.1**). Hair was taken from both flanks to ensure sufficient material for cortisol analysis. Hair cortisol concentration was determined based on the protocol by Davenport *et al.* (2006). In short, samples were washed and dried. Approximately 35mg of hair was then ground with a bead beater (TissueLyser II, QIAGEN Benelux B.V., Antwerp, Belgium) for a minimum of 2x 15 minutes at 30 Hz, in 2 mL tubes (Eppendorf Safe-Lock, Eppendorf Nederland B.V., Nijmegen, the Netherlands) containing three 2.3mm steel beads (BioSpec, Lab Services B.V., Breda, the Netherlands). One mL of methanol was added to ground samples and they were incubated for 24h with slow rotation to extract corticosteroids. 0.6 mL of the extract was dried using a vacuum centrifuge. The dried extracts were then dissolved in 0.3 mL phosphate buffer. A Salimetrics Salivary Cortisol ELISA kit was used to determine hair cortisol concentrations *in duplo*.

Statistical analysis

All statistical analyses were performed using R statistical software, version 3.4.2 (R Core Team, 2017). For linear mixed models, package nlme (Pinheiro *et al.*, 2018) was used. For each mixed model the random effect structure was assessed using Restricted Maximum Likelihood (REML) estimation. Final selection of random effect structure was based on Akaike's information criterion (AIC). Using the selected random effect structure, different fixed effect structures were assessed using Maximum Likelihood (ML) estimation. Selection of the final model was based on AIC. When a fixed effect did not improve the fit of a model (i.e. AIC was not lower after inclusion of the variable), it was taken as indication that this fixed effect was not of importance in explaining the data. Such fixed effects were excluded from the model. Statistical significance was set at $p < 0.05$. Effect size was calculated as Pearson's r . Unless indicated otherwise, results are presented as mean \pm SD .

Sows

The effect of parity on sows' hair cortisol concentrations was analyzed using Welch's t -test. The effects of parity on sows' salivary cortisol concentrations were analyzed using a linear mixed model with Parity and Sample as fixed effects and random intercepts for Group to account for a random effect of

group composition during separation from the main herd. Salivary cortisol concentrations were \log_{10} transformed to improve the distribution of residuals. Saliva samples from two sows (1 primiparous and 1 multiparous) were insufficient for cortisol analysis. Therefore, salivary cortisol analysis was performed on the remaining 30 samples. To analyze a correlation between chronic and acute stress, hair cortisol concentration was used as a measure of chronic stress and increase in salivary cortisol concentration from baseline to post-separation was used as a measure of acute stress. Spearman's rho was used for correlation analysis as increases in salivary cortisol concentrations were not normally distributed.

Average litter size of primi- and multiparous sows was compared using Welch's *t*-test. To assess whether litter size and sow hair cortisol were correlated, Pearson's product-moment correlation coefficients were calculated for all sows combined, and primi- and multiparous sows separately.

Piglets

Average birth weight of LBW and NBW pigs was compared using Welch's *t*-test. The effects of maternal stress and birth weight on piglets' hair cortisol concentrations were analyzed using a linear mixed model. Maternal stress (represented by sow hair cortisol concentration) and litter size did not improve the fit of the model and were therefore excluded from further analysis. The resulting mixed model had Birth weight, Sex and Birth weight x Sex interaction as fixed effects and random intercepts for Litter. A reciprocal transformation was used on hair cortisol concentrations to improve distribution of residuals.

To assess whether LBW piglets occur more frequently in larger litters, the proportion of LBW piglets within litters was compared. Litter size (LS) was categorized in four categories: 13 total born or less ($LS_{\leq 13}$), 14-16 total born (LS_{14-16}), 17-18 total born (LS_{17-18}), 19 total born or more ($LS_{\geq 19}$). As the proportion of LBW piglets was not normally distributed, a Kruskal-Wallis test (from package *pgirmess* (Giraudoux *et al.*, 2018)) was used to compare categories. To further assess if there was an increase in the proportion of LBW piglets with increasing litter size, a Jonckheere trend test (from package *clinfun* (Seshan, 2018)) was used.

RESULTS

Sows

Hair cortisol

No effect of parity was found on hair cortisol concentrations of sows (primiparous: 36.23 pg/mg \pm 8.97, multiparous: 35.90 pg/mg \pm 4.86; $t_{23,10} = 0.13$, $p = 0.898$).

Salivary cortisol

Separation from the main herd caused an increase in salivary cortisol concentration in all sows compared to baseline (Sample effect: $F_{2,82} = 58.81$, $p < 0.001$, $r = 0.58$; **Figure 2.2**). Salivary cortisol concentration in post-handling samples were comparable to baseline. Salivary cortisol concentrations were higher for primiparous sows than multiparous sows at all time points (Parity effect: $F_{1,82} = 4.22$, $p = 0.043$, $r = 0.22$; **Figure 2.2**).

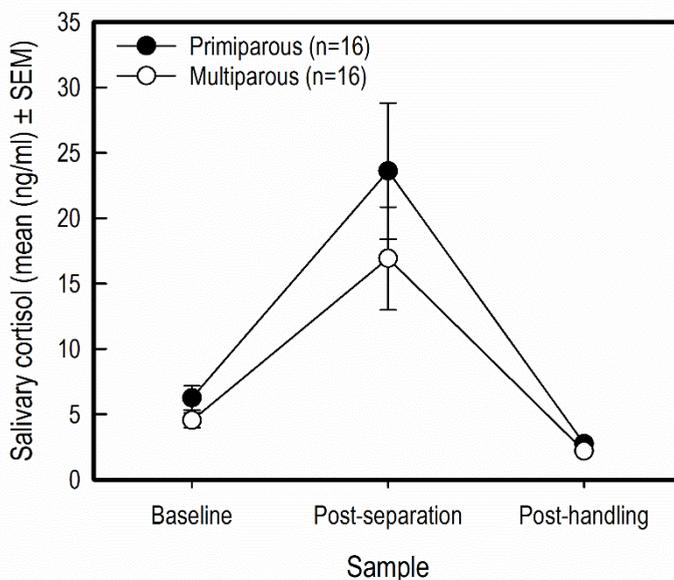


Figure 2.2. Average salivary cortisol concentrations of primi- and multiparous sows. Baseline samples were collected in group-housing, post-separation samples were collected after separating sows from the main herd prior to moving to the farrowing unit and post-handling samples were collected after sows were showered and weighed.

Correlation hair and salivary cortisol

No significant correlation was found between hair cortisol concentration and increase in salivary cortisol concentration after the sows were removed from the main group ($r_s = -0.07, p = 0.712$).

Litter size

Primiparous sows produced smaller litters than multiparous sows (primiparous: 13.69 ± 3.36 piglets, multiparous: 17.38 ± 2.45 piglets; $t_{27.41} = -3.55, p = 0.001, r = 0.56$). For all sows combined, no correlation between litter size and hair cortisol concentration was found ($r = 0.26, n = 32, p = 0.146$). However, when primi- and multiparous sows were analyzed separately, a positive correlation was found between litter size and hair cortisol concentration of multiparous sows (primiparous: $r = 0.23, n = 16, p = 0.387$; multiparous: $r = 0.58, n = 16, p = 0.018$).

Piglets

Birth weight

LBW piglets had on average a lower birth weight than NBW piglets (LBW: 0.97 ± 0.22 kg, NBW: 1.38 ± 0.19 kg; $t_{27.31} = -10.81, p < 0.001, r = 0.73$).

Hair cortisol

Female piglets had higher hair cortisol concentrations than male piglets, irrespective of birth weight (Sex effect: $F_{1,82} = 8.41, p = 0.005, r = 0.37$; **Figure 2.3**). Furthermore, birth weight had a sex-dependent effect on hair cortisol concentrations (Birth weight x Sex interaction: $F_{1,82} = 5.10, p = 0.027, r = 0.24$; **Figure 2.3**), with LBW females having higher hair cortisol concentrations than LBW males. No main effect of birth weight on hair cortisol concentrations was found.

Birth weight and litter size

Mean proportion of LBW piglets differed across different litter size categories ($H_3 = 12.83, p = 0.005$), with an increasing proportion of LBW piglets with increasing litter size ($J = 2067.5, p < 0.001$). Average proportion

of LBW piglets was $8.34\% \pm 12.24$ in $LS_{\leq 13}$ litters, $13.36\% \pm 10.20$ in LS_{14-16} litters, $15.52\% \pm 11.53$ in LS_{17-18} litters and $21.42\% \pm 11.95$ in $LS_{\geq 19}$ litters.

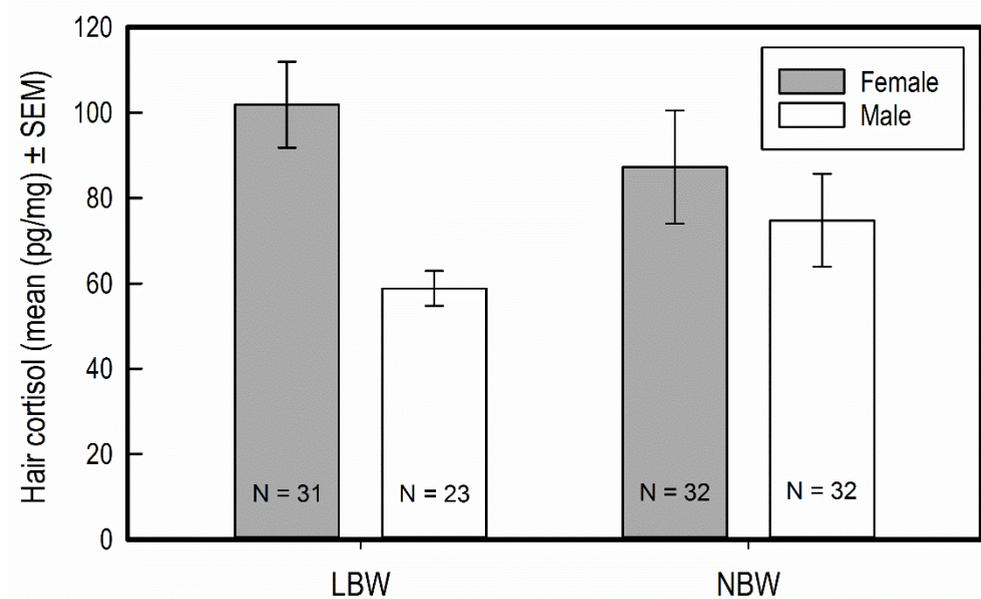


Figure 2.3 Average hair cortisol concentrations of LBW and NBW piglets at three days after birth.

DISCUSSION

In this study, we assessed acute and chronic stress in commercially housed sows, as well as HPA axis development of their low- and normal birth weight offspring. Both primi- and multiparous sows showed an acute stress response to separation from the group prior to being moved to farrowing crates, suggesting sows do not adapt to this stressor. Primi- and multiparous sows also showed comparable levels of chronic stress, as measured by hair cortisol concentration. To assess HPA axis development of piglets, hair cortisol concentration on day 3 after birth was assessed. Birth weight influenced this measure in a sex-dependent way, with female low birth weight (LBW) piglets showing increased hair cortisol concentration compared to LBW males.

Acute stress in sows

Primi- and multiparous sows showed a comparable acute stress response to a change in their (social) environment. Compared to baseline, salivary cortisol concentrations were higher after sows had spent time in a small subgroup of six to 10 animals, while separated from the main herd prior to moving to the farrowing crates. The stress experienced during this separation appears to be mostly of a social nature: the sows are forced to interact with a randomly selected group of conspecifics in a smaller pen with little (structural) enrichment material, without the possibility to avoid each other.

When housed in a large dynamic group, sows appear to maintain smaller subgroups of familiar animals with a stable dominance hierarchy (Durrell *et al.*, 2003). Establishing a dominance hierarchy with unfamiliar sows involves aggression (Spoolder *et al.*, 2009) and takes multiple days (Arey, 1999; Spoolder *et al.*, 2009). This makes it unlikely that aggressive interactions will have subsided while sows are in the separation pen. The larger dimensions of barns used for large groups allow sows to perform avoidance behavior, which is an important aspect of non-aggressive maintenance of social relationships (Turner and Edwards, 2004; Greenwood *et al.*, 2014). Overcrowding by decreasing available space per sow can lead to social stress, as shown by increased aggression and plasma cortisol concentration (Hemsworth *et al.*, 1986; Weng *et al.*, 1998). Allowing sows to perform avoidance behavior by providing barriers behind which they can hide may result in decreased aggression (Arey and Edwards, 1998). Both unfamiliarity of pen mates and an inability to avoid aggression are present when sows are separated from the herd prior to farrowing, making it likely that social stressors played a role in the observed acute stress response.

Aside from changes in social setting, there are other changes in environment that could have contributed to the sows' stress response after being moved to the separation pen. Mainly, the new pen is barren, whereas lying areas with straw are available in the main herd's housing. Straw can improve pigs' comfort when lying down (Tuytens, 2005) and, more importantly, can be a suitable substrate for certain behavioral needs such as rooting behaviors. As such, the provision of straw has been shown to reduce aggression as more time is spent in explorative behaviors (Tuytens, 2005; Day *et al.*, 2008). However, straw may also function as a limited resource to be defended (Morgan *et al.*, 1998). Future research comparing separation pens containing different environmental elements (e.g. structural enrichment to allow

avoidance of conspecifics such as barriers (Bulens *et al.*, 2017), straw) in combination with behavioral observations (e.g. of aggressive interactions) will allow assessment of which (combination of) elements are responsible for sows' on-farm stress response.

Handling procedures prior to sows' move to the farrowing crates (i.e. showering and weighing) did not cause an acute stress response. Post-handling salivary cortisol concentrations of both primi- and multiparous sows were comparable to baseline level as measured in group housing. This suggests that handling by the animal caretakers was not experienced as stressful in our study. Previous studies have shown that human-animal interactions can be a source of stress for sows (Waiblinger *et al.*, 2006; Powell *et al.*, 2016). However, this is likely dependent on the caretaker's disposition. For example, positive changes in caretaker's attitude towards pigs have resulted in a decrease in pigs' fearful behavior (Hemsworth *et al.*, 1994). Our study confirms that human-animal interactions are not necessarily a source of stress for sows. It is important to note that the handling procedures assessed in our study did not consist solely of contact with caretakers. Sows were showered, weighed and escorted to the farrowing unit. Showering of pigs is frequently applied in lairage, prior to slaughter, and is considered a means of distracting the pigs and decreasing abnormal behavior (Weeding *et al.*, 1993; Warriss, 2003). Therefore, it is possible that showering the sows lowers their stress, possibly compensating for any negative effects from handling by the caretakers. This could be addressed by future studies assessing the impact of separate components of handling procedures.

No effect of parity on acute stress response

To further our knowledge of sows' potential for adaptation to stress, the acute stress response of primi- and multiparous sows was compared. Primiparous sows had on average higher salivary cortisol concentrations, but showed a comparable increase in cortisol to multiparous sows after a stressor. It is likely that the higher cortisol concentrations for younger sows are an age effect, because baseline cortisol levels decrease as pigs mature (Ruis, 1997). The comparable acute stress response for primi- and multiparous sows suggests that sows do not adapt to the stress of staying in the separation pen prior to farrowing.

Previous studies have shown that sows do adapt to social stressors. However, these studies have frequently based their results on experimental designs with

social environments that are quite different from sows' actual circumstances on a commercial farm (e.g. smaller group sizes of 2-8 animals, different physical environment (van Putten and Buré, 1997; van der Staay *et al.*, 2008; Couret *et al.*, 2009; Ison *et al.*, 2014)). Our study assessed acute stress in sows in on-farm conditions, whereas previous studies applied experimental conditions (minimizing confounding effects of elements besides the stressor of interest). This has resulted in a combination of potential stressors being responsible for sows' stress response in our study (e.g. contact with unfamiliar animals, decrease in space allowance, lack of enrichment). Additionally, on-farm exposure to separation from the group occurs only once per reproductive cycle. Perhaps the long time-interval between successive exposures is too long to facilitate adaptation. Previous studies showing adaptation to social stressors used intervals of several days (van der Staay *et al.*, 2008; Couret *et al.*, 2009; Ison *et al.*, 2014) or weeks (van Putten and Buré, 1997) between repeated exposures. A negative relationship between inter-stressor interval and adaptation to the stressor has previously been shown in rats (De Boer *et al.*, 1990). Our study shows that on-farm stressors (i.e. presented as a combination of stressors with a long time-interval between repeated exposures) can remain stressful, even as sows gain experience with them.

Chronic stress in sows

Primi- and multiparous sows had comparable hair cortisol concentrations. As hair cortisol reflects accumulation of cortisol over a long-term period (Davenport *et al.*, 2006), this finding indicates that sows across parities experience comparable levels of chronic stress. This finding is supported by a previous study finding no change in hair cortisol concentration in sows over two successive reproductive cycles (Bacci *et al.*, 2014).

Based on comparable hair cortisol concentrations alone, we cannot state whether sows on commercial farms are chronically stressed, i.e. it is also possible that primi- and multiparous sows show a comparable lack of chronic stress. However, based on previous studies this seems unlikely. Sows on commercial farms show signs of chronic stress such as stereotypic behavior (Zhang *et al.*, 2017), increased adrenal weight (van der Staay *et al.*, 2010) and altered HPA axis functioning (Jarvis *et al.*, 2006a). Furthermore, our finding of a comparable acute stress response for primi- and multiparous sows suggests that sows continue to be affected by on-farm stressors. Apparently,

increased familiarity with stressors does not necessarily lead to adaptation and a resulting decrease in markers of chronic stress. Future research comparing hair cortisol concentrations of sows experiencing fewer or less severe stressors to those in common commercial conditions could provide further evidence that commercial conditions are a source of chronic stress.

We found no correlation between salivary cortisol increase after a stressor (i.e. acute stress response) and hair cortisol concentration (i.e. chronic stress). This suggests that sows showing a stronger acute stress response do not necessarily have a higher cortisol accumulation over a longer time-period. Such a lack of correlation between salivary and hair cortisol levels has previously been reported for male growing pigs (Casal *et al.*, 2017). These findings are likely due to the large difference in time period reflected by the different samples. In humans, hair and salivary cortisol levels only correlate when saliva samples repeatedly collected over a longer time period were included in analysis (D'Anna-Hernandez *et al.*, 2011). In our study, salivary cortisol increase in response to a single stressor was used, whereas sows on farms face a multitude of stressors regularly (Bench *et al.*, 2013). All of these stressors influence hair cortisol concentration, explaining why measures of acute and chronic stress may not always be related.

Prenatal stress

In our study, sows' hair cortisol concentration did not have an influence on piglet hair cortisol concentration at three days after birth. This suggests that maternal stress did not influence piglet HPA axis development. Previous studies have shown that maternal stress influences the development of the HPA axis in piglets (Hausmann *et al.*, 2000; Jarvis *et al.*, 2006b; Rutherford *et al.*, 2009; Ison *et al.*, 2010). However, in all these studies, there was a difference between stressed and control sows leading to an effect in their offspring. In our study, no such difference between sows' stress levels was found. This resulted in an inability to properly assess the effects of different levels of maternal stress.

In addition to maternal stress, we evaluated the effects of birth weight on HPA axis development by comparing hair cortisol concentration of LBW and normal birth weight (NBW) piglets. LBW piglets had a significantly lower birth weight than NBW piglets. As birth weight is the main read-out parameter of intra-uterine growth restriction in pigs (Wu *et al.*, 2006), this

result suggests that the LBW piglets assessed in our study were most likely affected by suboptimal prenatal conditions.

Instead of the expected general influence of birth weight on HPA axis development, its effects were sex-dependent. Female LBW piglets had an increased hair cortisol concentration compared to LBW males. This finding is corroborated by earlier studies of sex differences in HPA axis activity in LBW piglets. For example, female LBW piglets have increased adrenal weight and plasma cortisol concentration at three days after birth (Klemcke *et al.*, 1993). There has also been a report of a general effect of LBW on postnatal acute stress responses, with LBW piglets showing exaggerated HPA axis function (Poore and Fowden, 2003). However, the authors also reported that it is possible that different mechanisms are responsible for the development of enhanced HPA axis activity of female and male LBW pigs. For example, a relationship between plasma cortisol concentration during an acute stress response and adrenal size at the age of three months (prior to puberty) was found for male, but not female, LBW pigs. The effects of prenatal stress in general have also been found to be sex-specific, with increased responsiveness of the HPA axis often reported for females in a variety of species, including pigs (Weinstock, 2007; Rutherford *et al.*, 2014; McGowan and Matthews, 2018). Our study provides further evidence that prenatal stress, in the form of intra-uterine growth retardation, can have sex-specific effects on HPA axis development.

Litter size

We confirmed that LBW piglets are more common in larger litters, with the proportion of LBW piglets and litter size being positively correlated. This finding is in support of previous studies reporting an increased incidence of LBW piglets in larger litters (Quiniou *et al.*, 2002; Rutherford *et al.*, 2013). Litter size did not influence hair cortisol concentration of piglets, suggesting that aside from the found effects of LBW, HPA axis development of NBW piglets in larger litters is not negatively impacted. Comparable baseline and post-stressor salivary cortisol concentrations have previously been found for NBW piglets from small (≤ 13 piglets) and large (≥ 18 piglets) litters (Fijn *et al.*, 2016).

As multiparous sows produced larger litters than primiparous sows, their piglets are more likely to experience intra-uterine crowding. This not only impacts the piglets through increased incidence of LBW and its associated

effects on HPA axis development, but also the sows themselves. For multiparous sows a positive correlation between litter size and hair cortisol concentration was found. This finding suggests that litter size could be a risk factor for chronic stress in sows. Unfortunately, the effects of litter size on sows' stress during gestation have not yet received scientific attention. A study with mice has shown increased behavioral signs of anxiety in females carrying a larger litter (D'Amato *et al.*, 2006). As selection for increased litter size is ongoing on commercial farms, future studies examining the effects of larger litters on sows' chronic stress are encouraged.

CONCLUSION

A comparable acute stress response to separation from the group prior to farrowing was found for primi- and multiparous sows. This finding suggests that sows do not adapt to this stressor as they gain on-farm experience. Further support for a lack of adaptation to on-farm stressors is provided by comparable chronic stress responses of primi- and multiparous sows, as measured by hair cortisol concentration. For multiparous sows, a positive correlation between fetal litter size and hair cortisol concentration was found. This suggests that the selection for increased litter size on commercial farms could have detrimental effects on sows. Litter size did not only affect the sows, but also their offspring, as larger litters contained more LBW piglets. For these piglets, a sex-dependent effect on HPA axis development was found. Female LBW piglets were shown to have higher neonatal hair cortisol concentrations than LBW males.

3

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

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Under review

ABSTRACT

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

INTRODUCTION

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

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

Assessing whether LBW is a risk factor for impaired brain development in pigs is of relevance for their welfare on commercial farms. First, if LBW pigs are more vulnerable to negative emotions, as has been found in humans, this could be a risk factor for negative animal welfare (Dantzer, 2002). Second,

assessing neurological functioning of LBW piglets could provide a better understanding of the risk factors for their increased mortality (Rutherford *et al.*, 2013). If LBW piglets suffer from delayed neurological development, it is possible that they are less able to appropriately respond to their environment during the neonatal period.

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

The present study aimed to assess neurological functioning and emotional development in LBW piglets. For an assessment of neurological functioning, we performed a basic veterinary neurological examination, consisting of various tests of behavioral abnormalities, reflexes and proprioception. In addition, we assessed piglets' coordination and balance during locomotion to collect further information on their motor skills. For an assessment of emotional development, we compared behavioral responses of LBW and NBW piglets in a human approach test (HAT). We included both female and male piglets, as in humans the development of emotional disorders is more common in (LBW) females (Kessler, 2003; Van Lieshout and Boylan, 2010). Behaviors such as latency to approach an unfamiliar human and vocalization rate were used as indicators of fear (Forkman *et al.*, 2007; Murphy *et al.*, 2014).

Based on results from human studies, we expected LBW piglets to display a delayed neurological development, as indicated by poorer outcomes in the neurological examination. In the HAT, we expected LBW piglets to show increased fear-related behaviors compared to NBW piglets.

METHODS

Ethical note

All methods that demanded the handling of live animals were reviewed and approved by the local animal welfare body (Animal Welfare Body Utrecht) and were conducted in accordance with the recommendations of the EU directive 2010/63/EU.

Animals

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

Selected piglets were housed with their own litter and sow in a farrowing pen (2.4 x 1.8 m), in which the sow was restrained in a centrally positioned farrowing crate (1.90 x 0.85 m). The floor of the pen was partially solid with floor heating for the piglets, and partially slatted for waste disposal. In addition, each pen was equipped with a heat lamp. Temperature inside the farrowing unit was maintained at 24°C until the piglets were approximately one week old, after which it was reduced to 20°C. Piglets were provided with supplemental feed according to supplier's recommendations, starting with milk replacer (Milkiwean BabyMilk, Trouw Nutrition, Nutreco N.V., the

Netherlands) when they were two to three days old. Water was available *ad libitum*. To improve survival of piglets in larger litters, cross-fostering was regularly applied, but only when piglets were two to four days old to ensure colostrum intake.

Neurological examination

Balance beam apparatus

Balance and coordination were assessed using a balance beam consisting of a 120 cm wooden beam covered with a thin rubber mat as a walking surface (**Figure 3.1**). Beam width was selected based on the size of the piglets, with the beam being approximately 5 cm wider than the piglet's shoulder width. Piglets with a shoulder width <6 cm were tested on a 10 cm wide beam, piglets with a shoulder width of 6-7 cm were tested on a 12 cm wide beam, and piglets with a shoulder width ≥ 8 cm were tested on a 15.5 cm wide beam. Wooden board walls were positioned alongside, but not touching, the balance beam. Each wall was placed approximately 2.5 cm away from the beam. This created a narrow space alongside each side of the beam where a piglet could safely misstep without falling.

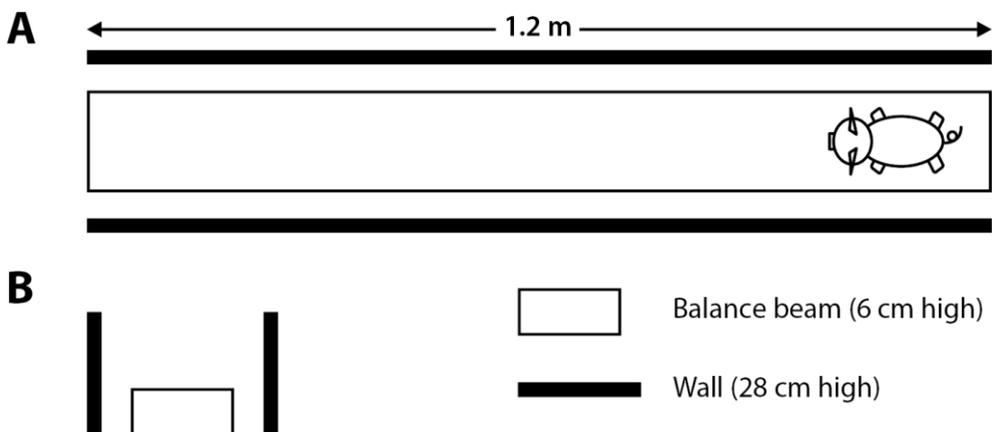


Figure 3.1 Schematic overview of the balance beam apparatus, with top view (**A**) and front view (**B**).

Protocol

Piglets were subjected to multiple tests of neurological functioning when they were 1-2 days old. Only vital piglets were examined, i.e., piglets which actively participated in feeding from the sow and performed independent locomotion. Neurological tests were performed in order of supposed increasing discomfort for the piglet, to minimize effects of stress. These were, in order of testing:

1. **Assessment of abnormal behaviors** which are considered major clinical signs of nervous dysfunction (Constable *et al.*, 2017). Presence of the following symptoms were scored:
 - a. Involuntary head or limb movements
 - b. Gait abnormalities such as circling (assessed during locomotion in farrowing pen)
 - c. Abnormal (head) posture
 - d. Muscle tremors
 - e. Pathological nystagmus, i.e. uncontrolled, repetitive eye movements (assessed using a penlight)

2. **Assessment of menace response** as a sign of cranial nerve function. This response consists of closing of the eyelids after registering the rapid approach of an object (Constable *et al.*, 2017):
 - a. Closing of the eyelid was provoked by rapidly stabbing a finger towards the piglet's eye, without making contact. The test was then repeated for the other eye. Absence or presence of the menace response was scored.

3. **Assessment of palpebral reflex** as a sign of cranial nerve function. This reflex consists of closing of the eyelids when the skin near the eyes is touched (Constable *et al.*, 2017; Nordquist *et al.*, 2017a):
 - a. The skin near the inside corner of each eye was touched, without touching the eyelashes. Absence or presence of palpebral reflex was scored.

4. **Assessment of proprioception** as a sign of peripheral nervous system function (Jackson and Cockcroft, 2002; DeLahunta *et al.*, 2015):
 - a. A wheelbarrow test was performed by raising the piglet's front limbs off the ground and forcing it to walk backwards while

balancing on its hind limbs. It was scored whether the piglet stepped backwards to keep its balance. The wheelbarrow test was then repeated by making the piglet walk forwards while balancing on its front limbs.

- b. A hopping response test was performed by raising three of the piglet's limbs and gently pushing it laterally in the direction of the supporting limb. It was scored whether the piglet hopped on the supporting limb to maintain its balance. The hopping response test was repeated for each of the front and hind limbs.
 - c. The righting response was tested by placing the piglet recumbent on its side. It was scored whether the piglet immediately corrected to an upright position.
5. **Assessment of coordination and balance** during locomotion as a sign of overall motor control, including both central and peripheral nervous system function (DeLahunta *et al.*, 2015):
- a. The piglet had to run across the balance beam three times in close succession. Each misstep (piglet placing foot next to the beam) was scored. After a misstep, the piglet was placed back onto the beam to continue its run. Piglets were encouraged to move towards a litter mate placed near the end of the beam.
6. **Assessment of withdrawal reflex** as a sign of spinal nerve function. This reflex consists of withdrawal of a limb after application of a noxious stimulus (DeLahunta *et al.*, 2015; Nordquist *et al.*, 2017a):

The coronary band of the claw of each hind limb was pinched using only the examiner's fingers. Presence or absence of rapid limb withdrawal was scored. In addition, presence or absence of crossed extensor reflex, i.e. extension of the opposite limb, was scored.

Human approach test

Arena

The arena used for the HAT measured 2 m x 1 m and was created by using two synthetic barriers (1.5 m high) as walls in a corridor with a concrete floor. The arena could be entered by temporarily removing one of the barriers. The

arena was divided into three segments using black cloth tape (1.5 cm wide): a back and middle segment measuring 0.5 m x 1 m each and a front segment measuring 1 m x 1 m (**Figure 3.2**). A camera (Sony HDR-AS50) positioned on top of one of the synthetic barriers recorded the entire arena continuously during testing.

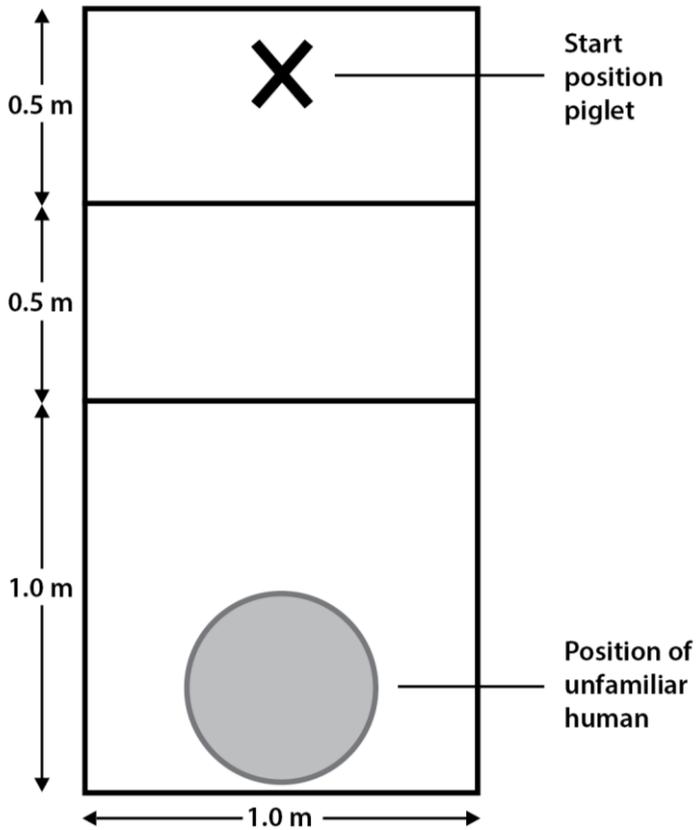


Figure 3.2 Overview of the arena used for the human approach test, showing division of arena into three segments: front segment with position of unfamiliar human, middle segment, and back segment with start position of piglets.

Testing

Piglets were assessed in the HAT when they were approximately 3 weeks of age (all piglets were 20-22 days of age at time of testing). A piglet was taken from the farrowing pen and placed in the back segment of the test arena, situated in a room next to the farrowing unit. Here, the piglet was left for one minute (habituation), after which a person that was unfamiliar to the piglet entered the arena. The person crouched down in the front segment, leaning against the back wall of the arena, and remained there for a duration of three minutes. The unfamiliar person did not encourage the piglet to approach and/or make contact, i.e. they stayed motionless and silent. After three minutes had passed, the piglet was returned to the farrowing pen.

Behavioral variables

The following behavioral variables were scored from the video recordings of the human approach test:

- Total number of **line crossings** (a crossing was scored as soon as both front limbs were placed in a new segment)
 - Scored separately for habituation and HAT
- Total number of **vocalizations**
 - Scored separately for habituation and HAT
- **Latency to approach**, i.e. the time in seconds elapsed between the unfamiliar person entering the arena and the piglet approaching within 0.5m of the human by placing both front limbs in the front segment where the human resided. Piglets which did not approach the unfamiliar human were given a score of 180 seconds.
- **Latency to touch**, i.e. the time in seconds elapsed between the human entering the arena and the piglet making physical contact. Piglets which did not make physical contact with the unfamiliar human were given a score of 180 seconds.
- **Time in back segment** and **time in front segment**, scored as the proportion of time the piglet was scored as being in the segment furthest away from the unfamiliar human and the proportion of time the piglet was scored as being in the segment containing the unfamiliar human, respectively (**Figure 3.2**).
- **Frequency of contact**, scored as total number of times the piglet made physical contact with the human

Statistical analysis

All statistical analyses were performed using R statistical software, version 3.4.2 (R Core Team, 2017). For chi-square analyses, package *gmodels* (Warnes *et al.*, 2018) was used. For linear mixed models, package *nlme* (Pinheiro *et al.*, 2018) was used. Statistical significance was set at $p < 0.05$. Unless indicated otherwise, results are presented as mean \pm *SD*.

Birth weight

Average birth weight of LBW and NBW piglets was compared separately for piglets selected for the neurological examination and HAT using an independent samples *t*-test. For piglets tested in the HAT, average body weight of LBW and NBW piglets at three weeks old was also compared.

Neurological examination

For the neurological examination, all variables that describe the presence of a response (i.e., outcomes of abnormal behaviors and responses, presence of reflexes) were analyzed using a Pearson's chi-square test if expected frequencies were >5 . If expected frequencies for a variable were ≤ 5 , Fisher's exact test was used.

Average number of missteps on the balance beam of LBW and NBW piglets was compared using a linear mixed model with Birth Weight as fixed effect. Random effect structure consisted of random intercepts for Litter.

Human approach test

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

A dependent *t*-test was used to compare time spent in back segment with time spent in front segment, to assess whether piglets spend relatively more time closest to or furthest from the unfamiliar human. Average number of line crossings per minute and the frequency of vocalizations per minute during habituation and HAT were compared using dependent *t*-tests to assess potential effects of the presence of a human on these behaviors.

RESULTS

Neurological examination

Birth weight

LBW piglets selected for neurological examination had on average a lower birth weight than NBW piglets (LBW: 0.76 kg \pm 0.12, NBW: 1.32 kg \pm 0.14; $t_{118} = -23.49$, $p < 0.001$).

Neurological tests

In most neurological tests, all piglets from both birth weight groups displayed a normal response:

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

Five LBW piglets failed to successfully perform hind limb wheelbarrowing, while none of the NBW piglets failed to perform this response. Based on Fisher's exact test, this difference between groups was not significant ($p = 0.057$). LBW piglets did have a higher occurrence of absent withdrawal reflex (LBW: 13 piglets without withdrawal reflex, NBW: 4 piglets without withdrawal reflex; $X^2(1) = 5.55$, $p = 0.018$). LBW piglets made on average more missteps on the balance beam (LBW: 3.23 \pm 0.29, NBW: 1.85 \pm 0.18; $F_{1,91} = 22.08$, $p < 0.001$).

Human approach test

Birth weight

LBW piglets selected for the HAT had on average a lower birth weight than NBW piglets (LBW: $0.86 \text{ kg} \pm 0.10$, NBW: $1.43 \text{ kg} \pm 0.18$; $t_{118} = -21.54$, $p < 0.001$). This difference in body weight was still present when the piglets were tested at three weeks of age (LBW: $3.70 \text{ kg} \pm 1.18$, NBW: $6.28 \text{ kg} \pm 1.32$; $t_{118} = -11.31$, $p < 0.001$).

Behavioral variables

No effects of Birth Weight, Sex or their interaction were found on any of the behavioral variables scored during the HAT (**Table 3.1**). No difference was found between the time piglets spent in the furthest segment and time spent in the segment where the human resided (time spent in furthest square: $67.78 \text{ s} \pm 3.37$, time in closest square: $73.12 \text{ s} \pm 3.13$; $t_{119} = -0.90$, $p = 0.368$). Average number of line crossings per minute decreased when the human entered the arena (habituation: 7.63 ± 5.17 , HAT: 5.20 ± 2.55 ; $F_{1,200} = 25.44$, $p < 0.001$). Average number of vocalizations per minute increased when the human entered the arena (habituation: 36.23 ± 25.14 , HAT: 42.84 ± 25.24 ; $F_{1,200} = 5.16$, $p = 0.024$).

DISCUSSION

In the present study we assessed the effect of birth weight on neurological and emotional development in pigs. To do this, we compared the performance of low birth weight (LBW) and normal birth weight (NBW) piglets in a battery of neurological tests and a human approach test (HAT). Based on findings of delayed neurological development in LBW children (Arcangeli *et al.*, 2012; Savchev *et al.*, 2013; Tosun *et al.*, 2017) and previous neurological assessments in pigs (Vallet and Miles, 2012; Radlowski *et al.*, 2014; Vanden Hole *et al.*, 2018), we expected LBW piglets to have lower scores in their neurological examination. This expectation was partially confirmed, with NBW piglets being more likely to display a withdrawal reflex in response to a painful stimulus and having improved coordination and balance compared to LBW piglets. In the HAT, we expected LBW piglets to show increased fear responses compared to NBW piglets, due to an increased

vulnerability to negative emotions, as has been found in humans (Lahti *et al.*, 2010). However, no differences were found between LBW and NBW piglets for any of the behaviors scored during the HAT.

Table 3.1 Performance of low birth weight and normal birth weight piglets in the human approach test, including measures scored during habituation phase.

Measure	Phase	Birth weight (BW)			Sex			BW x Sex		
		F	df	P \leq	F	df	P \leq	F	df	P \leq
Line crossings	Hab	0.02	1,78	0.878	0.00	1,78	0.985	2.30	1,78	0.133
	HAT	0.05	1,78	0.818	0.02	1,78	0.880	1.58	1,78	0.212
Vocalization frequency	Hab	0.45	1,78	0.505	0.44	1,78	0.510	0.06	1,78	0.802
	HAT	0.14	1,78	0.709	0.00	1,78	0.958	0.16	1,78	0.695
Latency to approach	HAT	0.18	1,78	0.676	0.66	1,78	0.420	0.04	1,78	0.834
Latency to touch	HAT	0.48	1,78	0.488	0.49	1,78	0.486	0.32	1,78	0.574
Time in back segment	HAT	0.18	1,78	0.670	1.11	1,78	0.295	0.26	1,78	0.612
Time in closest segment	HAT	0.11	1,78	0.742	0.09	1,78	0.767	0.14	1,78	0.711
Frequency of contact	HAT	0.00	1,78	0.974	0.13	1,78	0.722	0.18	1,78	0.675

Abbreviations: Hab, habituation; HAT, human approach test.

Neurological development

We subjected a large sample of neonatal piglets to a neurological examination at day one after birth. This allows us to describe some general features of functional neurodevelopment in pigs. First, all pigs successfully completed (most) tests of proprioception. These tests forced the pigs to use postural reactions to maintain their balance (DeLahunta *et al.*, 2015). The ability to coordinate the movement of joints and muscles to maintain posture is known to develop quickly after birth in precocial species such as sheep and horses (Fox, 1964; Nauwelaerts *et al.*, 2013). Based on our findings, postural reactions develop within the first day of life in pigs. None of the piglets displayed a menace response, that is, piglets did not respond to the sudden approach of an object towards the eye by immediate closure of the eyelids. This finding can be explained by the fact that the menace response is a learned response which develops over time (Constable *et al.*, 2017). In other precocial species such as sheep, goats and horses, the menace response only develops after multiple days (horse: ~9 days; Enzerink, 1998; sheep: ~8 days, goat: ~14 days; Raofi *et al.*, 2011). To assess whether development of this response is delayed in LBW piglets, daily follow-up assessments over the first weeks of life are recommended. All piglets displayed a palpebral reflex, which relies on cranial nerve function, in particular the facial nerve (responsible for the motor component of the reflex) and the trigeminal nerve (responsible for the sensory component of the reflex; Constable *et al.*, 2017). This finding was expected as the palpebral reflex is a brain stem reflex which is only absent in unconscious animals (Verhoeven *et al.*, 2015).

When comparing the neurological assessment of LBW and NBW piglets, results of two tests suggest that LBW delays neurological development. The LBW piglets in our study were less likely to display a withdrawal reflex in response to a painful stimulus and made more missteps on the balance beam. Based on these findings alone, we cannot state which specific neurological functions are delayed. The withdrawal reflex is a spinal nerve reflex involving sensory neurons, motor neurons, and nociception pathways in the central nervous system (DeLahunta *et al.*, 2015). Performance on the balance beam relies on a combination of neuromotor control (including brainstem, spinal cord and peripheral nerve function; Constable *et al.*, 2017), as well as the muscle strength required to maintain locomotion. It is important to note that a number of the neurological tests we performed are influenced (to some degree) by muscle strength, as they require muscle movement. Multiple studies have reported that LBW pigs have fewer muscle fibers compared to

NBW pigs (Rehfeldt and Kuhn, 2006; Beaulieu *et al.*, 2010; Berard *et al.*, 2010). This makes it possible that muscle strength is a confounding factor in studies assessing functional neuromotor development in pigs. Future studies are required to establish which exact (neurological or physical) impairments are responsible for the results found in the present study.

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

Human approach test

We used the HAT to elicit LBW and NBW piglets' fear responses to the presence of an unfamiliar human. Human interaction paradigms such as the HAT are a commonly used method to assess fearfulness in different species of farm animals, including pigs (Forkman *et al.*, 2007; Murphy *et al.*, 2014). We scored behavioral measures such as latency to approach an unfamiliar human, line crossings, and vocalizations as indicators of the piglets' fearfulness during the test. However, the validity of some of these measures have been questioned. For example, latency to approach an unfamiliar human is assumed to reflect fear, where less fearful pigs will be more likely to approach the human. However, such approaches could also be motivated by aggression or a desire for social support from the human (Waiblinger *et al.*,

2006; Murphy *et al.*, 2014). Line crossings do not always correlate with other fear-related variables in behavioral tests such as the elevated plus maze or light/dark test (Andersen *et al.*, 2000). Rather, it seems that this measure is often confounded by the general activity level of an animal, independent of emotion (Murphy *et al.*, 2014). Vocalization appears to be the behavioral response which is least likely to be confounded in the HAT. The majority of vocalizations we observed during the HAT were grunts with low tonality and high-pitched screams (personal, non-systematic observation). Such vocalizations in pigs represent contact calls to (re-)establish social contact with group mates and calls to communicate a current stressed state, respectively (Manteuffel *et al.*, 2004). For example, high-pitched screams are elicited in response to social isolation or painful procedures (Weary and Fraser, 1995; Döpjan *et al.*, 2008; da Silva Cordeiro *et al.*, 2013). Therefore, based on the presence of such vocalizations we can assume that we were successful in eliciting an emotional response in our piglets using the HAT. Future studies measuring vocalization frequencies are encouraged, as these can objectively distinguish between different call types and potentially provide information about the pig's emotional state (Leliveld *et al.*, 2017).

