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SOCIAL RECOGNITION IN NEONATAL PIGS AND THE EFFECTS OF ACUTE STRESSORS ON IT

By

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ABSTRACT

SOCIAL RECOGNITION IN NEONATAL PIGS AND
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Social recognition (SR), used here to refer to the ability to discriminate a previously encountered individual from a novel individual, is of fundamental importance for a wide range of social behaviors and, in many species, is sensitive to interference by acute stressors. In pigs, failure in SR may increase aggression, yet the effects of acute stressors on SR of neonatal pigs have not been investigated. This PhD thesis therefore presents protocols to assess short- and long-term SR in neonatal pigs and the effect of acute stressors on it.

The 'habituation-dishabituation' paradigm was used in 11-day old female piglets to assess short-term SR and the effect of acute increases in cortisol on it. Although an intra-muscular hydrocortisone injection administered 15 minutes prior to testing significantly increased salivary cortisol levels, it did not interfere with short-term SR. This protocol involved considerable handling and fighting among subject and stimuli piglets, which could interfere with the outcome of the subsequent studies.

Therefore, a novel method to test SR abilities in pigs in which handling is minimized and fighting during the familiarization phase is prevented was developed and tested. This protocol proved to be suited for assessing SR in neonatal pigs, since SR could also be assessed in both 11-day-old and 21-day-

old animals, and the latter showed SR for up to 24 hours after the last familiarization prodecure.

The last study was based on the results of the previous three and investigated whether weaning age (D11 or D21) and an acute stressor (15 minutes of social isolation) interfered with the ability of female piglets to recognize familiar conspecifics. SR deficit was only observed in isolated D11 pigs, while non-isolated D11 and isolated and non-isolated D21 pigs did not show this impairment. This was taken as an indication that acute social stress may impair SR ability of D11 pigs, if so it may explain at least in part the increase in aggression during the first day of post-mixing in D11 animals that was previously reported by our lab.

I dedicate this dissertation to my god-father and god-mother João e Constância

they gave me great support throught my studies

although they left from this life before this work was concluded

they were always in my thoughts, heart and prayers

Eu dedico esta dissertação aos meus padrinhos João e Constância

eles me deram grande apoio durante os meus estudos

apesar deles terem partido desta vida antes que esta obra fosse concluída

eles sempre estiveram nos meus pensamentos, coração e preces

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"An education isn't how much you have committed to memory, or even how much you know. It's being able to differenciate between what you know and what you don't."

-Malcolm Forbes

A dissertation is more than only one among a number of requirements to conquer a PhD title. I see it as a product of systematic evaluations of an individual in his/her desire to find answers that can contribute to a better understanding of the world (fundamental research) and/or to solve existing problems (applied research).

My dissertation marks the end of a long and eventful journey and without mentioning those who have contributed to it would leave it incomplete. I admit that this list is just an attempt to acknowledge all those who have directly or indirectly contributed to this work, and that in writing this I have probably left out people who in one way or another did contribute to it. Please forgive me for that.

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CHAPTER 1

GENERAL INTRODUCTION

The significance of the domestic pig for our society has considerably increased since its domestication. There now is a high demand for pigs not only for food supply, but also as an animal model for various types of research in biomedicine and agriculture. Society has responded to this increased demand by further optimizing pig production systems which considerably differ from the natural environment in which they live, and the conditions under which they were domesticated. In order to increase efficiency, farms have adopted drastic changes in both the social and physical environment of pigs. There is growing evidence that certain management practices have led to behavioral and physiological alterations of the animals that indicate problems in coping with the living conditions. The higher levels of aggression after mixing in pigs that were weaned at a very young age that was reported by our lab (Yuan et al., 2004) constituted the main motivation for starting this PhD research. The main purpose of this introductory chapter therefore is to briefly summarize natural pig behavior and the conditions under which pigs have been domesticated, focusing on social structure, and relate these to the environments that modern production systems have imposed on pigs. It follows by a description of data generated in our lab that led