To increase our understanding of piglets' responses in the HAT, we used additional analysis of behavioral measures to assess whether the piglets experienced the presence of an unfamiliar human as stressful. We found that piglets increased their vocalization rate after the human entered the test arena, as compared to the habituation phase prior to the HAT. This could suggest that piglets became more fearful in the presence of an unfamiliar human (Manteuffel *et al.*, 2004). However, if this were the case, it would also be expected that the piglets would avoid the human. We compared the proportion of time piglets spent in the segment furthest away from the human's position in the test arena to the time spent in the segment containing the human. No difference was found, suggesting the piglets did not actively avoid being near the unfamiliar human. Rather, it seems that the increase in vocalization rate is due to stress caused by other conditions inherent to the HAT, such as being in an unfamiliar environment and being isolated from the sow for a prolonged period of time. Such conditions are known to cause stress (and associated vocalizations) in piglets (Hötzel *et al.*, 2011; Iacobucci *et al.*, 2015). One study found isolated piglets to double their vocalization rate in response to calls from the sow, suggesting that the vocalizations made by nursing piglets in isolation are contact calls directed at the sow (Weary *et al.*, 1997). It appears that the main stressor provided by the HAT in nursing

piglets is the isolation from the sow and their familiar environment, and not the presence of an unfamiliar human.

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

In addition to a comparable fear response of LBW and NBW piglets, we found no difference between female and male LBW piglets. In humans, LBW females are more likely to develop emotional disorders than LBW males (Van Lieshout and Boylan, 2010). However, these disorders develop after the onset of puberty, suggesting the piglets in our study were too young to detect a sex-specific difference in emotion. Previous studies with LBW pigs also did not report differences in acute or chronic stress responses between females and males (Poore and Fowden, 2003; Roelofs *et al.*, 2018). Interestingly, Poore and Fowden (2003) report that while the *in utero* programming of the HPA axis differs for female and male LBW pigs, the cortisol increase in response to an acute stressor did not. This supports our finding of a comparable acute stress response for female and male LBW piglets.

CONCLUSION

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

Part II

Post-weaning effects of low birth weight

4

Female and male pigs' performance in a spatial holeboard and judgment bias task

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ABSTRACT

Studies of the cognitive abilities of pigs are increasing in number, due to their relevance for the fields of animal welfare and biomedical research. While both female and male pigs have been used in cognitive tasks, possible sex differences in performance have not yet received extensive attention. This is of interest, as sexual dimorphism in cognitive abilities has been documented for a variety of species. The aim of this study was to assess the effects of sex on pigs' performance in two cognitive tasks. Spatial learning and memory of ten female and ten male pigs was compared in a spatial holeboard task. Working and reference memory, as well as measures of motivation and exploration were assessed. Both females and males acquired the task and no differences were found between sexes for any measures of spatial memory. However, female pigs performed more successfully during reversal trials (shorter latency to first reward, higher number of rewards found), indicating greater response flexibility. This difference between sexes was transient, with males eventually reaching the same level of performance as the females. Judgment bias, a cognitive measure of affective state, was subsequently assessed using an active choice judgment bias task. Pigs were trained to respond differently to a negative and a positive stimulus, signaling either a small or a large reward. During judgment bias testing, pigs were presented with ambiguous cues and their trained 'positive' and 'negative' responses were recorded as optimistic or pessimistic choices, respectively. Both females and males displayed a slightly optimistic judgment bias. Optimistic choosing decreased with repeated testing for both groups. It is likely the pigs learned about the unrewarded outcome of ambiguous cues, rendering them no longer ambiguous. Further improvement of the judgment bias task as a cognitive measure of affective state is deemed necessary. Overall, our results indicate that sex is not a confounding factor when measuring baseline performance of pigs in the spatial holeboard or judgment bias task. Sex effects were only found when subjecting the pigs to a reversal task, warranting further study of sex differences in response flexibility. Such a difference would have implications for pig welfare, as it suggests that males are slower to cope with changes in their environment.

INTRODUCTION

Cognitive research in domestic pigs (*Sus scrofa*) has recently gained interest for a number of reasons. First, in commercial farming, pigs are reared for meat production under conditions which have given rise to concerns about their welfare. Measures of cognition are increasingly used as tools in welfare assessment (Mendl and Paul, 2004; Paul *et al.*, 2005; Boissy *et al.*, 2007). Furthermore, pigs are also considered suitable for biomedical research, and viewed as a promising animal model for human brain disorders (Lind *et al.*, 2007; Gieling *et al.*, 2011; Kornum and Knudsen, 2011). Therefore, a better understanding of pig cognition will benefit both animal welfare and biomedical research.

Sexual dimorphism in cognitive task performance has been reported for a variety of species, including humans, rats, mice, grey seal, voles and guppies (Beck *et al.*, 2007; Andreano and Cahill, 2009; Healy *et al.*, 2009; Lucon-Xiccato *et al.*, 2016). Such differences in cognitive abilities have been explained by differences in environmental demands experienced by males and females, such as differences in home range size, complexity of social life, or means of food provision (Beck *et al.*, 2007; Healy *et al.*, 2009; Lucon-Xiccato *et al.*, 2016). Possible sex-based differences in the cognitive abilities of pigs have not yet been extensively investigated. As both sexes of pigs (in single-sex and mixed-sex groups) have previously been used in cognitive tasks (Gieling *et al.*, 2011), such knowledge would be valuable. Most studies using both sexes have reported a comparable performance of their male and female pigs for different cognitive abilities such as spatial, operant and discrimination learning tasks (e.g. de Jong *et al.*, 2000; Sneddon *et al.*, 2000; Bolhuis *et al.*, 2004; Moustgaard *et al.*, 2005). However, several studies do mention an effect of sex on cognitive task performance (e.g. spatial learning: Siegford *et al.*, 2008; Elmore *et al.*, 2012). Based on these findings, it seems premature to assume comparable cognitive abilities for female and male pigs.

It is possible that sex-based differences in cognition have only been found in certain studies due to task-dependent parameters. These may stimulate specific cognitive and psychological processes with different functioning between the sexes, as has been shown for humans and rats (Andreano and Cahill, 2009; Faraji *et al.*, 2010). For example, it has been suggested that males and females have a preference for different cognitive strategies of navigation, possibly influencing their performance in spatial learning tasks. Females appear to be more dependent on the presence of landmarks, whereas males

appear to rely more on geometric cues, such as angles and distances between objects in their environment (Keeley *et al.*, 2013). Stress experienced before or during testing can be another task-dependent influence on results, when testing conditions serve as a stressor. Such stress may have either a positive or a negative effect on an animal's cognitive performance. For example, high stress can decrease attention during cognitive tasks, resulting in an increase of errors (Mendl, 1999; Sandi, 2013). On the other hand, mild stress has been shown to facilitate performance in classical conditioning tasks (Sandi, 2013). Stress, experienced before or during testing, can have sexually dimorphic effects on cognition. For example, acute stress often has a more detrimental effect on the cognitive performance of female rats compared to males (Healy *et al.*, 2009). Acute stress has even been shown to enhance the performance of male rats (Shors, 2001). Chronic stress appears to have a stronger negative effect on the cognitive performance of males compared to females in rodents (Healy *et al.*, 2009; ter Horst *et al.*, 2012). In fact, there have been reports of improved cognitive performance after chronic stress for female rats (Bowman *et al.*, 2001; Kitraki *et al.*, 2004; cf. Conrad *et al.*, 2003). Together, these findings suggest that sex-based influences on results cannot be generalized across tasks and species. To avoid false conclusions, the results of each task must separately be assessed for sex effects.

Two cognitive tasks for pigs that have been developed in recent years are the spatial holeboard task and the judgment bias task. The spatial holeboard is a free choice maze-task where animals are allowed to make revisits as they search for the locations of multiple hidden food rewards in an open arena (van der Staay *et al.*, 2012). Hereby, it simultaneously assesses reference and working memory. Reference memory holds information that remains relevant across successive trials, such as the maximum number of rewards to be found, where the rewards are hidden and which action is required to access them (Olton and Papas, 1979). This can be operationalized as the ratio between visits to rewarded and unrewarded locations (van der Staay *et al.*, 2012). Working memory holds information that is only relevant within a single trial, such as which locations have already been visited (Olton and Papas, 1979; Dudchenko, 2004). This can be operationalized as the ratio between first visits and all visits (including revisits) to the rewarded locations (van der Staay *et al.*, 2012). The holeboard task is considered particularly suitable for studying (pig) cognition, as it allows for collection of data on several domains such as spatial learning and memory, motivation and exploration (Gieling *et al.*, 2011; van der Staay *et al.*, 2012). Sex effects on holeboard performance have been found for rodents, with males

outperforming females (e.g. Faraji *et al.*, 2010; Arp *et al.*, 2014). This was likely due to task-dependent parameters, as stress responses were experimentally altered in one study (Arp *et al.*, 2014), while females' preferred navigational strategy (use of landmarks) was not possible during the other (Faraji *et al.*, 2010). The holeboard task has been successfully applied to pigs of both sexes, using either all-female groups (e.g. Arts *et al.*, 2009; Gieling *et al.*, 2012, 2013; Clouard *et al.*, 2016; Grimberg-Henrici *et al.*, 2016; van der Staay *et al.*, 2016), all-male groups (e.g. Haagensen *et al.*, 2013b; Antonides *et al.*, 2015b; Fijn *et al.*, 2016) or mixed-sex groups (e.g. Bolhuis *et al.*, 2013; Haagensen *et al.*, 2013a; Gieling *et al.*, 2014; Antonides *et al.*, 2015a, 2016). For the studies using both male and female pigs, possible sex effects were often not analyzed (e.g. Gieling *et al.*, 2014; Antonides *et al.*, 2015a, 2016). One study found no sex effects (Bolhuis *et al.*, 2013). Haagensen *et al.* (2013a) found a slightly better performance of female (mini)pigs for one of several spatial memory measures studied. This difference was attributed to the error-free performance of one of their female pigs (out of six females total).

Judgment bias tasks are used to obtain a cognitive measure of animal affective state (Mendl *et al.*, 2009; Baciadonna and McElligott, 2015; Roelofs *et al.*, 2016). Animals are trained to distinguish between two stimuli and to associate one with a positive outcome (reward) and the other with a negative outcome (punishment or lack of reward). A distinct behavior has to be performed in response to each type of stimulus. When testing judgment bias, the animals are presented with intermediate stimuli that are expected to be ambiguous, i.e. neither associated with a positive nor negative outcome. Animals in a positive affective state are expected to show an 'optimistic' response, by displaying their learned response to the positive stimulus. Animals in a negative affective state are expected to interpret ambiguous stimuli as being similar to the negative stimulus. In this manner, the valence (both positive and negative) of animal affective states can be assessed. Sex effects on judgment bias have been reported for goats and rats, with (stressed) females behaving more optimistically than males (Briefer and McElligott, 2013; Barker *et al.*, 2016). In pigs, judgment bias has been measured using all-female groups (e.g. Douglas *et al.*, 2012; Döpjan *et al.*, 2013, 2017; Murphy *et al.*, 2013b), all-male groups (e.g. Carreras *et al.*, 2015; Murphy *et al.*, 2015) and mixed-sex groups (e.g. Scollo *et al.*, 2014; Brajon *et al.*, 2015; Asher *et al.*, 2016; Carreras *et al.*, 2016a). Only two studies (Asher *et al.*, 2016; Carreras *et al.*, 2016a) analyzed their data for a possible sex effect on judgment bias. No differences in performance between females and males were found.

The aim of the present study was to assess possible sex-based differences in cognitive performance in pigs, as measured by the holeboard task and the judgment bias task. Baseline sex effects were tested for by minimizing possible (sexually dimorphic) effects of stress prior to and during testing. To this end, female and male pigs were socially housed in an enriched environment and carefully habituated to the requirements of both tasks. The pigs were first trained and tested in a spatial holeboard task. No sex effects were expected as female and male pigs experience similar environmental demands on spatial cognitive abilities in the wild. For example, both sexes explore large home ranges for food (Podgórski *et al.*, 2013). Also, the holeboard arena provides both geometric and landmark cues, allowing for different navigational strategies to be successful. This should allow both males and females to successfully complete the task, using their preferred navigational strategy (Keeley *et al.*, 2013). The pigs were subsequently tested in an active choice judgment bias task, where active behavioral responses were required in response to both the positive and the negative stimulus. Enriched housing conditions have been shown to lead to an optimistic judgment bias in pigs (Douglas *et al.*, 2012). Also, a previous study has found no sex effects on judgment bias for pigs (Carreras *et al.*, 2016a). Therefore, it was expected that the male and female pigs in our study would show a comparable, optimistic judgment bias.

METHODS

Ethical note

All methods that demanded the handling of live animals were reviewed and approved by the local ethics committee (dierexperimentcommissie (DEC) Utrecht) and were conducted in accordance with the recommendations of the EU directive 2010/63/EU.

Animals

Ten pairs of piglets [(Terra x Finnish Landrace) x Duroc] from eight different litters, all born within the same week, were selected from the commercial pig breeding farm of Utrecht University. All piglets from each of the litters were weighed at weaning and the average female and average male weight was calculated per litter. From each litter, the female and the (entire) male closest

in weight to the litter average were selected. From two litters, an additional pair of average weight piglets was selected.

Housing

After weaning at approximately four weeks of age, the selected pigs were moved to the research facility (located next to the commercial farm). Pigs were housed in two adjacent pens (both approximately 4×5 m) in a naturally ventilated building, with females and males being housed separately. Pen floors were concrete and covered with straw bedding that was replaced daily. Each pen contained a covered piglet nest (with rubber mats in addition to straw bedding) and different toys (balls, chains, chewing sticks). Heat lamps warmed the nest box until the pigs were approximately eight weeks old. Transparent plasticized PVC slats, hanging in front of the entrance to the nest box protected the piglets from the cold. Pens were cleaned daily. Minimum and maximum temperatures were recorded daily and ranged from 7°C to 38°C (to avoid testing animals under heat stress, pigs were only tested after voluntarily entering the testing apparatus). Pigs received food and water *ad libitum*. To facilitate individual recognition of the pigs during testing, each pig was marked with a letter sprayed on its back.

Spatial holeboard task

Apparatus

The holeboard apparatus (manufactured by Ossendrijver B.V., Achterveld, The Netherlands) was a square arena (5.3×5.3 m) with 16 holes (food bowls) placed in a 4×4 matrix (**Figure 1.2A**). The holeboard had a synthetic slatted floor and synthetic walls (80 cm high) surrounding the arena. Each wall contained a guillotine door which could be operated from outside the holeboard by a rope and pulley system. The pigs could enter the holeboard via a corridor (40 cm wide) surrounding the arena. Using four different starting positions has been suggested (Arts *et al.*, 2009) and applied (e.g. Antonides *et al.*, 2015a; Fijn *et al.*, 2016) as a means of avoiding fixed search patterns being developed by the pigs (van der Staay *et al.*, 2012). Start positions were randomly selected prior to each trial. Extra-maze cues were available (such as the position of the experimenter outside the apparatus) for orientation inside the holeboard.

Chocolate candies (M&M's® Milk Chocolate) were used as rewards. To avoid discrimination between baited and non-baited holes based on scent, each food bowl was fixed with a false bottom beneath which four candies were placed (**Figure 1.2B**). All food bowls were covered with synthetic red balls (JollyBall Dog Toy, ø24 cm, 1400 g, Jolly Pets, Ohio, USA) to avoid visual discrimination between baited and non-baited holes. Pigs could only determine whether a bowl contained a reward by lifting the ball with their snout (**Figure 1.2B**). To avoid the development of scent cues, the entire holeboard apparatus was cleaned daily. Additionally, the holeboard was rinsed immediately after a pig soiled it during testing.

All hole visits were automatically recorded using custom made software (Blinq Systems, Delft, The Netherlands). Lifting of a ball interrupted the connection between a magnet in the ball and a sensor in the food bowl. This signal was registered by an interface (LabJack) and sent to a PC. A revisit was only recorded if another hole was visited in between or if 10s passed in between successive visits to the same hole. A trial was started as soon as the pig had moved both front legs into the arena. A trial ended when a pig had found all rewards or when 7.5 min passed, whichever occurred first.

Training and testing

Training in the spatial holeboard task started when the pigs were approximately four weeks of age. All pigs were first habituated to the presence of and being handled by the researcher. The pigs were then gradually habituated to the hallway leading to the holeboard and to the holeboard apparatus itself. Pigs were initially allowed to explore the holeboard arena in groups, the size of which were gradually decreased until they explored the holeboard individually. When all pigs were able to lift the balls off the food bowls, testing was started. All pigs were tested in the holeboard in three consecutive phases: habituation (four trials), acquisition (44–60 trials) and reversal (20 trials). Pigs always performed two consecutive trials daily (massed trials). Habituation trials started when the pigs were about seven weeks of age. During habituation trials, all 16 holes were baited. For the acquisition trials, each pig was assigned to and trained on one of four possible reward configurations with only four baited holes (**Figure 1.3**). A pig completed the acquisition phase when it had reached a reference memory score of at least 0.7 (see section **Holeboard data** in this chapter for RM score calculation) for two consecutive training days (i.e. at least four consecutive

trials) or after 60 trials, whichever occurred first. However, each pig received a minimum of 44 acquisition trials, i.e. a pig that reached the criterion of 0.7 before the 44th trial continued training until 44 trials were completed. These minimum and maximum numbers of acquisition trials were based on previous holeboard experiments with pigs (e.g. Gieling *et al.*, 2012, 2013) and were expected to allow most of the pigs to reach the specified criterion level of performance. For the reversal phase, the configuration of baited holes was changed. Each pig was now trained on a rotation of their previous configuration (A switched to C, B switched to D and vice versa).

Judgment bias task

Apparatus

The judgment bias apparatus consisted of a rectangular arena (3.6×2.4 m) connected to a start box (1.2 m^2) via an antechamber (**Figure 1.1**). Entrance of pigs from the start box to the arena was controlled by a guillotine door, operated by a rope and pulley system. Goal-boxes where the pigs could obtain rewards were situated at the back wall of the arena, near the corners. Each goal-box (40 cm wide) contained a food bowl identical to those used in the holeboard apparatus (**Figure 1.2B**). Goal-boxes could be opened and closed using guillotine doors which could be operated from outside the apparatus by rope and pulley systems. Tone-cues were generated using the open source software Audacity (<http://audacity.sourceforge.net/>). Speakers mounted to the back of the arena (Logitech z-313, Logitech Europe S.A., Morges, Switzerland) were used to play the tone-cues. Chocolate candies (M&M's[®] Milk Chocolate) were used as rewards and also placed underneath the false bottom of the food bowls to avoid influence of scent cues. Additionally, the apparatus was cleaned daily and rinsed immediately after an animal soiled it during testing.

Pre-training

Pre-training for the judgment bias task started when the pigs were approximately 4.5 months old. Pigs were first habituated to the new testing apparatus, until they explored it individually. They then had to perform forced trials, where they had to wait in the start box until the door was opened. Then, they were allowed to enter the arena and retrieve a single candy from one of the goal-boxes (only one was open per trial). After retrieving the

reward, the goal-box was closed and the pig returned to the start box. Pigs were trained until they could perform a series of 12 consecutive trials. Next, 'positive' and 'negative' cues were introduced. Two tone-cues were used during training: a 1000 Hz (high) and a 200 Hz (low) pure tone (waveform: sine, amplitude: 1). Pigs were trained to associate one of the tones with a large reward (four candies) in one goal-box and the other tone with a small reward (one candy) in the other goal-box. The meanings (positive or negative) of the tones were counterbalanced across animals, as were the associated goal-boxes. Tone training (four sessions of 12 trials) consisted of six positive and six negative trials in pseudorandom order (no more than two identical trials in a row). During a trial, a tone-cue was started while the pig was in the start box. Only the appropriate goal-box was open and baited with the appropriate reward. The tone was stopped when the pig lifted the ball inside the goal-box to obtain its reward. Training commenced with two sessions of 12 open choice trials during which both goal-boxes were open but only the correct one was baited. If a pig made a correct choice (by visiting the rewarded goal-box), the tone was stopped and the pig could return to the start box for its next trial. If it made an incorrect choice, the door of the incorrect goal-box was closed and the tone kept playing until the pig visited the correct goal-box to find its reward. After three weeks of pre-training, the pigs were ready to start their formal discrimination training trials.

Discrimination training

During discrimination training, each pig performed one daily session of 13 trials. The first three trials were forced trials where only the correct goal-box was open. The first trial was always negative, followed by a positive and negative trial in random order, changing daily. These forced trials were followed by five negative and five positive free trials in a daily changing pseudorandom order (no more than two identical trials in a row). During free trials, both goal-boxes were open and an incorrect choice was followed by closing both goal-boxes. The pig was 'punished' with a 90s time-out, i.e. it had to wait in the main chamber before it could return to the start box for the next trial. If a pig failed to choose a goal-box within 30s (recorded as an omission), a 90s time-out punishment was applied. Pigs continued discrimination training until they reached a criterion of at least four (out of five) correct choices for both positive and negative free trials in three consecutive sessions (with a maximum of 30 discrimination training sessions). During every fifth session the first three positive and first three

negative free trials were replaced by open choice trials, to maintain the association between cues and goal-boxes.

Judgment bias testing

Judgment bias testing started a day after a pig had reached its training criterion. Each pig performed four testing sessions, consisting of 16 trials each. The setup of a testing session was the same as a training session, except that three extra ambiguous trials were added. During each of these trials, a different ambiguous tone-cue was played instead of the learned positive and negative tones. These cues were pure tones of frequencies at equal intervals between the training tones on a logarithmic scale: 299.07 Hz, 447.21 Hz and 668.74 Hz. Depending on the value of the high and low training tones, these ambiguous cues represented a ‘near-negative’ ambiguous tone (most similar to the learned negative tone), an ‘intermediate’ ambiguous tone (447.21 Hz) and a ‘near-positive’ ambiguous tone (most similar to the learned positive tone). During a testing session, trials 6, 11 and 16 were the ambiguous trials. Pigs were always presented with the intermediate ambiguous tone-cue during trial 6, while trials 11 and 16 alternated between the near-negative and near-positive ambiguous tone-cues. Whether an ambiguous cue was preceded by a negative or positive trial was counterbalanced across testing sessions, to balance any possible effects of the preceding trials on judgment bias. During ambiguous trials, the goal-boxes were not baited.

Statistical analysis

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

Holeboard data

The following variables were calculated per trial (van der Staay *et al.*, 2012):

- **Working memory** was calculated as the number of visits that yielded a food reward divided by the total number of visits (including revisits) to the baited set of holes.

- **Reference memory** was calculated as the total number of visits to baited holes divided by the total number of visits to all holes.
- **Trial duration** and **latency to first reward** were calculated as average time in seconds elapsed between entering the holeboard and performing the required action. A maximum time of 450s was assigned to a latency measure when a pig failed to perform the required action.
- **Inter-visit interval** was calculated as the average time in seconds between two successive hole visits.
- **Total number of visits** and other frequency measures (**number of rewards found** and **number of locations visited**) were scored as absolute counts.

For all variables in the acquisition and reversal phases, means of four successive trials (trial blocks) were calculated. The effects of sex on these variables were analyzed using a mixed model ANOVA (SAS PROC MIXED) with litter as random effect and trial blocks and sex as fixed effects. All analyses were performed for the different phases of the experiment: habituation (4 trials analyzed separately), acquisition (first 44 trials; trial blocks 1–11), reversal (20 trials; trial blocks 12–16) and transition (final four trials of acquisition and first four trials of reversal).

Judgment bias data

The following variables were calculated per pig:

- **Sessions to criterion** was calculated as the number of discrimination training sessions needed to complete the discrimination training phase.
- **Optimistic choice percentage** was calculated as the percentage of optimistic choices made during testing sessions, per cue type. Optimistic choices are approaches to the goal-box that would have contained a large reward during training.
- **Latency to respond** was calculated as average time in seconds elapsed between leaving the start box (both front legs outside the start box) and lifting a ball in a goal-box, per cue type.

The effects of sex on all variables were analyzed using a mixed model ANOVA (SAS PROC MIXED) with litter as random effect and cue type and sex as fixed effects. Additionally, optimistic choice percentage was analyzed separately for the first and last two test sessions to assess a possible effect of repeated testing on judgment bias performance. These data were analyzed with test sessions as an additional fixed effect.

RESULTS

Spatial holeboard task

Habituation trials

During the habituation trials, sex differences were found for two measures describing exploration. Female pigs had a higher total number of visits (female average: 28.1 visits; male average: 23.6 visits; $F_{1,65} = 8.87, p = 0.004$) and a higher number of locations visited (female average: 15.3 locations; male average: 13.9 locations; $F_{1,65} = 5.96, p = 0.017$) than male pigs.

Spatial learning and memory

Working memory (WM; see **Figure 4.1**) performance improved for female and male pigs during the acquisition phase (Trial blocks: $F_{10,190} = 4.09, p < 0.001$) and the reversal phase (Trial blocks: $F_{4,83} = 26.46, p < 0.001$). WM scores decreased for all pigs during the transition phase (Trial blocks: $F_{1,29} = 43.85, p < 0.001$). No sex effects on WM scores were found.

Reference memory (RM; see **Figure 4.1**) performance improved for all pigs during the acquisition phase (Trial blocks: $F_{10,191} = 40.55, p < 0.001$) and the reversal phase (Trial blocks: $F_{4,83} = 63.59, p < 0.001$). During the transition phase RM scores decreased for both female and male pigs (Trial blocks: $F_{1,29} = 225.27, p < 0.001$). No sex effects on RM performance were found.

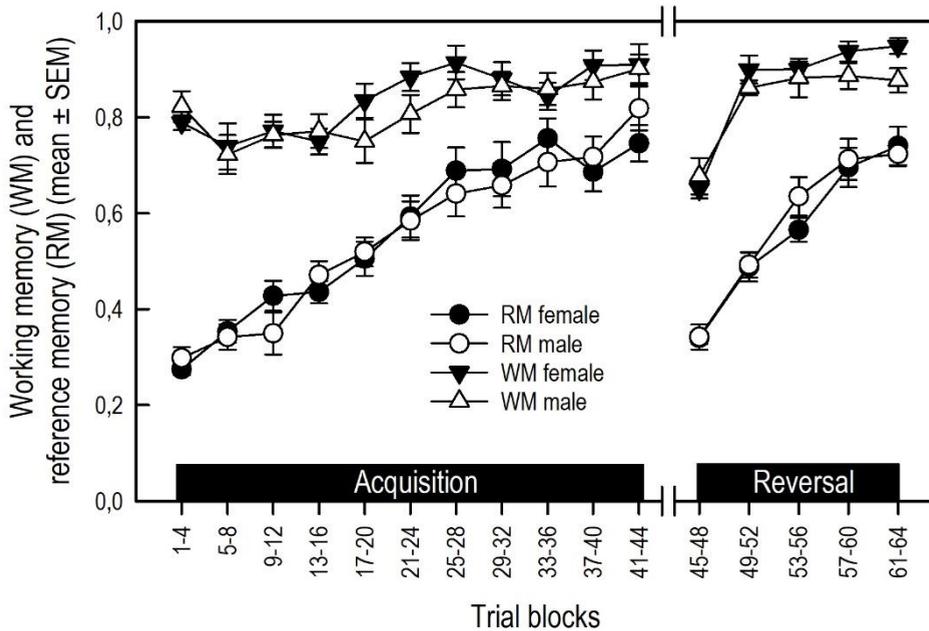


Figure 4.1 Working memory (WM) and reference memory (RM) scores of female and male pigs in the spatial holeboard task.

Duration measures

Trial duration (TD; see **Figure 4.2**) decreased during the acquisition phase (Trial blocks: $F_{10,191} = 13.97$, $p < 0.001$) and the reversal phase (Trial blocks: $F_{4,83} = 88.41$, $p < 0.001$) for all animals. TD increased during the transition phase (Trial blocks: $F_{1,29} = 292.76$, $p < 0.001$). No sex effects on TD were found.

Inter-visit interval (IVI) decreased during the acquisition (Trial blocks: $F_{10,191} = 2.87$, $p = 0.002$) and reversal phases (Trial blocks: $F_{4,83} = 6.16$, $p < 0.001$), and increased during the transition phase (Trial blocks: $F_{1,29} = 21.72$, $p < 0.001$) for all pigs. No sex effects on IVI were found.

Latency to first reward (LFR; see **Figure 4.2**) decreased for both males and females during the acquisition phase (Trial blocks: $F_{10,191} = 7.36$, $p < 0.001$)

and the reversal phase (Trial blocks: $F_{4,83} = 38.54, p < 0.001$). LFR increased for all animals during the transition phase (Trial blocks: $F_{1,29} = 129.32, p < 0.001$). The rate at which the LFR changed for females and males differed for all phases (Sex by Trial blocks interaction: Acquisition, $F_{10,191} = 2.04, p = 0.031$; Transition, $F_{1,29} = 11.76, p = 0.002$; Reversal, $F_{4,83} = 4.32, p = 0.003$), due to males having higher latencies at the start of the acquisition and reversal phases. This difference disappeared in the final trial blocks of both phases. A sex effect on LFR was found for the transition phase: male pigs had a higher LFR during the first trial blocks of the reversal phase (Sex: $F_{1,29} = 8.46, p = 0.007$).

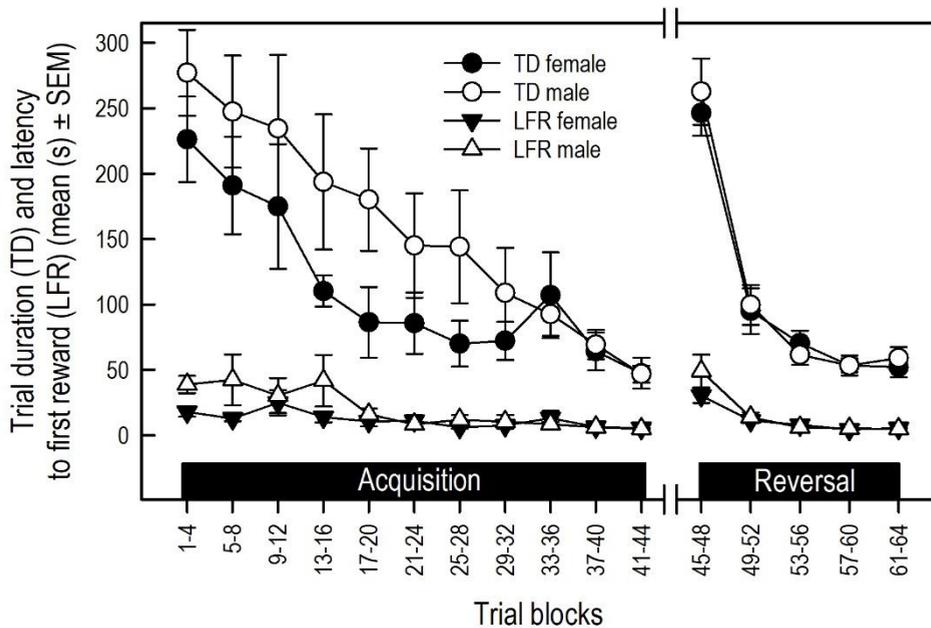


Figure 4.2 Trial duration (TD) and latency to first reward (LFR) of female and male pigs in the spatial holeboard task.

Exploration measures

The total number of visits (TV) decreased for all pigs during the acquisition phase (Trial blocks: $F_{10,191} = 15.09$, $p < 0.001$) and the reversal phase (Trial blocks: $F_{4,83} = 25.91$, $p < 0.001$). TV increased for all pigs during the transition phase (Trial blocks: $F_{1,29} = 75.02$, $p < 0.001$). The decrease in TV during the acquisition phase differed for males and females, with females having a higher TV during the first trial blocks (Sex by Trial blocks interaction: $F_{10,191} = 1.93$, $p = 0.043$).

The number of locations visited (LOC; see **Figure 4.3**) decreased for both females and males during the acquisition (Trial blocks: $F_{10,191} = 16.05$, $p < 0.001$) and reversal (Trial blocks: $F_{4,83} = 23.55$, $p < 0.001$) phases, and increased during the transition phase (Trial blocks: $F_{1,29} = 64.22$, $p < 0.001$). The rate of decrease in LOC during the reversal phase differed for males and females, with females visiting more locations during the first trial block (Sex by Trial blocks interaction: $F_{4,83} = 2.87$, $p = 0.028$). During the transition phase, females had a higher average LOC (Sex: $F_{1,29} = 8.64$, $p = 0.006$).

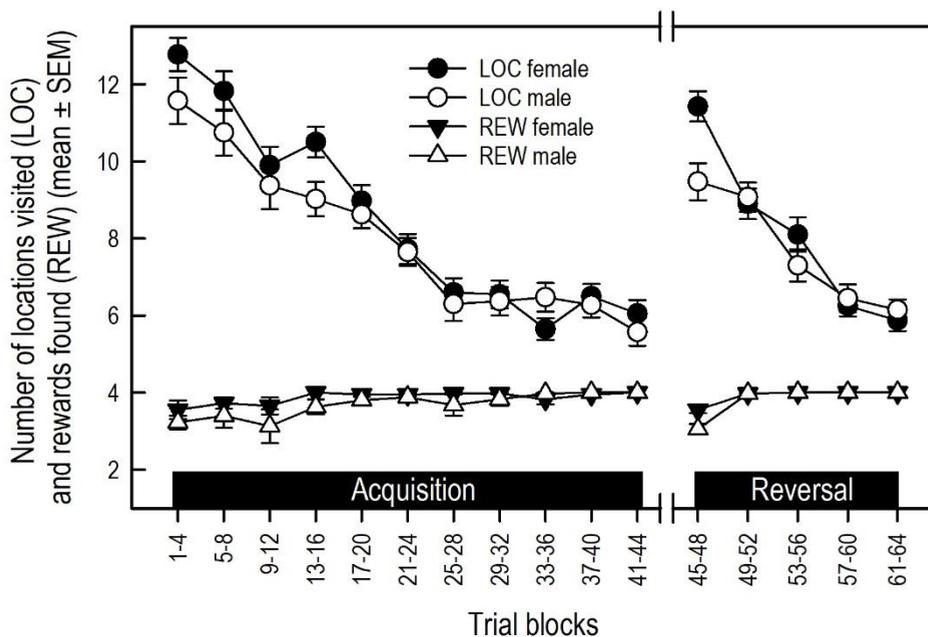


Figure 4.3 Number of locations visited (LOC) and number of rewards found (REW) for female and male pigs in the spatial holeboard task.

The number of rewards found (REW; see **Figure 4.3**) increased for all animals during the acquisition phase (Trial blocks: $F_{10,191} = 3.12, p < 0.001$) and the reversal phase (Trial blocks: $F_{4,83} = 70.30, p < 0.001$). REW increased during the transition phase (Trial blocks: $F_{1,29} = 72.00, p < 0.001$). Sex effects on REW were found for the transition and reversal phases. Female pigs found more rewards (Sex: Transition, $F_{1,29} = 9.18, p = 0.005$; Reversal, $F_{1,83} = 5.86, p = 0.018$) and displayed a different rate of change for REW (Sex by Trial blocks interaction: Transition, $F_{1,29} = 9.18, p = 0.005$; Reversal, $F_{4,83} = 9.25, p < 0.001$). Both findings appear to be the result of females finding more rewards during the first trial block of the reversal phase.

Judgment bias task

Discrimination training

Of the 20 piglets that were trained, one female and one male failed to reach criterion level within the maximum of 30 discrimination training sessions. For the remaining piglets, no difference was found for the average number of training sessions required (Mean \pm SD: females 22.33 ± 6.16 ; males 22.11 ± 5.99) to complete the discrimination training phase of the experiment (Sex: $F_{1,9} = 0.01, p = 0.940$).

Judgment bias testing

Cue type affected the percentage of optimistic choices (OC; see **Figure 4.4**) during judgment bias testing (Cue type: $F_{4,73} = 33.98, p < 0.001$). OC increased with increasing similarity to the learned positive cue. No effect of sex on OC was found. When OCs were separated for the first and last two judgment bias test sessions, an effect of repeated testing on OC was found (Test session: $F_{1,153} = 6.59, p = 0.011$; see **Figure 4.5**). OC decreased with repeated testing. Again, no sex effect was found for the separate test sessions.

The latency to respond (**Figure 4.6**) during judgment bias testing was affected by cue type (Cue type: $F_{4,73} = 6.69, p < 0.001$). Latency to respond decreased as similarity to the learned positive cue increased. No effect of sex on latency to respond was found.

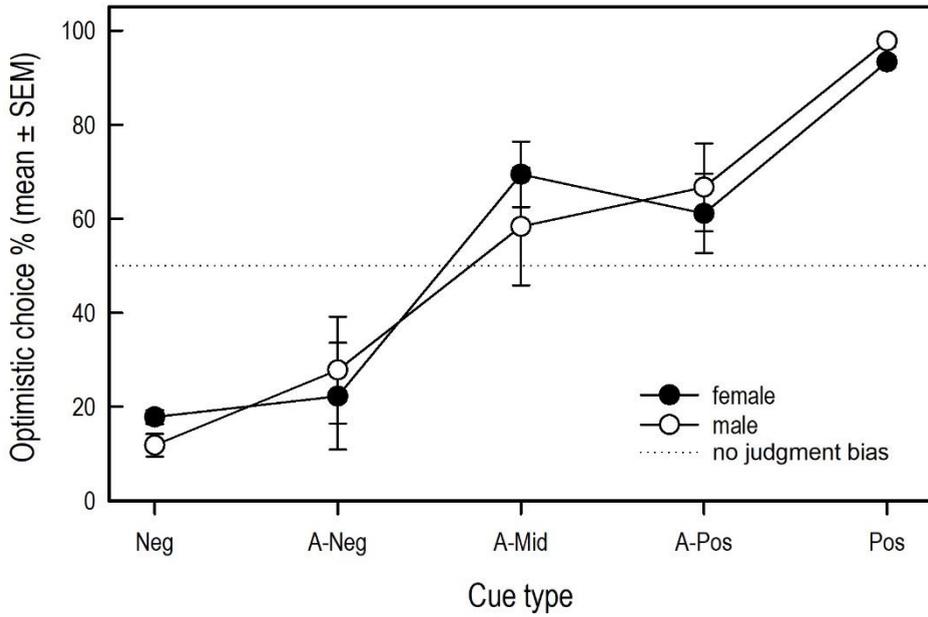


Figure 4.4 Optimistic choice percentage of female and male pigs in the judgment bias task.

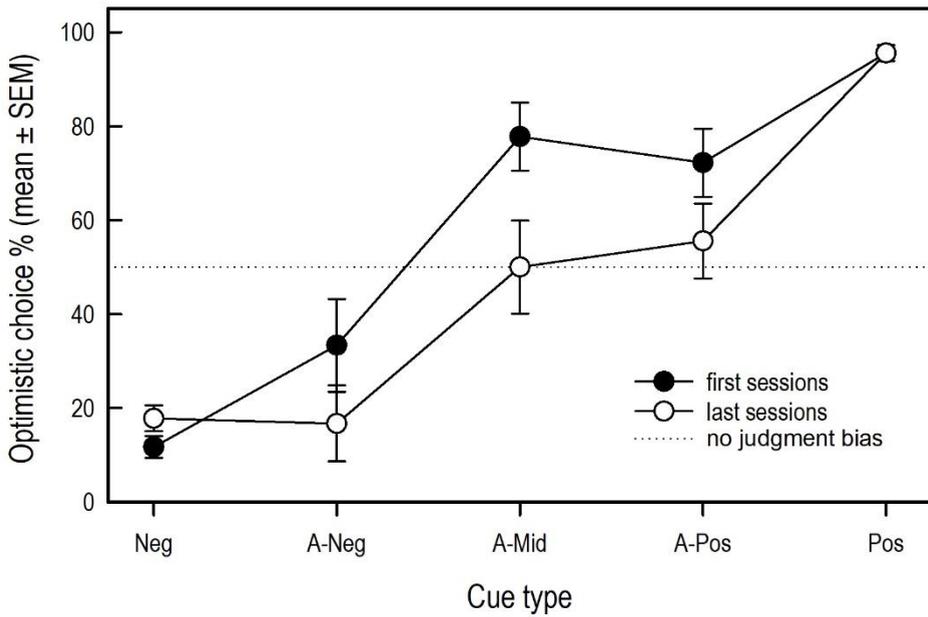


Figure 4.5 Optimistic choice percentage of all pigs combined for first and last test sessions in the judgment bias task.

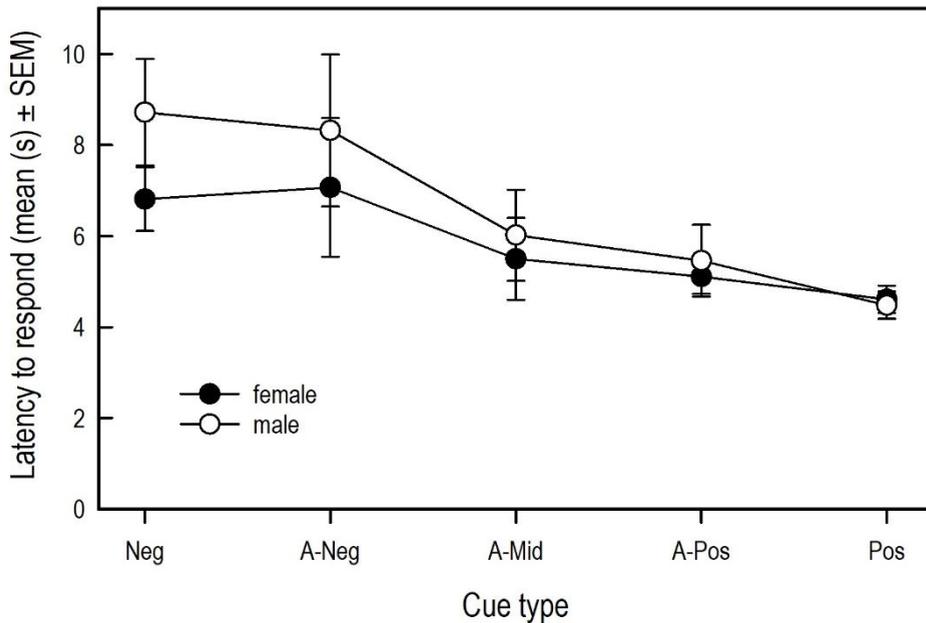


Figure 4.6 Latency to respond of female and male pigs in the judgment bias task.

DISCUSSION

In the present study, the cognitive performance of female and male pigs was compared. It was expected that males and females would show a comparable performance in the spatial holeboard task. This was true for most measures of spatial learning and memory, such as working and reference memory. However, contrary to expectations, female and male pigs differed in their response to the reversal phase of the task. In a judgment bias task, it was expected that both groups of pigs would show a comparable, optimistic judgment bias. Indeed, no sex effects were found on judgment bias. However, the measured level of optimism was low. An effect of repeated testing was the cause for this unexpected find.

Spatial holeboard task

Spatial learning and memory of female and male pigs were compared in the appetitively motivated holeboard task. All pigs successfully acquired the task,

as was expected based on previous holeboard studies with pigs (e.g. Arts *et al.*, 2009; Bolhuis *et al.*, 2013; Antonides *et al.*, 2016; Grimberg-Henrici *et al.*, 2016). All pigs improved their memory scores over the course of the experiment. Additional measures of successful acquisition of the task, such as latency scores and exploration measures, also showed an improvement over time. Both female and male pigs became faster and more efficient at performing in the holeboard task.

Spatial learning and memory

No differences were found between female and male pigs for any of the spatial memory measures that were examined. Working memory (WM) and reference memory (RM), forms of short- and long-term memory, respectively, are the main indicators of spatial learning and memory in the holeboard task. Both groups of pigs had comparable WM and RM scores throughout both the acquisition and the reversal phases of the experiment. These results are in concordance with a previous study which used both sexes to assess pigs' spatial cognition in the holeboard and reported a lack of differences between sexes in average performance (Bolhuis *et al.*, 2013). As the present study is the first to compare baseline performances of male and female conventional pigs (Haagensen *et al.* (2013a) used Göttingen minipigs), it provides an important step in validating studies which did not test for sex as a possible confounding influence on their results (e.g. Gieling *et al.*, 2014; Antonides *et al.*, 2015a, 2016). Also, a comparable spatial memory performance in the holeboard task of males and females implies that results from studies using pigs of only one sex can be generalized to describe the performance of the other sex as well.

It is important to note that in the present study, we assumed that both groups of pigs did not experience their housing and/or testing conditions as stressful. The pigs were housed in enriched pens and were extensively habituated to being tested in the holeboard. Therefore, no conclusions can be drawn about a possible sex difference caused by stress on pigs' WM and RM scores as measured by the holeboard task. Stress responses during spatial learning tasks have been found to differentially affect males and females in rats (e.g. Perrot-Sinal *et al.*, 1996; Beiko *et al.*, 2004; Harris *et al.*, 2008). Differences in WM and RM scores between males and females disappeared when stress prior to and during testing was eliminated by familiarizing animals with testing conditions (Perrot-Sinal *et al.*, 1996; Beiko *et al.*, 2004).

This suggests that sex differences in pigs' performance in a spatial holeboard task may only occur when experimental treatment and/or testing conditions act as stressors. Apparent differences in stress response between female and male pigs have been reported. For example, females have higher levels of plasma cortisol after mixing and handling stress at the slaughterhouse (D'Eath *et al.*, 2010; Guàrdia *et al.*, 2012). These findings indicate that future studies comparing the stress response of female and male pigs in general and its effects on spatial holeboard performance in particular would be useful. Ruling out the possible sexually dimorphic effects of stress will validate the use of pigs of either sex (or both) in spatial holeboard tasks.

Sex difference in reversal learning

Unexpectedly, the present study found a sex-based difference in the response to the reversal phase of the spatial holeboard task. Female piglets were faster to find their first reward, found a higher number of rewards and visited a higher number of locations during the first trials of the reversal phase. These findings are indicative of a difference between females and males in response flexibility (as is commonly measured using reversal training, e.g. Bolhuis *et al.*, 2004). This explanation suggests that when there was an unexpected change of rewarded locations, female pigs were quicker than the males to explore other, previously unrewarded, locations. Differences between females and males in response flexibility have been reported for numerous other species (e.g. zebra finches: Brust *et al.*, 2013; guppies: Lucon-Xiccato and Bisazza, 2014; rats: Noschang *et al.*, 2012; mice: Stack *et al.*, 2008). Sex effects on reversal learning in pigs have been examined using a T-maze task (Bolhuis *et al.*, 2004; Elmore *et al.*, 2012) and an 8-arm radial maze task (Dilger and Johnson, 2010). Elmore *et al.* (2012) found that when their pigs were started on the reversal phase (where a reward was moved to the opposite arm in the T-maze), they were less likely to choose an arm. Interestingly, a sex effect was found for this non-compliance, with control males failing to make a choice more often than control females. The other studies (Bolhuis *et al.*, 2004; Dilger and Johnson, 2010) found no influence of sex on reversal learning. Two holeboard studies using pigs of both sexes tested their animals in a reversal phase of the task (Antonides *et al.*, 2015a, 2016). However, a possible sex effect was not explored.

Personality type or coping style has been shown to influence both behavioural flexibility and explorative behavior in pigs (Bolhuis *et al.*, 2004;

Jansen *et al.*, 2009). Pigs classified as ‘high-resisting’, based on number of escape attempts during a backtest, are less flexible in their behavior, performing relatively poorly during reversal tasks (Bolhuis *et al.*, 2004). They are also characterized by a less thorough exploration of their environment (Jansen *et al.*, 2009; Bolhuis *et al.*, 2013). These findings lead to the question whether sex could be of influence on personality traits in pigs. Unfortunately, most studies on pig personality have compared females and castrated males. Often, no sex effects were found (e.g. Hessing *et al.*, 1993; Brown *et al.*, 2009; Camerlink *et al.*, 2014; cf. Spake *et al.*, 2012). However, when comparing females and intact males (as were used in our study), males were found to have a more high-resisting personality type. Male pigs struggled more often, vocalized more and started struggling faster than female pigs during the backtest (Kranendonk *et al.*, 2006). Whether there is a general relationship between sex and personality type in pigs must be addressed in future studies.

Further investigation of a possible sex effect on reversal training in additional tasks would be valuable to assess whether there is a general difference in response flexibility. A difference in response flexibility between female and male pigs would have potential consequences for pig welfare, as it implies that males are slower to cope with changes in their environment. Common management practices in pig farms include such changes [e.g. fattening pigs commonly move through several different housing systems during their life (Marchant-Forde, 2009)]. Understanding differences in behavioral flexibility between female and male pigs will allow for the welfare needs of both sexes to be taken into account.

Judgment bias task

In an active choice judgment bias task, female and male pigs’ cognitive measures of affective state were compared. Most pigs reached criterion performance level during discrimination training, as is required for judgment bias testing. This high success rate is comparable to those of previous studies using the same task (Murphy *et al.*, 2013b, 2015).

Judgment bias

As expected, no sex effects were found on measures of judgment bias in pigs. Optimistic choice percentage (OC) and latencies to respond did not differ between female and male pigs, for any of the ambiguous tone cues. In other

studies assessing sex effects on judgment bias, latencies to respond were also comparable for female and male pigs (Asher *et al.*, 2016; Carreras *et al.*, 2016a). As both groups in the current study were housed in similar, enriched environments, a difference in baseline judgment bias would have been an unexpected finding. Our comparison of female and male pigs is therefore another step towards validating the use of pigs of either sex (or both) when using a judgment bias task. This could be of particular value to welfare research on finishing pigs. Both female and male piglets raised for slaughter experience mostly similar conditions throughout their lives. The present study's results show that both sexes could be used as an appropriate model for this category of pigs as far as judgment bias tasks are concerned. However, it is important to note that we only compared the performance of animals that were presumed to be experiencing good welfare. It still needs to be investigated whether female and male piglets respond differently to situations assumed to induce poor welfare. For example, it has been suggested that sex could be an influencing factor on susceptibility to negative affective states (Carrier and Kabbaj, 2012; Sachs *et al.*, 2014). From these studies, it appears that females are more likely to develop a negative affective state in response to a stressor. Interestingly, the only animal studies reporting a sex difference in judgment bias found that females from a poor welfare group responded more optimistically than their male counterparts (Briefer and McElligott, 2013; Barker *et al.*, 2016). Future studies comparing the performance of female and male pigs experiencing negative affective states would therefore be valuable for further understanding of results of judgment bias tasks.

Effect of repeated testing

Both male and female pigs were only slightly more likely to make an optimistic choice than a pessimistic one following presentation of the intermediate ambiguous tone. An optimistic bias was expected as the pigs were housed socially in an enriched environment. Contact with conspecifics and the presence of rooting material are assumed to provide pigs with a good welfare state and resulting positive affective state (Studnitz *et al.*, 2007; D'Eath and Turner, 2009). A previous study applying a judgment bias task to pigs also found an optimistic bias in animals housed in enriched conditions (Douglas *et al.*, 2012). By contrast, Döpjan *et al.* (2013) do not mention any enrichment for their study subjects and they found their pigs to respond optimistically to all ambiguous stimuli. A study by Murphy *et al.*, which used

the same active choice task and housing conditions as the present study, found their group of conventional pigs to respond to the middle ambiguous tone-cue with an average OC of 46% (Murphy *et al.*, 2013b). A later study by Murphy *et al.* (2015) found much higher OC values, with normal birth weight animals (comparable to those used in the current study) choosing optimistically in response to the middle ambiguous tone in over 80% of trials.

Animals learning about the outcome of ambiguous testing trials appears to be an important reason for unexpected pessimistic biases. When ambiguous trials are not rewarded, as is the case with most judgment bias tasks (Mendl *et al.*, 2009; Roelofs *et al.*, 2016), animals could learn to associate an unfamiliar stimulus with the absence of reward. This could lead to a decrease in optimistic responses, irrespective of the animals' affective state. Learning about the outcome of ambiguous trials is especially likely when rewards were always present during discrimination training. The lack of reward during ambiguous trials will then likely be unexpected, which facilitates learning of the contingency between this cue-type and non-reward (Jamieson *et al.*, 2012). Several judgment bias studies have reported on the effects of repeated unrewarded ambiguous trials (e.g. Brilot *et al.*, 2010; Doyle *et al.*, 2010b). Animals were observed to develop a reluctance to respond to ambiguous cues. Murphy *et al.* (2013b) reported similar findings, with their pigs reducing optimistic choices with repeated testing. This could have been a possible explanation for their animals displaying a rather low optimistic choice percentage in spite of their housing conditions. Similarly, our pigs showed a decrease in OC with repeated testing. During the first test sessions, OC was 78%, which dropped to 51% for the final test sessions. Possibly, learning about the absence of rewards during ambiguous testing trials caused our animals to display a rather low level of optimistic judgment bias. The relatively high OCs found by Douglas *et al.* (2012) and Döpjan *et al.* (2013) could then be explained by the fact that they presented their pigs with fewer ambiguous trials. This provided their animals with less opportunities to learn about the outcome of ambiguous trials. The high OC found by Murphy *et al.* (2015) can be explained by their practice of rewarding ambiguous trials. Pigs learning about a positive outcome of ambiguous trials could influence results in a manner that is similar to learning about the absence of reward, i.e. ambiguous trials are no longer ambiguous. Although limiting exposure to ambiguous stimuli seems an effective measure to prevent loss of ambiguity, it also limits the amount of data collected. Other suggested measures to avoid an effect of repeated testing on judgment bias are the use of secondary reinforcers and partial reinforcement schedules during discrimination

training (Roelofs *et al.*, 2016). Döpjan *et al.* (2017) recently reported promising results using a partial reinforcement schedule when testing judgment bias in pigs.

CONCLUSION

No sex effects were found for most measures of learning and memory in the spatial holeboard. A difference in performance between females and males was only detected when the pigs were presented with a reversal task. This finding suggests that females have a higher level of response flexibility than males. Performance in the judgment bias task was comparable for female and male pigs. However, an effect of repeated testing was found, which warrants further investigation to improve the implementation of judgment bias tasks. As differences in cognitive performance between the sexes may be task-dependent, future studies comparing the performance of female and male pigs in other tasks are encouraged.

5

Discrimination learning and judgment bias in low birth weight pigs

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ABSTRACT

Low birth weight (LBW) is a risk factor for cognitive and emotional impairments in humans. In pigs, LBW is also a common occurrence, but unlike in humans, its effects on cognition and emotion have received only limited scientific attention. To assess whether LBW pigs suffer from impaired cognitive and emotional development, we trained and tested 21 LBW and 21 normal birth weight (NBW) pigs in a judgment bias task. Judgment bias is a cognitive measure of emotional state which reflects the influence of emotion on an animal's interpretation of ambiguous stimuli. In the judgment bias task, pigs first received discrimination training, allowing assessment of cognitive performance. Pigs were trained to perform a specific behavioral response to two auditory stimuli, predicting either a positive or negative outcome. Once pigs successfully discriminated between these stimuli, they were presented with intermediate, ambiguous stimuli. The pigs' responses to ambiguous stimuli were scored as optimistic (performance of 'positive' response) or pessimistic (performance of 'negative' response). Optimistic interpretation of an ambiguous stimulus is indicative of a positive emotional state, whereas a pessimistic interpretation is indicative of a negative emotional state. We found LBW pigs to require more discrimination training sessions than NBW pigs to reach criterion performance, suggesting that LBW causes a mild cognitive impairment in pigs. No effects of LBW on judgment bias were found, suggesting a similar emotional state for LBW and NBW pigs. It is possible the enriched housing conditions applied during our study influenced these results.

INTRODUCTION

Low birth weight (LBW) is a known risk factor for impaired cognitive and emotional development in humans. Children who were small for gestational age at birth are more likely to experience learning difficulties (O’Keeffe *et al.*, 2003; Yu and Garcy, 2018) and show impaired academic performance (Strauss 2000; Larroque *et al.*, 2001; Lindström *et al.*, 2017) throughout childhood and adolescence. In terms of emotional development, lower birth weight is associated with increased likelihood of anxiety in adulthood (Lahti *et al.*, 2010). Furthermore, being small for gestational age increases the risk of developing emotional disorders (e.g. anxiety disorder, depression) in preterm babies (Boyle *et al.*, 2011; Lahat *et al.*, 2017). Together, these studies show that LBW can have long-lasting effects on the highly associated processes of cognitive and emotional functioning (Lazarus, 1982).

LBW is also becoming a common occurrence in commercially housed pigs. This is a result of sows producing increasingly large litters (Rutherford *et al.*, 2013) and being unable to provide sufficient oxygen and nutrients for proper development of all of the fetuses (Père and Etienne, 2000; Wähner and Fischer, 2005). The resulting intra-uterine growth restriction is very comparable to how LBW develops in humans (Cox and Marton, 2009; Gayatri *et al.*, 2017). Unlike in humans, however, the long-term effects of LBW on cognition and emotion have not yet been extensively studied in pigs. Such potential effects are of interest, as pigs depend on both learning and memory as well as emotional processes for successful coping with their environment (Held *et al.*, 2002; Boissy *et al.*, 2007).

The effects of LBW on post-weaning cognitive performance in pigs are not fully understood, as the studies that have been done so far have produced contradictory results. For example, LBW pigs have been reported to show impaired (Gieling *et al.*, 2012; Radlowski *et al.*, 2014; Roelofs *et al.*, 2018), similar (Gieling *et al.*, 2014) or even improved (Antonides *et al.*, 2015a) spatial learning and memory compared to normal birth weight (NBW) pigs. Studies aimed at the post-weaning emotional functioning of LBW pigs have mostly relied on physiological measures of stress, mainly plasma cortisol concentration (Rutherford *et al.*, 2013). Results of these studies suggest that LBW pigs react more strongly to acute stressors than NBW pigs (e.g., Poore *et al.*, 2002; Poore and Fowden, 2003). Behavioral measures of emotional state in pigs, which can provide a better indication of the valence of experienced

emotions (Murphy *et al.*, 2014), have not yet been widely applied to compare LBW and NBW pigs.

Judgment bias is one such behavioral measure of emotional state in animals. It describes the influence of emotion on the interpretation of ambiguous, i.e. emotionally neutral, stimuli (Paul *et al.*, 2005; Mendl *et al.*, 2009). To measure judgment bias, animals are first trained to successfully discriminate between a stimulus signaling a positive outcome (e.g., a food reward) and a stimulus signaling a negative outcome (e.g., punishment or a smaller food reward). The animal has to perform a different behavioral response to each stimulus, and these responses are then used to assess judgment bias. If an animal performs the behavior it has learned to associate with a positive outcome after being presented with an ambiguous stimulus (often intermediate between the positive and negative stimuli), this is scored as an optimistic response. If it performs the behavior it has learned to associate with a negative outcome, this is scored as a pessimistic response. An animal which makes more optimistic responses, is assumed to be in a more positive emotional state than an animal which makes more pessimistic responses (Mendl *et al.*, 2009; Roelofs *et al.*, 2016). Judgment bias tasks have been successfully applied to a variety of species, including humans and pigs (Miranda and Mennin, 2007; Roelofs *et al.*, 2016). For example, pigs respond more optimistically to ambiguous stimuli when housed in enriched conditions which were assumed to improve their emotional state (Douglas *et al.*, 2012). Because discrimination training is a necessary component of a judgment bias task, it allows for successive assessment of cognition (i.e., discrimination learning) and emotional state (i.e., judgment bias).

Murphy *et al.* have previously compared LBW and NBW pigs in a judgment bias task (Murphy *et al.*, 2015). They found LBW pigs to be equally capable of mastering the conditional discrimination task, but LBW pigs displayed a more negative judgment bias than NBW pigs. However, in their study only male pigs were tested. Accounting for a potential difference between females and males is relevant when training and testing LBW pigs in a judgment bias task. First, LBW pigs have been found to display altered stress responses compared to NBW pigs (Poore and Fowden, 2003). Stress is known to influence learning and memory abilities, and such effects can be sex-specific (Bowman *et al.*, 2003; Healy *et al.*, 2009). Second, the increased risk of emotional disorders due to LBW found in humans appears to affect females more than males (Costello *et al.*, 2007; Van Lieshout and Boylan, 2010). Therefore, repeating a judgment bias study with both female and male LBW

pigs is relevant for assessing the potential differential effect of sex on the performance of LBW in pigs.

The aim of the present study was to assess the effects of birth weight on discrimination learning and judgment bias in pigs. This was assessed by an active choice judgment bias task, where the pigs had to perform active behavioral responses to both a positive and a negative stimulus. Several improvements to a previous study assessing judgment bias in LBW pigs were applied. First, we assessed a larger sample size consisting of both female and male pigs to account for possible differential effects of stress on LBW pigs' cognition and emotional state. Second, to further assess a potential difference in stress response between LBW and NBW pigs, markers of acute and chronic stress (salivary and hair cortisol concentrations, respectively) were included. We expected LBW pigs would show impaired discrimination learning and a more pessimistic judgment bias compared to NBW pigs. Additionally, LBW pigs were expected to have an exaggerated stress response, with increased hair cortisol concentrations and a stronger salivary cortisol response to an acute stressor.

METHODS

Compliance with ethical standards

All methods that demanded the handling of live animals were reviewed and approved by the local animal welfare body (Animal Welfare Body Utrecht) and were conducted in accordance with the recommendations of the EU directive 2010/63/EU.

Animals

Pigs [(Yorkshire x Dutch Landrace) x Duroc] were selected from the commercial pig breeding farm of Utrecht University. From 14 different litters, 21 LBW-NBW sibling pairs were selected (11 female pairs and ten male pairs). The experiment took place in two separate rounds due to limited availability of LBW piglets, with 20 piglets selected for the first round (10 LBW-NBW pairs, trained and tested in March-June 2017) and 22 piglets selected for the second round (11 LBW-NBW pairs, trained and tested in August-November 2017). For each selection round, all piglets born over a period of one week were weighed on the day of birth. Three criteria were

used to select LBW piglets: 1) birth weight was a minimum of 1 *SD* below the litter average, 2) birth weight was a minimum of 1 *SD* below the study population average, yielding a maximum birth weight of 1050 grams, and 3) litter size was a minimum of 10 piglets. For each LBW piglet, a NBW piglet was selected from the same litter based on two criteria: 1) piglet had the same sex as the selected LBW piglet, and 2) birth weight was closest to litter average. To improve chances of survival for LBW piglets, non-selected siblings were cross-fostered when litter size exceeded the sow's number of functional teats. Furthermore, milk replacer was provided when piglets were two to three days old. One female LBW piglet was euthanized due to complications from a rectal prolapse during the early stages of discrimination training in the judgment bias task. Her data was excluded from analysis, resulting in a final sample size of 41 pigs.

Housing

At approximately four weeks of age, piglets were weaned and moved to the research facility, which was located next to the breeding farm. Per round, pigs were housed in two adjacent pens (measuring $\sim 4 \times 5$ m), with LBW and NBW piglets housed separately. Pens had concrete floors and were supplied daily with fresh straw bedding. To protect piglets from the cold, the pens contained a covered piglet nest equipped with rubber mats covered by straw and plasticized PVC slats hanging in front of the entrance. The nests also contained heat lamps until the pigs were approximately eight weeks old. The research facility was naturally ventilated. Minimum and maximum temperatures ranged from 3 to 34°C over the course of the experiment. Pigs were only tested if they voluntarily entered the testing apparatus, to avoid testing animals in heat stress. Pigs received $1/3$ of their daily food ration in the morning (prior to training) and the remaining $2/3$ in the afternoon (after training). Water was provided *ad libitum*. Individual recognition of animals was facilitated by a letter sprayed on the pigs' backs.

Judgment bias task

Apparatus

The judgment bias apparatus consisted of a main arena (3.6 x 2.5m) connected to a start box (1.2m²) by an antechamber (**Figure 1.1**). The start box was equipped with a guillotine door, operated by a rope and pulley

system, through which pigs could enter the arena. Two goal-boxes (40cm wide) were located near the corners at the back of the arena. Each of these contained a food bowl from which the pigs could obtain a reward (M&M's® Milk Chocolate candies). The food bowls were equipped with a false bottom, beneath which additional candies were placed to avoid discrimination between goal-boxes based on scent cues (for details of food bowls, see **Figure 1.2B**). Each food bowl was covered by a synthetic ball (JollyBall Dog Toy, ø 24 cm, 1400 g, Jolly Pets, Ohio, USA), so pigs could not see which bowl contained a food reward. The goal-boxes were equipped with guillotine doors which could be operated from outside the apparatus by rope and pulley systems. Tone-cues were generated using open source software (Audacity; <http://audacity.sourceforge.net/>) and played by speakers mounted to the back of the arena (Logitech z-313, Logitech Europe S.A., Morges, Switzerland). The entire judgment bias apparatus was cleaned daily and rinsed immediately if a pig soiled it during training.

Habituation and pre-training

After moving the pigs to the research facility, they were first habituated to being handled by the researchers over a period of 1 week. The pigs were then gradually habituated to the judgment bias apparatus by letting them explore the apparatus in increasingly smaller groups. Habituation finished when all pigs explored the apparatus individually and were able to lift the balls off the food bowls.

Pre-training started with forced trials, which consisted of a pig entering the start box and waiting there for the door to the arena to open. When the door was opened, they could enter the arena and retrieve a single candy from one of the goal-boxes. Only one of the goal-boxes was open and baited per trial, with the location of the reward alternating between the left and right goal-box. After retrieving the reward, the goal-box was closed and the pig returned to the start box for its next trial. Number of trials was gradually increased from six trials until each pig performed a session of 12 consecutive trials.

Next, pigs received four sessions (one session daily) during which they were introduced to a 'positive' tone-cue predicting a large reward (four candies) in one goal-box and a 'negative' tone-cue predicting a small reward (one candy) in the other goal-box. Two pure tones were used as tone-cues: a 1000 Hz (high) and a 200 Hz (low) tone (waveform: sine, amplitude: 1). The valence

of the tone-cues (positive or negative) and the associated goal-boxes (large reward in left or right goal-box) were counterbalanced across animals, for both birth weight and sex. A session of forced trials now consisted of six positive and six negative trials in a pseudorandom order with no more than two identical trials in a row. At the start of a trial, a tone-cue was played while the pig was in the start box. When the pig entered the arena, it could retrieve the appropriate reward from the appropriate goal-box (the other goal-box remained closed). When the pig lifted the ball covering the food bowl to gain access to the reward, the tone-cue was stopped.

The final phase of pre-training consisted of two sessions of ‘open choice’ trials, during which both goal-boxes were open and the pig had to choose a goal-box after hearing the tone-cue. If a pig chose the rewarded goal-box (correct choice), the tone-cue stopped playing, the pig consumed the reward and returned to the start box for its next trial. If a pig chose the unrewarded goal-box (incorrect choice), this goal-box was closed and the tone-cue kept playing until the pig visited the correct goal-box to retrieve the reward. In total, habituation and pre-training took approximately six weeks.

Discrimination training

Discrimination training consisted of daily sessions of 13 trials each. The first three trials were ‘forced’ trials where only the correct goal-box was open. The first of these forced trials was always a negative trial, followed by a negative and positive trial in a random, daily changing order. Forced trials were followed by five negative and five positive ‘free’ trials in pseudorandom order, with no more than two identical trials in a row. Each daily session had a different order of free trials. Free trials were comparable to open choice trials, except that an incorrect choice was followed by closing both goal-boxes, after which the pig had to return to the start box without receiving a reward. The same consequences applied if a pig did not choose a goal-box within 30 seconds (recorded as an omission to choose). During every fifth discrimination training session, the first three negative and first three positive free trials were replaced by open choice trials, to allow all pigs to maintain an association between tone-cues and goal-boxes. Discrimination training continued until a pig reached a criterion score of at least four out of five correct choices for both negative and positive free trials for three consecutive training sessions. Pigs that did not reach criterion within a maximum number of 45 training sessions did not proceed to judgment bias testing.

Judgment bias testing

For judgment bias testing, each pig performed four sessions, consisting of 16 trials each. A testing session was similar in setup to a training session, with an additional three ambiguous trials. During each of these ambiguous trials, a different ambiguous tone-cue was played. Ambiguous cues were intermediate between the learned positive and negative tones, with frequencies at equal intervals between the training tones on a logarithmic scale: 299.07 Hz, 447.21 Hz and 668.74 Hz. Depending on whether the high or low frequency training tone was used as the positive stimulus, the ambiguous tones represented a ‘near-negative’ ambiguous cue (most similar to the negative tone-cue), an intermediate ambiguous cue (the 447.21 Hz tone) and a ‘near-positive’ ambiguous cue (most similar to the positive tone-cue). For each daily testing session, trials 6, 11 and 16 were the ambiguous trials. The intermediate tone-cue was always presented during trial 6, while the near-negative and near-positive tone-cues alternated between trials 11 and 16. Whether an ambiguous trial was preceded by a negative or positive trial was counterbalanced across testing sessions, to control for potential effects of a preceding trial and its consequences on pigs’ expectations. Ambiguous trials were always unrewarded.

Preventing loss of ambiguity

Previous judgment bias studies have shown that leaving ambiguous test trial unrewarded can result in a loss of ambiguity, where animals learn to associate ambiguous stimuli with a lack of reward (Roelofs *et al.*, 2016). Several measures were applied in this study to prevent pigs from learning about the outcome of ambiguous trials. First, from tone introduction onwards, pigs were trained on a partial reinforcement ratio schedule, with an 80% reinforcement ratio as suggested by Döpjan *et al.* (2017). As a result, one negative and positive trial per session were unrewarded. Unrewarded sessions were randomly determined, however the first and last positive and negative trial of a session were always reinforced. Second, to maintain responsiveness of pigs during unrewarded trials, a secondary reinforcer was used. During training, every correct choice was reinforced with a clicker, as a signal of correct responding even during unrewarded trials.

Variables

The following variables were calculated per pig during discrimination training:

- **Sessions to criterion** was calculated as the number of discrimination training sessions needed to reach criterion level and proceed to judgment bias testing.
- **Number of correct choices** was calculated as the number of correct choices made during a training session, per cue type (excluding forced trials).

The following variables were calculated per pig during judgment bias testing:

- **Optimistic choice** was calculated as the proportion of optimistic choices (i.e. approaching the location associated with a large reward) made during testing sessions, per cue type (negative, positive and ambiguous).
- **Latency to choose** was calculated as average time in seconds elapsed between a pig leaving the start box and lifting a ball in a goal-box, per cue type (negative, positive and ambiguous).

Cortisol analysis

Hair cortisol

Hair samples were collected twice: at weaning and at the end of the experiment, when the pigs were approximately 4.5 months old. Hair was collected from the left flank of each pig with a disposable razor (single edged disposable prep razor, Kai Medical, Solingen, Germany), using a new razor for each sample. Determination of hair cortisol concentration was based on a protocol by Davenport *et al.* (2006). In short, samples were washed and dried after collection. Approximately 35 mg of hair was ground with a bead beater (TissueLyser II, QIAGEN Benelux B.V., Antwerp, Belgium) for a minimum of 2x 15 minutes at 30 Hz, in 2 mL tubes containing three 2.3 mm steel beads (BioSpec, Lab Services B.V., Breda, the Netherlands). Corticosteroids were extracted by adding 1 mL methanol to the ground hair and incubating samples for 24h with slow rotation. Of the extract, 0.6 mL was dried using a vacuum centrifuge. Dried extracts were dissolved by adding

0.3 mL phosphate buffer. Hair cortisol concentrations were then determined in duplo using a Salimetrics Salivary Cortisol ELISA kit. Intra-assay and inter-assay coefficients of variation (CV) were 7.2 and 7.7, respectively.

Salivary cortisol

Saliva samples were collected from each pig prior to and after their first individual pre-training session (consisting of six forced trials). Pre-stressor samples were collected at approximately 14:00 in the afternoon in their home pens. Post-stressor samples were taken approximately 20 minutes after a pig's pre-training session, to allow for the peak in cortisol response to develop (Merlot *et al.*, 2011). Saliva samples were collected by allowing each pig to chew on two cotton swabs (Cotton Swabs 150 mm × 4 mm WA 2PL; Heinz Herenz, Hamburg, Germany) until they were sufficiently moistened. Saliva was collected from the swabs by centrifuging them in saliva collection tubes (Salivette, Sarstedt, Germany) at around 3524 g for 10 minutes at 10°C. Saliva samples were stored at -20°C until salivary cortisol concentration was determined in duplo using a Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics B.V., The Hague, the Netherlands). Intra-assay and inter-assay CVs were 4.4 and 8.5, respectively.

Statistical analysis

All statistical analyses were performed using R statistical software, version 3.4.2 (R Core Team, 2017). For mixed models, package lme4 (Bates *et al.*, 2015) was used. For linear mixed models the random effect structure was assessed using Restricted Maximum Likelihood (REML) estimation, fixed effect structure was assessed using Maximum Likelihood (ML) estimation. Model selection was based on Akaike's information criterion (AIC), using package MuMIn (Barton, 2018). Round (first or second round of selected animals) did not improve fit of mixed models based on AIC, suggesting it did not have explanatory value in the models. Therefore, this factor was not included in analysis. Confidence intervals were calculated as 95% parametric bootstrap intervals with 1000 samples. Type III and Wald tests were used to test significance (with $\alpha = .05$) of fixed effects of linear models and generalized linear/logistic regression models, respectively. Unless indicated otherwise, results are presented as mean \pm SEM.

Birth weight and growth

Average birth weight of LBW and NBW pigs was compared using Welch's *t*-test. To compare weekly weight gain of LBW and NBW pigs, a linear mixed model was used with Birth Weight, Week and Birth weight x Week interaction as fixed effects. Random effect structure consisted of random intercepts for Subjects nested in Litter.

Discrimination training

Sessions to criterion for LBW and NBW pigs were compared using a negative binomial generalized linear mixed model to account for overdispersion in the data. Only birth weight was included as a fixed effect, as inclusion of Sex did not improve the fit of the model based on AIC. Random effect structure consisted of random intercepts for Litter. For number of correct choices during training sessions, LBW and NBW pigs were compared using a logistic regression model with Session as fixed effect. Inclusion of Birth weight, Sex or interaction terms did not improve the fit of the model based on AIC. Random effect structure consisted of random intercepts for Pig nested in Litter.

Judgment bias

For optimistic choice and latency to choose, means were calculated across test sessions per cue type. Factors assessed during model selection were Birth weight, Sex, Cue type (negative, near-negative ambiguous, intermediate ambiguous, near-positive ambiguous or positive), Session (first two sessions combined versus last two sessions combined), Training duration (discrimination training was completed within 25 sessions or required more than 25 sessions) and all two-way interactions as fixed effects. Pig, Litter and Pig nested in Litter were assessed as potential random effects.

Effect of birth weight on optimistic choice was assessed using a logistic regression model. Based on AIC, a model with Cue type, Session and Cue type x Session interaction as fixed effects was selected. This suggests neither Birth Weight nor Sex were important to the model. Random effect structure consisted of random intercepts for Pig.

Latency to choose was analyzed using a linear mixed model with Cue type and Session as fixed effects. Inclusion of additional fixed effects (such as Birth Weight or Sex) did not improve the fit of the model based on AIC. Random effect structure consisted of random intercepts for Pig nested in Litter. Latencies to choose were \log_{10} transformed to improve distribution of residuals.

Cortisol concentrations

Factors assessed during model selection for hair and salivary cortisol were Birth weight, Sex and its interaction as fixed effects. For salivary cortisol, an additional factor of Sampling time (prior to or post stressor) was assessed. Pig, Litter and Pig nested in Litter were assessed as random effects.

The effects of birth weight on pigs' hair cortisol concentrations were analyzed using a linear mixed model with Birth weight, Sex and Birth weight x Sex interaction as fixed effects and random intercepts for Litter. The effects of birth weight on salivary cortisol concentrations before and after a pig's first individual pre-training session were analyzed using a linear mixed model with Birth weight as fixed effect and random intercepts for Pig.

To assess a potential relationship between judgment bias and chronic stress, mean optimistic choice percentage in the judgment bias task and mean hair cortisol at the end of the experiment were compared using Spearman's correlation as hair cortisol concentrations were not normally distributed.

RESULTS

Birth weight and growth

LBW piglets had on average a lower birth weight than NBW piglets (LBW: 0.83 ± 0.10 , NBW: 1.47 ± 0.23 ; $t_{28.45} = -11.76$, $p < 0.001$, 95% CI [-0.75, -0.53]). LBW piglets continued to have lower body weight throughout the duration of the experiment (Birth weight: $F_{1,26} = 95.54$, $p < 0.001$, 95% CI [-7.13, -1.57]) and had a slower growth rate than the NBW piglets (Birth weight x Week: $F_{13,487} = 6.42$, $p < 0.001$, 95% CI [-0.86, -0.47]; **Figure 5.1**).

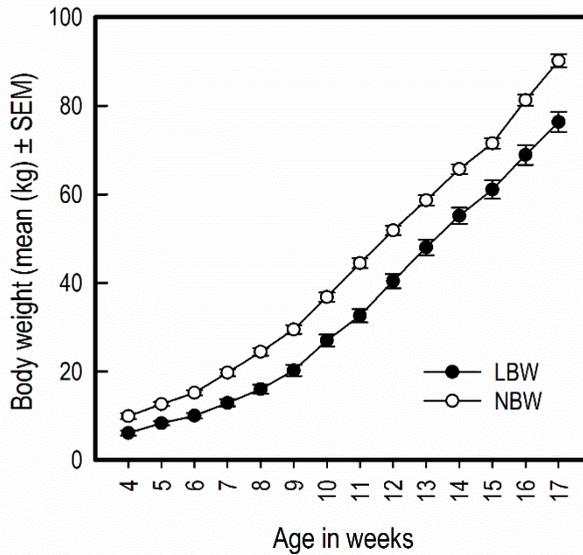


Figure 5.1 Average body weight in kilograms of LBW and NBW pigs from weaning until the end of the experiment.

Discrimination training

Of the 41 pigs that started discrimination training, a total of 5 pigs did not reach criterion performance: two LBW pigs (one female and one male) and three NBW pigs (two females and one male). Of the pigs that completed discrimination training, LBW pigs required a higher number of sessions to reach criterion compared to NBW piglets (LBW: 29.44 ± 8.77 , NBW: 24.83 ± 8.89 ; $X^2 = 4.62$, $df = 1$, $p = 0.032$, 95% CI [0.02, 0.36]).

During discrimination training sessions, the pigs initially only visited the location of the high reward, resulting in very few errors during positive trials, and very few correct choices during negative trials (**Figure 5.2**). On the number of correct choices during training sessions, only Session was found to have an effect. Pigs started to make more correct choices during negative trials as training progressed ($X^2 = 269.77$, $df = 1$, $p < 0.001$, 95% CI [0.24, 0.31]; **Figure 5.2**). As the pigs started increasing their visits to the location of the low reward, correct choices during positive trials slightly decreased ($X^2 = 12.26$, $df = 1$, $p < 0.001$, 95% CI [-0.10, -0.03]; **Figure 5.2**).

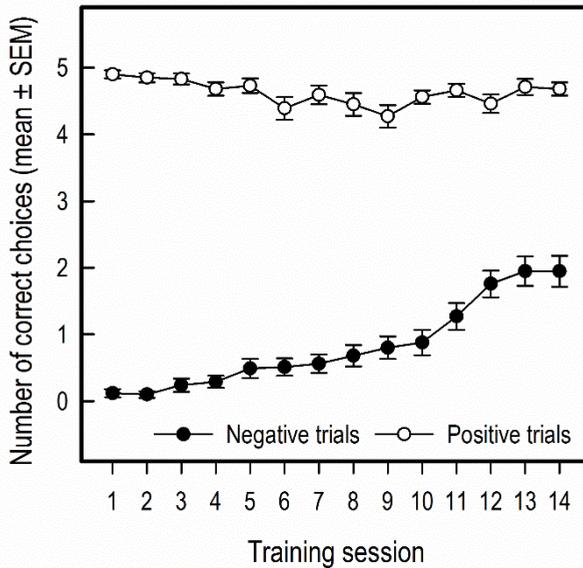


Figure 5.2 Mean number of correct choices for negative and positive trials during the first 14 sessions of discrimination training. As no effects of birth weight or sex were found, the data for all pigs have been combined. After 14 sessions, the first pigs reached criterion performance and moved on to judgment bias testing.

Judgment bias

Cue type affected optimistic choice, with all pigs increasing their optimistic choices as similarity to the positive tone cue increased ($X^2 = 305.18$, $df = 4$, $p < 0.001$, negative vs. positive tone 95% CI [3.64, 4.63]; **Figure 5.3A**). As inclusion of Birth weight or Sex did not lower model AIC, we can conclude that these factors did not influence optimistic choice during the judgment bias task (**Figure 5.3A**). No general effect of Session was found ($X^2 = 0.24$, $df = 1$, $p = 0.627$, 95% CI [-0.48, 0.29]), however the Session x Cue type interaction did affect optimistic choice ($X^2 = 12.00$, $df = 4$, $p = 0.017$), implying pigs changed their responses to certain tone cues over the course of testing. This was confirmed for the intermediate ($X^2 = 9.89$, $df = 1$, $p = 0.002$, 95% CI [-1.93, -0.46]) and near-positive ($X^2 = 8.87$, $df = 1$, $p = 0.003$, 95% CI [-2.01, -0.43]) ambiguous cues, with pigs reducing their optimistic choice as testing sessions progressed (**Figure 5.3B**).

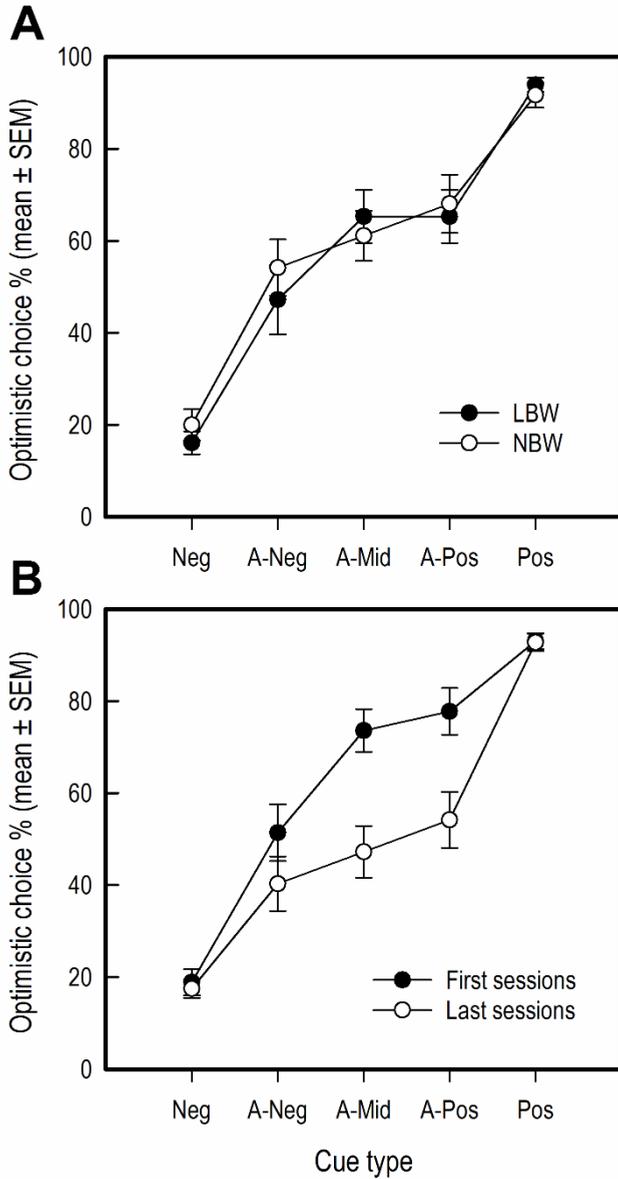


Figure 5.3 **A** Optimistic choice percentage of LBW and NBW pigs during judgment bias testing. As no effect of sex was found, data for female and male pigs are combined. **B** Optimistic choice percentage of all pigs combined for first and last test sessions.

For latency to choose, only effects of Cue type and Session were found. Latency to choose decreased as similarity to the positive tone cue increased ($F_{4,319} = 31.99$, $p < 0.001$, negative vs. positive tone 95% CI of \log_{10} transformed latencies [-0.28, -0.19]). Furthermore, pigs increased their latency to choose during the last test sessions, compared to the first ($F_{1,319} = 25.96$, $p < 0.001$, 95% CI [0.04, 0.10]), providing further indication of loss of ambiguity. As inclusion of any other factors did not lower model AIC, we can conclude that neither birth weight nor sex influenced latency to choose during the judgment bias task.

Cortisol concentrations

Hair cortisol

No effects of birth weight were found on hair cortisol concentrations, either at weaning ($F_{1,24} = 2.16$, $p = 0.155$, 95% CI [-9.43, 0.37]) or at the end of the experiment, at five months of age ($F_{1,24} = 0.02$, $p = 0.876$, 95% CI [-5.37, 5.39]). Sex did not influence hair cortisol concentrations, neither as a main effect (at weaning: $F_{1,24} = 0.00$, $p = 0.980$, 95% CI [-7.54, 3.68]; at 5 months old: $F_{1,24} = 0.29$, $p = 0.595$, 95% CI [-5.14, 7.63]), nor in interaction with birth weight (at weaning: $F_{1,24} = 1.25$, $p = 0.275$, 95% CI [-3.01, 10.83]; at 5 months old: $F_{1,24} = 0.02$, $p = 0.904$, 95% CI [-7.06, 8.15]).

No correlation was found between hair cortisol concentrations at the end of the experiment and mean optimistic choice percentage in the judgment bias task ($r_s = -0.06$, $p = 0.730$).

Salivary cortisol

As sampling time did not influence salivary cortisol concentrations, we can conclude that the pigs did not show an acute stress response to their first individual training session. Although the model with Birth weight as fixed effect had the lowest AIC, no difference between LBW and NBW pigs was found ($F_{1,39} = 1.02$, $p = 0.318$, 95% CI of \log_{10} transformed concentrations [-0.39, 0.13]).

DISCUSSION

In the present study we assessed the effects of low birth weight (LBW) and sex on discrimination learning and judgment bias in pigs. We were successful in selecting piglets with a significantly lower birth weight than the selected normal birth weight (NBW) pigs. This is important, as LBW is currently the best read-out parameter of intra-uterine growth restriction and its associated effects on brain development in pigs (Wu *et al.*, 2006; Gieling, 2013). Based on previous studies with humans and pigs, we expected to find both a cognitive impairment (O’Keeffe *et al.*, 2003; Gieling *et al.*, 2012; Radlowski *et al.*, 2014; Roelofs *et al.*, 2018; Yu and Garcy, 2018) and a more negative judgment bias (Lahti *et al.*, 2010; Murphy *et al.*, 2015) in LBW pigs. During discrimination training, LBW pigs were slower to consistently perform the correct behaviors in response to the correct cues. However, no differences between LBW and NBW pigs were found in judgment bias. This finding of a similar emotional state in both groups of pigs is supported by similar hair cortisol concentrations, which were used as a marker for chronic stress.

Discrimination learning

Birth weight had a mild effect on pigs’ cognitive abilities, based on the number of discrimination training sessions required to reach criterion performance. LBW pigs took longer to finish discrimination training compared to NBW pigs. This finding indicates that the LBW pigs had more difficulty learning the rules of the task, i.e., they took longer to consistently perform the correct responses to the training cues. Such a cognitive impairment was expected based on the substantial difference in birth weight between groups, as well as the lack of catch-up growth displayed by the LBW pigs. Catch-up growth has been shown to limit the risks for cognitive impairment in humans (Lindström *et al.*, 2017). It is unlikely that this difference in performance between LBW and NBW pigs was due to a difference in motivation to perform the task. Both groups of pigs showed comparable latencies to respond to training cues during judgment bias testing. Furthermore, LBW and NBW pigs do not differ in their food motivation in operant conditioning tasks (van Eck *et al.*, 2016).

Our finding of a post-weaning cognitive impairment in LBW pigs is comparable to results of multiple human studies. These studies also show impaired cognitive performance in LBW children which persists well beyond

childhood (e.g., Kormos *et al.*, 2014; Lindström *et al.*, 2017). In humans, the negative cognitive effects of LBW can be limited by ameliorating circumstances such as positive family attitudes (Yu and Garcy, 2018). Perhaps the enriched housing conditions applied during our study also had such an ameliorating effect on the pigs' cognitive performance (Sneddon *et al.*, 2000; Grimberg-Henrici *et al.*, 2016), resulting in only a mild impairment compared to the NBW pigs. Similar mild effects have also been found for other cognitive domains in LBW pigs, such as spatial learning and memory (Gieling *et al.*, 2012; Radlowski *et al.*, 2014; Roelofs *et al.*, 2018; cf. Antonides *et al.*, 2015a). However, the only other studies comparing LBW and NBW pigs in a discrimination training task found no effects of birth weight, with both groups of pigs requiring a similar number of training sessions to complete the task (Murphy *et al.*, 2013a, 2015).

The difficulty in the discrimination training task lies in correct responding to the negative cue. Pigs show a strong preference for the larger reward available after presentation of the positive cue, causing them to respond incorrectly during negative trials. During the first training sessions, pigs almost exclusively approach the positive goal-box, irrespective of the presented tone-cue. This has also been found in previous discrimination training tasks with pigs (e.g., Murphy *et al.*, 2013a), as well as in a delay discounting task (Melotti *et al.*, 2013). In the delay discounting task, the pigs' preference for a larger reward was so strong that instead of performing a response leading to a small reward, they resorted to omissions and failed to gain a reward at all.

The training protocol used in our study could have led to a more difficult cognitive challenge for the pigs, compared to the protocol used in the studies by Murphy *et al.* (2013a, 2015). In those studies, continuous reinforcement was applied during discrimination training, where a correct response (i.e., approaching the correct goal-box) was always rewarded. In our study, we applied a partial reinforcement schedule with one out of six training trials going unrewarded, irrespective of a pig's response. A possible difference in task difficulty between our study and those of Murphy *et al.* is reflected by the difference in required training sessions until the pigs reach criterion performance. In our study, pigs required an average of 25-30 training sessions, compared to an average of 16 training sessions needed in the study by Murphy *et al.* (2015).

In theory, both our study and those by Murphy *et al.* (2013a, 2015) applied a differential outcome paradigm (Holden and Overmier, 2014). In such a

paradigm, the two types of stimulus-response sequences (positive cue followed by approach to positive goal-box and negative cue followed by approach to negative goal-box) are followed by specific, different rewards (i.e., different quantities of food reward). However, it is possible the LBW pigs in our study had more difficulty reaching criterion performance because correct responses were not consistently rewarded. Task acquisition in general has been shown to slow down when partial reinforcement is applied (Sangha *et al.*, 2002; Grady *et al.*, 2016), especially when training sessions are widely spaced, as was the case in our study with one training session per day (Robbins, 1971). In addition, the unrewarded trials during each session had non-differential outcomes (i.e., both stimulus-response sequences lead to the same outcome of no reward; Holden and Overmier, 2014). A negative effect of non-differential outcomes has also been shown in LBW children, who only reach comparable performance to NBW children when trained on a differential outcome paradigm (Martínez *et al.*, 2012).

Judgment bias

We found no effect of birth weight or sex on judgment bias. LBW and NBW pigs displayed similar rates of optimistic choices in response to ambiguous tone-cues. Additionally, both groups had similar latencies to approach a goal-box after being presented with an ambiguous cue. These findings suggest that the LBW pigs in our study did not suffer from a more negative emotional state than the NBW pigs. This is also suggested by the similar hair and salivary cortisol concentrations found for LBW and NBW pigs. Human studies have reported an increased risk for emotional disorders such as anxiety and depression in LBW children (Lahti *et al.*, 2010; Boyle *et al.*, 2011; Lahat *et al.*, 2017). Similarly, LBW pigs in a previous study showed a more pessimistic judgment bias in response to ambiguous stimuli than NBW pigs (Murphy *et al.*, 2015). It is possible that we did not find a more negative emotional state in LBW pigs because they were housed in enriched conditions, which are known to have a positive effect on emotional state (de Jong *et al.*, 2000; Douglas *et al.*, 2012). In pigs, LBW has been shown to lead to increased vulnerability to stressors (Poore and Fowden, 2003), and in humans this vulnerability is more pronounced in females (Van Lieshout and Boylan, 2010). However, based on our housing conditions and the results from hair and salivary cortisol analysis, neither the LBW nor the NBW pigs were stressed. This lack of stress could explain why we were unable to detect (sex-specific) effects of LBW on emotional state. Furthermore, several differences

in study design could have contributed to the discrepancy in results between our study and that performed by Murphy *et al.* (2015).

First, our results are based on a bigger sample size of 18 LBW versus 18 NBW pigs, compared to eight LBW versus eight NBW pigs tested by Murphy *et al.* (2015). Using a larger sample size decreases the probability of chance findings (Taborsky, 2010; Button *et al.*, 2013). As a result of that, confounding factors influencing emotional state, such as personality (Krause *et al.*, 2017), are less likely to affect a study's results. That individual differences within groups could be a confounding factor is supported by the high variability shown in our study. Within the LBW and NBW groups, there were considerable differences in optimistic choice percentage, with certain animals being more optimistic than others irrelative of birth weight. A study based on a smaller sample size could therefore lead to statistically significant findings that do not reflect a true effect, if by coincidence a subset of more pessimistic LBW pigs were chosen.

Besides a difference in sample size, it is possible that the LBW pigs tested by Murphy *et al.* (2015) were more stressed than those tested in our study due to differences in social environment. First, their LBW and NBW pigs were housed in mixed groups, whereas the pigs in our study were housed separately per birth weight category. A pig's social rank and body weight are correlated, with larger pigs often having a higher position in the dominance hierarchy (Litten *et al.*, 2003; O'Connell *et al.*, 2004). As LBW pigs remain smaller than their NBW siblings, it is likely that when they are housed together, NBW pigs will have a higher social rank than the LBW pigs. This could have resulted in a more negative emotional state for the LBW pigs tested by Murphy *et al.* (2015). Such an effect has previously been found in rats, with lower ranking female rats showing a more negative judgment bias than those with a higher social rank (Barker *et al.*, 2018). In pigs, only indirect measures of emotional state have been assessed in correlation with their social status. These studies also suggest that in stable hierarchies, lower ranking pigs may have a more negative emotional state, as they have more injuries, lose competition over food and display more fearful behavior in a novel object test (O'Connell *et al.*, 2004; Boumans *et al.*, 2018).

Second, Murphy *et al.* (2015) tested only male pigs, whereas the current study included both sexes, housed in mixed-sex groups. Group composition may affect the emotional state of pigs, as males and females may have different social behavior. For example, male pigs are reported to initiate more

aggressive interaction and to perform higher rates of mounting behavior than females (Clark and D'Eath 2013; Puls *et al.* 2017). Mounting behavior is a likely cause of stress, as recipients produce high-pitched screams (Hintze *et al.*, 2013). Further indication that group composition may influence emotional state in pigs comes from a finding of impaired behavioral flexibility in male pigs when housed in a single-sex group (Roelofs *et al.*, 2017b). When this study was repeated with mixed-sex housing, no difference in behavioral flexibility between females and males was found (Roelofs *et al.*, 2018).

It is unlikely that the higher number of discrimination training sessions applied in our study influenced judgment bias, as no effect of training duration on optimistic choice percentage was found. This is corroborated by a previous study showing that the number of discrimination training sessions is independent from optimistic choosing in the judgment bias task (Roelofs *et al.*, 2017a). Overall, it appears that differences in social environment between our study and the study by Murphy *et al.* (2015) influenced the emotional state of the pigs, either through a difference in social rank of the LBW pigs or a difference in group composition. This makes it difficult to directly compare the results of these studies. Future studies assessing the emotional state of pigs with different positions in the dominance hierarchy and comparing mixed- and single-sex housing are encouraged.

Loss of ambiguity

Multiple judgment bias studies have reported a loss of ambiguity due to repeated testing (e.g., Doyle *et al.*, 2010b; Karagiannis *et al.*, 2015). Loss of ambiguity can occur when animals learn about the outcome of ambiguous trials, i.e. trials during which they are presented with an ambiguous stimulus (Roelofs *et al.*, 2016). Most often in judgment bias tasks, ambiguous trials go unrewarded while during training, rewards were always present after a correct response. As a result, the lack of reward during ambiguous trials stands out, facilitating learning about the outcome of such trials (Jamieson *et al.*, 2012). Once animals learn to associate ambiguous trials with a specific outcome (i.e., absence or presence of reward), they could adjust their responses accordingly (e.g., display a pessimistic response when they know no reward is available). Thereby, loss of ambiguity can influence results of judgment bias tasks (Doyle *et al.*, 2010b). Such effects of loss of ambiguity have also been reported for pigs (Murphy *et al.*, 2013b; Scollo *et al.*, 2014; Roelofs *et al.*, 2017b). To prevent loss of ambiguity, partial reinforcement schedules have been

successfully applied during discrimination training (e.g., Bateson *et al.*, 2015; Bethell and Koyama, 2015; Döpjan *et al.*, 2017). In these studies, a pre-defined percentage of training trials goes unrewarded, increasing similarity between training and testing conditions.

In our study, applying partial reinforcement of training trials was not successful as a means of avoiding loss of ambiguity. Both LBW and NBW pigs decreased their optimistic choice percentage as judgment bias testing sessions progressed. This suggests the outcome of ambiguous trials still stood out to the pigs, in spite of their experience with unrewarded trials during discrimination training. Perhaps this would not have been the case if the ratio of reference trials to ambiguous trials had been higher. Currently, three out of 16 testing trials were ambiguous and unrewarded. If the ambiguous trials are less frequent, the pigs are provided with less opportunity to learn about their outcome. A recent study successfully applied a study design where testing sessions consisted of 50 reference trials and three ambiguous trials (Hintze *et al.*, 2018). To speed up the process of training and testing a sufficient number of animals on such a considerable number of daily trials, a task was used where animals could ‘opt out’ of negative trials by initiating a new trial. This way, the long latencies to respond during negative trials are avoided. Validation of such a judgment bias task for pigs could potentially increase the reliability of results by avoiding loss of ambiguity.

CONCLUSION

Our results show that LBW causes a mild impairment of conditional discrimination learning in pigs. No effects of birth weight on judgment bias were found, suggesting that LBW pigs do not necessarily have a more negative emotional state than NBW pigs. This finding was supported by similar hair and salivary cortisol concentrations for LBW and NBW pigs. It is likely that the enriched housing conditions applied during our study contributed to these findings. Finally, the use of partial reinforcement during discrimination training was unsuccessful in avoiding loss of ambiguity during judgment bias testing. Further improvement of judgment bias task designs for pigs are therefore encouraged.

6

Low birth weight impairs acquisition of spatial memory task in pigs

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ABSTRACT

In commercial pig farming, an increasing number of low birth weight (LBW) piglets are born, due to selection for large litter sizes. While LBW piglets have a higher risk of pre-weaning mortality, a considerable number of these piglets survive to slaughter age. In humans, LBW is a risk factor for long-term cognitive impairments. In pigs, studies examining the post-weaning effects of LBW on cognition have reported contradictory results. Therefore, the current study aimed to assess the effects of LBW on cognitive development in pigs using an improved study design, by (1) testing a larger sample size than previous studies, (2) assessing acute and chronic stress responses to account for a potential altered stress response in LBW pigs, and (3) testing both female and male pigs to account for potential confounding effects of sex. Learning and memory of 20 LBW pigs and 20 normal birth weight (NBW) pigs, both groups consisting of 10 females and 10 males, were compared using a spatial holeboard task. In this task, pigs had to learn and remember the locations of hidden food rewards. After a pig had successfully acquired the task, it was presented with two successive reversal phases during which it was presented with a new configuration of reward locations. The holeboard allows for simultaneous assessment of working and reference memory, as well as measures of motivation, exploration, and behavioral flexibility. Mixed model ANOVAs revealed a transiently impaired reference memory performance of LBW pigs, implying they had more difficulty learning their reward configuration in the holeboard. Also, LBW piglets showed increased pre-weaning hair cortisol concentrations compared to their NBW siblings. No other effects of LBW were found. Sex had no direct or interaction effects on any measures of holeboard performance or stress. It is possible that the enriched housing conditions applied during our study had an ameliorating effect on our pigs' cognitive development. Overall, our results suggest LBW has a negative effect on post-weaning cognitive performance in pigs. This could have welfare consequences as cognitive skills are required for pigs to learn how to correctly respond to their environment.

INTRODUCTION

Piglets born with low birth weight (LBW) are an increasingly common occurrence on commercial pig farms. This is a result of selection for increased sow fecundity, leading to larger litters. With increasing litter size, sows may be unable to provide sufficient nutrients and oxygen for the optimal development of all fetuses (Père and Etienne, 2000; Wähler and Fischer, 2005). This explains the more frequent occurrence of LBW piglets in larger litters (Rutherford *et al.*, 2013). Besides a sub-optimal prenatal development, LBW piglets also have a higher risk of pre-weaning mortality (Galiot *et al.*, 2018). While this results in a relatively higher number of LBW piglets dying during the farrowing stage compared to piglets with normal birth weight (NBW), there is still a considerable number of LBW piglets surviving to slaughter age at approximately 6 months old (Calderón Díaz *et al.*, 2017).

The sub-optimal development of LBW offspring has been associated with postnatal cognitive impairments in a variety of species. In humans, LBW has been linked to learning difficulties throughout adolescence (Lindström *et al.*, 2017; Yu & Garcy, 2018). Impaired cognitive development associated with LBW has also been studied in a variety of animal models, most frequently in rats and sheep (although contrary to pigs and humans, LBW has to be experimentally induced in these models; Hunter *et al.*, 2016). For example, LBW has been linked to spatial memory deficits in rats (Camprubí Camprubí *et al.*, 2017). Together, these findings suggest that LBW could have a long-term impact on cognitive functioning. Pigs' prenatal brain development has similarities to humans', including a period of rapid brain growth *in utero* (Dobbing & Sands, 1979). It is possible that the cognitive impairments resulting from a sub-optimal intra-uterine environment are also comparable.

Understanding whether LBW also causes a long-term impairment of cognitive functioning in pigs is crucial, as such an impairment may influence their abilities to cope with housing and rearing conditions. Pigs are presented with multiple challenges to their learning and memory abilities in the common conditions of a commercial farm (Wechsler & Lea, 2007). For example, piglets have to learn how to acquire food from a feeder after being weaned (Magowan *et al.*, 2008), be able to recognize conspecifics and remember the organization of the dominance hierarchy to avoid unnecessary aggression (Coutellier *et al.*, 2007; Li & Wang, 2011) and if available, be able to successfully interact with cognitive enrichment (Zebunke *et al.*, 2013). Pigs need to be able to learn and remember how to interact with their

environment, creating predictability, and controllability, which have been shown to reduce stress (Wiepkema & Koolhaas, 1993).

Studies examining the effects of LBW on cognitive development in pigs have produced contradictory results. A study comparing pre-weaning spatial learning abilities of LBW and NBW piglets found LBW to be associated with the expected impaired performance (Radlowski *et al.*, 2014). After weaning, one study found LBW to be associated with the predicted cognitive impairments, with LBW pigs showing impaired reversal learning in a spatial learning task compared to NBW pigs (Gieling *et al.*, 2012). Other studies reported a comparable performance of LBW and NBW pigs, finding no effects of LBW on spatial learning ability (Gieling *et al.*, 2014) or associative learning (Murphy *et al.*, 2013a, 2015). There has also been a report of improved cognitive performance in LBW pigs, with LBW being associated with improved spatial learning (Antonides *et al.*, 2015a). Together, these studies do not provide a consensus on the long-term impact of LBW on the cognitive development of pigs. Rather, they show the need for further replication of cognitive studies with LBW pigs, applying methodological improvements to increase the quality of results.

None of the studies assessing the long-term effects of LBW on cognitive development took a potential effect of their study subjects' sex into account. Although it is unlikely that sex in itself has an influence on baseline cognitive performance in pigs (Roelofs *et al.*, 2017b), it is possible that females and males perform differently under the influence of stress. For example, spatial learning and memory is typically impaired as a consequence of chronic stress (Conrad, 2010). Such negative effects of chronic stress appear to be more prominent for males than females (Luine *et al.*, 2007). The opposite has been found for acute stress, which causes a more detrimental effect on females' cognitive performance (Healy *et al.*, 2009). Possible sex-dependent effects of stress are relevant when assessing cognition in LBW animals, as LBW may lead to altered functioning of the HPA-axis. For example, LBW piglets show increased plasma cortisol concentrations throughout the first week after birth (Wise *et al.*, 1991; Klemcke *et al.*, 1993), and show an exaggerated cortisol response to a physiological stressor (administration of insulin or ACTH) at 3 months of age (Poore & Fowden, 2003). Together, these results suggest that stressors may have a more detrimental effect on pigs with LBW. Considering that females and males may be differentially affected by such stressors, taking both sex of the study subjects and measures of stress into account is of importance when assessing the effects of LBW on cognition.

A suitable task to assess the effects of LBW on learning and memory in pigs is the spatial holeboard task. The holeboard is a free-choice maze task consisting of an open arena in which pigs have to learn and remember the locations of hidden food rewards (van der Staay *et al.*, 2012). Such a task is highly ecologically relevant for pigs, as it is based on their natural foraging behavior (Graves, 1984). Furthermore, the spatial holeboard allows for simultaneous assessment of multiple behavioral variables. The most important cognitive measures provided by the spatial holeboard are reference and working memory. Reference memory is required for information that remains relevant over a longer time period, such as how well a pig remembers the locations of rewards and how many locations contain a reward (Olton & Papas, 1979). Reference memory can be quantified as the ratio between visits to rewarded and unrewarded locations (van der Staay *et al.*, 2012). Working memory is required for information that is relevant for a shorter time span, such as which locations have already been visited within a single training trial (Dudchenko, 2004). This information is irrelevant in subsequent trials and consequently, working memory must be reset between trials. Working memory can be quantified as the ratio between first visits and all visits (including revisits) to a location (van der Staay *et al.*, 2012). Besides measures of spatial learning and memory, the holeboard can also be used to assess motivation (by measuring latency to first visit or the time interval between visits), exploration (by measuring which locations are visited), and behavioral flexibility (by applying a reversal of the task – van der Staay *et al.*, 2012). The spatial holeboard task has already successfully been applied to assess spatial cognition in pigs, showing it is sensitive enough to detect even mild cognitive impairments (e.g., Grimberg-Henrici *et al.*, 2016).

The current study aimed to assess the long-term effects of LBW on learning and memory in pigs, as assessed by the spatial holeboard task. Several improvements to previous studies were applied. First, a larger sample size was included, with 20 LBW and 20 NBW pigs being tested. This doubles the sample size used in previous studies to assess baseline effects of birth weight on post-weaning cognition (Gieling *et al.*, 2012, 2014; Antonides *et al.*, 2015a). Second, as LBW pigs may suffer from an altered stress response, hair and salivary cortisol concentrations were included as measures of chronic and acute stress, respectively. Finally, female and male pigs were tested to account for a potential confounding effect of sex. Based on studies assessing cognitive effects of LBW in humans (Lindström *et al.*, 2017; Yu & Garcy, 2018) and earlier studies with pigs at various ages (Gieling *et al.*, 2012; Radlowski *et al.*, 2014), it was expected that LBW would cause an impaired cognitive

development in pigs. This would result in decreased performance in the spatial holeboard, compared to NBW pigs. Furthermore, LBW pigs were expected to show an altered stress response, resulting in higher basal hair cortisol concentrations compared to NBW pigs and an exaggerated salivary cortisol increase after a stressor.

METHODS

Ethical note

All methods that demanded the handling of live animals were reviewed and approved by the local animal welfare body (Animal Welfare Body Utrecht) and were conducted in accordance with the recommendations of the EU directive 2010/63/EU.

Animals

Twenty pairs of piglets [(Yorkshire × Dutch Landrace) × Duroc] from 15 different litters were selected from the commercial pig breeding farm of Utrecht University, resulting in 20 LBW pigs and 20 NBW pigs (10 pairs of females and 10 pairs of males). Selection occurred in two separate rounds of 20 piglets (ten LBW-NBW pairs) to ensure availability of LBW piglets. During each selection round, all piglets born over a period of one week were weighed within 24 hours after birth. LBW piglets were selected based on three criteria: (1) a minimum of 1 *SD* below the average birth weight of the litter, (2) a minimum of 1 *SD* below the average birth weight of the study population, yielding a maximum birth weight of 1,050 grams, and (3) from a minimum litter size of 10 piglets. For each LBW piglet, a NBW sibling was selected based on two criteria: (1) of the same sex as the selected LBW piglet, and (2) a birth weight closest to the litter average. To increase food intake and thereby survival rates of LBW piglets, cross-fostering of non-selected siblings was applied when litter size exceeded the sow's number of functional teats. Additionally, all litters were provided with milk replacer at 2–3 days of age. One female LBW piglet and one male NBW piglet died of natural causes during the early stages of training in the holeboard. Their data was excluded from analysis, resulting in a final sample size of 38 pigs. Of these, one female LBW piglet could not participate in the second reversal phase due to lameness.

Housing

The selected pigs were weaned and moved to the research facility (located next to the commercial farm), at approximately 4 weeks of age. They were housed in four adjacent pens ($\sim 4 \times 5\text{m}$) in a naturally ventilated building. For each selection round, LBW and NBW pigs were housed separately. Pens had concrete floors and contained a covered piglet nest. Each day, the pens were cleaned and supplied with fresh straw bedding. To protect the piglets from the cold, the nest was equipped with rubber mats on the floor and transparent polyvinyl chloride (PVC) slats hanging in front of the entrance. Additionally, piglet nests contained heat lamps until the pigs were approximately 8 weeks old. Minimum and maximum temperatures were recorded daily outside the piglet nest and ranged from 0 to 27°C. To avoid effects of heat stress, pigs were only tested if they voluntarily entered the holeboard apparatus. Pigs received 1/3 of their daily food ration in the morning (prior to testing) and the remaining 2/3 in the afternoon (after testing). Water was provided *ad libitum*. Each pig had a number sprayed on its back to facilitate individual recognition of the pigs.

Spatial holeboard task

Apparatus

The holeboard apparatus (manufactured by Ossendrijver B.V., Achterveld, The Netherlands) consisted of a square arena ($5.3 \times 5.3\text{m}$) with a synthetic slatted floor, surrounded by synthetic walls (80 cm high). The holes in the arena consisted of 16 food bowls placed in a 4×4 matrix (**Figure 1.2A**), in which food rewards could be hidden. Pigs could enter the arena to search for these rewards via a surrounding corridor (40 cm wide), which gave access to one of four guillotine doors (operated by a rope and pulley system) placed in the walls surrounding the arena. By using four different starting positions, pigs cannot rely on a fixed search pattern to solve the task (van der Staay *et al.*, 2012). Instead, pigs had to rely on extra-maze cues (such as the position of the experimenter outside the arena) to orient themselves inside the holeboard. A baited hole would contain two chocolate candies (M&M's® Milk Chocolate) as a reward. Each food bowl was fixed with a false bottom, beneath which four candies were placed to avoid providing the pigs with scent cues about the locations of the baited holes (**Figure 1.2B**). Additionally, each bowl was covered with a synthetic red ball (JollyBall Dog Toy, $\varnothing 24\text{ cm}$,

1400 g, Jolly Pets, Ohio, USA) to avoid visual discrimination between baited and non-baited holes. The pigs were trained to lift the ball off a food bowl in order to obtain the food reward. If a pig soiled the holeboard during testing, it was rinsed immediately to avoid the development of scent cues. Additionally, the entire holeboard was rinsed daily. During testing, visits to holes were automatically recorded using custom made software (SeaState5, Delft, The Netherlands). When a ball was lifted off a food bowl, the connection between a magnet in the ball and a sensor in the bowl was interrupted. This signal was registered by an interface (LabJack) and sent to a laptop. A revisit to a hole was only recorded if a pig visited another hole in between or if 10 seconds passed in between successive visits to the same hole.

Training and testing

After the pigs were moved to the research facility, training started by habituating the pigs to the presence of and being handled by the researchers. The pigs were then gradually habituated to being inside the holeboard apparatus. Initially, pigs were allowed to explore the holeboard in groups of ten. Group size was then gradually decreased until the pigs explored the holeboard individually.

Testing trials started when all pigs were able to lift the balls off the food bowls (at this point, pigs were approximately 8 weeks old). Each pig performed two consecutive trials daily. At the start of a trial, a pig was let into the corridor surrounding the holeboard arena. When it reached an open entrance into the arena (one of four entrances was randomly chosen prior to each trial), it could freely search for food rewards by visiting holes (i.e., lifting the ball covering a food bowl). A trial ended when a pig managed to find all rewards or when a maximum trial duration of 7.5 minutes had passed, whichever occurred first.

The holeboard experiment consisted of four consecutive phases: habituation (four trials), acquisition (44–76 trials), first reversal (24–44 trials) and second reversal (20 trials). During habituation trials, all 16 holes contained a reward. This encouraged the pigs to visit as many holes as possible during each habituation trial. After the habituation trials, the acquisition phase started. Each pig was assigned one of four possible reward configurations (**Figure 1.3**). A reward configuration consisted of a subset of four baited holes (the remaining 12 holes did not contain a food reward). Each pig continued

training on its assigned reward configuration for the duration of the acquisition phase. Reward configurations were randomly assigned but balanced for birth weight category and sex.

There were two criteria for a pig to complete the acquisition phase, based on previous holeboard studies with pigs (Gieling *et al.*, 2012; Roelofs *et al.*, 2017b): a pig had to (1) complete a minimum of 44 acquisition trials, and (2) reach a reference memory score of at least 0.7 (see section **Behavioral variables** in this chapter for calculation of reference memory) for two consecutive training days (consisting of four consecutive trials). This criterion performance indicated a pig had successfully learned the locations of the four baited holes. After completing the acquisition phase, a pig was assigned a new reward configuration for the first reversal phase (**Table 6.1**). The pigs now had to learn to retrieve their rewards in a new set of holes (e.g., a pig that was trained on configuration A during acquisition was now switched to configuration C). Pigs could complete the first reversal phase after a minimum of 24 reversal trials and reaching the same criterion level for reference memory performance that was set during the acquisition phase. After completing the first reversal phase, a second reversal was applied where pigs were again trained on a new reward configuration (**Table 6.1**).

Table 6.1 Combinations of reward configurations used for the holeboard experiment.

Combination	Phase		
	Acquisition	1 st reversal	2 nd reversal
1	A	C	B
2	B	D	C
3	C	A	D
4	D	B	A

Behavioral variables

For each trial in the holeboard, the following variables were analyzed:

- **Working memory**, calculated as the number of visits that yielded a reward divided by the total number of visits (including revisits) to baited holes.
- **Reference memory**, calculated as the total number of visits to baited holes divided by the total number of visits to all holes. Reference memory was further divided into components reflecting spatial orientation and spatial pattern learning:
 - **Rotational reference memory** reflects rotational errors made while a pig was orienting himself after entering the holeboard, i.e. reference memory mistakes made prior to finding the first reward (Cheng & Newcombe, 2005). This measure was calculated as 1 divided by the total number of visits to all holes up to and including the first rewarded visit.
 - **Spatial pattern reference memory** reflects a pig's ability to successfully complete the spatial pattern formed by the reward configuration, i.e., reference memory mistakes made after finding the first reward (Brown & Wintersteen, 2004). This measure was calculated as total number of visits to baited holes divided by the total number of visits to all holes, excluding visits made before finding the first reward.
- **Trial duration, latency to first visit and latency to first reward**, calculated as average time in seconds elapsed between entering the holeboard and performing the required action. When a pig failed to perform the required action, a maximum value of 450 seconds was assigned.
- **Inter-visit interval**, calculated as the average time in seconds between two successive visits.
- **Total number of visits, number of different locations (holes) visited and number of rewards found**, calculated as absolute counts.

Additionally, **trials to criterion** was calculated as the number of trials needed to reach criterion performance for the acquisition and first reversal phase.

Cortisol analysis

Hair cortisol

Hair samples were collected at weaning and at the end of the experiment, when the pigs were approximately 5 months old. Hair was taken from the left flank of each pig with a razor (single edged disposable prep razor, Kai Medical, Solingen, Germany; a new razor was used for each sample). Hair cortisol concentration was determined based on the protocol by Davenport *et al.* (2006). In short, samples were washed and dried, after which ~35mg of hair was ground with a bead beater (TissueLyser II, QIAGEN Benelux B.V., Antwerp, Belgium) for a minimum of 2×15 min at 30Hz, in 2mL tubes containing three 2.3mm steel beads (BioSpec, Lab Services B.V., Breda, the Netherlands). After grinding, 1mL methanol was added and samples were incubated for 24 h with slow rotation to extract corticosteroids. Of the extract, 0.6mL was dried using a vacuum centrifuge. Dried extracts were dissolved in 0.3mL phosphate buffer. Hair cortisol concentrations were then determined in duplo using a Salimetrics Salivary Cortisol ELISA kit. Intra-assay and inter-assay coefficients of variation (CV) were 7.1 and 23.1%, respectively. The higher inter-assay CV implies plate-to-plate variation (i.e., different plates produced different cortisol concentrations for the same sample). To avoid an influence of inter-assay CV on group comparisons, samples were balanced across plates for birth weight and sex.

Salivary cortisol

Saliva samples were collected from each pig prior to and after their first individual habituation trial in the holeboard. Pre-stressor samples were collected at ~14:00 in the afternoon in their home pens. Post-stressor samples were taken ~20 min after a pig's trial in the holeboard, to allow for the peak in cortisol response to develop (Merlot *et al.*, 2011). Saliva was collected by allowing each pig to chew on two cotton swabs (Cotton Swabs 150mm \times 4mm WA 2PL; Heinz Herenz, Hamburg, Germany) until they were sufficiently moistened. Then, the swabs were centrifuged using saliva collection tubes (Salivette, Sarstedt, Germany) at around 3,524 g for 10min at 10°C. Saliva samples were stored at -20°C until salivary cortisol concentration was determined in duplo using a Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics BV, The Hague, the

Netherlands). Intra-assay and inter-assay CVs were 4.8 and 1.6%, respectively.

Statistical analysis

All statistical analyses were performed using R statistical software, version 3.4.2 (R Core Team, 2017). For linear mixed models, package nlme (Pinheiro *et al.*, 2018) was used. For each mixed model the random effect structure was assessed using Restricted Maximum Likelihood (REML) estimation. Final selection of random effect structure was based on Akaike's information criterion (AIC). Round (first or second round of selected animals) did not improve fit of mixed models and was therefore dropped from further analysis. Statistical significance was set at $p < 0.05$. Effect size was calculated as Pearson's r based on contrasts. Unless indicated otherwise, results are presented as mean \pm SEM.

Birth weight and growth

Average birth weight of LBW and NBW pigs was compared using Welch's t -test. The effect of birth weight on pigs' weekly weight gain from weaning until 5 months of age was analyzed using a linear mixed model with Birth weight, Week and Birth weight \times Week interaction as fixed effects. Random effect structure consisted of random slopes and intercepts for Subject nested within Litter.

Holeboard data

For all variables scored during the acquisition and reversal phases, means of four successive trials (trial blocks) were calculated. Furthermore, to assess the effect of transitioning to a reversal phase, the last trial block of the acquisition phase was compared to the first trial block of the first reversal phase. The same was done for the transition from first to second reversal phase. The effect of birth weight on pigs' learning curves during acquisition, transition and reversal phases for all holeboard variables were analyzed using a linear mixed model with Birth Weight, Sex, Trial Block, and their two-way interactions as fixed effects. Random effect structure consisted of random intercepts for Subject nested within Litter and a first order autoregressive correlation structure for residuals to account for repeated measures within subjects. The habituation phase was analyzed similarly, but with Trial as a

fixed effect instead of Trial Block. Durations and latencies were \log_{10} transformed to improve the distribution of residuals. Finally, trials to criterion for the acquisition and first reversal phase were compared using a linear mixed model with Birth Weight, Sex, and Birth Weight \times Sex interaction as fixed effects and random intercepts for Litter.

Cortisol concentrations

The effects of birth weight on pigs' hair cortisol concentrations at weaning and 5 months of age were analyzed using a linear mixed model with Birth weight, Sex, and Birth weight \times Sex interaction as fixed effects and random intercepts for Litter. Hair samples collected at weaning from three pigs (1 LBW male, 1 NBW male, and 1 NBW female) were insufficient for cortisol analysis. Therefore, hair cortisol analysis on samples at weaning was performed on the remaining 35 samples.

The effects of birth weight on salivary cortisol concentrations before and after a pig's first individual trial in the holeboard were analyzed using a linear mixed model with Birth Weight, Sex, Sample, and all two-way interactions as fixed effects and random slopes and intercepts for Subject. Salivary cortisol concentrations were \log_{10} transformed to improve distribution of residuals. Saliva collected from one LBW male was insufficient for cortisol analysis. Therefore, salivary cortisol analysis was performed on samples collected from the remaining 37 animals.

RESULTS

Birth weight and growth

LBW piglets had on average a lower birth weight than NBW piglets (LBW: $0.81 \text{ kg} \pm 0.02$, NBW: $1.45 \text{ kg} \pm 0.05$; $t_{27,31} = -11.63$, $p < 0.001$; $r = 0.91$). LBW piglets continued to have lower body weight throughout the duration of the experiment (Birth weight: $F_{1,22} = 34.30$, $p < 0.001$; $r = 0.79$; **Figure 6.1**) and had a slower growth rate than the NBW piglets (Birth weight \times Week: $F_{15,482} = 10.39$, $p < 0.001$; **Figure 6.1**).

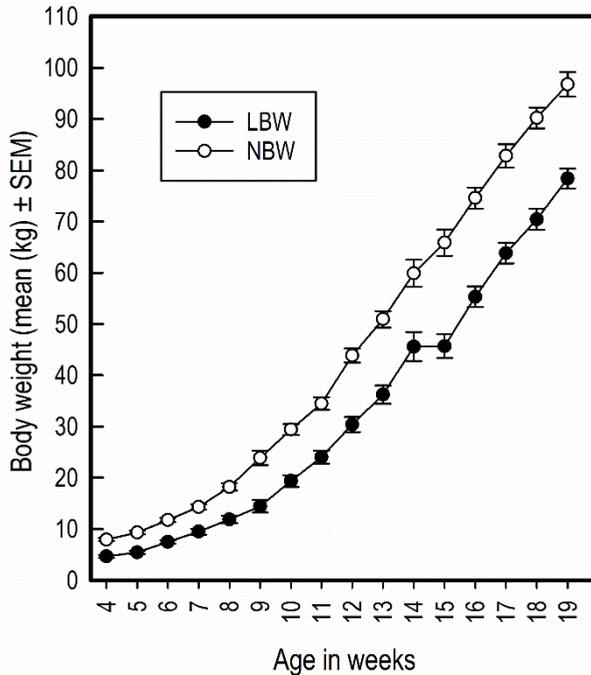


Figure 6.1 Average body weight in kilograms of LBW and NBW pigs from weaning until the end of the experiment. For week 15, data from only ten LBW pigs was available, causing an appearance of lack of growth from week 14 to 15. This is an artefact due to missing data.

Spatial holeboard task

Habituation

Birth weight and sex did not influence pigs' performance during the habituation trials (**Table 6.2**). During habituation, all pigs showed a comparable exploration of the holeboard based on total visits and locations (holes) visited.

Spatial learning and memory

Working memory

Neither birth weight nor sex had an effect on working memory (WM) scores during any phase of the experiment (**Figure 6.2; Table 6.2**). All pigs

Table 6.2 Performance of low birth weight and normal birth weight piglets in the spatial holeboard task, during a habituation (Hab), acquisition (Acq), first transition (Trans I), first reversal (Rev I), second transition (Trans II) and second reversal (Rev II) phase. Effects printed in bold have associated probabilities of < 0.05. Effects printed in italics have associated probabilities of 0.10 > *p* ≥ 0.05. *For habituation phase, 4 separate trials were analyzed instead of trial blocks. Abbreviations: WM, working memory; RM, reference memory; rRM, rotational reference memory; sRM, spatial pattern reference memory; TD, trial duration; LFV, latency to first visit; IVI, inter-visit interval; LFR, latency to first reward; TV, total number of visits; LOC, total number of locations visited; REW, number of rewards found.

Measure Phase	Birth weight (BW)			Sex			BW x Sex			Trial blocks			BW x Trial blocks			Sex x Trial blocks		
	F	df	P≤	F	df	P≤	F	df	P≤	F	df	P≤	F	df	P≤	F	df	P≤
WM																		
Acq	2.22	1,20	.152	0.18	1,20	.674	1.63	1,20	.217	16.13	10,350	<.001	1.03	10,350	.422	1.25	10,350	.258
Trans I	0.08	1,20	.780	0.47	1,20	.450	1.45	1,20	.242	139.19	1,34	<.001	0.56	1,34	.459	0.06	1,34	.803
Rev I	0.02	1,20	.894	0.00	1,20	<.999	0.37	1,20	.549	55.39	5,174	<.001	0.20	5,174	.964	0.39	5,174	.855
Trans II	1.45	1,19	.243	0.03	1,19	.858	3.32	<i>1,19</i>	<i>.084</i>	175.11	1,34	<.001	1.22	1,34	.277	0.01	1,34	.934
Rev II	0.73	1,19	.402	0.16	1,19	.691	0.46	1,19	.505	89.16	4,136	<.001	<i>2.01</i>	<i>4,136</i>	<i>.096</i>	0.34	4,136	.854
RM																		
Acq	5.76	1,20	.026	1.03	1,20	.322	0.93	1,20	.344	104.71	10,350	<.001	0.58	10,350	.832	1.04	10,350	.407
Trans I	0.21	1,20	.649	0.00	1,20	.978	0.01	1,20	.931	1787.92	1,35	<.001	0.08	1,35	.783	0.37	1,35	.549
Rev I	0.01	1,20	.926	0.66	1,20	.427	0.01	1,20	.920	173.34	5,175	<.001	0.07	5,175	.996	0.86	5,175	.508
Trans II	0.00	1,19	.976	0.54	1,19	.470	0.00	1,19	.979	1882.18	1,34	<.001	1.63	1,34	.210	0.30	1,34	.589
Rev II	1.65	1,19	.214	1.75	1,19	.201	0.24	1,19	.633	54.74	4,136	<.001	1.10	4,136	.362	2.35	4,136	.058
rRM																		
Acq	0.45	1,20	.509	0.23	1,20	.640	0.10	1,20	.758	49.90	10,350	<.001	2.04	10,350	.029	0.73	10,350	.695
Trans I	0.10	1,34	.749	1.23	1,34	.276	0.08	1,34	.778	567.30	1,35	<.001	5.27	1,35	.028	4.15	1,35	.049
Rev I	2.54	1,20	.126	0.86	1,20	.366	0.83	1,20	.374	82.78	5,175	<.001	1.34	5,175	.249	1.57	5,175	.172
Trans II	0.48	1,19	.497	3.26	<i>1,19</i>	<i>.087</i>	1.43	1,19	.246	164.70	1,34	<.001	0.05	1,34	.829	0.00	1,34	.945
Rev II	1.63	1,19	.217	0.01	1,19	.914	1.42	1,19	.248	43.10	4,136	<.001	0.03	4,136	.999	0.41	4,136	.804

Table 6.2 Continued.

Measure Phase	Birth weight (BW)			Sex			BW x Sex			Trial blocks			BW x Trial blocks			Sex x Trial blocks		
	F	df	P≤	F	df	P≤	F	df	P≤	F	df	P≤	F	df	P≤	F	df	P≤
sRM																		
Acq	5.51	1,20	.029	1.22	1,20	.283	1.24	1,20	.279	108.61	10,350	<.001	0.60	10,350	.811	0.97	10,350	.472
Trans I	0.08	1,20	.782	1.38	1,20	.254	1.72	1,20	.204	1198.26	1,35	<.001	0.33	1,35	.569	0.00	1,35	.988
Rev I	0.22	1,20	.647	0.10	1,20	.763	0.04	1,20	.850	140.71	5,175	<.001	0.23	5,175	.948	1.06	5,175	.385
Trans II	0.01	1,19	.906	0.40	1,19	.533	0.75	1,19	.396	977.78	1,34	<.001	1.35	1,34	.254	0.67	1,34	.419
Rev II	1.30	1,19	.269	1.59	1,19	.222	0.16	1,19	.697	152.19	4,136	<.001	1.21	4,136	.310	2.97	4,136	.022
TD																		
Hab*	3.27	1,20	.086	0.08	1,20	.784	3.01	1,20	.098	5.14	3,105	.002	0.15	3,105	.930	1.28	3,105	.286
Acq	3.34	1,20	.083	0.81	1,20	.378	0.50	1,20	.487	35.19	10,350	<.001	0.84	10,350	.586	1.22	10,350	.275
Trans I	0.29	1,20	.594	0.28	1,20	.606	0.10	1,20	.751	858.47	1,35	<.001	0.14	1,35	.707	0.04	1,35	.840
Rev I	0.14	1,20	.711	0.05	1,20	.835	0.12	1,20	.739	142.47	5,175	<.001	0.26	5,175	.934	0.56	5,175	.730
Trans II	0.07	1,19	.798	0.58	1,19	.455	3.47	1,19	.078	577.78	1,34	<.001	0.60	1,34	.443	1.18	1,34	.285
Rev II	0.79	1,19	.385	1.14	1,19	.230	0.60	1,19	.450	109.61	4,136	<.001	2.34	4,136	.058	1.98	4,136	.101
LFV																		
Hab*	3.80	1,20	.065	0.00	1,20	<.999	1.79	1,20	.196	5.58	3,105	.001	0.17	3,105	.917	0.79	3,105	.504
Acq	1.04	1,20	.320	0.48	1,20	.498	0.01	1,20	.913	13.35	10,350	<.001	1.55	10,350	.120	0.92	10,350	.512
Trans I	0.42	1,20	.524	0.04	1,20	.850	0.11	1,20	.741	3.09	1,35	.087	0.04	1,35	.850	0.00	1,35	.994
Rev I	0.35	1,20	.559	0.16	1,20	.696	0.06	1,20	.802	8.53	5,175	<.001	0.23	5,175	.949	0.75	5,175	.586
Trans II	0.19	1,19	.664	0.20	1,19	.658	1.04	1,19	.320	0.26	1,34	.615	0.06	1,34	.803	0.98	1,34	.329
Rev II	0.27	1,19	.607	0.22	1,19	.646	0.75	1,19	.398	2.33	4,136	.059	0.31	4,136	.868	1.48	4,136	.210
IVI																		
Hab*	3.08	1,20	.095	0.09	1,20	.761	3.39	1,20	.081	5.28	3,105	.002	0.40	3,105	.750	0.39	3,105	.758
Acq	2.84	1,20	.108	3.13	1,20	.092	1.32	1,20	.264	5.20	10,350	<.001	1.83	10,350	.054	0.68	10,350	.739
Trans I	1.43	1,20	.246	0.01	1,20	.917	0.00	1,20	.961	33.48	1,35	<.001	0.66	1,35	.423	0.01	1,35	.939
Rev I	0.60	1,20	.448	0.00	1,20	.973	0.00	1,20	.976	39.46	5,175	<.001	0.76	5,175	.583	0.46	5,175	.804
Trans II	0.10	1,19	.756	2.04	1,19	.170	0.07	1,19	.798	24.78	1,34	<.001	0.36	1,34	.551	4.01	1,34	.053
Rev II	0.04	1,19	.845	1.55	1,19	.228	0.21	1,19	.650	18.78	4,136	<.001	1.81	4,136	.130	4.07	4,136	.004

Table 6.2 Continued.

Measure Phase	Birth weight (BW)			Sex			BW x Sex			Trial blocks			BW x Trial blocks			Sex x Trial blocks		
	F	df	P _≤	F	df	P _≤	F	df	P _≤	F	df	P _≤	F	df	P _≤	F	df	P _≤
LFR																		
Acq	6.40	1,20	.012	0.77	1,20	.389	0.13	1,20	.725	27.07	10,350	<.001	1.33	10,350	.214	0.84	10,350	.595
Trans I	0.05	1,20	.824	0.12	1,20	.730	0.07	1,20	.791	325.04	1,35	<.001	0.78	1,35	.384	3.67	1,35	.064
Rev I	0.23	1,20	.636	0.46	1,20	.504	0.20	1,20	.660	120.27	5,175	<.001	0.49	5,175	.781	1.31	5,175	.262
Trans II	0.46	1,19	.507	0.99	1,19	.332	0.00	1,19	.977	203.14	1,34	<.001	0.57	1,34	.455	0.01	1,34	.918
Rev II	0.63	1,19	.438	0.71	1,19	.410	0.16	1,19	.696	71.90	4,136	<.001	0.05	4,136	.996	0.22	4,136	.928
TV																		
Hab*	0.00	1,20	.968	0.01	1,20	.909	0.11	1,20	.747	0.88	3,105	.453	0.83	3,105	.480	1.91	3,105	.132
Acq	3.36	1,20	.082	0.18	1,20	.675	0.00	1,20	.997	116.56	10,350	<.001	1.09	10,350	.372	1.75	10,350	.069
Trans I	2.03	1,20	.170	0.49	1,20	.494	0.77	1,20	.390	247.69	1,35	<.001	1.13	1,35	.295	0.20	1,35	.654
Rev I	1.21	1,20	.284	0.18	1,20	.678	0.37	1,20	.551	112.93	5,175	<.001	0.94	5,175	.457	0.30	5,175	.911
Trans II	0.82	1,19	.378	1.53	1,19	.231	4.31	1,19	.052	449.54	1,34	<.001	0.95	1,34	.338	1.86	1,34	.182
Rev II	0.00	1,19	.947	1.36	1,19	.258	0.04	1,19	.853	212.76	4,136	<.001	1.11	4,136	.353	1.80	4,136	.132
LOC																		
Hab*	2.20	1,20	.154	0.00	1,20	.965	0.83	1,20	.373	2.50	3,105	.063	1.53	3,105	.211	0.46	3,105	.711
Acq	3.57	1,20	.073	0.13	1,20	.719	0.83	1,20	.373	193.31	10,350	<.001	1.23	10,350	.271	1.66	10,350	.089
Trans I	2.11	1,20	.162	0.36	1,20	.558	0.92	1,20	.350	240.42	1,35	<.001	1.14	1,35	.293	0.22	1,35	.638
Rev I	1.22	1,20	.283	0.08	1,20	.785	0.43	1,20	.520	69.99	5,175	<.001	0.67	5,175	.650	0.42	5,175	.837
Trans II	0.01	1,19	.916	2.89	1,19	.106	1.45	1,19	.244	1075.92	1,34	<.001	0.24	1,34	.629	3.64	1,34	.065
Rev II	0.66	1,19	.426	0.07	1,19	.793	0.37	1,19	.550	255.37	4,136	<.001	0.78	4,136	.539	2.84	4,136	.027
REW																		
Acq	3.23	1,20	.087	0.20	1,20	.657	0.05	1,20	.829	3.81	10,350	<.001	2.13	10,350	.022	0.09	10,350	<.999
Trans I	0.35	1,20	.563	0.06	1,20	.806	0.05	1,20	.825	35.37	1,35	<.001	0.32	1,35	.574	0.10	1,35	.757
Rev I	0.39	1,20	.539	0.00	1,20	.982	0.09	1,20	.770	35.43	5,175	<.001	0.25	5,175	.941	0.92	5,175	.472
Trans II	0.49	1,19	.493	0.59	1,19	.452	0.88	1,19	.360	26.26	1,34	<.001	0.35	1,34	.557	1.33	1,34	.258
Rev II	0.20	1,19	.661	0.22	1,19	.646	1.28	1,19	.272	21.52	4,136	<.001	0.77	4,136	.548	1.69	4,136	.156

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improved their WM scores as training progressed during the acquisition phase, first reversal and second reversal (Trial blocks: Acquisition, $F_{10,350} = 16.13$, $p < 0.001$; First reversal, $F_{5,174} = 55.39$, $p < 0.001$; Second reversal, $F_{4,136} = 89.16$, $p < 0.001$). After a transition to a new configuration of baited holes, all pigs showed an initial decrease in WM scores (Trial blocks: First transition, $F_{1,34} = 139.19$, $p < 0.001$; Second transition, $F_{1,34} = 175.11$, $p < 0.001$).

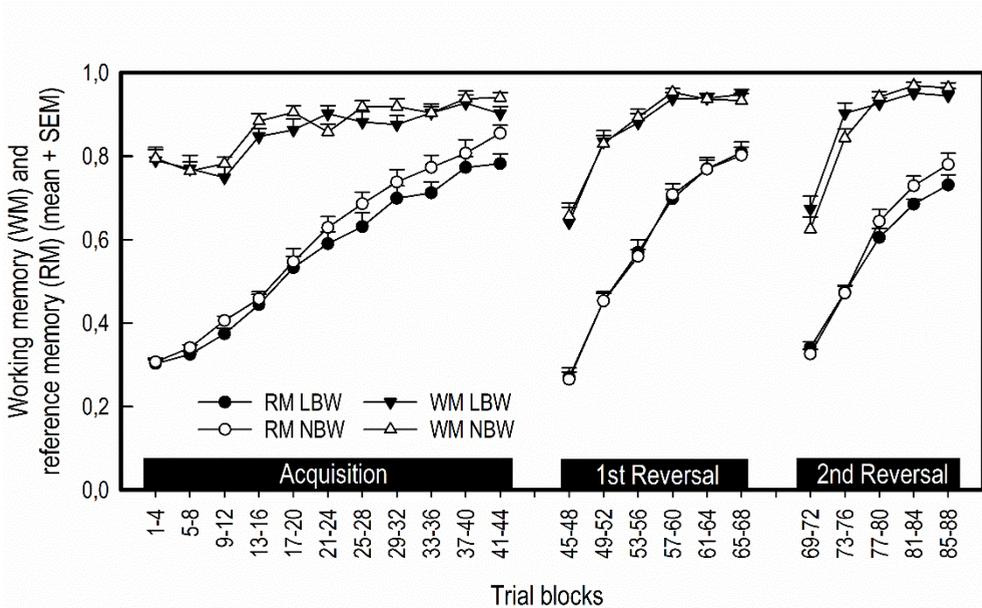


Figure 6.2 Average working memory (WM) and reference memory (RM) scores of LBW and NBW pigs in the spatial holeboard task per trial block. There was no significant effect of sex on holeboard performance, either as a main effect or interaction with birth weight. Therefore, the data for males and females have been combined.

Reference memory

Birth weight affected reference memory (RM) scores during the acquisition phase [Birth weight: $F_{1,20} = 5.76$, $p = 0.026$, $r = 0.12$; **Figure 6.2**; **Table 6.2**), with LBW piglets scoring lower than NBW piglets. This difference was transient, with no effect of birth weight on RM scores during either the first or second reversal phase (Birth weight: First reversal, $F_{1,20} = 0.01$, $p = 0.926$; Second reversal, $F_{1,19} = 1.65$, $p = 0.214$). Similarly, there was a trend for LBW

piglets to require a higher number of trials to complete the acquisition phase compared to NBW piglets (LBW: 53.26 ± 2.27 , NBW: 48.00 ± 1.70 ; $F_{1,20} = 4.19$, $p = 0.054$). No effect of birth weight was found on the number of trials required to complete the first reversal phase (LBW: 29.26 ± 1.38 , NBW: 29.79 ± 1.08 ; $F_{1,20} = 0.08$, $p = 0.776$). Sex had no effect on trials to criterion during the acquisition phase (Sex: $F_{1,20} = 2.46$, $p = 0.132$; Sex \times Birth weight: $F_{1,20} = 0.46$, $p = 0.504$) or the first reversal phase (Sex: $F_{1,20} = 0.08$, $p = 0.775$; Sex \times Birth weight: $F_{1,20} = 1.41$, $p = 0.249$), nor did it influence RM scores during any phase of the experiment (**Table 6.2**). All pigs improved their RM scores during the acquisition, first reversal and second reversal phases (Trial blocks: Acquisition, $F_{10,350} = 104.71$, $p < 0.001$; First reversal, $F_{5,175} = 173.34$, $p < 0.001$; Second reversal, $F_{4,136} = 54.74$, $p < 0.001$). After each transition to a reversal phase, all pigs showed an initial decrease in RM scores (Trial blocks: First transition, $F_{1,35} = 1787.92$, $p < 0.001$; Second transition, $F_{1,34} = 1882.18$, $p < 0.001$).

RM scores can be separated into a rotational and a spatial pattern component. Birth weight influenced rotational RM (rRM) scores for certain specific trial blocks during the acquisition phase and transition to the first reversal phase, as indicated by Birth weight \times Trial blocks interactions. For the acquisition phase, LBW pigs had lower rRM scores for trials 21–24 and 25–28 (Birth weight \times Trial blocks: $F_{10,350} = 2.04$, $p = 0.029$). For the first transition phase, LBW pigs had higher rRM scores for the final trial block of the acquisition phase and lower rRM scores for the first trial block of the first reversal phase (Birth weight \times Trial blocks: $F_{1,35} = 5.27$, $p = 0.028$). Together, these findings do not represent a systematic difference between LBW and NBW pigs for rRM scores (**Table 6.2**). However, a general effect of birth weight was found for spatial pattern RM scores during the acquisition phase (Birth weight: $F_{1,20} = 5.51$, $p = 0.029$, $r = 0.12$), suggesting the difference found between LBW and NBW pigs in RM performance reflects a difference in spatial pattern learning. No effect of birth weight on subsequent phases was found (Birth weight: First reversal, $F_{1,20} = 0.22$, $p = 0.647$; Second reversal, $F_{1,19} = 1.30$, $p = 0.269$). A difference between female and male pigs was only found for certain trial blocks during the transition from acquisition to first reversal phase and during the second reversal phase. Male pigs had higher rRM scores for the final trial block of the acquisition phase (Sex \times Trial blocks: $F_{1,35} = 4.15$, $p = 0.049$) and higher sRM scores for the final trial block of the second reversal phase (Sex \times Trial blocks: $F_{4,136} = 2.97$, $p = 0.022$). Similar to the general RM scores, both rotational and spatial pattern RM scores improved

within training phases, but initially decreased when pigs were transitioned to a reversal phase (**Table 6.2**).

Duration measures

Birth weight had an effect on the latency to first reward during the acquisition phase, with LBW pigs taking longer than NBW pigs to find their first reward (Birth weight: $F_{1,20} = 6.40$, $p = 0.012$, $r = 0.35$). This finding was due to a difference between groups for the first trial blocks and thus does not reflect a systematic difference in performance between LBW and NBW pigs. Similarly, female pigs had higher inter-visit intervals compared to male pigs during the first trial block of the second reversal phase (Sex \times Trial blocks: $F_{4,136} = 4.07$, $p = 0.004$). No other effects of birth weight or sex on duration measures were found (Trial duration, Latency to first visit, Latency to first reward and Inter-visit interval; **Table 6.2**). Most duration measures decreased as training progressed during the acquisition, first reversal and second reversal phase, with pigs needing less time to finish a trial. Latency to first visit increased during the acquisition phase, likely due to pigs learning to approach a rewarded location for their first visit, instead of simply visiting the nearest hole upon entering the holeboard (Trial block: Acquisition, $F_{10,350} = 13.35$, $p < 0.001$). Most duration measure scores initially increased after a transition to a reversal. The only exception was latency to first visit, which remained stable after the start of both reversal phases (Trial block: First transition, $F_{1,35} = 3.09$, $p = 0.087$; Second transition, $F_{1,34} = 0.26$, $p = 0.615$). As the second reversal progressed, only a trend for an increase in latency to first visit was found (Trial block: Second reversal, $F_{4,136} = 2.33$, $p = 0.059$).

Exploration measures

No systematic effects of birth weight or sex were found for any of the exploration measures assessed (Total number of visits, Number of locations visited and Number of rewards found). LBW pigs found less rewards than NBW pigs during the first trial blocks of the acquisition phase (Birth weight \times Trial blocks: $F_{10,350} = 2.13$, $p = 0.022$). Female pigs visited more locations than male pigs during the final trial blocks of the second reversal phase (Sex \times Trial blocks: $F_{4,136} = 2.84$, $p = 0.027$). No other effects of birth weight or sex were found. Scores for all exploration measures improved as pigs progressed during the acquisition, first reversal and second reversal phases (**Table 6.2**). As training progressed, pigs required fewer total visits, visited

fewer locations, and found a higher number of rewards. The opposite was true when pigs were subjected to the reversal phases.

Cortisol concentrations

Hair cortisol

At weaning, cortisol concentration in flank hair of LBW piglets was higher than that of NBW piglets (LBW: 33.20 pg/mg \pm 1.68, NBW: 29.26 pg/mg \pm 1.41; $F_{1,18} = 5.38$, $p = 0.032$, $r = 0.34$). Sex did not influence hair cortisol concentration at weaning (Sex: $F_{1,18} = 0.00$, $p = 0.975$; Sex \times Birth weight: $F_{1,18} = 0.04$, $p = 0.847$). The difference between birth weight categories was no longer present in hair samples collected at 5 months of age, at the end of the experiment (LBW: 20.61 pg/mg \pm 1.14, NBW: 21.84 pg/mg \pm 1.88; $F_{1,20} = 0.33$, $p = 0.575$). Again, sex did not influence hair cortisol concentration (Sex: $F_{1,20} = 2.96$, $p = 0.101$; Sex \times Birth weight: $F_{1,20} = 0.52$, $p = 0.480$).

Salivary cortisol

Performing the first individual trial in the spatial holeboard task caused an increase in salivary cortisol concentration for all piglets ($F_{1,34} = 31.53$, $p < 0.001$; **Figure 6.3**). No effects of birth weight (Birth weight: $F_{1,33} = 2.55$, $p = 0.120$; Birth weight \times Sample: $F_{1,34} = 0.01$, $p = 0.924$; **Figure 6.3**) or sex (Sex: $F_{1,33} = 1.15$, $p = 0.292$; Sex \times Sample: $F_{1,34} = 0.18$, $p = 0.677$; Sex \times Birth weight: $F_{1,33} = 1.21$, $p = 0.279$) were found on salivary cortisol concentrations.

DISCUSSION

The present study assessed the effects of LBW on post-weaning cognitive performance in pigs. To this end, it is important that the LBW piglets selected for our study actually represented a different population than the selected piglets with NBW. Indeed, LBW piglets had significantly lower birth weights than NBW piglets. Furthermore, the LBW piglets selected for our study had birth weights comparable to or smaller than those of piglets assessed in other LBW studies (e.g., Gieling *et al.*, 2012, 2014; Antonides *et al.*, 2015a). In pigs, birth weight is the main measure used to determine whether intra-uterine growth restriction has occurred (Wu *et al.*, 2006; Gieling, 2013). Head morphology has been suggested as an additional measure, where a relatively

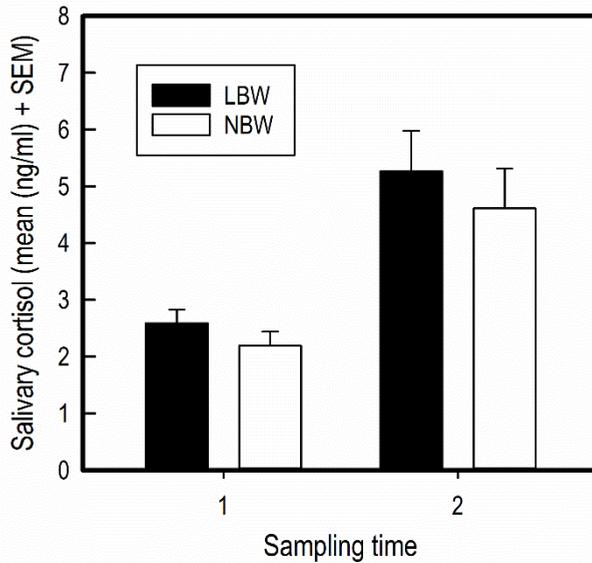


Figure 6.3 Average salivary cortisol concentrations of LBW and NBW pigs before and after a stressor. There was no significant effect of sex on cortisol concentrations, either as a main effect or interaction with birth weight. Therefore, the data for males and females have been combined.

large head is considered a sign of the so-called “brain sparing effect”, i.e., placental insufficiency resulting in prioritized brain development (Amdi *et al.*, 2013; Antonides *et al.*, 2015a). However, head morphology has been shown to correlate with birth weight (Amdi *et al.*, 2013) and can also be confounded by differences in head shape between different pig breeds (own, non-systematic observations). Therefore, birth weight remains the best indicator that the LBW pigs used in our study suffered from limited nutrients and oxygen in utero. The found difference in body weight of LBW and NBW pigs persisted throughout the duration of the experiment. This shows LBW pigs continued to experience impaired weight gain well beyond weaning. Long-term effects on growth have also been shown in previous studies with LBW pigs (e.g., Rehfeldt & Kuhn, 2006; Antonides *et al.*, 2015a). Such a lack of catch-up growth shown by LBW offspring is considered an additional risk factor for cognitive impairment in humans (Lindström *et al.*, 2017).

Based on previous studies assessing the effects of LBW in both humans and pigs (e.g., Gieling *et al.*, 2012; Radlowski *et al.*, 2014; Lindström *et al.*, 2017), it was expected that LBW pigs would have an impaired cognitive development

as shown by lower memory scores in the spatial holeboard task compared to NBW pigs. Both groups of pigs were able to acquire the task, producing similar learning curves to previous holeboard studies with pigs (e.g., Arts *et al.*, 2009; Roelofs *et al.*, 2017b). Pigs improved their performance as training progressed, as shown by increasing memory scores and decreasing latencies and exploration. In line with our expectation, LBW pigs had lower reference memory (RM) scores during the acquisition phase of the experiment. Additionally, LBW pigs had higher average hair cortisol concentrations (HCC) than NBW pigs in samples taken at weaning. This implies LBW pigs experienced more chronic stress during pre-weaning development. Both the cognitive impairment and the increased HCC found for LBW pigs were transient, likely due to the enriched housing conditions applied during this study.

Effects of low birth weight on spatial learning and memory

Birth weight was found to cause a mild cognitive impairment, based on spatial learning and memory in the holeboard task. Compared to NBW pigs, LBW pigs showed lower RM scores as the acquisition phase of the experiment progressed. This finding indicates that LBW pigs had more difficulty learning and remembering the locations of food rewards in the holeboard. It is unlikely this effect of LBW was caused by a difference in motivation between LBW and NBW pigs to perform the task. Both groups showed comparable scores on measures of motivation, such as the latency to first visit and the inter-visit interval. This is corroborated by an earlier study comparing food motivation of LBW and NBW pigs (van Eck *et al.*, 2016).

Our finding of impaired cognitive development is supported by earlier studies showing decreased learning and memory associated with LBW in pigs (Gieling *et al.*, 2012; Radlowski *et al.*, 2014). Similarly, LBW in humans has been shown to cause learning difficulties throughout adolescence (Lindström *et al.*, 2017; Yu & Garcy, 2018), as well as impaired spatial learning (Leitner *et al.*, 2005). However, there have also been studies which have shown LBW pigs to have a comparable, or even superior, cognitive performance compared to NBW pigs (Murphy *et al.*, 2013a, 2015; Gieling *et al.*, 2014; Antonides *et al.*, 2015a). Several factors could have contributed to this discrepancy in results.

First, it is difficult to compare the results found by Antonides *et al.* (who reported improved cognitive performance of LBW pigs) to those of other

studies assessing post-weaning cognition in LBW pigs, including the current study. This is due to large differences in housing conditions. Their pigs were removed from the sow at 4–6 days of age, whereas other studies applied weaning at 4 weeks of age, comparable to standard commercial practice. Abrupt changes in neonatal environment have been shown to impact piglet development, resulting in increased behavioral and physiological signs of stress (van Nieuwamerongen *et al.*, 2017; de Ruyter *et al.*, 2017). Additionally, there was a considerable difference in stocking density. Antonides *et al.* provided 0.625–1.25 m² space per pig, whereas the other studies provided a minimum of ~2 m² per pig (Gieling *et al.*, 2012, 2014; Murphy *et al.*, 2013a, 2015). A higher stocking density affects pig welfare mainly through increased aggression (Fu *et al.*, 2016). Such effects of stocking density could have impacted NBW pigs more, as they remain larger than LBW pigs. Taken together, these differences in housing conditions may have influenced the pigs' early development, hindering direct comparison of results.

Second, the findings of previous studies examining LBW pigs have all been based on smaller sample sizes than applied in our study. Smaller sample sizes increase the probability of chance findings (Button *et al.*, 2013), potentially leading to contradictory results in replication studies. Other factors influencing cognitive abilities, e.g., personality (Mazza *et al.*, 2018), could then lead to a significant difference between groups that does not reflect the effects of birth weight. In particular, several studies reporting comparable cognitive performance of NBW and LBW pigs have based their results on relatively small sample sizes. For example, Murphy *et al.* (2013a) compared six NBW to five LBW pigs in a conditional discrimination task, where both groups were equally capable of learning the task. Similarly, Gieling *et al.* (2014) found comparable spatial holeboard performance of LBW and NBW pigs by using litter as the experimental unit in data analysis (i.e., average performance of LBW or NBW litter mates was analyzed instead of individual performance of each pig). This resulted in a loss of statistical power by reducing the effective sample size. Interestingly, visual inspection of the RM scores of their pigs show a similar pattern to the current study. Control LBW pigs (half of the animals were prenatally treated with an anti-oxidative drug) have lower average RM scores toward the end of the acquisition phase.

Finally, it is possible that LBW has not consistently been found to impair cognition in pigs due to the use of different cognitive tasks in different studies. Cognitive development of LBW pigs has been assessed using measures of spatial learning (Gieling *et al.*, 2012, 2014; Radlowski *et al.*, 2014;

Antonides *et al.*, 2015a) and associative learning (Murphy *et al.*, 2013a, 2015). Spatial learning in a holeboard task and associative learning in a conditional discrimination task have previously been found to be independent measures of cognition in pigs (Roelofs *et al.*, 2017a). Perhaps no effects of LBW were found in associative learning studies with pigs because they assessed a cognitive domain that is less vulnerable to impairment as a result of LBW. Such specific effects of LBW, with varying effects on different cognitive tasks, have previously been reported for humans (Ford *et al.*, 2017) and rats (Caprau *et al.*, 2012). That LBW does not have a general negative effect on cognitive development in pigs is also supported by our finding of decreased RM, but not working memory (WM) scores. This is corroborated by earlier holeboard studies (Gieling *et al.*, 2012, 2014; Antonides *et al.*, 2015a), where LBW and NBW pigs show very comparable WM learning curves during the acquisition of the holeboard task (although one study found an effect of LBW on WM scores during reversal learning, implying impaired behavioral flexibility; Gieling *et al.*, 2012). Furthermore, when separating the general RM scores into rotational RM scores based on the ability of orientation within the environment (Cheng & Newcombe, 2005) and spatial pattern RM scores based on spatial pattern learning (Brown & Wintersteen, 2004), our results show that birth weight only affected spatial pattern RM scores. After entering the holeboard, LBW and NBW pigs were equally capable of orienting themselves and locating a baited hole. However, completing the spatial pattern of baited holes after finding this first reward was impaired in LBW pigs. This provides further evidence that LBW could have specific effects on different cognitive domains. It would be relevant for future studies to assess the effects of LBW on additional cognitive domains in pigs, mainly those that are relevant for their welfare. For example, as social behavior is of such importance to pig welfare (Coutellier *et al.*, 2007), testing the effects of LBW on social cognition is recommended.

Effects of low birth weight on pre-weaning chronic stress

Previous studies with pigs have shown that LBW causes an altered functioning of the HPA-axis. LBW pigs show increased baseline cortisol levels, both pre- and post-weaning (Klemcke *et al.*, 1993; Poore *et al.*, 2002). Furthermore, LBW pigs show an exaggerated acute stress response (Poore & Fowden, 2003). It was expected that the LBW pigs in our study would suffer from a similar increase in HPA-axis activity, namely an increase in hair cortisol concentration (HCC) compared to NBW pigs, indicative of chronic

stress and an exaggerated increase in salivary cortisol compared to NBW pigs after exposure to an acute stressor. These expectations were only partially confirmed.

HCC was used as a non-invasive measure of chronic stress (Meyer & Novak, 2012). As cortisol is incorporated into the growing hair shaft, HCC allows for assessment of HPA-axis activity over a longer time period than other biomarkers of stress. HCC as a measure of chronic stress has previously been assessed in pigs (e.g., Antonides *et al.*, 2015; van der Staay *et al.*, 2017). For example, barren housing leads to a higher HCC in pigs (van der Staay *et al.*, 2017). These results are comparable to chronic stress assessment using HCC in other species, with long-term stress leading to increased HCC (Davenport *et al.*, 2006).

At weaning, LBW pigs showed an increased HCC compared to NBW pigs. This suggests that LBW pigs experienced more chronic stress while in the farrowing pens. It is known that LBW piglets experience more physiological stressors after farrowing. For example, LBW piglets suffer from impaired thermoregulation (Herpin *et al.*, 2002) and are less likely to acquire a (desirable) teat when suckling (Le Dividich *et al.*, 2017) compared to their NBW siblings. However, in our study the effects of such stressors were mitigated by providing extra heating in the farrowing pens (both heat lamps and floor heating), as well as applying cross-fostering to ensure the number of piglets in a litter did not exceed the sow's number of teats. It is possible that in addition to the increased physiological stressors, LBW piglets display an exaggerated response to chronic environmental stressors. Housing piglets in farrowing pens, in which the sow is constrained in a farrowing crate, can lead to behavioral signs of decreased welfare, such as decreased play behavior (Held & Špinka, 2011). This could be due to the lack of space and limited opportunities for sow-piglet interaction (Singh *et al.*, 2017). Based on our results, LBW piglets are more chronically stressed in the farrowing environment than their NBW siblings. Future studies are required to establish which specific physiological or environmental stressors are responsible for LBW piglets' chronic stress.

Ameliorating effects of enrichment

Only a mild impairment of spatial cognition was found, along with a transient increase in chronic stress. It is likely that the effects of LBW found were ameliorated by the enriched housing conditions applied during this study.

Enriched housing has been shown to improve cognitive performance in pigs (Sneddon *et al.*, 2000; Grimberg-Henrici *et al.*, 2016). Furthermore, it has been suggested that training animals - in particular if training extends over a longer time period - may act as cognitive enrichment (Manteuffel *et al.*, 2009). Together, the environmental and cognitive enrichment applied in the current study may have alleviated the effects of adverse conditions, such as LBW. Therefore, it is possible that the LBW pigs in our study performed better than they would have done if they had been housed in the barren conditions that are standard practice on most commercial farms. Future studies exploring the post-weaning cognitive abilities of LBW pigs in different housing conditions are encouraged.

Several indications were found that enrichment also had an ameliorating effect on LBW pigs' stress response. First, in contrast to pre-weaning HCC, post-weaning HCC was not influenced by birth weight. This suggests both groups of pigs were experiencing similar levels of chronic stress once they were moved to the research facilities. Previous studies have shown enriched housing conditions decrease stress as measured by HCC and serum cortisol concentration in pigs (Carreras *et al.*, 2016b; van der Staay *et al.*, 2017). Therefore, it is likely that in our study, neither LBW nor NBW pigs experienced chronic stress after weaning. Similar findings have been reported by Murphy *et al.*, who compared mean salivary cortisol concentration (SCC) and found no difference between LBW and NBW pigs housed in enriched conditions (2015; cf. Antonides *et al.*, 2015a).

Second, we found no exaggerated acute stress response in LBW pigs. In our study, SCC was used as a non-invasive measure of acute stress (Hellhammer *et al.*, 2009). Both LBW and NBW pigs showed increased SCC after performing the first individual trial in the holeboard, indicating the applied stressor was successful (Merlot *et al.*, 2011). However, this increase in SCC was similar for LBW and NBW pigs. This provides further suggestion that the enrichment applied during our study had an ameliorating effect on LBW pigs' stress response, as a previous study has found LBW pigs to show an exaggerated response to acute stress (Poore and Fowden, 2003). Future research aimed at the comparison of HPA axis functioning between LBW and NBW pigs housed in standard commercial conditions is encouraged. A longitudinal study of hair cortisol on commercial pig farms should be feasible, as collection of samples is non-invasive. The acute stress response of LBW and NBW pigs could be compared by collecting saliva samples prior to and

after common stressors on commercial farms, such as ear tagging and tail docking at a few days old or mixing animals after weaning.

No sex effects on cognition or cortisol

Our study is the first to control for an effect of sex on the cognitive development of LBW pigs. This was done to account for a possible sex-dependent effect of stress on learning and memory, as has been found in other species (Luine *et al.*, 2007; Healy *et al.*, 2009). Such effects were expected to be exaggerated in LBW pigs, due to their altered HPA axis functioning (Wise *et al.*, 1991; Klemcke *et al.*, 1993; Poore *et al.*, 2002; Poore and Fowden, 2003). However, sex did not systematically influence any of the measures for spatial learning and memory in the holeboard, possibly because we failed to find an exaggerated stress response in our LBW pigs (see section **Ameliorating Effects of Enrichment** in this chapter).

In a previous study examining the effects of sex on spatial holeboard performance, male pigs showed impaired behavioral flexibility when faced with a reversal of the task (Roelofs *et al.*, 2017b). This result was not corroborated in the current study. Independent of birth weight, female and male pigs showed a similar exploration of the holeboard and were equally able to find the rewarded holes during the reversal phase. These contrasting results could be due to a difference in housing conditions between the two studies, with the previous study housing females and males separately. In our study, pigs were grouped according to birth weight category, resulting in mixed-sex groups. The effects of mixed- vs. single-sex housing in pigs in relation to their behavior and stress response has not yet received extensive scientific attention. Whether being housed in a mixed-sex group is more or less stressful may differ for males and females, as male pigs have been shown to engage in more aggressive behavior than females (Colson *et al.*, 2006; Clark and D'Eath, 2013). It has also been shown that male aggression is provoked more in mixed-sex groups than when males are housed separately from females (Colson *et al.*, 2006). As group composition appears to influence aggressive interactions and thereby social stress (Camerlink *et al.*, 2016), it is possible that it also impacts pigs' behavioral flexibility.

CONCLUSION

Our results show that LBW causes a transient cognitive impairment in weaned pigs, as measured by a spatial holeboard task. An impaired development of spatial cognition could have adverse effects on the welfare of LBW pigs, as they require spatial learning and memory to correctly respond to their environment. For example, remembering specific locations, such as food and water sites, preferred areas for resting or the preferred areas of dominant conspecifics, is relevant to pig welfare (Mendl *et al.*, 1997; Held *et al.*, 2002). LBW pigs also showed a transient increase in HCC, implying increased chronic stress in the farrowing environment. It is likely the effects of LBW found were mitigated by the enriched housing conditions applied during this study. Therefore, future studies assessing the cognitive development and stress responses of LBW pigs in commercial housing conditions are encouraged.

Part III

Judgment bias as a
measure of emotional
state

7

Making decisions under ambiguity: judgment bias tasks for measuring emotional state in animals

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ABSTRACT

Judgment bias tasks (JBTs) are considered promising tools in the assessment of emotional states of animals. JBTs provide a cognitive measure of optimism and/or pessimism by recording behavioral responses to ambiguous stimuli. For instance, a negative emotional state is expected to produce a negative or pessimistic judgment of an ambiguous stimulus, whereas a positive emotional state produces a positive or optimistic judgment of the same ambiguous stimulus. Measuring an animal's emotional state or mood is relevant in both animal welfare research and biomedical research. This is reflected in the increasing use of JBTs in both research areas. We discuss the different implementations of JBTs with animals, with a focus on their potential as an accurate measure of emotional state. JBTs have been successfully applied to a very broad range of species, using many different types of testing equipment and experimental protocols. However, further validation of this test is deemed necessary. For example, the often extensive training period required for successful judgment bias testing remains a possible confounding factor on results. Also, the issue of ambiguous stimuli losing their ambiguity with repeated testing requires additional attention. Possible improvements are suggested to further develop JBTs in both animal welfare and biomedical research.

INTRODUCTION

Within the framework of animal welfare studies as well as in biomedical studies, assessment of the emotional state of an animal can yield highly relevant information. The majority of studies on animal emotions (most of them using rodent species) address anxiety, which is assessed with classical tests such as the open field test, the light-dark test and the plus-maze test (for a recent critique of these tests, see Ennaceur, 2014). These tests measure the unconditioned response of an animal to an unfamiliar situation (the testing environment) that contains elements which the animal perceives as adverse/threatening (such as open space and/or high light intensities). They may be less suited for assessing emotion in non-rodent species such as pigs (see Murphy *et al.*, 2014). Instead of looking at the animal's response to unconditioned stimuli, one may use cognitive tests to assess emotion in animals, such as judgment bias tasks (JBTs; Harding *et al.*, 2004; Paul *et al.*, 2005; Murphy *et al.*, 2014), or (variants of) decision-making tasks (Murphy *et al.*, 2015). Affective reactions may provide useful feedback, both explicitly and implicitly, from emotional appraisal processes (Storbeck and Clore, 2007). According to Marchant-Forde (2015), the most influential recent studies measuring emotional state as an index of animal welfare are those assessing judgment (cognitive) bias. Bateson and Nettle (2015) consider JBTs as the 'gold standard' for measuring the mood of animals. In the area of biomedical research, judgment bias research is still in its infancy, although the number of studies using this type of task is growing. Besides its value for the purpose of welfare assessment, within the area of biomedical research, the affective state of an animal may be a confound for other behavioral tests and a source of uncontrolled variation (Bateson and Nettle, 2015). Knowledge of the animals' emotional state may contribute to understanding test results.

Emotion, cognition and judgment bias

Emotions are adaptive processes that help individuals react adequately to internal or external stimuli, thereby avoiding harm and seeking valuable resources, while cognition can be described as information processing mechanisms. Emotions cannot be regarded separately from cognition. Emotional states affect cognitive processes and conversely cognitive processes are often the initiators of emotions (Lazarus, 1982; Dolcos, 2015). The interdependence of emotion and cognition is reflected in the definition by Kleinginna and Kleinginna, (1981 p. 355):

“Emotion is a complex set of interactions among subjective and objective factors, mediated by neural–hormonal systems, which can

- a) give rise to affective experiences such as feelings of arousal, pleasure/displeasure;
- b) generate cognitive processes such as emotionally relevant perceptual effects, appraisals, labeling processes;
- c) activate widespread physiological adjustments to the arousing conditions; and
- d) lead to behavior that is often, but not always, expressive, goal-directed, and adaptive.”

The brain cannot be divided in cognitive and affective regions, since ‘affective’ brain regions are also involved in cognition and brain regions that are viewed as cognitive are also involved in emotions. Cognition and emotion are integrated in the brain (Pessoa, 2008). Brain structures at the heart of the neural circuitry for emotion (e.g., the amygdala) impact cognitive processing, from early attention allocation (Holland and Gallagher, 1999), through perceptual processing, to memory (Phelps, 2006). Similarly, brain structures involved in the neural circuitry for cognition, such as the dorsomedial and ventrolateral prefrontal cortex, have an intrinsic role in the experience of emotion (Barrett *et al.*, 2007).

If one regards emotion as a result of an anticipated, experienced, or imagined outcome of an adaptationally relevant transaction between organism and environment, cognitive processes are always crucial in the elicitation of an emotion (Lazarus, 1982). Cognitive processes are closely linked to emotional states as they are, for example, necessary for the appraisal of environmental cues and for the ‘production’ of emotions (Lazarus, 1982; Mathews and MacLeod, 1994). On the other hand, emotional states influence information processing in the brain, which helps individuals to react appropriately within a certain context (Mathews *et al.*, 1997). Emotional influences on cognition are defined as cognitive biases, of which three types can be distinguished: attention biases, memory biases, and interpretation or judgment biases (see Paul *et al.*, 2005). Attention bias occurs in threatening situations as a result of an anxious emotional state and is characterized by an increased attention to negative and threatening cues (Mathews and MacLeod, 1994; Mogg and Bradley, 1998). Memory bias refers to the phenomenon that events associated with positive or negative emotions are more readily remembered than neutral events, and includes memory storage, consolidation and retrieval processes

(Cahill and McGaugh, 1996; Hamann *et al.*, 1999). It is likely though, that the effects on memory are caused by high arousal and not by the valence of the emotion (Bradley *et al.*, 1992). Judgment bias or interpretation bias (from now on referred to as judgment bias) refers to the influence of emotions on the interpretation of ambiguous information (Mathews *et al.*, 1989; Eysenck *et al.*, 1991; Richards and French, 1992).

There are numerous operational definitions of judgment bias. Combining definitions of Boleij *et al.* (2012) and Bateson and Nettle (2015),

A judgment bias is a relative reaction to an ambiguous stimulus, expressing an ‘interpretation’ of this stimulus and an ‘expectation’ about the consequences of the reaction (Boleij *et al.*, 2012). In JBTs “(...) animals that respond to the ambiguous stimuli similarly to the positive stimulus are interpreted as displaying a high expectation of reward in the presence of ambiguous information, and hence an ‘optimistic’ cognitive style indicative of a positive affective state. In contrast, animals that respond to the ambiguous stimuli similarly to the negative stimulus are interpreted as displaying a higher expectation of punishment or lower expectation of reward, and hence a more ‘pessimistic’ cognitive style indicative of a more negative affective state”. — (Bateson and Nettle, 2015, p.3).

The processing of current information and the resulting behavioral choices are affected by optimism and pessimism (Dember *et al.*, 1989). In JBTs, optimism is operationally defined “as a higher proportion of responses to an ambiguous cue as if it were the cue predicting the positive outcome, and pessimism as a higher proportion of responses to an ambiguous cue as if it were the cue predicting the negative outcome” (Douglas *et al.*, 2012, p. 66). JBTs are thus believed to provide a cognitive measure of mood (Bateson *et al.*, 2015; Mellor, 2015).

Aim of this review

Since its introduction as a test for use in animals just a decade ago (Harding *et al.*, 2004), a considerable number of JBTs has been developed and applied in scientific studies, using a broad range of procedures and test equipment, in a large number of species. For continued (translational) research on animal emotions using these tasks, JBTs have to be validated and adapted to the abilities of each of these species (Anderson *et al.*, 2012). Here, we review the different implementations of this task, and a number of questions to be

solved, such as the cues and test arenas used, measuring response latencies vs. categorizing responses as Go/No-go, and species-specific modifications. We discuss the potential of JBTs as tools for measuring animals' emotional state and to assess the effects of experimental manipulations of the emotional state. Outstanding questions for future research on measuring judgment bias in animals relevant for both animal welfare research and biomedical research are discussed.

Discrimination learning

To successfully perform in a JBT, an animal has to first learn to discriminate between a stimulus (or set of stimuli) that predicts a positive consequence (S^+) and a stimulus (or set of stimuli) that predicts a negative consequence (S^- ; see **Figure 7.1**). Once the animal has mastered this discrimination, at least one ambiguous stimulus is introduced that lies somewhere between the original stimuli, i.e., judgment bias is tested in situations where animals make decisions under ambiguity (Mendl *et al.*, 2011; for an example, see **Figure 7.2**).

Two classes of JBTs can be distinguished: Go/Go and Go/No-go tasks. Go/No-go requires suppression of response at S^- , whereas in Go/Go tasks the animal responds to both types of stimuli with an active response (Murphy *et al.*, 2013b). In both Go/Go and Go/No-go tasks, the animal learns to discriminate between:

1. A *favorable reward* (large reward, immediate reward) and a *less favorable reward* (small reward, delayed reward), or
2. A *positively valenced outcome* (e.g., a large food reward) and a *negatively valenced outcome* (e.g., a small or less palatable food reward, food with an aversive taste, no reward). In some cases, the negative outcome consists of exposure to aversive noise or a frightening stimulus, such as a mild electric foot shock (e.g., Harding *et al.*, 2004), a blower surrounded with plastic (e.g., Destrez *et al.*, 2013), a dog (e.g., Doyle *et al.*, 2011a), swaying a plastic bag in front of the animal (e.g., Douglas *et al.*, 2012), i.e., consequences on a different modality than the consequences associated with the S^+ .

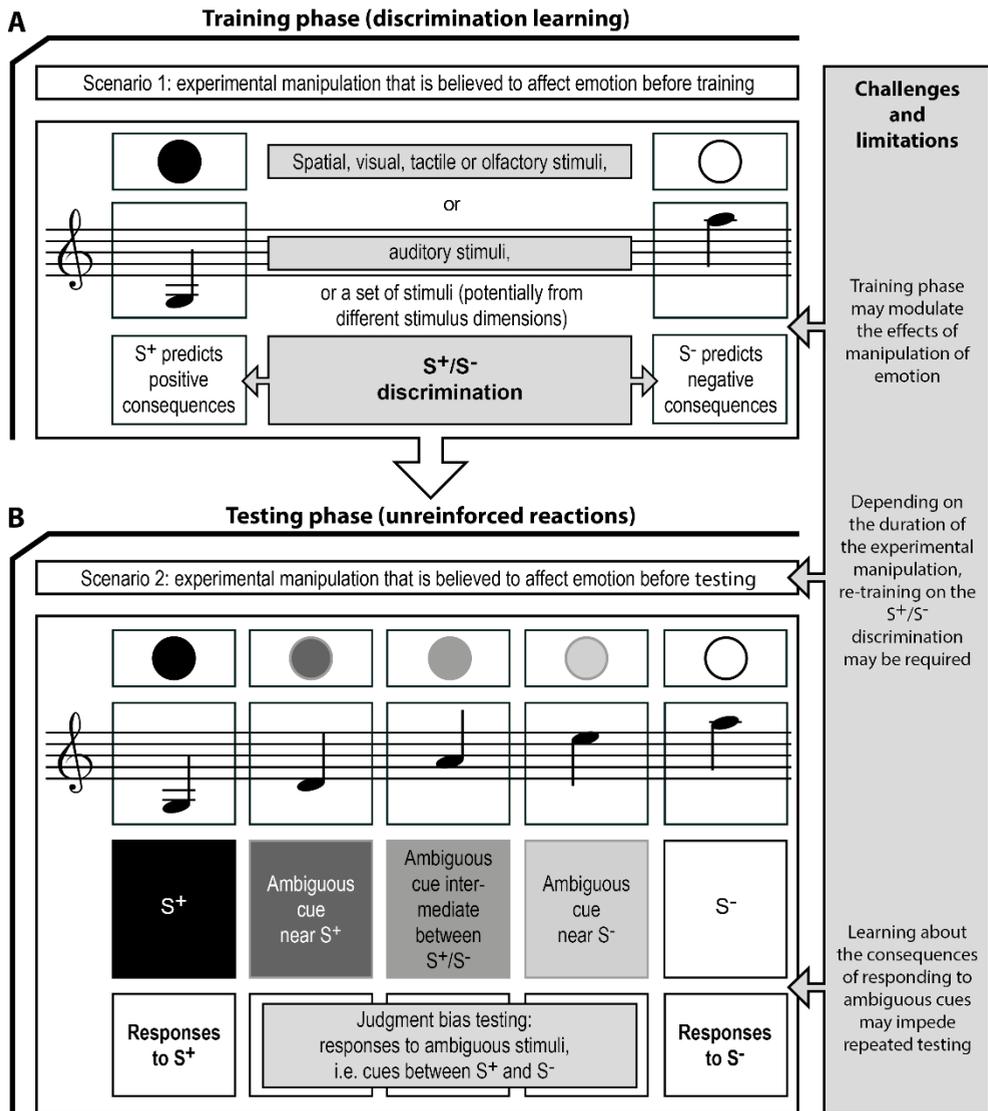


Figure 7.1 Schematic representation of judgment bias training and testing (inspired by Bateson *et al.*, 2011; Mendl *et al.*, 2011). The experimental manipulation that is believed to affect emotion precedes the training phase (**A**; Scenario 1) or the testing phase (**A**; Scenario 2). Retraining on the discrimination acquired during the training phase may be necessary, if the experimental manipulation preceding phase **B** lasts for a longer time period. An example of Scenario 1 is studying the effects of growing up in different housing systems, whereas Scenario 2 may be applied in a study assessing the effects of shorter lasting experimental manipulations, such as confinement, on emotion. Phase **B** may be repeated multiple times (e.g., Douglas *et al.*, 2012) to test the effects of different experimental manipulations in the same animal. Specific challenges and limitations may be connected to the different phases.

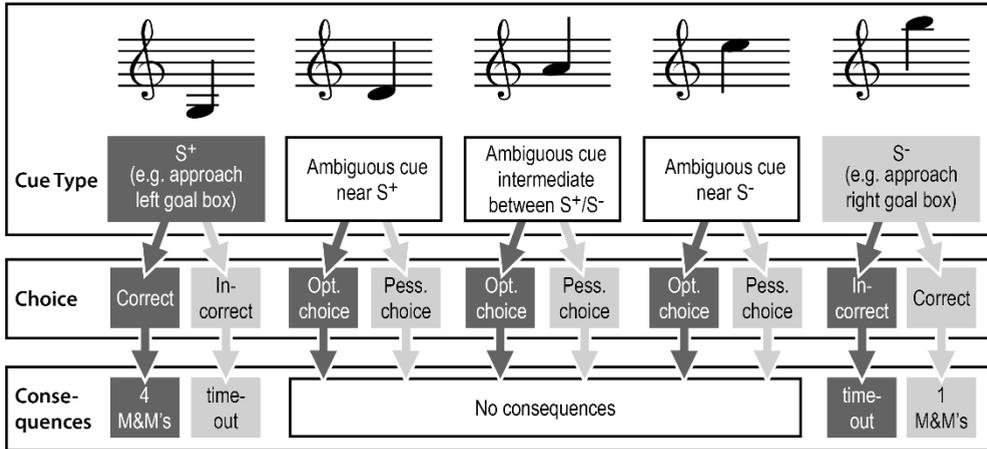


Figure 7.2 Example of the exact contingencies connected with responding to the different cues presented during the testing phase (see **Figure 7.1B**) in a judgment bias task (consequences of choices as used in Murphy *et al.*, 2013b). Under these or similar testing conditions, the animal already has been trained to respond correctly to the S⁺ and S⁻. As the responses to the ambiguous cues do not yield reward, the animal may learn that these cues represent a new class of stimuli.

Judgment bias tasks

Cues used in JBTs are spatial (e.g., Briefer and McElligott, 2013; Destrez *et al.*, 2013; Kis *et al.*, 2015); visual (e.g., Salmeto *et al.*, 2011); auditory (e.g., Murphy *et al.*, 2013b), tactile (e.g., Barker *et al.*, 2016), olfactory (e.g., Boleij *et al.*, 2012; see **Figure 7.1**), or a combination of different stimulus classes (Douglas *et al.*, 2012). In the latter case it may be difficult to define ambiguous cues and their scaling on the continuum from S⁻ to S⁺. A large variety of testing equipment is used for judgment bias testing, such as the home cage (e.g., Boleij *et al.*, 2012), runways (e.g., Salmeto *et al.*, 2011), open fields (e.g., Destrez *et al.*, 2012), or mazes with arms radiating from a start box (e.g., Briefer and McElligott, 2013). Owing to the large range of animal species that has been tested in JBTs, species-specific modifications are necessary concerning the size and layout of the testing arena (if any; e.g., dogs have been tested in their owner's home: Karagiannis *et al.*, 2015), the stimuli (cues) used; the type of response required (Go/Go, Go/No-go); the type of experimental manipulation used to affect emotion, and the type of consequences as result of the response to a cue (**Figure 7.2**; for an example see also Murphy *et al.*, 2013b).

PEAK SHIFT

When considering the use and results of JBTs, it should also be taken into account that basic psychological mechanisms such as generalization gradients and peak shift may play a role in responses to ambiguous stimuli in judgment bias paradigms. Generally, judgment bias paradigms start with the acquisition of a simple discrimination task, in which one stimulus provides a desired outcome (S^+) and another stimulus provides an undesired outcome (S^-). Thus, in Go/No-go tasks, responding (in whatever form the task requires) to the S^+ increases, while responding to the S^- decreases. This does not apply to Go/Go tasks, where maintenance of active responding to both the S^+ and the S^- is required.

It has been shown in a number of species, including humans, that when animals are trained using one S^+ and then tested using stimuli similar to but not exactly the same as the S^+ , responses will be highest to the stimuli nearest to the original S^+ . This is called a generalization gradient (see **Figure 7.3**; Cheng *et al.*, 1997). The response rate to intermediate stimuli found between an S^+ and S^- is thought to be predicted by the interaction between the two generalization gradients (Hanson, 1957; Kalish and Guttman, 1957, 1959).

The distribution of responses in a generalization gradient around the S^+ are usually symmetrical if only one stimulus is used. However, if a second stimulus is used (as in most JBTs), the peak of responses to the S^+ may shift to a cue further from the S^- (Ghirlanda and Enquist, 2003), a process termed peak shift. This particularly occurs if the S^+ and S^- are relatively similar to start with. A complicating factor for Go/No-go tasks is that it is difficult to assess whether there is a shift in the generalization gradient surrounding the S^- , as there is generally a low response rate or no response at all to the S^- , which predicts an undesired outcome (such as no reward). Results from studies specifically analyzing the responses to the S^- seem to indicate that there is also a peak-shift in S^- responses (Hanson, 1959), though it is not clear whether this is to the same degree as the influence on S^+ responding. If peak shift differentially affects generalization gradients surrounding the S^+ and S^- , then responding to ambiguous stimuli surrounding the S^+ and S^- may also be differentially affected.

For a better understanding of the processes underlying judgment bias, it may be necessary to address the generalization gradients around the discriminative stimuli used. This may include presenting ambiguous stimuli that are outside

the value-range between S^+ and S^- (see **Figure 7.3**) to determine the role that peak shift may play in responding in JBTs (Ghirlanda and Enquist, 2003, p. 20).

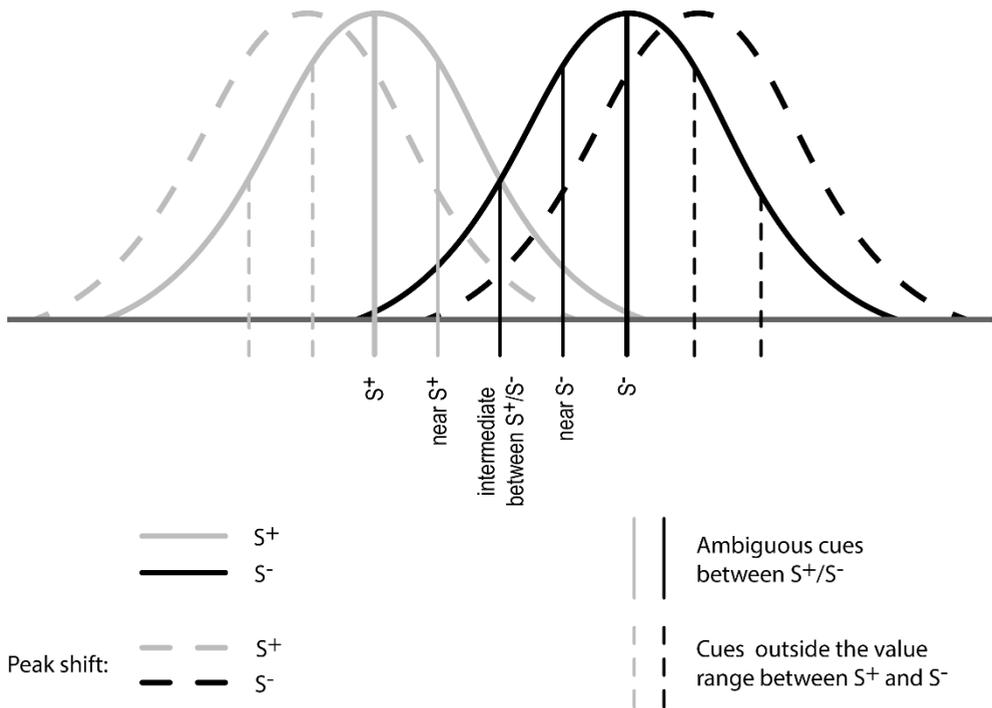


Figure 7.3 Discrimination between S^+ and S^- ; gradients around S^+ and S^- are depicted as Gaussian distributions. This distribution reflects the phenomenon of ‘generalization’, in which stimuli that are more similar to an S are more likely to elicit a similar response. Generalization gradients, and specifically interactions between the gradients around the S^+ and S^- , may play a role in responding in JBTs, independent of the judgment bias of the subject. Peak shift, in which the peak of a generalization gradient surrounding the S^+ shifts away from the S^- and vice versa (see dotted lines), may also influence responding to ambiguous stimuli.

STATE VS. TRAIT

Faustino et al. (2015) suggest that judgment bias may reflect either a state or a trait. However, JBTs have commonly been used to measure the affective *state* of an animal. Modulation of judgment bias through situational or contextual factors which can be observed as within-individual variability (e.g., by providing an enriched living environment, stress, or mood-enhancing drugs) is characteristic of a state. Emotional *trait* can be considered as a constant that is a permanent feature of the individual (Ramos and Mormède, 1998), i.e., may be the expression of a specific phenotype of an individual (Faustino *et al.*, 2015). Similarly, Strelau (2001, p. 311) defined trait as a relatively stable and individual-specific generalized tendency to behave or react in a certain way expressed in a variety of situations (see also **Figure 7.4**). In order to assess a trait (which is stable over time), the test(s) used must yield highly replicable results (Carter *et al.*, 2013). A trait is thus considered a permanent characteristic, whereas a state is considered as a transient condition that is only observable at particular moments (see also Fridhandler, 1986; Koski, 2011; Carter *et al.*, 2013). However, traits are not entirely static; they can change gradually over time under the influence of environmental factors (Strelau, 2001).

Anxiety, for example, can be seen as both a trait and a state. Trait anxiety is defined as the intrinsic basal anxiety characteristic of an individual, which does not vary from moment to moment, while state anxiety is defined as the anxiety that an individual experiences at a particular moment in time (Lister, 1990). Trait anxiety is determined by genetic factors, environmental influences and gene by environment interactions. Theoretically animals that have a higher trait anxiety respond to dangerous situations more frequently and with a greater intensity than individuals lower in trait anxiety (Spielberger *et al.*, 1984). More anxious individuals thus have a higher level of trait anxiety and in threatening situations probably also higher levels of state anxiety. The difficulty with state anxiety is that the level of anxiety that is measured depends on both the situation and the level of trait anxiety of the individual. The most reliable measure of the anxiety characteristic of an animal would thus be a measure of trait anxiety, and not of state anxiety. In animals it remains to be investigated how to make a distinction between state and trait anxiety. Human studies revealed that judgment bias is influenced by trait anxiety (as measured by questionnaires; see for example Eysenck *et al.*, 1991;

Mathews and MacLeod, 1994), as well as state anxiety (Mathews and MacLeod, 1994; Anderson *et al.*, 2012).

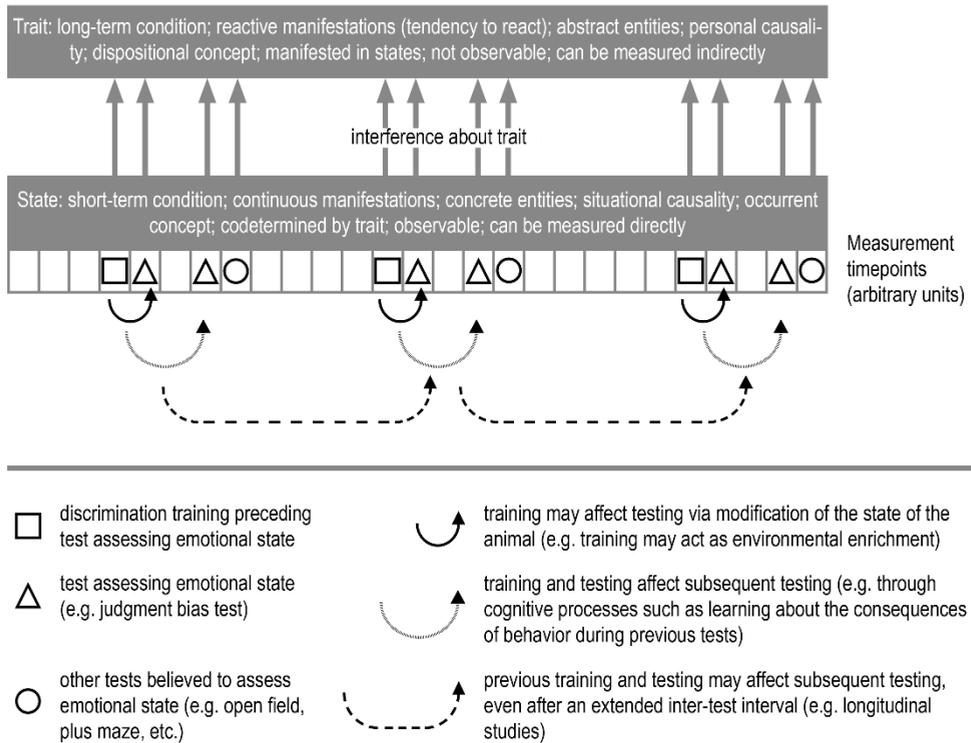


Figure 7.4 Measuring trait vs. state. Repeatedly testing emotional state (e.g., across the lifespan of an animal) may yield information about emotional trait, i.e., the behavior indicative for a particular trait needs to be repeatable (Carter *et al.*, 2013). In this hypothetical example, the JBT and some other tasks that are believed to assess emotional state are applied multiple times (for an example, see Bethell and Koyama, 2015). Possible consequences of repeated testing are summarized. *The description of the concepts trait and state are from Strelau (2001 p. 317, Table 2).

For further validation of JBTs, the animal's behavior during the testing phase should be correlated with behavior in other tasks that are believed to assess emotional states and/or traits. For rodents, these may be tests such as the open field test, the light-dark test, the elevated plus-maze, the novel object test, and/or the modified holeboard, to name a few (e.g., van der Staay *et al.*, 1990b; Duncan and Keller, 2011; see **Figure 7.4**). In non-rodent species, these tasks may be less adequate and other tasks validated for those particular species must be applied. Some studies have compared JBT performance to other tests of emotionality. Judgment bias has been shown to correlate with anxiety in pigs as measured by a novel object test (Carreras *et al.*, 2016a). Pessimistic judgment bias was positively correlated with a more fearful response during the novel object test. Rats which laugh when tickled (a confirmed behavioral signal of positive emotional state) have a more positive judgment bias than rats which don't (Rygula *et al.*, 2012). Destrez *et al.* (2012) found that lambs treated with an anxiolytic showed a positive judgment bias and were less fearful during isolation and suddenness tests. When studying the responses of laying hens in different tests of emotionality, some correlations were found between measured parameters during a JBT, a novel object test and an anticipation test. However, no clear relationship between the tests was found (Wichman *et al.*, 2012). To test specifically for the effects of emotional traits on judgment bias, examining possible correlations with tasks that measure personality traits is necessary. For example, repeated comparisons of baseline judgment bias of individuals with high vs. low trait anxiety would be valuable. Individual differences in baseline judgment bias have been reported (e.g., Starling *et al.*, 2014). Repeated judgment bias testing has been applied to a small sample of chimpanzees ($n = 3$). Individual differences in judgment bias were found, which remained stable across five test sessions (spanning a time period of 1–2 weeks; Bateson and Nettle, 2015). When a similar study was performed with pigs, no consistent results were found between two test sessions (with a 5-week intermittent period; Carreras *et al.*, 2015). In rats, repeated testing of baseline judgment bias has produced stable results, which correlate with traits such as motivation and sensitivity to stress (Rygula *et al.*, 2013, 2015b).

Though replicability and stability of results is a basic requirement of a trait, this may be difficult to demonstrate empirically. Unfortunately, the order of testing may affect behavior in subsequent tests (e.g., McIlwain *et al.*, 2001; Blokland *et al.*, 2012). This is an observation that may also complicate a correlational approach (e.g., factor analysis) to validating JBTs. To the best of our knowledge, neither the correlations between different tests that are

believed to assess emotion with JBTs, nor the effects of repeated (or longitudinal) assessment has yet been studied systematically.

CUES ON ONE OR MULTIPLE STIMULUS DIMENSIONS

There are several potential concerns related to the choice of stimulus dimension(s) when preparing a JBT design. There may be variation between animals in their capabilities to differentiate between cues. For example, when using auditory cues, the accuracy of perceiving differences between tones may be different for good and poor listeners in learning the original tone discrimination (see, e.g., Amitay *et al.*, 2005). For olfactory stimuli, it needs to be ensured that mixtures of the S⁺ and S⁻ odors are distinguishable as such, i.e., are not simply regarded as a novel odor, but as intermediates between S⁺ and S⁻ (Dreumont-Boudreau *et al.*, 2006). Differences in ability to discriminate between learned and ambiguous stimuli may similarly affect studies using visual or tactile cues. Additionally, there may be a non-linear relationship between the perception of the originally acquired S⁺/S⁻ and the intermediate stimuli, i.e., due to the sensory capabilities of the species studied, the scaling of cues may not be perceived as intended. For example, what is intended to be an intermediate ambiguous cue may be perceived as having a higher similarity to S⁺ than to S⁻. The dimension and scaling of the cues used thus may affect performance in JBTs by affecting ambiguity. Therefore, it is important to adjust the dimension and scaling of cues to the species studied (e.g., auditory cues used in several rat studies are adapted to the species' audiogram (e.g., Enkel *et al.*, 2010; Rygula *et al.*, 2013)).

A number of studies used cues from different stimulus dimensions. Although such a methodology might make discrimination between cues easier, they limit the interpretation of JBT results (Nogueira *et al.*, 2015). Graded stimuli on a unidimensional scale allow for the prediction of response patterns (see **Figure 7.5**). When different dimensions are used for S⁺ and S⁻, ambiguous cues can no longer be considered as intermediate. For example, Salmeto *et al.* (2011) used a series of chicken to owl morphs, where the S⁺ was the mirror image of the tested chicken, whereas the S⁻ and the different morphs were silhouettes, printed on cards (the mirror image added an extra dimension to the S⁺, namely movement). When comparing responses to a previous experiment using a chicken silhouette as S⁺, it became clear that the chicks responded differently to the mirror than to printed stimuli, with decreased latencies to respond to the moving mirror images. When the ambiguous

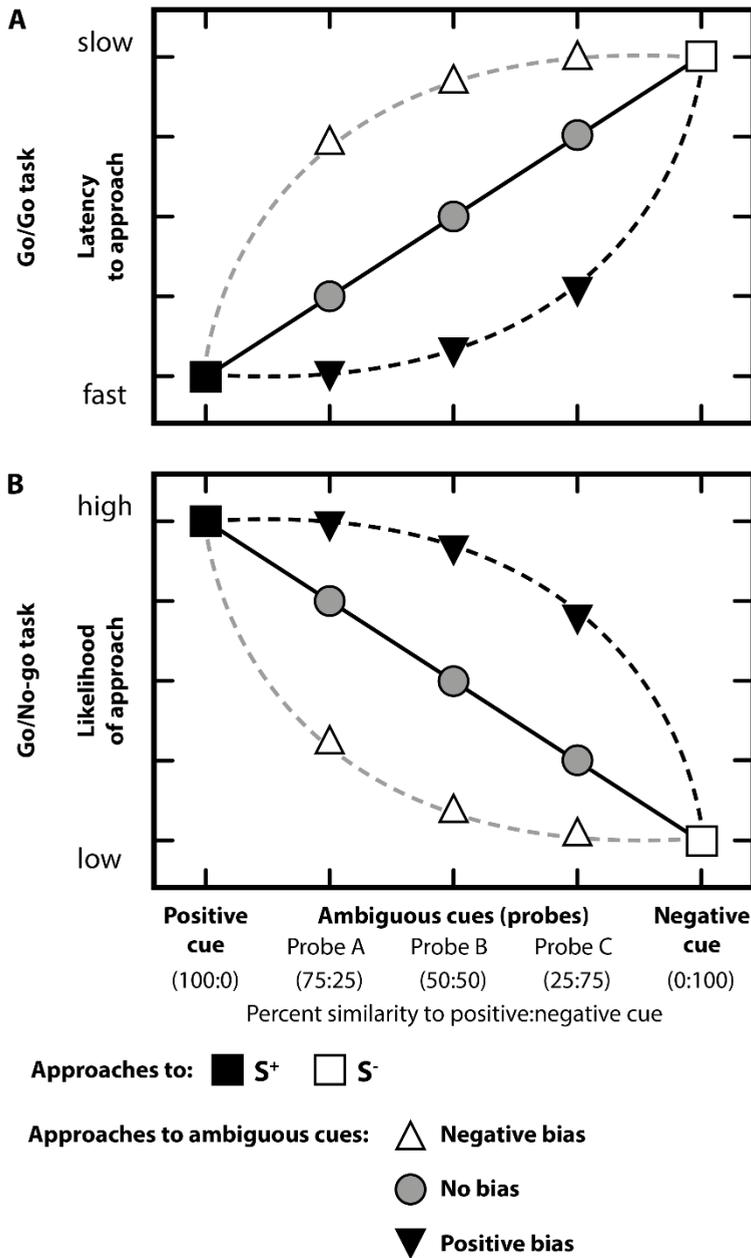


Figure 7.5 Judgment bias represented schematically. In Go/Go tasks, the latency to approach a cue is usually analyzed (A), whereas the likelihood (or the proportion of animals in a treatment group) is usually analyzed in Go/No-go tasks (B). Note that rather often, experiments conceived as Go/No-go tasks report latencies to approach, because animals didn't learn to suppress responding in the no-go trials (e.g., Bateson *et al.*, 2015). Similarly, results of Go/Go tasks can be reported as likelihoods of approaching/not approaching (e.g. Murphy *et al.*, 2015).

stimuli are unrelated to the trained reference cues, there is a risk of measuring response to novelty instead of ambiguity. For example, using wild peccary as subjects, Nogueira *et al.* (2015) used categorically different auditory stimuli (whistle and horn as S⁺ and S⁻, bell as ambiguous). Ambiguity is characterized by the possibility to interpret a situation or stimulus in two (or more) distinct ways, i.e., in the case of JBTs ambiguous cues can be interpreted as predicting a similar outcome to either S⁺ or S⁻. In order to obtain results which are interpretable as responses to ambiguity, it is suggested to only use cues on a single dimension.

LOSS OF AMBIGUITY

It is common practice in JBTs to leave test trials (i.e., presentations of ambiguous stimuli) unrewarded (see **Figure 7.2**). Such a lack of reward will stand out after extensive training where rewards were always present. This will facilitate learning about unrewarded ambiguous trials (Jamieson *et al.*, 2012). As a result, repeated testing in JBTs could lead to a loss of ambiguity, as the animals will learn to associate the ambiguous stimuli with a specific outcome. This could influence the animals' subsequent choices during test trials and thereby lead to false conclusions of measured judgment bias (Doyle *et al.*, 2010b). Such possible confounding effects of unrewarded testing trials have been recognized in numerous studies. Brilot *et al.* (2010) found that their study subjects (starlings) increased the response latencies as testing progressed, while failing to detect a cognitive bias. They concluded that their birds quickly learned that the ambiguous trials were never rewarded and therefore became slower to respond to ambiguous cues. Multiple other studies report a loss of ambiguity as a possible cause for increased response latencies of their study subjects (Doyle *et al.*, 2011b; Sanger *et al.*, 2011; Starling, 2012; Destrez *et al.*, 2014; Starling *et al.*, 2014; Verbeek *et al.*, 2014; Karagiannis *et al.*, 2015). In addition to an increased response latency, Murphy *et al.* (2013b) found that their pigs also decreased the number of optimistic responses with repeated testing. Doyle *et al.* (2010a) suggested that a loss of ambiguity could even explain why stressed sheep responded more pessimistically than their non-stressed controls. As mild stress may enhance learning (Mendl *et al.*, 2009), the stressed animals could simply have learned about the lack of rewards during ambiguous trials faster than the control group. Similar conclusions have been drawn in other studies (Destrez *et al.*, 2012, 2013; Scollo *et al.*, 2014). A study dedicated solely to the effect of repeated testing in absence of any experimental manipulations or changes in

environment found that sheep develop a reluctance to respond during ambiguous test trials (Doyle *et al.*, 2010b). As there was no explanation for this change in behavior related to a change in their emotional state, an increase in pessimism seems unlikely. Rather, this study supports the notion that animals may learn about the outcome of ambiguous trials with repeated testing and change their responses accordingly.

Several possible solutions to the problem of loss of ambiguity have been suggested. Use of a secondary reinforcer during training and testing was successfully applied in a study by Keen *et al.* (2014). In addition to a high and low food reward, their bears were also reinforced with a clicker to maintain responsiveness. During ambiguous testing trials, no food rewards were given, but reinforcement with the clicker continued. A secondary reinforcing audio cue has also been used in a study with Rhesus macaques (Bethell *et al.*, 2012). Another measure to reduce learning about the outcome of test trials is a partial reinforcement ratio schedule for training and control trials. For example, Neave *et al.* (2013) used partial reinforcement of positive trials during training. Although the punishment rate for negative trials remained 100%, they reduced the reward rate for positive trials to 50%. Using this training procedure, their calves learned to have lower expectations of reward during ambiguous trials. Partial reinforcement of training and control trials has also been successfully applied in various other studies (Bateson and Matheson, 2007; Matheson *et al.*, 2008; Bethell *et al.*, 2012; Richter *et al.*, 2012; Neave *et al.*, 2013; Daros *et al.*, 2014; Bateson *et al.*, 2015; Bethell and Koyama, 2015).

The number of learning opportunities about the outcomes of trials during judgment bias testing can also be reduced by minimizing the number of ambiguous test trials. In a study by Vögeli *et al.* (2014), sheep were subjected to three test sessions of five trials, with each session containing only one ambiguous trial. No reduction in visits to unrewarded ambiguous probes was reported. Similarly, studies by Rygula *et al.* (2013, 2015b) report stable judgment bias for their rats by using a relatively small number of ambiguous trials in comparison to positive/negative control trials. Although these studies support the notion that limiting the exposure to unrewarded ambiguous probes can prevent loss of ambiguity, this measure would also reduce the number of trials that can be used to estimate effects of experimental manipulations on judgment bias. A reduced number of ambiguous trials may make the JBT results more prone to chance findings.

Using a between-subjects design would at least minimize the number of exposures to ambiguous stimuli per animal (Brilot *et al.*, 2010).

A final suggestion has been to reward ambiguous trials (Carreras *et al.*, 2015; Murphy, 2015, pp. 185–187). This was shown to lead to a maintenance of optimistic choosing throughout test sessions, whereas unrewarded test trials lead to a decrease in optimistic choice. However, such a design may still lead to associative learning concerning the outcomes of ambiguous trials, rendering them no longer ambiguous.

Surprisingly, Bateson and Nettle (2015) used no specific measures to avoid loss of ambiguity, yet found no effects of repeated testing in three chimpanzees with respect to the latencies to react to intermediate stimuli. Consequently, they conclude that their JBT is suited for longitudinal assessment of welfare in this species. The authors ascribe their apparent maintenance of ambiguity to the difficulty of their discrimination task (paper cones of 20% vs. 60% gray, with intermediate shades as ambiguous stimuli). However, their very small sample size ($n = 3$) increases the chance of false positive findings (Tversky and Kahneman, 1971). Therefore, repeated testing with a larger sample size would be beneficial to further support their method of preventing loss of ambiguity.

Loss of ambiguity can become a considerable limitation to JBTs, as it renders them ineffective for the detection of changes in affective state (Brilot *et al.*, 2010). Rather, it may cause animals to base their performance on associative learning as testing progresses. This could lead to incorrect conclusions about the effects of experimental manipulations on an animal's affective state. Therefore, it is recommended to implement precautions against loss of ambiguity, such as the use of a partial reinforcement schedule during training and testing (e.g., Bateson *et al.*, 2015). The specific design of a study will determine which precautions are the most suitable. Also, it is important to exclude loss of ambiguity as a possible explanation for results. This can be done by testing for changes in response to ambiguous stimuli in the absence of experimental manipulation (Neave *et al.*, 2013; Daros *et al.*, 2014).

GO/NO-GO VS. ACTIVE CHOICE TASKS

In an article by Baciadonna and McElligott (2015), of the judgment bias publications reviewed, approximately 70% (22 of the 32 publications) were designed as a Go/No-go task. Of the studies evaluated for this review,

approximately 50% was of the Go/No-go type. Matheson *et al.* (2008) developed an active choice task for starlings in which the subject must respond to the S⁺ and S⁻ with the same operant behavior (e.g., pecking a key (S⁺) associated with immediate reward, or a key (S⁻) associated with delayed reward). Other variants have since been developed, such as an active choice task for pigs, in which responding by approaching the goal box associated with the S⁺ yields a large reward and approaching the goal box associated with the S⁻ yields a small reward (Murphy *et al.*, 2013b).

Theoretically, the main difference between Go/Go tasks and Go/No-go tasks is that in Go/Go or active choice tasks the animal is required to make an active response to both the S⁺ and S⁻, whereas in Go/No-go tasks, the animal is required to suppress a response to the S⁻. In Go/No-go tasks, a cut off criterion is defined to distinguish between the two response classes. Usually, trials in which an animal did not respond within a pre-determined cut-off time are scored as No-go responses (see **Figure 7.5**). Alternatively, the median response time—an approach that determines the cut-off within a preset period empirically—serves to distinguish between Go (latency to respond below median latency) and No-go (latency to respond above median) responses (e.g., Wichman *et al.*, 2012). In both instances, the proportion of animals responding in one of the two classes (Go, No-go) is analyzed. The selected cut-off time may determine the discriminating ability of the test.

The response suppression required for Go/No-go tasks may influence JBT results, as behavioral inhibition is thought to be influenced by emotion (Cyders and Smith, 2007). Moreover, in Go/No-go tasks, No-go responses could be considered an omission to react, rather than a pessimistic response (Guldimann *et al.*, 2015). Active choice tasks circumvent this possible confounding factor of motivation by requiring active responses for both optimistic and pessimistic choices (Hales *et al.*, 2014). As active choice tasks do not require behavioral inhibition and allow for omissions to be measured separately from optimistic/pessimistic responses, they may be more suited for measuring judgment bias than Go/No-go tasks (Murphy *et al.*, 2013b). It should be noted that, in practice, a Go/No-go and a Go/Go task are not necessarily mutually exclusive (see **Figure 7.5**). If latencies to respond are recorded in an active choice task, then the data can also be analyzed as reflecting Go/No-go responses (e.g., see Douglas *et al.*, 2012).

Many different criteria have been used for mastering the basic discrimination task preceding judgment bias testing. In both Go/Go and Go/No-go tasks similar criteria are used, usually based on accuracy (60% to 90% correct responding to S⁺ and S⁻, Anderson *et al.*, 2013; Keen *et al.*, 2014; Rygula *et al.*, 2015a), latency (shorter latencies to respond to S⁺ than to S⁻, Briefer Freymond *et al.*, 2014; Kis *et al.*, 2015), or running speed (faster to S⁺ than S⁻, Karagiannis *et al.*, 2015). Specific to No-go trials, a predetermined number of no-approaches to S⁻ (Sanger *et al.*, 2011) has been used for determining when animals have learned the basic discrimination. The learning criterion must be reached over a predetermined number of training days, trials, or trials within a number of days. All animals that did not reach the criterion within this maximum are excluded from testing with ambiguous cues (e.g., Müller *et al.*, 2012). Sometimes additional criteria, such as that the animal makes no omissions in a fixed number of trials (Anderson *et al.*, 2013), are used.

In some studies, differences between the responses to the S⁺ and S⁻, confirmed statistically by Wilcoxon test (e.g., Kis *et al.*, 2015) or Mann-Whitney U-test (e.g., Starling, 2012) were used as criterion. It has not yet been investigated how the learning criterion, i.e., the level of mastering the original S⁺/S⁻ discrimination, affects the sensitivity of subsequent testing with ambiguous cues. It is conceivable that a weak criterion decreases the likelihood to detect a judgment bias.

TRAINING AND TESTING IN ISOLATION VS. IN THE GROUP

Though not unique for JBTs, testing social animals individually, without direct contact with its group, may increase the stress level and/or decrease the willingness of an animal to learn the task and/or perform the required responses. Extensive habituation and pre-training may be necessary before judgment bias can be individually assessed (see Krasheninnikova and Schneider, 2014). For example, pigs need extensive habituation before they can be trained and tested individually in JBTs (Murphy *et al.*, 2013b, 2015).

Training and testing in a group setting is another solution to problems associated with individual testing of social animals. However, group testing is likely also accompanied by methodological issues. To date, only one study has used group training for a JBT. Training white-lipped peccary in isolation was unsuccessful in a study by Nogueira *et al.* (2015), necessitating training within a group setting. During discrimination training, the animals responded

to the S⁺ (approached a food bowl containing rewards) as a group. A similar method was used to acquire correct No-Go responses to the S⁻. The authors mention that extra food rewards were provided when higher-ranking individuals were monopolizing the food bowl. This implies that higher-ranking individuals received more rewards for correct responses than lower-ranking animals, possibly influencing results of discrimination training. Also, differential expectations of reward could have been established, with higher-ranking individuals experiencing a bigger contrast between rewarded and non-rewarded trials. Only animals which reached a criterion level of performance were used for individual judgment bias testing. Another potential limitation of training in a group setting is the difficulty of determining individual performance. Did all animals truly acquire the discrimination between S⁺ and S⁻, or were some individuals simply copying the responses of their group members? This can only be established by acquiring individual results (e.g., by individual training or by evaluating responses to reference tones during individual testing). No systematic comparisons of JBT training and testing in isolation and in social groups have been reported so far. Due to the many potential limitations of training and testing in a group setting, it does not seem likely to be advantageous over individual habituation of social animals.

The effects of (short-term) isolation of social animals, applied as an experimental manipulation of the emotional state preceding judgment bias testing, have been explored in several species. Social isolation affected judgment bias in chicks, with duration of the isolation period having specific effects on JBT performance. A pre-testing isolation period of 5 min induced increased pessimistic responses, while an isolation period of 60 min also decreased optimistic responding (Salmeto *et al.*, 2011; Hymel and Sufka, 2012). For pigs and laying hens, no effect of short-term social isolation on JBT performance was found (Düpjan *et al.*, 2013; Murphy *et al.*, 2013b; Hernandez *et al.*, 2015). Only the study by Murphy *et al.* (2013b) mentions habituation of the animals prior to training and testing, possibly explaining why no effect of social isolation was found. When male rats are moved from group housing to individual cages, their rate of optimistic responding decreased (no effect was found for female rats). However, as enrichment and available shelter were also removed when moving the rats, these could have been confounding factors in this study (Barker *et al.*, 2016). Together, these studies suggest that habituation of social animals to the training and testing conditions is sufficient to avoid a confounding influence of stress during testing.

EXCLUDING NON-LEARNERS

Many judgment bias studies report the exclusion of animals that failed to reach a required criterion during training (e.g., Starling, 2012; Starling *et al.*, 2014; Verbeek *et al.*, 2014; Bethell and Koyama, 2015; Hernandez *et al.*, 2015). For example, Wichman *et al.* (2012) reported 10 out of 38 chickens were unable to acquire the discrimination between rewarded and unrewarded trials, in spite of a long training period. These animals could therefore not be subjected to judgment bias testing. Similarly, in a group of 18 white-lipped peccaries, four adult individuals did not learn the basic discrimination in a Go/No-go auditory discrimination task and were consequently not tested in the subsequent JBT (Nogueira *et al.*, 2015). In a study by Brajon *et al.* (2015), only 59% of their 54 pigs completed the training preceding judgment bias testing. Consequently, all results and conclusions from JBTs are based on the study subjects that were capable of learning the discrimination task. If not all animals are able to reach the preset learning criterion, the samples are biased toward ‘learners’. The larger the proportion of excluded ‘non-learners’ is, the more biased a study is, and consequently, the less the results can be generalized. Development of tests that need less pre-training, e.g., by ensuring the discrimination training is better suited to the natural abilities/behaviors of the studied species, may allow for more animals to participate in subsequent judgment bias testing. Developing discrimination tasks which the studied animals are able to master fairly easily may also prevent selective loss of animals in experimentally manipulated groups. For example, possible effects of stress on learning could lead to animals undergoing a particular treatment (e.g., induced anxiety) being more likely to fail to pass the training phase (Mendl *et al.*, 2009; Conrad, 2010). Increasing the difficulty of discrimination training may increase the likelihood of a larger proportion of non-learners in a specific treatment group.

USABILITY FOR ASSESSING ANIMAL WELFARE

In animal welfare research, JBTs have been applied to a wide range of species that are commonly kept in captivity for a variety of reasons (e.g., production animals such as pigs, Brajon *et al.*, 2015; laboratory animals such as rats, Burman *et al.*, 2008; zoo animals such as Grizzly bears, Keen *et al.*, 2014; companion animals such as dogs, Titulaer *et al.*, 2013). Most of these judgment bias studies have been aimed at investigating the effects of common conditions inherent to life in captivity. For example, the effects of

providing environmental enrichment have been studied extensively (e.g., Douglas *et al.*, 2012; Bethell and Koyama, 2015). Also, the effects of common handling procedures have been frequently tested using JBTs, such as dehorning procedures in cattle (e.g., Neave *et al.*, 2013).

According to Bateson and Matheson, (2007 p. 36), “to be practically useful as a measure of how animals feel, cognitive bias needs to be easy to measure in applied settings”. However, many studies needed extensive training on the basic discrimination task before judgment bias could be assessed, decreasing the practical applicability of JBTs as a form of welfare assessment. Additionally, an extensive training period could mask potential detrimental effects of experimental manipulation and is considered one of the most confounding factors in judgment bias test paradigms (Novak *et al.*, 2015). Acting as cognitive enrichment, training could improve the welfare/affective state of the study subjects (Carlstead and Shepherdson, 2000; Puppe *et al.*, 2007; Pomerantz and Terkel, 2009; Zebunke *et al.*, 2011; Guldemann *et al.*, 2015). In spite of negative affect manipulations, this could lead to optimistic responses from subjects (Düpjan *et al.*, 2013). In line with this expectation, in preparation of judgment bias testing, Svendsen (2012) trained farmed mink categorized as fearful or as explorative, to induce a positive affective state. Whereas the fearful mink behaved more explorative at the end of training, an opposite effect of training was found in the mink categorized as explorative: these animals were rated as less positive post-training, possibly due to frustration about the absence of expected rewards during later training sessions. Svendsen *et al.* (2012, p. 366) caution that “(...) studies that involve induced affective states and a lot of training of the animals to assess their welfare, such as the cognitive bias method, need to be interpreted carefully as the handling and training has a different effect on animals in different affective states”. Consequently, future research should focus on the question of whether training for a JBT itself modulates the animal’s emotional state.

The sensitivity of judgment bias to detect effects of experimental manipulations on emotions has not yet unequivocally been established. For example, in a study by Keen *et al.* (2014) the JBT was unable to detect differential effects of environmental enrichment methods in bears. Although behavioral observations showed that the different types of enrichment were valued differently by the bears (some items were interacted with more than others), this did not result in differences in measured judgment bias. It is possible that providing enrichment did not produce a measurable increase in judgment bias because the JBT was not sensitive enough to detect this change

in affective state. However, lack of effect to be detected is another possibility, as the bears were already housed in enriched environments. The addition of an extra enrichment item may not have produced a measurable improvement in affective state to begin with. Similarly, another judgment bias study did not discriminate between the welfare of short and long term kennelled dogs (Titulaer *et al.*, 2013). These similar results between animals which are assumed to be experiencing different levels of welfare, could have been considered a result of the lack of sensitivity of JBTs. However, additional measures of welfare (such as behavioral observations and stress hormone levels) did not differ between the groups either. This study shows the importance of validating judgment bias results by comparing them with other measures of welfare.

Reviewing JBTs as tools to assess welfare in farm animals, Baciadonna and McElligott (2015) conclude that these tasks are sensitive to manipulations that induce negative emotions, whereas experimental evidence for sensitivity to manipulations that induce positive emotions is yet weak. This lack of evidence for sensitivity to positive judgment bias could be due to a lack of scientific attention. The majority of judgment bias studies measure the effects of manipulations which are expected to produce a negative affective state. Studies investigating optimistic judgment bias are much less common. For example, Rygula *et al.* (2012) have shown that laughing rats (displaying a clear behavioral indication of positive affective state) have a more positive judgment bias than rats which don't laugh when tickled. As improvement of animal welfare relies on both the reduction of negative emotions and the promotion of positive emotions (Boissy *et al.*, 2007), studies aimed at the sensitivity of JBTs to positive emotions are important.

USABILITY FOR BIOMEDICAL RESEARCH

JBTs have been used as tools for affective state assessment in biomedical research. The majority of these studies have used rodents as their subjects (e.g., mice, Boleij *et al.*, 2012; rats, Kregiel *et al.*, 2016), reflecting the common use of rodents as animal models in biomedical research. In most of the biomedical studies, experimental manipulations were performed prior to testing (Scenario 2 in **Figure 7.1**). In such experiments, all study subjects experience similar conditions during training. This is in contrast to many welfare studies, which alter housing conditions, etc., prior to training. Studies which have different conditions for experimental groups prior to or during

training, apply experimental manipulations that could affect both the discrimination training preceding judgment bias testing and responding in the JBT proper.

The main aim of judgment bias studies in biomedical research has been to investigate effects of experimental manipulations expected to affect mood in animal models of mood disorders such as depression and/or anxiety. Most of these studies have tested the effects of anxiolytics and/or anti-depressants on judgment bias performance (e.g., Doyle *et al.*, 2011a; Destrez *et al.*, 2012; Hymel and Sufka, 2012; Anderson *et al.*, 2013; Rygula *et al.*, 2014a,b, 2015c).

It is important that results of JBTs are generalizable to other species, e.g., results should simulate the clinical condition of depression/anxiety in humans and inform about the effects of therapeutics believed to modulate these clinical conditions (van der Staay, 2006). If this is not the case, the translational value of judgment bias measurements in non-human animals may be limited. JBTs appear to be a useful tool for studying animal models of depression and anxiety. Multiple studies have found responses comparable to those found in human studies of judgment bias (Enkel *et al.*, 2010; Salmeto *et al.*, 2011; Hymel and Sufka, 2012; Richter *et al.*, 2012; Papciak *et al.*, 2013; Kloke *et al.*, 2014; Rygula *et al.*, 2014a, 2015a; Kregiel *et al.*, 2016).

JBTs appear to have particular potential to differentiate between anxiety disorders and depression. Although both mood disorders result in a negative affective state, they produce different response profiles in the JBT. In humans, depression is expressed by a decrease in optimistic responses combined with an increase in pessimistic responses. Individuals suffering from anxiety only display increased pessimistic responding (MacLeod and Byrne, 1996; Miranda and Mennin, 2007). These findings have been replicated in judgment bias studies using a chick model of anxiety and depression (Salmeto *et al.*, 2011; Hymel and Sufka, 2012). Chicks in an anxiety-like state displayed more pessimistic behaviors in response to ambiguous aversive cues (i.e., ambiguous cues near S⁻) and to intermediate ambiguous cues. Chicks in a depression-like state behaved similarly, but in addition displayed less optimistic behaviors in response to ambiguous cues near the S⁺. These results highlight the importance of using a spectrum of ambiguous stimuli, ranging from near-negative to near-positive. Different same-valence affective states (such as depression and anxiety) may produce different responses to these different forms of ambiguous cues (Kloke *et al.*, 2014).

The translational value of biomedical judgment bias studies is of particular importance, as results are used for comparison with humans and/or other model species. Therefore, differences between species in baseline responding during a JBT require attention (e.g., is a pessimistic response caused by induction of a negative affective state or by a trait of the studied species, see **State vs. trait** section in this chapter). Several studies have reported a baseline judgment bias of their study subjects. Using test designs with reward and punishment, both rats and BALB/c mice displayed a baseline negative judgment bias. These animals made pessimistic choices by showing punishment avoidance during ambiguous cue presentation (Boleij *et al.*, 2012; Anderson *et al.*, 2013; Rygula *et al.*, 2015c). These findings could have been a direct result of the test design and comparisons to studies using a discrimination task based on favorable/less favorable reward would be valuable. One study found a baseline positive judgment bias in rats and ascribed this optimism to the favorable testing conditions, i.e., the possibility of food reward and exploration of a novel environment, further indicating effects of test design on baseline judgment bias performance (McGuire *et al.*, 2015). Rygula *et al.* (2014b) reported a difference in baseline judgment bias between groups of rats used for separate experiments, citing this as a possible reason for differences in results found after experimental manipulation. This finding implies that even within-species/strain differences in baseline judgment bias are a possibility that needs to be taken into account. In support of this argument, several studies mention individual variation in judgment bias as a possible influence on their results (Verbeek *et al.*, 2014; Kis *et al.*, 2015).

Biomedical studies have commonly used JBTs to assess effects of drug treatments. When tested drugs have side effects, this could influence behavior in JBTs. One common pharmacological side effect is a reduction or increase of appetite. Most JBTs use food as a reward for correct responses to S⁺, with numerous studies also using (less palatable) food as punishment predicted by S⁻. When treated study subjects experience a decrease in appetite, their performance of optimistic behaviors (i.e., collecting a food reward) may be reduced independent of their affective state. Two studies have mentioned a decrease in appetite as a possible side effect of drug treatments and both used food reinforcers as part of their experimental design (Anderson *et al.*, 2013; Rygula *et al.*, 2014b). An increase in appetite after treatment with the anxiolytic diazepam was discussed as a possible explanation for the observed negative judgment bias (pessimistic responses required the intake of food items with low palatability; Boleij, 2013). Observing animals' responses to S⁺

and S⁻ could provide an indication of appetite-related side effects affecting treated subjects. For instance, when responding during food-rewarded positive trials remains high, it is unlikely that a change in appetite is responsible for a change in responding during ambiguous trials. Using an alternative to food reinforcers will rule out treatment-induced differences in food motivation altogether (e.g., Kloke *et al.*, 2014). When food reinforcers have been used, the possibility of side effects of treatment on the consummatory behavior of study subjects should be ruled out (Mendl *et al.*, 2009). There are other common side effects of pharmacological manipulation to reckon with that potentially influence behavior in JBTs (e.g., locomotor activity, lethargy). Doyle *et al.* (2011a), for example, examined possible side effects affecting the results of dose response trials in a JBT by examining the behavior and physiology of their animals in simple tests of food motivation, reactivity and locomotion.

CONCLUSIONS AND FUTURE DIRECTIONS

JBTs may be suited to assess the emotional state of an animal. Provided that judgment bias can be repeatedly tested in the same animal over a longer time period (see **Loss of ambiguity** section in this chapter), it may also be suited to assess emotional trait in animals.

Judgment bias can be tested in a very broad range of species, from insects to humans, i.e., may allow comparison between species, and may be suited for translational research. There is a large variation in test equipment and testing procedures between and within species (for a recent review see Bethell, 2015). A huge variation in criteria is applied for mastering the basic discrimination task. Also, a large range of computational and statistical methods to analyze the judgment bias data has been used. Recently, Gygax (2014) reviewed these methods and gave recommendations, which already have been critically commented upon (Bateson *et al.*, 2015). This gamut of statistical analyses hampers comparisons within and between studies and species.

JBTs have been used in the field of animal welfare research and in biomedical research. These tasks need to be further developed and adapted to the species of animals used and the research questions to be addressed. In welfare assessment studies, modifications may include the applicability under non-laboratory conditions, testing of social animals in groups and increase of the efficacy to train animals on the basic discrimination task. Owing to the

extensive training preceding judgment bias testing, this task appears to be less suited for routine monitoring of animal welfare.

In biomedical research, a lengthy training period preceding testing with ambiguous stimuli may be less of a concern, as drug treatments usually start after completion of learning the basic discrimination between S⁺/S⁻. However, the problem that ambiguous stimuli may lose their ambiguity very quickly, enabling collection of data in a few trials only, needs to be solved. Also, we need to assess whether the task is suited for repeated testing in a longitudinal design (see also **Figure 7.4**).

Many open questions, addressed in the present article, still need to be answered before JBTs may be considered as a validated, useful tool in the toolbox of researchers interested in measuring animal emotions in both the context of animal welfare studies and biomedical studies.

8

Judgment bias in pigs is independent of performance in a spatial holeboard task and conditional discrimination training

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ABSTRACT

Biases in judgment of ambiguous stimuli, as measured in a judgment bias task, have been proposed as a measure of the valence of affective states in animals. We recently suggested a list of criteria for behavioral tests of emotion, one of them stating that responses on the task used to assess emotionality should not be confounded by, among others, differences in learning capacity, i.e. must not simply reflect the cognitive capacity of an animal. We performed three independent studies in which pigs acquired a spatial holeboard task, a free choice maze which simultaneously assesses working memory and reference memory. Next, pigs learned a conditional discrimination between auditory stimuli predicting a large or small reward, a prerequisite for assessment of judgment bias. Once pigs had acquired the conditional discrimination task, optimistic responses to previously unheard ambiguous stimuli were measured in the judgment bias task as choices indicating expectation of the large reward. We found that optimism in the judgment bias task was independent of all three measures of learning and memory indicating that the performance is not dependent on the pig's cognitive abilities. These results support the use of biases in judgment as proxy indicators of emotional valence in animals.

INTRODUCTION

Cognitive processes and emotional processes are closely related. Although originally considered separate disciplines, the interaction between emotion and cognition has been demonstrated in both human and animal research (Lazarus, 1982; Dolcos, 2015). Cognitive processes are those that involve some form of information processing in the brain, such as memory, attention, problem-solving and planning (Pessoa, 2008). Some authors divide cognition into lower- and higher-level cognitive processes with the higher-level ones including judgment, reasoning and decision-making (Blanchette and Richards, 2010). Emotions are adaptive processes linked to the avoidance of harm and the seeking out of valuable resources, which are reflected by cognitive, behavioral and/or physiological changes (Paul *et al.*, 2005). Since the “functional relationships between cognition and emotion are bidirectional” (Lazarus, 1991, p. 353), the link between emotion and cognition can be studied in different ways; by assessing how emotion affects cognitive processes or how cognition can impact upon emotional responses.

Biases in judgment of ambiguous stimuli have recently been proposed as a method for establishing the valence of emotional states in animals; optimistic judgments are said to indicate positively valenced emotions, while pessimistic judgments indicate negative emotions (Harding *et al.*, 2004; Paul *et al.*, 2005; Mendl *et al.*, 2009). This approach is receiving increasing attention in animal welfare science as suggested by the number of papers and variety of species to which it has been applied (Roelofs *et al.*, 2016). For example, treatments such as cage enrichment (Burman *et al.*, 2008; Brydges *et al.*, 2011), social stress (Papciak *et al.*, 2013) and unpredictable mild stress (Harding *et al.*, 2004; Novak *et al.*, 2016) influence judgment bias in rodents. We recently proposed a list of criteria for behavioral tests of emotion, one of which is that a task should “specifically and unambiguously capture emotion-related behaviors” (Murphy *et al.*, 2014, p. 27). This means that responses on the task should not be confounded by differences in learning capacity, activity levels or motivation. Although the judgment bias task assesses emotion through cognitive processes, its results must not simply reflect the cognitive capacities of an animal.

Judgment bias can be measured in animals by training them to discriminate between two stimuli: one predicting a positive outcome and the other predicting a negative or less positive outcome (Mendl *et al.*, 2009; Roelofs *et al.*, 2016). Animals are trained to display a specific behavior in response to

each of these cues. After successful discrimination training, the animal is presented with ambiguous stimuli, often with qualities intermediate between the positive and negative cues. Then, the behavioral responses to the ambiguous stimuli are taken to indicate an animal's expectation of the positive or negative outcome (Mendl *et al.*, 2009).

We have developed an active choice task to assess judgment bias in pigs (Murphy *et al.*, 2013a, b, 2015; Roelofs *et al.*, 2017b). Prior to judgment bias testing, pigs are trained in an audio-spatial conditional discrimination task. Tone-cues of different frequencies are used as positive and negative stimuli, predicting the presence of either a large food reward (positive outcome) or a small food reward (negative outcome) in a left or right goal-box. Put simply, the pigs need to learn: if the positive tone-cue, predicting a large reward, is presented, go to the left goal-box; if the negative tone-cue, predicting a small reward, is presented, go to the right goal-box (Murphy *et al.*, 2013a). Once pigs reach a predefined criterion level of performance on the conditional discrimination task, a series of ambiguous tone-cues is presented, of intermediate frequencies between the previously trained tones. Responses indicating expectation of the large reward (i.e. approaches to the 'positive' goal-box) are considered optimistic responses and recorded as a measure of judgment bias.

Spatial holeboard tasks have been established as valid instruments in cognitive research as they allow measuring multiple facets of cognition simultaneously in one test (van der Staay *et al.*, 2012). The holeboard is a free choice maze for assessing spatial learning and memory. It contains a number of 'holes', potential reward locations, of which only a subset is baited. An animal can search freely for the baited holes within a certain time period. The holeboard allows for the assessment of both working memory and reference memory. Working memory is a short-term memory that contains information which is only relevant within a testing trial (Dudchenko, 2004). In the holeboard, working memory allows animals to remember which holes have already been visited during a trial, so unrewarded revisits of holes are avoided. This information is no longer relevant once the trial has ended (Dudchenko, 2004; van der Staay *et al.*, 2012). Reference memory is a long-term memory that contains "the general rules of a task" (van der Staay *et al.*, 2012, p. 383). This information remains relevant across trials. In the holeboard, reference memory allows animals to remember which subset of holes is baited.

The holeboard task was first adapted for use with pigs by Arts *et al.* (2009), who found that it was suitable for measuring spatial learning in this species. Further studies have validated the suitability of the holeboard for simultaneous measurement of task-specific reference memory and trial-specific working memory in pigs (Gieling *et al.*, 2012, 2013, 2014; Bolhuis *et al.*, 2013; Haagensen *et al.*, 2013a, b; Antonides *et al.*, 2015b; Fijn *et al.*, 2016; Roelofs *et al.*, 2017b).

Cognitive performance in pigs can be described by different measures, examples of which are working and reference memory in spatial learning tasks, or auditory spatial memory in the conditional discrimination task preceding judgment bias testing. Therefore, when assessing whether judgment bias is independent of cognitive abilities, it is preferable to include a variety of cognitive measures in the analysis. This requires animals that have been subjected to multiple learning and memory tasks (Zanghi *et al.*, 2015). In previous experiments, no correlation was found between pigs' performance in conditional discrimination training prior to judgment bias testing and optimism measured in the judgment bias task (Murphy *et al.*, 2013b). Also, no correlation was found between performance in a pig gambling task (measuring decision-making under risk) and optimism in the judgment bias task (Murphy *et al.*, 2015).

The question whether measures of spatial working and reference memory in holeboard-type tasks are independent has been addressed in a similar manner in only a small number of studies. No correlation was found between working and reference memory of rats trained in the holeboard or cone field, a modification of the holeboard (van der Staay *et al.*, 1990b; Blokland *et al.*, 1992; Prickaerts *et al.*, 1999; van der Staay, 1999). Further evidence supporting this notion comes from a factor analysis of the cognitive and behavioural performance of inbred mice in the modified holeboard (Ohl *et al.*, 2003). This study revealed that working and reference memory loaded on different factors. Separate factor analyses performed in an age-comparison study of C57/BL mice yielded evidence that working memory and reference memory were independent in the oldest (24-month-old), but not in middle-aged (16-month-old) or young adult (4-month-old) mice (Weiss *et al.*, 1998).

A problem with these findings is that a lack of correlation between variables does not provide evidence for their independence. Standard statistical analysis provides a P value which, when it falls below a specified threshold (often 0.05), allows for the rejection of the null hypothesis in favor of the

alternative hypothesis. Unfortunately, a P value of >0.05 does not provide evidence for the null hypothesis (Wagenmakers, 2007; Rouder *et al.*, 2009). In the case of correlation analysis, the null hypothesis stating that there is no correlation between variables cannot be proven using standard tests. To provide evidence for independence of variables (shown by a lack of correlation), Bayesian statistics are preferable, as these provide an estimate of support for the null hypothesis (Gallistel, 2009; Wetzels and Wagenmakers, 2012; Wagenmakers *et al.*, 2016).

In order to assess whether optimism of pigs in an active choice judgment bias task was related to their learning ability, we used the results of three studies in which pigs were first tested in the spatial holeboard task (Gieling *et al.*, 2013, 2014; Roelofs *et al.*, 2017b), followed by the judgment bias task (Murphy *et al.*, 2013b; Roelofs *et al.*, 2017b). Firstly, we expect that optimism, as measured in the judgment bias task, will be found to be independent of learning ability, as measured by working and reference memory performance in the holeboard task and the acquisition of the conditional discrimination task preceding judgment bias testing. Second, we expect that working and reference memory as measured in the spatial holeboard task will be independent of one another, i.e. that they represent different memory domains, as shown previously in studies using rodents as subjects. Third, reference memory may be important for successful performance in conditional discrimination training as well as the spatial holeboard. During discrimination training, the animals have to memorize general rules of the task, for example: ‘if cue A, response A; if cue B, response B’ (Murphy *et al.*, 2013a). Therefore, we expect that reference memory in the spatial holeboard task and acquisition of the conditional discrimination task are correlated.

METHODS

The animals and methods used in this study are described in detail elsewhere: Experiment 1 in Gieling *et al.* (2013) and Murphy *et al.* (2013a, b); Experiment 2 in Gieling *et al.* (2014) and Murphy *et al.* (2015); Experiment 3 in Roelofs *et al.* (2017b).

Subjects and housing

Information on the subjects used in each study is provided in **Table 8.1**. Shortly after weaning at approximately 4 weeks of age, pigs were moved to

the research stables. Pigs were group housed in two straw-bedded pens (15–20 m², 8–10 pigs per pen) in naturally lit and ventilated stables. Each pen contained a covered nest area and was provided with toys for enrichment. Pigs had access to water *ad libitum* and were fed twice per day (Holeboard: 33% morning, 66% evening; Judgment bias task: 25% morning, 75% evening), amounts according to the recommendations of their breeders.

Holeboard task

Apparatus

The same spatial holeboard apparatus (**Figure 1.2**) was used in the three studies and is described in detail by Gieling *et al.* (2012, 2013, 2014). The holeboard was a square arena (5.3 x 5.3 m) with 1-m-high walls. The entire arena was surrounded by a narrow corridor (40 cm wide) leading to four entrances into the arena, one in the middle of each side, which could be opened by the experimenter using pulley-operated guillotine doors. Within the arena was a 4 x 4 matrix of food bowls (Road Refresher, Jolly Pet), the ‘holes’ of the holeboard, which had a false bottom underneath which rewards could be placed to control odor cues. To control visual cues, a large hard-plastic ball (24 cm diameter) covered each food bowl. A pig could easily raise the ball off the food bowl using their snout to gain access to rewards underneath. Guide rails ensured that the ball could not be knocked off the bowl and that it returned to cover the bowl once the pig had retracted its snout. Rewards used were chocolate M&M’s® (Mars Nederland B.V., Veghel, The Netherlands).

Habituation

Full details on habituation and training of animals for Studies 1 and 2 can be found in Gieling *et al.* (2013, 2014) and for Study 3 in Roelofs *et al.* (2017b). In short, pigs were gradually exposed to the presence of experimenters and the testing apparatus during daily habituation sessions. Pigs initially explored the holeboard in groups, which gradually decreased in size until they were comfortable exploring the holeboard individually.

Table 8.1. Overview of subjects used, age in weeks at start of holeboard task training and conditional discrimination training preceding the judgement bias task and number of trials (trial blocks) of the acquisition of the holeboard task in studies 1, 2, and 3. Habituation sessions and pre-training sessions preceded formal training (not included in table). Abbreviations: HBT, holeboard task; CDT, conditional discrimination task; JBT, judgement bias task.

Study	Subjects	N; Sex	Approximate age in weeks at start of formal training/testing			Trials (trial blocks) in acquisition phase
			HBT	CDT	JBT [§]	HBT
1	Miniature pigs	8 ♀	13	26	28	104 (26)
	Conventional pigs	7 ♀	13	26	28	104 (26)
2 ^a	LBW* Allopurinol [†]	10; 4♀, 6♂	8	20	24.5	40 (10)
	LBW* Control [†]	8; 1♀, 7♂	8	20	24.5	40 (10)
	NBW* Allopurinol [†]	10; 5♀, 5♂	8	20	24.5	40 (10)
	NBW* Control [†]	9; 1♀, 8♂	8	20	24.5	40 (10)
3	Female pigs [‡]	10 ♀	9	21	28	40–60 (10–15)
	Male pigs [‡]	10 ♂	9	21	28	40–60 (10–15)

^a Performed in two replicates.

* All piglets from 10 litters were weighed at birth and the litter mean calculated. Low-birth-weight (LBW) pigs weighed at least 1SD below the litter mean, while normal-birth-weight (NBW) pigs were those closest to an adjusted litter mean (mean excluding LBW pig weights) as in Gieling *et al.* (2014).

[†] Five sows had been treated with 15 mg.kg⁻¹ Allopurinol for the 30 days (± 2 days) before farrowing.

[‡] All piglets from 8 litters were weighed at birth. One or two male piglets and one or two corresponding female piglets weighing closest to the litter mean (calculated separately for male and female piglets) were selected from each litter.

[§] As pigs learned at different rates, this age refers to the mean age at starting the JBT

Training and testing

After habituation to the experimenter, rewards and apparatus, formal training in the holeboard began. Each animal was randomly assigned to one of four configurations of four rewarded holes (containing 2 M&M's®; **Figure 1.3**). In each trial, a pig was let into the corridor surrounding the holeboard and walked the perimeter of the arena until it found the open entrance into the arena, the location of which was chosen randomly per trial. Pigs could then search the arena for the rewarded holes. Trials ended after all four rewards were obtained or when a maximum trial duration of 10 minutes (Studies 1 and 2) or 7.5 minutes (Study 3) had elapsed. In each trial, working memory and reference memory were recorded. As they reduce the bias induced by incomplete trials, ratio measures for working memory and reference memory were used (van der Staay *et al.*, 2012). Working memory was measured as the number of visits to holes which resulted in a reward (maximum of four) divided by the total number of visits to these same holes, i.e. a score of 1 would imply that a pig had not revisited any of the baited holes after obtaining a reward. Reference memory was measured as the number of visits to the baited holes, divided by the total number of visits to all holes, i.e. a score of 1 would imply that a pig had only made visits to baited holes. To get an overall measure of working memory and reference memory performance over time, data were averaged across four trial blocks. Number of trial blocks needed for acquisition of the holeboard task per study subject is provided in **Table 8.1**.

Judgment bias task

Apparatus

The same judgment bias apparatus, described in detail by Murphy *et al.* (2013a, b, 2015), was used in all three studies (**Figure 1.1**). A start box (1.2 m²) was connected via a small antechamber (1.2 m²) to a rectangular test arena (3.6 m x 2.4 m). Two goal-boxes were attached to the back wall of the test arena, each of which contained a food bowl system as described above for the holeboard apparatus. Entrance to the test arena from the start box and access to each goal-box were regulated by the experimenter using pulley-operated guillotine doors. Tones were used to cue the location of rewards in the judgment bias task. These tones were generated using the open-source software Audacity (<http://audacity.sourceforge.net/>) and played on an MP3

player (Archos 18 Vision, 4 GB, Archos GmbH, Grevenbroich, Germany) through speakers (Logitech z-313, Logitech Europe S.A., Morges, Switzerland) attached at the back of the testing area. The training tone-cues used were a 30-s-long 200 and 1000 Hz pure tone (Waveform: Sine; Amplitude: 1). Ambiguous tone-cues were generated at equal intervals between the training tone-cues on a logarithmic scale: 299.07, 447.21, and 668.74 Hz. Rewards used were chocolate M&M's® (Mars Nederland B.V., Veghel, The Netherlands).

Habituation and training

Full details on habituation and training of animals for Studies 1 and 2 can be found in Murphy *et al.* (2013a, b) and Murphy (2015, chapter 6), and for Study 3 in Roelofs *et al.* (2017b).

After habituation to the experimenter, rewards and apparatus, pigs were trained in a conditional discrimination task to distinguish between the two training tone-cues. In a 'positive' trial, a tone-cue (CS⁺) predicted a large reward (4 M&M's®) in the associated 'positive' goal-box, while in a 'negative' trial a tone-cue (CS⁻) predicted a small reward (1 M&M's®) in the associated 'negative' goal-box. Tone frequency and goal-box location in positive and negative trials were counterbalanced across animals. Each pig received one training session daily, and sessions consisted of 13 trials: three forced trials (2 negative; 1 positive), where only the correct goal-box was available, followed by 10 free trials (5 negative; 5 positive), where both goal-boxes were available but only the correct goal-box, as predicted by the tone-cue, contained a reward. Upon presentation of a tone-cue, the pig was released from the start box and had up to 30 s to choose between the two goal-boxes. A choice was defined as any lift/push of the ball covering a food bowl in a goal-box with enough force to cause the ball to move. If a pig failed to choose within this time (omission) or made an incorrect choice, both goal-boxes were closed and the pig remained in the test arena for a 90-s time-out penalty. In Studies 2 and 3, the first 3 positive and 3 negative trials in every fifth session were 'open choice' trials, where an incorrect choice resulted in the closing of the incorrect goal-box only and pigs could still visit the correct goal-box to collect a reward. This was used to remind pigs that rewards were available in every trial. Pigs were trained until they responded correctly four out of five times to both positive and negative tone-cues (free trials) in three consecutive training sessions. The number of sessions needed to reach this criterion level

of performance was taken as a measure of conditional discrimination learning.

Testing

Judgment bias was then assessed over four testing sessions. Each daily session consisted of 16 trials; three forced and 10 free trials, as before, and three ambiguous trials where one of the three previously unheard ambiguous tone-cues was played in lieu of one of the training tone-cues. In ambiguous trials, both goal-boxes were open. In Studies 1 and 3 neither contained a reward, while in Study 2 ambiguous trial choices were rewarded as expected, i.e. 4 M&M's® in the positive goal-box and 1 M&M's® in the negative goal-box. Once a pig had chosen a goal-box/eaten the reward, the trial was ended and the pig returned to the start box for the next trial. Each of the three ambiguous cues was presented once per day (trial numbers 6, 11 and 16). The order of trials was counterbalanced so that each ambiguous trial occurred after equal numbers of positive and negative trials. The percentage of 'optimistic' choices, i.e. choice for the positive goal-box, in response to each of the five cue types (CS⁻, ambiguous cue near CS⁻, ambiguous cue intermediate between CS⁺ and CS⁻, ambiguous cue near CS⁺, and CS⁺) was calculated per pig across the four test sessions. To get an overall measure of 'optimism' in the judgment bias task, the unweighted mean of optimistic choice percentages in response to the individual ambiguous cue types was calculated.

Statistical analysis

A set of variables was selected for both the holeboard task and the judgment bias task from the three studies (see **Table 8.2A**). The variables were taken as indices of optimism, average performance level and speed of learning and subjected to correlation analysis and Bayesian analysis to evaluate whether they were independent measures; that is, whether they reflect different aspects of different cognitive processes.

Holeboard task

Although most pigs received more than 40 acquisition trials in each of the three studies (**Table 8.1**), we only used the first ten trial blocks (each block representing the mean of four successive trials), because pigs usually

Table 8.2. Means, number of animals and standard error of the mean (*SEM*) of the measures that were used in the correlation analyses (**A**). The percent variation covered by the linear trend component calculated for all pigs of a study of acquiring the reference memory and working memory components of the holeboard task are listed in **B**. Abbreviations: HBT, holeboard task; CDT, conditional discrimination task; JBT, judgement bias task; RM, reference memory; WM, working memory.

A		Study 1			Study 2			Study 3		
		Mean	N	SEM	Mean	N	SEM	Mean	N	SEM
HBT	RM mean	0.480	16	0.019	0.497	37	0.013	0.535	20	0.017
	RM slope	0.044	16	0.004	0.049	37	0.003	0.052	20	0.004
	WM mean	0.838	16	0.015	0.837	37	0.008	0.820	20	0.012
	WM slope	0.009	16	0.005	0.018	37	0.002	0.016	20	0.002
CDT	Sessions to criterion	10.600	15	0.815	16.324	37	0.595	22.222	18	1.390
JBT	Optimistic choice %	38.333	15	5.040	75.450	37	2.693	50.926	18	4.451

B	Study 1	Study 2	Study 3
	Percent explained variation		
HBT RM lin. trend component of the within subjects variation	98.34 %	99.04 %	96.18 %
HBT WM lin. trend component of the within subjects variation	59.14 %	82.98 %	67.92 %

approached ceiling performance levels after 10 trial blocks, i.e. performance in later trial blocks reveals little additional information about the acquisition of the task (see **Figure 8.1** for working memory and reference memory learning curves per study).

For the holeboard, repeated measures analysis with the successive trial blocks as within-subjects factor was performed for each study, supplemented by polynomial contrasts (SAS PROC GLM, SAS 9.4, SAS Institute Inc., Cary, NC, USA). These orthogonal trend components define dimensions in terms of which differences in shape of learning curves can be described (Winer 1971, pp. 577–594). We determined the percent variation explained by the

linear trend component of the learning curves (Winer 1971; Cotton 1998) in the holeboard task over the successive trial blocks (van Luijtelaa *et al.*, 1989; Spowart-Manning and van der Staay, 2005) using appropriate sets of trend coefficients (from SAS PROC IML). The percent variation in the learning curves explained by the linear trend component was calculated as the percentage of the sum of squares of the linear component of the total within-subjects sum of squares. The slopes of both working memory and reference memory calculated over the same blocks per animal, representing the linear change over blocks, were used in the subsequent correlation analysis (**Table 8.2A**). Slopes were estimated using the SAS PROC REG procedure. As representative measure of the between-subjects average performance levels, the means of the 10 successive trial blocks of working memory and reference memory were used in the correlation analysis (**Table 8.2A**).

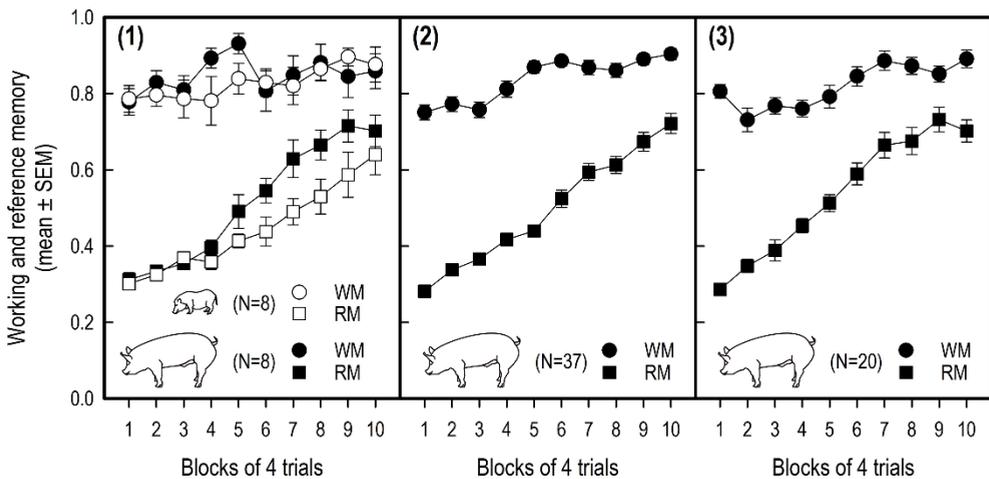


Figure 8.1. Increase in working memory (WM) and reference memory (RM) performance (mean \pm SEM) across successive trial blocks in the acquisition phase of the holeboard task for studies 1–3.

As a considerable percentage of the variation in the increase in working memory and reference memory in the holeboard task were covered by the linear trend components (see **Table 8.2B**), the linear components (or slopes) were considered as the measures that best reflect the increase in performance across the first 10 trial blocks, i.e. the improvement can adequately be described as a linear regression line of the form: $y = ax + b$ (a: slope, b: intercept). The faster the acquisition of the working memory and reference memory components of the holeboard task, the steeper the slope is. Thus, the slopes of both working memory and reference memory are representative of the overall progress of acquiring the holeboard task. The means of reference memory and working memory calculated across all trial blocks were considered as representative of overall performance level in the holeboard task. Steeper slopes will also result in a higher average performance, and consequently, slopes and mean performance may be positively correlated.

Judgment bias task

Two measures per pig of the judgment bias task were used for analysis: the number of trials needed to reach the learning criterion in the conditional discrimination task (sessions to criterion), and optimism (mean optimistic choice percentage across the three ambiguous tone-cues used in the judgment bias task; see **Table 8.2A**). Shapiro–Wilk statistics (SAS PROC UNIVARIATE) confirmed that all variables of the holeboard and judgment bias task except the optimistic choice percentage of Study 2 met the criterion to be treated as being normally distributed.

Correlation analysis

For each study, the variables derived from the holeboard task (slopes and mean performance of working memory and reference memory) and the judgment bias task (sessions to criterion, optimistic choice percentage) were subjected to correlation analysis (SAS PROC CORR). The Pearson product-moment correlation coefficients were calculated. The optimistic choice percentage of Study 2 did not fulfil the assumption of normality. Therefore, we also determined the Spearman rank correlations (see **Table 8.3B**) between the optimistic choice percentage and the other measures in Study 2. The correlation analyses were performed separately for each study, because in pooled data, the differences in age and breed of the animals and modifications in the testing procedures between the three studies may artificially increase or decrease the correlations (van der Staay *et al.*, 1990b).

Bayesian analysis

In order to quantify the relative support provided by each study for the two competing hypotheses (i.e. whether there is (H_1) or is not (H_0) a correlation between the variables presented in the correlation analysis above), a Bayes factor was computed for each correlation, for each study. A Bayes factor is the ratio of the likelihood of finding the data under the conditions of the two hypotheses, H_1 and H_0 (Spiegelhalter *et al.*, 2004). In this paper, we use Jeffreys' exact solution (Jeffreys, 1998; Ly *et al.*, 2016) to compute the Bayes factors $p(D|H_0)/p(D|H_1)$, based on sample size and the observed Pearson correlation coefficients. A Bayes factor larger than 1 indicates support for H_0 (i.e. there is no correlation between the variables).

The computed Bayes factors were then used to calculate the probability of H_0 for each possible correlation (e.g. optimistic choice and sessions to criterion, optimistic choice and mean working memory). For Bayesian hypothesis testing, a posterior odds $p(H_0|D)/p(H_1|D)$ for the two competing hypotheses can be obtained by combining a pre-specified prior odds $p(H_0)/p(H_1)$ with a Bayes factor calculated from the data. When multiple similar studies are available, under a Bayesian framework, evidence can be naturally updated by subsequently combining the studies. For each correlation, the following analysis was performed:

Prior odds x Bayes factor Study 1 = posterior odds Study 1

Posterior odds Study 1 x Bayes factor Study 2 = posterior odds Study 2

Posterior odds Study 2 x Bayes factor Study 3 = posterior odds Study 3

The prior odds represent the probability of H_0 over H_1 before observing the data. Before observing the data from Study 1, we assigned the value 1 to the prior odds, implying both hypotheses are equally likely. The uninformative prior odds are then updated using data from Study 1, resulting in posterior odds from Study 1. The posterior odds from Study 1 were used as informative prior odds to the Bayesian analysis of Study 2. Likewise, the prior odds for the analysis of Study 3 are the posterior odds from the analysis of Study 2. Eventually, the posterior odds $p(H_0|D)/p(H_1|D)$ resulting from Study 3 can be used to compute the final posterior probabilities for H_0 : $p(H_0|D)$.

RESULTS

The mean, number of animals and standard error of the mean (*SEM*) of the measures that were used in the correlation analyses are listed in **Table 8.2A**. The product-moment correlation coefficients and Bayes factors are shown in **Table 8.3**. The posterior probabilities for H_0 (no correlation between variables) are shown for each correlation in **Table 8.4**. The largest percentage of the variation in the improvement in working memory and reference memory in the holeboard task was covered by the linear trend components (see **Table 8.2B**). Therefore, the slopes of both working memory and reference memory were considered as the measures that best reflect learning across trials of the acquisition phase.

Optimism, learning and memory

Our data strongly support the null hypothesis that optimistic choice percentage in the judgment bias task is independent of all other cognitive measures (**Table 8.4**). The posterior probabilities of H_0 for individual correlations with optimistic choice percentage ranged from 0.878 to 0.962.

Working memory, reference memory and discrimination learning

In the holeboard task, overall performance (mean) was highly correlated to task acquisition (slope) with respect to reference memory in all three studies, i.e. pigs which learned the rules of the task faster had a better overall performance in the holeboard. These findings were supported by a <0.001 posterior probability for the null hypothesis that there is no correlation between these two variables. Overall performance and task acquisition of the working memory component were not found to be correlated in any of the studies. This was supported by a 0.917 posterior probability of independence based on the Bayesian analysis.

Our data do not fully support independence of reference memory and working memory in the holeboard task. Several correlations were found between the slope of the working memory learning curve and both reference memory measures (**Table 8.3**). Bayesian analysis provided posterior probabilities of 0.043 and 0.016 for independence between these measures, suggesting our data provide strong evidence for correlations between working memory acquisition and both measures of reference memory in the

Table 8.3. Pearson product-moment correlation coefficients (r : all above diagonal), and Spearman rank correlations (ρ : all below diagonal), their associated p values, the number of animals (N) and Bayes factors (BF). **A)** Study 1; **B)** Study 2; **C)** Study 3. Correlations printed in bold italics have associated probabilities <0.05 . Correlations printed in italics have associated probabilities $0.05 \leq p < 0.10$. Bayes factors <0.33 (providing at least substantial evidence for H_A ; Wetzels and Wagenmakers, 2012) or >3 (providing at least substantial evidence for H_0 ; Wetzels and Wagenmakers, 2012) are printed in bold italics.

Abbreviations: HBT, holeboard task; JBT, judgement bias task; RM, reference memory; WM working memory; sess. to crit., sessions to criterion; % opt. choices, percent optimistic choices.

*: The JBT % optimistic choices in Study 2 (panel **B**) were not normally distributed (Shapiro Wilk test: $p = 0.003$). Therefore, below diagonal, the Spearman rank correlations (ρ) are shown.

Note that the product-moment correlations and the rank correlations are highly similar.

A		HBT		HBT		JBT	
		RM mean	RM slope	WM mean	WM slope	Sess. to crit.	% opt. choices
HBT RM mean	r	-	<i>0.957</i>	<i>0.661</i>	0.085	0.072	0.344
	ρ	-	<i><0.001</i>	<i>0.005</i>	0.755	0.798	0.210
	N	-	16	16	16	15	15
	BF	-	<i><0.001</i>	<i>0.093</i>	<i>3.096</i>	<i>3.052</i>	1.540
HBT RM slope	r	-	-	<i>0.687</i>	0.260	0.037	<i>0.446</i>
	ρ	-	-	<i>0.003</i>	0.331	0.895	<i>0.096</i>
	N	-	-	16	16	15	15
	BF	-	-	<i>0.065</i>	2.136	<i>3.097</i>	0.853
HBT WM mean	r	-	-	-	0.309	-0.099	0.329
	ρ	-	-	-	0.244	0.725	0.232
	N	-	-	-	16	15	15
	BF	-	-	-	1.712	2.970	1.622
HBT WM slope	r	-	-	-	-	0.013	0.291
	ρ	-	-	-	-	0.963	0.293
	N	-	-	-	-	15	15
	BF	-	-	-	-	<i>3.140</i>	1.896
JBT Sess. to crit.	r	-	-	-	-	-	0.300
	ρ	-	-	-	-	-	0.278
	N	-	-	-	-	-	15
	BF	-	-	-	-	-	1.830
JBT % opt. choices	r	-	-	-	-	-	-
	ρ	-	-	-	-	-	-
	N	-	-	-	-	-	-
	BF	-	-	-	-	-	-

B*		HBT RM mean	HBT RM slope	HBT WM mean	HBT WM slope	JBT Sess. to crit.	JBT % opt. choices
HBT RM mean	<i>r</i>	-	0.834	0.308	0.237	0.018	-0.040
	<i>p</i>	-	<0.001	0.064	0.157	0.917	0.813
	<i>N</i>	-	37	37	37	37	37
	<i>BF</i>	-	<0.001	0.932	1.867	4.861	4.756
HBT RM slope	<i>r</i>	0.830	-	0.258	0.266	0.175	0.023
	<i>p</i>	<0.001	-	0.124	0.111	0.300	0.895
	<i>N</i>	37	-	37	37	37	37
	<i>BF</i>	-	-	1.563	1.440	2.915	4.845
HBT WM mean	<i>r</i>	0.358	0.295	-	-0.233	0.024	0.042
	<i>p</i>	0.030	0.076	-	0.164	0.889	0.805
	<i>N</i>	37	37	-	37	37	37
	<i>BF</i>	-	-	-	1.926	4.841	4.746
HBT WM slope	<i>r</i>	0.174	0.231	-0.288	-	0.412	0.052
	<i>p</i>	0.304	0.168	0.084	-	0.011	0.762
	<i>N</i>	37	37	37	-	37	37
	<i>BF</i>	-	-	-	-	0.223	4.675
JBT Sess. to crit.	<i>r</i>	-0.003	0.170	0.033	0.405	-	0.076
	<i>p</i>	0.987	0.316	0.845	0.013	-	0.656
	<i>N</i>	37	37	37	37	-	37
	<i>BF</i>	-	-	-	-	-	4.442
JBT % opt. choices	<i>r</i>	-0.088	-0.073	0.108	0.026	0.023	-
	<i>p</i>	0.604	0.667	0.524	0.878	0.892	-
	<i>N</i>	37	37	37	37	37	-
	<i>BF</i>	-	-	-	-	-	-

C

HBT RM mean	<i>r</i>	-	0.750	0.458	0.008	-0.187	0.005
	<i>p</i>	-	<0.001	0.042	0.973	0.459	0.985
	<i>N</i>	-	20	20	20	18	18
	<i>BF</i>	-	0.004	0.524	3.611	2.658	3.432
HBT RM slope	<i>r</i>	-	-	0.564	-0.079	0.120	0.189
	<i>p</i>	-	-	0.010	0.741	0.637	0.451
	<i>N</i>	-	-	20	20	18	18
	<i>BF</i>	-	-	0.158	3.433	3.094	2.636
HBT WM mean	<i>r</i>	-	-	-	-0.095	0.054	-0.080
	<i>p</i>	-	-	-	0.689	0.830	0.751
	<i>N</i>	-	-	-	20	18	18
	<i>BF</i>	-	-	-	3.352	3.360	3.275
HBT WM slope	<i>r</i>	-	-	-	-	0.073	-0.426
	<i>p</i>	-	-	-	-	0.772	0.078
	<i>N</i>	-	-	-	-	18	18
	<i>BF</i>	-	-	-	-	3.301	0.811
JBT Sess. to crit.	<i>r</i>	-	-	-	-	-	0.354
	<i>p</i>	-	-	-	-	-	0.149
	<i>N</i>	-	-	-	-	-	18
	<i>BF</i>	-	-	-	-	-	1.304
JBT % opt. choices	<i>r</i>	-	-	-	-	-	-
	<i>p</i>	-	-	-	-	-	-
	<i>N</i>	-	-	-	-	-	-
	<i>BF</i>	-	-	-	-	-	-

Table 8.4. Posterior probabilities for H_0 (no correlation between variables) for each correlation, based on updated Bayesian hypothesis testing using 3 separate studies. Probabilities <0.50 indicate data do not support H_0 , whereas probabilities >0.50 indicate data support H_0 . Abbreviations: HBT, holeboard task; JBT, judgement bias task; RM, reference memory; WM working memory; sess. to crit., sessions to criterion; % opt. choices, percent optimistic choices.

	HBT RM mean	HBT RM slope	HBT WM mean	HBT WM slope	JBT Sess. to crit.	JBT % opt. choices
HBT RM mean	-	<0.001	0.043	0.954	0.975	0.962
HBT RM slope		-	0.016	0.913	0.965	0.916
HBT WM mean			-	0.917	0.980	0.962
HBT WM slope				-	0.698	0.878
JBT Sess. to crit.					-	0.914
JBT % opt. choices						-

holeboard task. However, for independence of mean working memory of both reference memory measures, posterior probabilities were >0.9 , suggesting there is no correlation (**Table 8.4**).

Our data support the null hypothesis of no correlation between learning in the conditional discrimination task (sessions to criterion) and performance in the holeboard (working/reference memory slopes and mean performance). Posterior probabilities for independence ranged from 0.698 to 0.980 (**Table 8.4**).

DISCUSSION

The present paper explored the (in)dependence of pigs' performance in a judgment bias task (optimism measured as mean optimistic choice percentage), a holeboard task (spatial learning measured as reference memory and working memory) and a conditional discrimination task (learning measured as sessions to criterion) preceding testing in the judgment bias task. As expected, data from three independent studies provided evidence that optimism in the judgment bias task is independent of cognitive abilities assessed in the holeboard task and conditional discrimination task. Contrary to expectation, our data do not support the hypothesis that working memory and reference memory are entirely independent measures. Finally, although a correlation between reference memory in the spatial holeboard task and performance in the auditory conditional discrimination task was expected, our data support the notion that these measures are independent.

Optimism, learning and memory

One of our criteria for behavioral tests of emotions in pigs is that “(...) the task should specifically and unambiguously capture emotion-related behaviors. For example, results can be confounded by differences in learning capacity (...)” (Murphy *et al.*, 2014, p. 12). One approach to assessing the construct validity of our judgment bias task is therefore to test the independence of responses to ambiguity from potential confounding factors such as learning ability. In the present study, we aimed to investigate whether ‘optimism’ in a judgment bias task, reflected by expectation of positive outcomes, was affected by differences in learning ability as measured by reference and working memory performance in a spatial holeboard task and by the sessions to criterion in the auditory conditional discrimination training prior to the judgment bias task in three pig studies. Validating the results of judgment bias tasks is of importance, as they are increasingly used to assess emotional state in animals (Roelofs *et al.*, 2016).

Many studies have attempted to assess the predictive validity of the judgment bias paradigm through the effects of treatments assumed to influence mood. For example, enrichment produces a more optimistic judgment bias in both pigs and rats (Brydges *et al.*, 2011; Douglas *et al.*, 2012), while chronic stress (induced pharmacologically or by repeated restraint) produces a more pessimistic judgment bias in rats (Enkel *et al.*, 2010; Rygula *et al.*, 2013).

However, few studies have attempted to assess what other processes may impact upon responses to ambiguity. We have previously demonstrated that optimism in the same judgment bias task as used for the current study was not related to decision-making under risk in a gambling task in pigs (Murphy *et al.*, 2015). Similarly, Bateson *et al.* (2015) stated that responses to ambiguous stimuli in a judgment bias task in another species, the European starling, did not reflect their cognitive abilities. They based this conclusion on their finding that while experimental treatment affected judgment bias, it did not affect operant or discrimination training prior to judgment bias testing. Such dissociation in effects on task acquisition and judgment bias has been reported more often (e.g. Enkel *et al.*, 2010; Parker *et al.*, 2014; Gordon and Rogers, 2015; Brajon *et al.*, 2015). Several studies specifically report a lack of correlation between performance during discrimination training and measured judgment bias as indication that they are independent (Murphy *et al.*, 2013b; d’Ettorre *et al.*, 2016). Such correlation analysis has also been extended to other cognitive tasks. For example, no correlation was found between judgment bias and performance in simple maze tasks in sheep (Destrez *et al.*, 2013; Coulon *et al.*, 2015).

Although these findings, taken together, can be taken as support for the hypothesis that results of judgment bias tasks are not confounded by cognitive abilities, none of these studies provide a measure of support for the null hypothesis. To do this, Bayesian analysis is required (Wagenmakers *et al.*, 2016). Therefore, the current study goes one step further towards determining whether judgment bias is a measure independent of learning and memory. It is the first to quantify evidence for the null hypothesis that optimism in the judgment bias task, learning and memory in the spatial holeboard task and acquisition of an auditory conditional discrimination task reflect different cognitive domains in the same animal. Based on this study, our active choice judgment bias task is not confounded by pigs’ cognitive abilities.

Working memory and reference memory

In the current study, we could not provide support for the hypothesis that working memory and reference memory are independent. Although the acquisition of the working memory component was independent of both measures of reference memory, the overall working memory performance was related to reference memory slope and overall performance. This finding

is in contrast with previous studies, which have shown that working and reference memory in the holeboard can be influenced independently of each other by experimental manipulations. For example, when assessing the effects of environmental enrichment on working and reference memory of pigs in a holeboard, Bolhuis *et al.* (2013) found that reference memory was unaffected by enrichment, whereas it improved working memory. Similar results have been found for rodents, where either reference or working memory was affected by experimental treatment (Blokland *et al.*, 1998; Prickaerts *et al.*, 1999; Kuc *et al.*, 2005; Bainbridge *et al.*, 2008). Also, reference memory and working memory are affected differently by chronic stress (Conrad, 2010). It is possible that we found working and reference memory to be correlated due to our testing conditions. There was no lasting effect of treatment on working or reference memory in any of the studies used (Gieling *et al.*, 2013, 2014; Roelofs *et al.*, 2017b). It is likely that when unaffected by treatment, pigs show unimpeded improvement in both working and reference memory, resulting in a positive correlation between these measures.

While both measures used to represent reference memory were highly correlated, our data suggest that the acquisition of the working memory component and overall working memory performance were independent. This unexpected finding was likely due to a lack of improvement shown in the learning curves of working memory. Some pigs already demonstrated a high level of working memory performance from the start of the holeboard task. The working memory component of the holeboard task is based on natural foraging behavior using a win-shift foraging strategy (Gustafsson *et al.*, 1999); pigs have previously been shown to acquire a win-shift task faster and perform it more accurately than a win-stay task (Laughlin and Mendl, 2000). This could explain why working memory learning curves for pigs display a ceiling effect quite quickly. Working memory slope may therefore not be as useful a measure to describe working memory learning in pigs as it is for other species with a steeper learning curve (e.g. mice: Kuc *et al.*, 2005).

Discrimination task and reference memory

Interestingly, auditory conditional discrimination learning in the judgment bias task, a task which entails an element of spatial discrimination and where rule learning is important for successful performance, was unrelated to reference memory in the holeboard task, a task which entails a more complex

spatial discrimination. Correct responding in the conditional discrimination task, however, may not entirely reflect reference memory capacity. Pigs which took longer to learn may have struggled to accept the inequality of reward between positive and negative trials, rather than failing to remember the rules of the task. Capuchins show more refusals when offered a less preferred food in the presence of an unobtainable preferred food (Dubreuil *et al.*, 2006). It is possible, therefore, that the pigs took some time to understand that when the small reward is signaled, the large reward is not available and that longer learning times also reflect greater frustration at the perceived inequality rather than purely discrimination abilities. This is supported by the fact that before pigs reach criterion on the conditional discrimination task in the judgment bias task, their latencies to respond in negative trials increase (own, non-systematic observations), suggesting they are already aware of the association between cue and reward size. Similarly, monkeys showed shorter response times in an operant task when preferred rewards were signaled than when less preferred rewards were signaled (Watanabe *et al.*, 2001). The conditional discrimination task used prior to judgment bias testing, as designed, does not allow us to distinguish between discrimination learning where correct choices are equally rewarded and discrimination learning when there is inequality between the choices.

CONCLUSION

The present study provides support for the notion that optimism measured in the judgment bias task is unrelated to the animals' cognitive abilities in the holeboard task and in the conditional discrimination task. Based on three separate studies, evidence was provided that optimism in the judgment bias task on the one hand, and working and reference memory performance in the holeboard task and the acquisition of the conditional discrimination task preceding judgment bias testing on the other, were independent. These results further validate the use of judgment bias as a proxy measure of emotional valence in animals.

9

General discussion

Low birth weight (LBW) piglets are increasingly common on commercial farms, as a result of selection for increasing litter size. These piglets may suffer from lifelong emotional and cognitive impairments, due to long-term effects of their suboptimal in utero development. Emotion and cognition are now recognized as important components of animal welfare, which depends on an animal's ability to adapt to the demands of its environment and reach a state that it perceives as positive (Ohl and van der Staay, 2012). Therefore, in this thesis we assessed the effects of birth weight on emotion and cognition in pigs, to better understand the welfare consequences of LBW on commercial pig farms.

First, we assessed pre-weaning effects of birth weight on emotional state by comparing HPA-axis development, fear responses and chronic stress in LBW and normal birth weight (NBW) piglets. We also evaluated neurological functioning of LBW piglets as an additional measure of brain development. Second, we assessed post-weaning effects of LBW on emotion and cognition. A spatial holeboard task was used to capture a variety of cognitive measures, while a judgment bias task allowed us to assess both discrimination learning and emotional state. In addition, we compared acute and chronic stress in LBW and NBW pigs. Third, we evaluated the use of judgment bias tasks as a measure of emotional state in pigs.

Combined, the results of these studies allow us to assess the relevance of LBW in pigs as a potential welfare problem. We provide a comprehensive overview of the effects of LBW on emotion and cognition in pigs. Combined, our findings lead to a more complete understanding of the welfare of LBW pigs, by adding psychological measures to the existing body of knowledge of the effects of LBW on piglets' physical health (Rutherford *et al.*, 2013). Finally, acknowledging the limitations of our studies, we provide suggestions for improved assessment of emotional state in commercially housed pigs.

LOW BIRTH WEIGHT IN COMMERCIALY HOUSED PIGS

Disproportionally more LBW piglets are born in larger than in smaller litters (Quiniou *et al.*, 2002). Because the average litter size on commercial pig farms is increasing as a consequence of selective breeding for large litters (Rutherford *et al.*, 2013), we expected that any potential welfare consequences of LBW are of relevance for a considerable number of animals. In **Chapter 2**, we weighed all live-born piglets from 92 litters within 24 hours after birth.

At least one LBW piglet was born in 74 of these litters, suggesting LBW piglets are indeed common. Multiple studies have pointed out that LBW piglets have an increased mortality compared to their NBW siblings (Milligan *et al.*, 2002; Fix *et al.*, 2010). A recent study estimated the chances of survival to slaughter age for LBW pigs at 28% (Calderón Díaz *et al.*, 2017). For the studies described in **Chapter 3**, over a period of 13 weeks we weighed all available LBW piglets soon after birth, along with an equal number of NBW siblings. We followed up on these piglets at three weeks of age, and found that 49% of LBW piglets had died, compared to only 6% of NBW piglets. This suggests that while many LBW piglets die during the early post-natal period, there is still a considerable number of LBW piglets that survives long enough for potential emotional or cognitive impairments to affect their welfare. It also seems that LBW pigs will continue to be a common occurrence on commercial farms. As discussed in **Chapter 2**, we found the proportion of LBW piglets in a litter to increase with litter size. Therefore, as long as selection for sow fecundity continues, the findings of this thesis will likely remain relevant and may even gain relevance.

Intra-uterine growth restriction

The studies in this thesis are based on the assumption that LBW pigs have suffered intra-uterine growth restriction (IUGR), with its associated negative effects on brain development. This assumption is based on studies of LBW and IUGR in humans. As discussed in **Chapter 1**, IUGR occurs naturally in both humans and pigs, due to an impaired supply of nutrients and oxygen to the fetus (Cox and Marton, 2009; Hunter *et al.*, 2016). Such placental insufficiency occurs during the later stages of pregnancy, when placental function cannot keep up with the increasing demands of the developing fetus, or of multiple fetuses as is the case in commercial pigs (Hunter *et al.*, 2016). As the peak of brain development starts during the late prenatal period in both humans and pigs (Dobbing and Sands, 1979; Conrad *et al.*, 2012), IUGR due to placental insufficiency can have an adverse effect on emotional and cognitive functioning.

However, it is important to note that when using LBW as the only read-out parameter, we cannot be sure that IUGR actually occurred. IUGR is “a failure of a fetus to reach its genetic growth potential” (Cox and Marton, 2009). However, as this growth potential varies between individuals, some LBW piglets may have been fully developed, but constitutionally small, individuals.

Consequently, they may not have experienced IUGR and its adverse effects on brain development (Cox and Marton, 2009; Iughetti *et al.*, 2017). In humans, IUGR due to placental insufficiency leads to prioritized brain development, with preferential blood flow to the brain at the expense of other organs. IUGR can then be determined by assessing relative brain size by measures such as head circumference, or by serial observations of *in utero* growth (Pollack and Divon, 1992; Iughetti *et al.*, 2017). In pigs, *in utero* observations are not practically feasible on commercial farms, as it requires both suitable ultrasound equipment and individual identification of each fetus *in utero*. While measures of brain size such as head morphology have been applied as markers for IUGR in pigs (Amdi *et al.*, 2013), we have found them to be confounded by breed-specific differences in head shape, because most commercially kept pigs, including those used in the studies of the present thesis, are crossbreds. Therefore, LBW appears to currently be the best available measure of IUGR. However, this means that the findings in this thesis are likely based on a heterogeneous population, consisting of both LBW pigs which have suffered IUGR and fully developed but constitutionally smaller pigs.

EMOTION IN LOW BIRTH WEIGHT PIGS

We defined an animal's emotional state as a combination of short-term emotions in response to specific stimuli and longer-term general moods (Mendl *et al.*, 2010). Emotional state can be assessed using physiological, behavioral and cognitive measures. Using a combination of these different components is encouraged (Leliveld *et al.*, 2016), as single measures of emotion may be confounded by other processes such as physical activity or stress caused by sample collection (Otvic, 2014). Therefore, to assess whether LBW affects pigs' emotional development, we used a variety of physiological, behavioral and cognitive measures, assessing both short-term emotional responses and long-term moods. Salivary cortisol concentration and behavioral responses in the human approach test (HAT) were assumed to reflect short-term emotional responses to negatively valenced stimuli. While these measures are likely influenced by a pig's overall mood (Mendl *et al.*, 2010), they do not necessarily reflect a pig's long-term emotional state (i.e. a short-term fear response to a stressor does not suggest a pig is also suffering from constant, long-term fearfulness). For assessment of such longer-term emotional moods, we used hair cortisol concentration as a physiological

marker for chronic stress, as well as behavioral responses to ambiguous stimuli in the judgment bias task.

In **Chapter 2**, we used neonatal hair cortisol concentration to assess in utero cortisol production as a marker of HPA-axis development. We found that LBW alters HPA-axis development of piglets in a sex-dependent way. Female LBW piglets had higher neonatal hair cortisol concentrations than male LBW piglets, suggesting females produced more cortisol in utero.

In **Chapter 3**, we compared LBW and NBW piglets' behavioral responses in a human approach test (HAT). No differences between groups were found, with LBW and NBW scoring similarly for variables such as vocalization frequency and latency to approach the unfamiliar human.

In **Chapter 5**, we used hair cortisol concentrations at weaning (approximately 4 weeks of age) and at slaughter (approximately 24 weeks of age) to assess chronic stress experienced in the farrowing unit and in the experimental facility. In addition, we assessed acute stress responses in post-weaning LBW and NBW pigs by determining salivary cortisol concentration before and after presenting the pigs with a stressor. We found no differences in acute or chronic stress between LBW and NBW pigs.

In **Chapter 6**, we repeated the same assessment of acute and chronic stress in post-weaning pigs. In this study, contrary to our previous findings in **Chapter 5**, we found that LBW pigs had slightly higher hair cortisol concentrations at weaning, suggesting they experienced increased chronic stress in the farrowing unit. No other differences in cortisol concentrations were found. In addition to these physiological measures, we also assessed judgment bias as a cognitive measure of emotional state. Again, no differences between LBW and NBW pigs were found.

Taken together, our results provide inconclusive evidence for the notion that LBW may affect pre-weaning emotional state in pigs. We found LBW to affect long-term cortisol production in utero and while the piglets were housed in the farrowing pens. These findings support previous studies showing that LBW can result in an increased vulnerability to (chronic) stress pre-weaning (Wise *et al.*, 1991; Klemcke *et al.*, 1993). As stated in **Chapter 1**, chronic stress is associated with a negative emotional state as it is induced by aversive situations (Paul *et al.*, 2005). We found no indications that LBW has a negative effect on post-weaning emotional state. This is in contrast to

previous studies reporting LBW pigs to be more pessimistic and anxious post-weaning (Gieling *et al.*, 2014; Murphy *et al.*, 2015). This difference in results was likely due to differences in (social) environment between studies.

Effects of environmental conditions

Our findings suggest that whether or not LBW affects emotional state in pigs is dependent on their environment. The only indications of a more negative emotional state for LBW pigs were found before weaning, when the piglets were housed in the farrowing unit of a commercial farm. After weaning, when the pigs were housed in our experimental facility, LBW and NBW pigs were consistently similar in emotional state, based on both physiological and cognitive measures of emotion.

In the experimental facility, the pigs were housed in enriched conditions, with approximately 2 m² of space per pig and daily provision of straw. Such conditions have been shown to improve emotional state in pigs. Pigs housed at a lower stocking density show physiological signs of improved emotional state, such as lower fecal cortisol concentrations (Cornale *et al.*, 2015). Similar findings are reported on the effects of environmental enrichment, with barren housing resulting in a blunted circadian cortisol rhythm, which is associated with chronic stress (de Jong *et al.*, 2000), and a more pessimistic judgment bias (Douglas *et al.*, 2012). The pigs in our studies were also trained in either the holeboard or judgment bias task almost daily. This provided them with a solvable cognitive challenge, leading to successful control over an aspect of their environment. Such cognitive enrichment is known to have a positive influence on emotional state (Clark, 2017). In pigs, provision of long-term cognitive enrichment results in reduced stereotypical behavior, another indicator of chronic stress, and reduced fear responses in behavioral tests (Puppe *et al.*, 2007; Zebunke *et al.*, 2013). Therefore, potential negative effects of LBW on emotional state could have been ameliorated by the enriched housing conditions applied in our studies.

It is possible that LBW pigs only show an impaired emotional state when they are housed in more stressful conditions. The studies which have reported effects of birth weight on physiological measures of emotional state have applied commercial housing conditions, sometimes even applying individual housing of pigs (Klemcke *et al.*, 1993; Poore and Fowden, 2003). Two other studies have assessed post-weaning effects of LBW on emotional

state using behavioral and cognitive measures. Murphy *et al.* (2015) found LBW pigs to have a more pessimistic judgment bias compared to NBW pigs, while Gieling *et al.* (2014) found LBW pigs to show increased anxiety in an open field test. As discussed in **Chapter 5**, it is possible that the LBW pigs in those studies were more stressed than the pigs tested in our studies due to differences in social housing conditions. Together, these findings make it premature to assume that LBW pigs housed in commercial conditions do not suffer from altered emotionality, particularly as many practices on commercial pig farms are known to have the potential to induce a negative emotional state (Nordquist *et al.*, 2017b).

COGNITION IN LOW BIRTH WEIGHT PIGS

In **Chapter 1**, we recommended measuring cognition in pigs using tasks which can capture a variety of different cognitive measures. Therefore, our main assessment of cognitive performance in LBW pigs is based on the spatial holeboard task, which allows us to measure different aspects of spatial learning and memory. In **Chapter 6**, we found an adverse effect of birth weight on pigs' reference memory performance, suggesting that LBW pigs had more difficulty remembering which food bowls were baited in the holeboard. Other measures of cognition, such as working memory and behavioral flexibility, were unaffected in LBW pigs. In addition to these findings, in **Chapter 5**, we compared LBW and NBW pigs in the auditory-spatial discrimination task, which the pigs must master at a high level of accuracy before judgment bias can be tested. Pigs had to learn to discriminate between two auditory stimuli, and to approach different goal-boxes in response to each tone. LBW pigs required a higher number of training sessions than NBW pigs to consistently perform the correct responses in this task.

Together, these findings suggest that LBW can have long-lasting effects on pigs' cognitive abilities. The cognitive impairments in the holeboard task and the auditory-spatial discrimination task were found post-weaning, when pigs were approximately 4-5 months of age. These findings are unlikely to be due to effects of stress on LBW pigs' cognitive performance (Conrad, 2010), as no differences between LBW and NBW pigs were found in post-weaning salivary or hair cortisol concentrations, used as markers for acute and chronic stress. Nor did our findings reflect a difference in food motivation between LBW and NBW pigs (which would have been a possible confounding factor

as both tasks use food rewards). In both tasks, all pigs showed a similar level of motivation based on measures such as speed of performing the task (van der Staay *et al.*, 2012). Rather, it seems that the found impaired cognitive abilities were indeed implications of LBW.

Cognitive impairments in LBW pigs were expected based on human studies showing LBW children experience long-term learning difficulties (Lindström *et al.*, 2017; Yu and Garcy, 2018). While indications of impaired cognition in LBW pigs have previously been reported (Gieling *et al.*, 2012; Radlowski *et al.*, 2014), there have also been studies reporting no negative effects of LBW on cognitive performance (Murphy *et al.*, 2013a, 2015; Gieling *et al.*, 2014; Antonides *et al.*, 2015a). In particular, studies using the spatial holeboard task to assess cognition in LBW pigs have produced contradictory results (Gieling *et al.*, 2012, 2014; Antonides *et al.*, 2015a). This is of interest, as both these studies as well as our own have used the same holeboard apparatus and training protocol. In **Chapter 6**, we discussed that the finding of improved cognitive performance of LBW pigs reported by Antonides *et al.* (2015a) was likely a result of the different housing conditions applied in that study. The results found by Gieling *et al.* are much more in line with the findings of this thesis, with one study reporting mildly impaired performance in the holeboard by LBW pigs (2012) and the other showing a mild impairment but being limited by a reduced statistical power compared to our study (2014).

General versus specific cognitive deficits

We found LBW pigs to have mild impairments in two different measures of cognition. Both spatial reference memory and discrimination learning were affected by birth weight. Although these results show that LBW can affect different cognitive domains, they do not necessarily suggest that LBW causes a general cognitive impairment. Studies in a variety of species have shown that performance in one cognitive domain does not necessarily predict performance in other domains (Boogert *et al.*, 2011; Qi *et al.*, 2018). Similarly, we cannot be sure that impairment due to LBW in one cognitive domain predicts impairments in other domains. We do not know how IUGR specifically affects brain development, i.e. whether certain cognitive skills are more vulnerable to impairment than others because they rely on different neuronal substrates, brain structures or brain circuits (Committeri *et al.*, 2004). This notion of specific cognitive deficits is supported by our findings of unimpaired working memory and behavioral flexibility in **Chapter 6**, and our

finding of independence of spatial learning in the holeboard and discrimination learning in the judgment bias task in **Chapter 8**.

As we cannot translate findings from one cognitive domain to another, this means that in order to better understand how LBW affects overall cognition, we have to separately assess the different cognitive domains which are likely to be most relevant for pigs. While spatial learning and memory are relevant because in the wild, pigs explore large territories for food (Podgórski *et al.*, 2013), pigs are also very social animals (Graves, 1984). However, potential effects of birth weight on pigs' social cognition have not yet received scientific attention. Cognitive skills such as being able to discriminate between different group members are crucial for establishment of dominance hierarchies (Souza *et al.*, 2006). Such skills have previously been assessed in pigs, for example by requiring them to only approach a familiar animal in a Y-maze (McLeman *et al.*, 2005). We suggest applying such tasks of social recognition to LBW pigs, to further expand our knowledge of their cognitive abilities.

WELFARE OF LOW BIRTH WEIGHT PIGS

By combining our findings of the effects of LBW on emotion and cognition, we can provide a more complete overview of LBW pigs' psychological wellbeing. Animal welfare is directly affected by negative emotional states, that is, an animal that is currently in a negative emotional state will always have lowered welfare, in that moment, compared to an animal in a positive emotional state (Duncan and Petherick, 1991). The combined results of our studies suggest that the negative effects of LBW on pig welfare can be overcome by providing LBW pigs with enriched housing conditions. After all, we found no indications of a more negative emotional state in LBW pigs when they receive environmental and cognitive enrichment.

Even in enriched housing conditions, we found cognitive impairments in LBW pigs. It is likely that these findings were ameliorated by the enriched housing conditions we applied. Previous studies have shown that enriched housing can have a positive influence on cognitive performance (de Jong *et al.*, 2000; Grimberg-Henrici *et al.*, 2016), whereas chronic stress can have a negative effect (Sandi, 2013). Therefore, it is possible that when LBW pigs are housed in commercial conditions, where they are subjected to a variety of

known stressors such as barren housing and overcrowding (Nordquist *et al.*, 2017b), their cognitive development will be more severely affected.

Such cognitive impairments could have welfare consequences on commercial farms. Whether this is actually the case will depend on the cognitive challenges that are present in the pigs' environment. As discussed in **Chapter 1**, pigs rely on spatial learning and memory for learning and remembering where specific locations of importance within their environment can be found. These can be feeding and drinking sites, but also their own preferred resting spots and the preferred resting spots of others', such as more dominant animals which are best avoided (Mendl *et al.*, 1997; Held *et al.*, 2002; Wechsler and Lea, 2007). As pigs often pass through multiple housing systems during the course of their production period (Marchant-Forde, 2009), spatial learning and memory could benefit them while adjusting to a new environment. The impaired spatial learning and memory displayed by LBW pigs could have negative consequences for their welfare on commercial farms, as it decreases predictability of their environment (Wiepkema and Koolhaas, 1993).

Viability of low birth weight piglets

It is important to note that not all LBW pigs may experience the same potential threats to their welfare. We found that LBW pigs vary considerably in their absolute birth weight. For example, the minimum birth weight found in piglets selected for post-weaning assessment of emotion and cognition, i.e. piglets that survived to weaning age, was 670 grams in **Chapter 5**, and 690 grams in **Chapter 6**. In contrast, the minimum birth weight found in piglets assessed within a few days after birth was 560 grams in **Chapter 2**, and 450 grams in **Chapter 3**. This suggests that LBW piglets born with an absolute birth weight below approximately 700 grams do not survive to weaning or slaughter age. This is supported by other studies showing pigs' probability of mortality increases as absolute birth weight decreases (Quiniou *et al.*, 2002; Fix *et al.*, 2010). These differences in viability within the LBW population have welfare implications.

It is unlikely that the smaller LBW piglets (those ranging from <500 to 700 grams in birth weight) are ever affected in their welfare by cognitive impairments. Rather, most of these piglets die from lack of colostrum or are crushed by the sow, as their impaired energy reserves and thermoregulation

affect their ability to compete over food and to respond to sow movements (Le Dividich *et al.*, 2005; Rutherford *et al.*, 2013; Muns *et al.*, 2016). As discussed in **Chapter 3**, IUGR could have an additional effect on this through impaired neurological development. The welfare of smaller LBW piglets on commercial farms is thus most likely affected by their ability to respond to hunger and potential injury (Ohl and van der Staay, 2012).

Cognitive impairments due to IUGR do not come into play as a potential welfare threat until the piglets start to rely on their cognition for successful interaction with their environment. For example, during the first days after birth, piglets can rely on the sow's acoustic signals for feeding (Špinka *et al.*, 2002). Only when they are provided with milk replacer at approximately three days of age do they require spatial learning and memory to locate their food. As mortality is highest during the first few days after birth (Alonso-Spilsbury *et al.*, 2007; KilBride *et al.*, 2012), most of the smaller LBW piglets will not be faced with such cognitive challenges.

Sex-specific welfare implications

Throughout this thesis, we have accounted for potential sex-specific effects of LBW in pigs. This was based on findings in human studies, where LBW females are more prone to develop emotional disorders than LBW males (Costello *et al.*, 2007; Van Lieshout and Boylan, 2010). Also, stress responses (and its effects on cognition) have been found to be sex-specific in rodents (Luine *et al.*, 2007; Healy *et al.*, 2009). In **Chapter 2**, we found female LBW piglets to have higher *in utero* cortisol production than males, suggesting HPA-axis development in LBW pigs is affected in a sex-specific manner. However, no further sex-specific emotional and/or stress responses in LBW pigs were found. Based on the findings reported in this thesis, the welfare implications of LBW seem to be similar in female and male pigs.

We did find indications that female and male pigs could be affected differently by social housing conditions. In **Chapter 4**, male NBW pigs were found to have impaired behavioral flexibility compared to females, as measured in a reversal task in the spatial holeboard. These findings were based on pigs housed in single-sex groups and suggest that males were slower to adjust their behavior in response to a change in their environment. During a subsequent holeboard study, described in **Chapter 6**, this impaired behavioral flexibility was not found for males housed in mixed-sex groups. Similarly, a previous study assessing judgment bias in male LBW pigs (housed

in an all-male group) found these pigs to be more pessimistic than their NBW siblings (Murphy *et al.*, 2015). In **Chapter 5**, we repeated this study with LBW males housed in a mixed-sex group and found no differences in their emotional state compared to the NBW pigs. As discussed in **Chapter 5**, both findings suggest that it is possible that single-sex housing of male pigs could be more stressful than mixed-sex housing due to an increased performance of aggressive and sexual behavior (Clark and D'Eath, 2013; Puls *et al.*, 2017). Whether sex composition of a group also affects female pigs requires further investigation, systematically comparing male-only, female-only and mixed-sex groups. It is possible that when housed with males, the increase in aggression and mounting will negatively affect the welfare of females. Based on our findings for male pigs, welfare recommendations for social housing on commercial farms need to take sex into account.

MEASURING JUDGMENT BIAS IN PIGS

As stated previously, an animal's emotional state directly affects its welfare. The main aim of this thesis was to assess whether LBW in pigs could be a risk factor for impaired welfare on commercial farms. Therefore, it is important that we can accurately assess emotional state of (LBW) pigs housed in commercial conditions. Judgment bias is considered a potentially useful measure, as it can reflect both the intensity and valence of emotional state (Murphy *et al.*, 2014). In **Chapter 7**, we provided an extensive literature review of judgment bias tasks, to assess their potential to provide an accurate measure of emotional state in animals. We discussed several different implementations of judgment bias tasks, and how adjustments in study design could lead to improved quality of results. Here, we assess how our judgment bias task for pigs measures up to several points discussed in that review.

Judgment bias as a measure of emotional state

We suggested in **Chapter 7** that when judgment bias truly reflects emotional state in animals, results from the judgment bias task must correlate with other measures of emotional state. For example, a previous study has shown that a pessimistic judgment bias in pigs is accompanied by increased fear-responses as measured in a novel object test (Carreras *et al.*, 2016a). In **Chapter 5**, we found no correlation between judgment bias and chronic stress (measured as hair cortisol concentration). However, this finding could be due to a lack of differences between groups. We couldn't assess whether LBW causes a more

negative emotional state based on both judgment bias and hair cortisol, as no differences between LBW and NBW pigs were found. Therefore, we can only conclude that individual differences in judgment bias do not correlate with individual differences in cortisol production. Individual variation in emotional measures such as baseline hair cortisol concentrations have previously been reported for pigs (Casal *et al.*, 2017), and were also found in **Chapters 2, 5 and 6** of this thesis. We strongly suggest to continue using multiple measures of emotional state in judgment bias studies, to verify a correlation between different tests when an effect of treatment is found.

Another criterium for judgment bias as a measure of emotion is its independence of confounding factors such as cognition. Judgment bias should reflect a pig's emotional state, without being influenced by the pig's cognitive abilities (Murphy *et al.*, 2014). As described in **Chapter 8**, we used Bayesian analysis to show that the results of our judgment bias task are independent of cognitive measures such as discrimination learning, reference memory and working memory.

Loss of ambiguity

Loss of ambiguity was suggested as a main confounding factor on results of judgment bias tasks. When animals learn about the outcome of ambiguous stimuli during judgment bias testing, this will influence their subsequent choices, i.e. a lack of reward will signal that a response was incorrect, whereas rewarding as expected will signal that a response was correct. As a result, an animal's responses can no longer be considered to be made under ambiguity and thus no longer reflect judgment bias (Doyle *et al.*, 2010b). In **Chapter 4**, we confirmed the presence of loss of ambiguity in pigs. This is supported by other studies showing that pigs quickly learn about the outcomes of ambiguous stimuli (Murphy *et al.*, 2013b; Scollo *et al.*, 2014). In **Chapter 5**, we attempted to avoid loss of ambiguity by applying partial reinforcement of training trials. This was expected to impede learning about the outcome of ambiguous trials, as the pigs would be familiarized with an occasional lack of reward during training (Jamieson *et al.*, 2012). As discussed, this change in study design did not prevent the occurrence of loss of ambiguity. Similar to our findings in **Chapter 4**, pigs decreased their optimistic choice percentage as testing progressed, likely because they still learned about the unrewarded outcome of testing trials. We conclude that, to avoid loss of ambiguity as a confounding factor in our judgment bias task for pigs, a partial reinforcement

schedule alone is not sufficient. Future studies could benefit from additional adjustments in study design. For example, increasing the ratio of reference trials to ambiguous trials will decrease the opportunity for pigs to learn about the outcome of ambiguous stimuli (Hintze *et al.*, 2018).

Learner bias

It was suggested that results from judgment bias tasks can be biased towards 'learners', i.e. only animals that manage to successfully complete discrimination training can be tested. For example, a previous judgment bias study with pigs started discrimination training with 54 animals, but only 32 managed to complete training successfully by reaching a preset criterion level of performance and proceed to judgment bias testing (Brajon *et al.*, 2015). Conclusions based on only a specific subset of animals may not be generalizable to the entire population. Therefore, it is necessary to use discrimination tasks which the majority of animals are able to master. In both judgment bias studies performed in this thesis, the majority of pigs managed to reach criterion performance during discrimination training (18 out of 20 pigs in **Chapter 4** and 36 out of 41 pigs in **Chapter 5**). Therefore, it is unlikely that results from our judgment bias task were affected by a bias towards learners. Even when the task was made more difficult by adding unrewarded training trials in **Chapter 5**, most pigs still successfully completed discrimination training.

Applicability in commercial farm setting

One limitation of using judgment bias tasks for welfare assessment of animals housed in commercial conditions is that a period of discrimination training is required (Brilot *et al.*, 2009). Although judgment bias can be measured in housing conditions similar to those on a commercial farm, the required discrimination training is still likely to act as cognitive enrichment (Zebunke *et al.*, 2011, 2013). Furthermore, as discussed in **Chapter 7**, individual training of social animals such as pigs requires extensive habituation (e.g. Murphy *et al.*, 2013a), further reducing practical applicability for welfare assessment under on-farm conditions. This suggests that shortening the habituation and discrimination training period, so that farm conditions can be simulated without providing cognitive enrichment, would make judgment bias tasks more suitable tools for assessment of emotional state in pigs.

In **Chapter 4**, pigs required on average 22 discrimination training sessions before being tested in the judgment bias task. In **Chapter 5**, this number increased to an average of 25 sessions for NBW pigs and an average of 30 sessions for LBW pigs. Likely, this was due to the increased difficulty presented by a partial reinforcement schedule. Previous studies using the same task design also reported that weeks of discrimination training were required before the pigs reached a preset criterion of performance (Murphy *et al.*, 2013a, 2015). However, there are also implementations of judgment bias tasks for pigs which required a substantially shorter training period. In a study by Döpjan *et al.* (2017), pigs were trained over a period of seven days, receiving 12 training trials per day. During this training period, all pigs successfully learned to discriminate between the positive and negative stimulus. It appears one important difference with our study design lies in the consequence of an incorrect choice during negative trials. In our task, pigs can obtain a small reward during negative trials, which they miss out on if they make an incorrect response to the negative stimulus. In the task designed by Döpjan *et al.*, pigs are trained to avoid punishment during negative trials, where an incorrect choice leads to exposure to a stressor (a plastic bag waved in front of the pig's face). This suggests that an increased cost of incorrect choices may result in a shorter training time. As discussed in **Chapter 5**, the long training time required for our judgment bias task is due to the pigs making errors during negative trials. Perhaps by applying (mild) punishment for incorrect choices, training time could be reduced. However, future studies are required to ensure that such punishment does not interfere with treatment by affecting the emotional state of the pigs.

In **Chapter 7**, training and testing animals in their social group was discussed as an alternative to the (often lengthy) individual training process associated with judgment bias tasks. If social animals such as pigs can be trained in a group setting, this would eliminate the necessary habituation prior to discrimination training. However, when applying group training, i.e. when all animals are trained simultaneously, it is difficult to establish which animals truly discriminate between the positive and negative stimuli and which animals were simply copying the behavior of group members. Recent judgment bias studies with dolphins have circumvented this issue by training a single animal in the presence of its social group (Clegg *et al.*, 2017; Clegg and Delfour, 2018). However, due to their notoriously inquisitive nature, this is unlikely to be a successful approach for training pigs. The closest solution to group training for pigs appears to be training them while in visual and auditory contact with their group, as has previously been applied to chickens

(Salmeto *et al.*, 2011). Whether this would allow for a shortening (or elimination) of the extensive habituation to individual training conditions will have to be assessed in future studies.

CONCLUSIONS AND IMPLICATIONS

LBW piglets are present in the majority of litters on commercial pig farms. Larger litters in particular are likely to contain one or more piglets suffering from IUGR. We found that while LBW piglets may suffer from altered HPA axis functioning, possibly making them more prone to suffer from chronic stress, this potentially increased vulnerability for negative emotional states can be overcome by providing the piglets with enriched housing conditions. However, such housing conditions are not sufficient to counter LBW pigs' impaired cognitive development. Although the cognitive impairments we found were mild, it is likely they were ameliorated by the presence of (cognitive) enrichment. LBW pigs on commercial farms may suffer from exaggerated deficits compared to those used in this thesis.

It is highly unlikely that current commercial pig farms can provide the enriched housing conditions which would enable LBW piglets to thrive as well as their NBW siblings. Furthermore, LBW piglets suffer from welfare challenges that cannot be prevented by adjusted housing conditions. Assisting LBW piglets with their impaired ability to avoid injury and compete for food is not feasible on commercial farms, as it requires personal attention for each piglet. Therefore, the most practical solution to avoid the welfare challenges faced by LBW pigs is to minimize the chances of IUGR. Halting the continued selection for increased litter size would decrease the occurrence of LBW piglets, and thereby result in a lower number of individuals whose welfare is affected by IUGR.

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Nederlandse samenvatting

(Dutch summary)

Het welzijn van landbouwhuisdieren is een relevant onderwerp voor wetenschappelijk onderzoek, wegens de huisvesting van een toenemend aantal dieren in intensieve productiesystemen. Deze systemen zijn met name ingericht op efficiënte productie en niet zozeer op de behoeften van het dier. Het evalueren van dierwelzijn in landbouwhuisdieren omvat niet alleen het fysieke functioneren van het dier (zoals honger status en gezondheid), maar ook psychologische processen als emotie en cognitie.

De emotionele staat van een dier beschrijft (subjectief) hoe het dier zijn omgeving ervaart. Dit proces is een combinatie van kortstondige emoties (zoals angst door een negatieve interactie met een diervorzorger) en algemene stemmingen van langere duur. De emotionele staat van een dier uit zich in zowel fysiologie, gedrag en cognitie. Bijvoorbeeld, een angstig dier zal een verhoogde hartslag vertonen, specifieke vocalisaties produceren, en meer aandacht hebben voor bedreigende elementen in zijn omgeving. Dierwelzijn en emotie zijn nauw verwante concepten. Een dier in een (langdurig) negatieve emotionele staat heeft verminderd welzijn vergeleken met een dier in een positieve emotionele staat.

De cognitieve vaardigheden van een dier beschrijven hoe het dier informatie over zijn omgeving verwerkt. Hiermee speelt cognitie een belangrijke rol in de interacties tussen een dier en zijn huisvesting. Deze interacties kunnen van groot belang zijn voor dierwelzijn. Bijvoorbeeld, dieren die onderdeel zijn van een sociale hiërarchie moeten zich bewust zijn van hun positie binnen de rangorde om onnodige agressie te voorkomen. Ook moeten dieren in staat zijn te leren en onthouden waar elementen als voedsel en rustplekken binnen hun huisvestingssysteem te vinden zijn.

EMOTIE EN COGNITIE IN VARKENS

Omdat emotie en cognitie van belang zijn voor dierwelzijn, is er een toenemende behoefte om deze processen te kunnen meten in landbouwhuisdieren. Varkens zijn een geschikte diersoort voor zulk onderzoek, omdat zij in groten getale gehouden worden in intensieve productiesystemen. Bovendien bieden varkens de mogelijkheid voor onderzoek naar de welzijnseffecten van selectief fokken. Selectieve fokprogramma's zijn in de varkenshouderij zeer succesvol gebleken, met als gevolg toename in zowel groeisnelheid van vleesvarkens als vruchtbaarheid van fokzeugen. Of dit soort selectie ook effecten heeft op het welzijn van de

varkens is nog niet uitgebreid onderzocht. Dit soort onderzoek is afhankelijk van correcte maten van emotioneel en cognitief functioneren.

Een potentiële maat van emotie in varkens is de zogenaamde 'beoordelingsbias'. Beoordelingsbias beschrijft een afwijking in de beoordeling van neutrale informatie onder invloed van de huidige emotionele staat. Een veelgebruikt voorbeeld van beoordelingsbias in mensen is of ze het glas als halfleeg of halfvol ervaren. Hierbij gaan we ervan uit dat een optimistische beoordeling van de situatie veroorzaakt wordt door een positieve emotionele staat, terwijl een pessimistische beoordeling het resultaat is van een negatieve emotionele staat. Beoordelingsbias kan worden gemeten in een variëteit aan diersoorten, waaronder varkens. Om dit te doen wordt het varken eerst getraind om twee verschillende stimuli te associëren met een positieve of negatieve uitkomst. Na het horen van een hoge geluidstoon kan het varken altijd in één van twee aanwezige voerbakken een grote beloning vinden. Na het horen van een lage geluidstoon kan het varken in de andere voerbak een kleine beloning vinden. Het varken wordt getraind tot hij consequent de juiste reacties vertoont na het horen van de tonen. Dat wil zeggen, het varken benaderd na het horen van de hoge toon de voerbak waarin de grote beloning beschikbaar is, en benaderd na het horen van de lage toon de voerbak met de kleine beloning. Na de training worden ook 'ambigue' tonen afgespeeld, welke tussen de hoge en lage toon in liggen. De reactie van het varken na het horen van een ambigue toon wordt als pessimisme of optimisme gescoord. Het benaderen van de voerbak behorend bij de grote beloning wordt als een verwachting van een positieve uitkomst geïnterpreteerd (optimisme – positieve emotionele staat), terwijl het benaderen van de voerbak behorend bij de kleine beloning als een verwachting van een negatieve uitkomst wordt geïnterpreteerd (pessimisme – negatieve emotionele staat).

Ook de cognitieve vaardigheden van varkens kunnen met behulp van een gedragstaak onderzocht worden. Een geschikte cognitieve taak voor varkens is het 'holeboard': een open arena met daarin 16 locaties welke een beloning kunnen bevatten. Het varken moet dagelijks binnen de arena op zoek naar vier beloningen, welke altijd op dezelfde locaties te vinden zijn. Hierdoor kan bijgehouden worden hoe snel een varken zijn prestaties in het holeboard verbeterd, door steeds minder bezoeken aan onbeloonde locaties te maken. Het holeboard geeft dus een indicatie van het ruimtelijk leervermogen van varkens. Ook geeft het holeboard informatie over de motivatie van de varkens (bijvoorbeeld door te meten hoe snel ze locaties bezoeken), over hun

exploratiedrag (hoeveel verschillende locaties worden bezocht) en hun gedragsflexibiliteit (hoe snel passen ze zich aan als de beloningen zich plotseling op nieuwe locaties bevinden).

LAAG GEBOORTEGEWICHT BIJ VARKENS

De selectieve fok op verhoogde vruchtbaarheid van fokzeugen op commerciële varkenshouderijen heeft gezorgd voor een toename in gemiddelde toomgrootte. Waar de voorouder van het gedomesticeerde varken, het wilde zwijn, tomen van gemiddeld vier tot zes biggen produceert, is de gemiddelde toomgrootte op commerciële varkensbedrijven inmiddels toegenomen tot 16 biggen per toom. De zeug is echter niet altijd in staat om alle foetussen van voldoende zuurstof en nutriënten te voorzien om ze optimaal te laten ontwikkelen. Hierdoor bevatten grotere tomen regelmatig biggen met een laag geboortegewicht (LG).

Van biggen met LG is bekend dat ze een groter risico lopen op verminderd welzijn door bijvoorbeeld hun verhoogde kans op ziekte en verwondingen. Of hun welzijn ook wordt beïnvloed door aangetast emotioneel en cognitief functioneren is nog niet duidelijk aangetoond. Op basis van bevindingen in humane studies kan LG wel degelijk negatieve effecten hebben op de emotionele en cognitieve ontwikkeling. Baby's met LG lopen een groter risico om emotionele stoornissen te ontwikkelen en hebben een grotere kans op leerproblemen op latere leeftijd vergeleken met baby's met een normaal geboortegewicht (NG). Als bij varkens sprake is van vergelijkbare effecten van LG, kan dit invloed hebben op hun welzijn in gevangenschap. In dit proefschrift is daarom onderzocht of LG in varkens van invloed is op hun emotie en cognitie.

EMOTIE IN VARKENS MET LAAG GEBOORTEGEWICHT

In **Hoofdstuk 2** van dit proefschrift is gekeken naar de concentratie van het stresshormoon cortisol in haren van pasgeboren biggen met LG en NG. Cortisol in haren geeft informatie over de lange termijn cortisol productie van een dier, en is daarmee een potentiële parameter van chronische stress. In pasgeboren dieren weerspiegelt het de productie van cortisol tijdens de prenatale ontwikkeling. Vrouwelijke biggen met LG hadden een hogere cortisol concentratie dan mannelijke biggen met LG, maar een algemeen effect van geboortegewicht werd niet gevonden.

In **Hoofdstuk 3** is de angstrespons van drie weken oude biggen met LG en NG vergeleken. Het gedrag van biggen tijdens een tijdelijke sociale isolatie en blootstelling aan de aanwezigheid van een onbekend mens werd gescoord. Biggen met LG en NG verschilden niet in hun reacties. Ze produceerden evenveel vocalisaties en waren even snel in het benaderen van de onbekende persoon.

In **Hoofdstuk 5** is gekeken naar de beoordelingsbias van varkens (na spenen) met LG en NG. Ook hier werden geen verschillen tussen de groepen gevonden, wat suggereert dat LG (op basis van beoordelingsbias) geen effect heeft op de emotionele staat van varkens. Het is waarschijnlijk dat de huisvesting van de varkens tijdens deze studie effect heeft gehad op onze resultaten. De varkens waren in ruime hokken met stro gehuisvest, en mochten hun hok dagelijks uit voor het trainen en testen in de taak. Eerder onderzoek met biggen suggereert dat LG wellicht voor een sterkere stressrespons zorgt. Het is mogelijk dat LG wel invloed heeft op emotie van varkens op een commercieel bedrijf, waar vele stressoren aanwezig zijn.

Een alternatieve verklaring voor onze resultaten is dat beoordelingsbias wellicht geen geschikte maat is voor emotie in varkens. Echter, meerdere studies in dit proefschrift suggereren dat beoordelingsbias wel degelijk beïnvloed wordt door emotionele staat en gemeten kan worden in varkens. In **Hoofdstuk 4** is aangetoond dat zowel mannelijke als vrouwelijke vleesvarkens in verrijkte huisvesting een optimistische beoordelingsbias vertonen. **Hoofdstuk 7** beschrijft een uitgebreide literatuurstudie naar beoordelingsbias in dieren. Hierin wordt geconcludeerd dat hoewel resultaten van beoordelingsbias studies zorgvuldig geïnterpreteerd moeten worden, deze wel de potentie hebben om de emotionele staat van een dier te weerspiegelen. In **Hoofdstuk 8** is gekeken naar een mogelijke correlatie tussen cognitie en beoordelingsbias. Als beoordelingsbias werkelijk een maat van emotie is, dan mag deze niet beïnvloed worden door andere processen zoals cognitie. Op basis van opnieuw geanalyseerde data van voorgaande studies waarin zowel beoordelingsbias als prestaties in het holeboard van varkens gemeten werden, kan geconcludeerd worden dat beoordelingsbias onafhankelijk is van verschillende cognitieve vaardigheden.

COGNITIE IN VARKENS MET LAAG GEBOORTEGEWICHT

In **Hoofdstuk 6** van dit proefschrift is gekeken naar de cognitieve prestaties van varkens met LG en NG. Hiervoor werden de varkens getraind in het holeboard. Varkens met LG hadden meer trainingssessies nodig om de locaties van de beloningen te onthouden dan varkens met NG. Geboortegewicht had geen effect op andere processen als motivatie, exploratie en gedragsflexibiliteit. Deze bevinding van een negatief effect van LG op cognitie wordt ondersteund door resultaten uit **Hoofdstuk 5**. Voordat de beoordelingsbias van de varkens getest kon worden, vond eerst een training plaats om de varkens succesvol te laten discrimineren tussen de positieve en negatieve stimuli. Ook tijdens deze studie hadden de varkens met LG een hoger aantal sessies nodig dan de varkens met NG om de discriminatietraining succesvol af te ronden.

Deze resultaten suggereren dat LG in varkens een langdurig negatief effect heeft op hun cognitieve vaardigheden (de varkens werden getest op een leeftijd van 4-5 maanden). Dit kan consequenties hebben voor het welzijn van varkens op commerciële bedrijven. Bijvoorbeeld, varkens zijn afhankelijk van hun ruimtelijk leervermogen om te kunnen leren en onthouden waar belangrijke locaties in hun omgeving te vinden zijn. Zulke locaties zijn bijvoorbeeld plekken waar water en voer aangeboden worden, maar ook waar dominante groepsgenoten zich vaak bevinden zodat agressie ontweken kan worden. Een aangetast ruimtelijk leervermogen in varkens met LG kan er dus voor zorgen dat hun omgeving minder voorspelbaar is, en daarmee een grotere bron van stress.

CONCLUSIE

De resultaten van dit proefschrift vergroten ons inzicht in de welzijnsconsequenties van LG, door studies over emotie en cognitie toe te voegen aan de bestaande kennis over het fysieke welzijn van varkens met LG. Dit is relevant voor de vele biggen met LG die geboren worden op commerciële varkensbedrijven. Hoewel veel van deze biggen snel na geboorte overleiden, is er ook een aanzienlijk aantal dat overleefd tot slachtleeftijd. Voor deze dieren hebben emotie en cognitie een grote invloed op hun welzijn.

Op basis van de resultaten van dit proefschrift kunnen de potentiële negatieve effecten van LG gecompenseerd worden door de verrijkte huisvesting van de varkens. Wij vonden geen indicaties van een verschil in emotionele staat tussen varkens met LG en NG in de tests die na speenleeftijd werden uitgevoerd. Deze varkens waren gehuisvest in verrijkte stallen en ontvingen dagelijkse cognitieve verrijking door middel van het trainen en testen in de gedragstaken. Echter, zelfs bij varkens in deze verrijkte huisvesting had LG een negatief effect op cognitief functioneren. Het is mogelijk dat onze resultaten niet direct vertaald kunnen worden naar varkens in commerciële huisvesting. Varkens op commerciële bedrijven komen in aanraking met een variëteit aan stressoren als kale huisvesting en overbezetting. Binnen zulke omstandigheden is het mogelijk dat de emotionele en cognitieve ontwikkeling van varkens met LG sterker beïnvloed wordt dan het geval was in onze studies.

Het is zeer onwaarschijnlijk dat commerciële varkensbedrijven kunnen voorzien in de verrijkte omstandigheden die varkens met LG de kans geven op vergelijkbaar welzijn als dat van varkens met NG. Bovendien is het niet haalbaar op een commercieel bedrijf om biggen met LG tijdens de neonatale periode individueel te monitoren op voldoende inname van voedsel en gezondheidsstatus. Daarom lijkt het zo veel mogelijk voorkomen van LG bij varkens, en daarmee de met LG geassocieerde welzijnsproblemen, de meest praktische oplossing. Het afremmen van de selectiedruk op toenemende toomgrootte bij fokzeugen kan ervoor zorgen dat er minder biggen met LG geboren worden. Hierdoor zullen minder varkens in aanraking komen met de bijbehorende negatieve effecten op welzijn.

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About the author

Sanne Roelofs was born on the 25th of May 1987 in Hengelo, the Netherlands. After graduating from high school, she obtained a Bachelor's degree in Biology at Utrecht University. She subsequently enrolled in the specialization track Behavioural Ecology as part of the Master's program Environmental Biology. During her Master's, Sanne used all opportunities for research internships to learn about the welfare of animals in captivity. She spent nine months studying personality traits of zoo-housed bonobos in different European zoos, performed a behavioral study on the cognitive abilities of commercially housed pigs, and wrote a literature review on the welfare of rabbits used as laboratory animals. These projects resulted in a Master's degree with the distinction *cum laude*.

After completing her Master's program, Sanne joined the Behavior and Welfare group at the Department of Farm Animal Health, Faculty of Veterinary Medicine in Utrecht for a PhD project on emotion and cognition in pigs. The results of that project are presented in this thesis. As of August 2018, she is working as a postdoctoral researcher at the University of Pennsylvania in the United States. As part of the Swine Teaching and Research team, her work focuses on improving and validating the welfare assessment of commercially housed breeding sows.

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- **Roelofs, S.**, Meijer, E., Baas, V., Dobrovolskiy, G., van der Staay, F.J., Nordquist, R.E. (under review). Neurological functioning and fear responses in low and normal birth weight piglets.
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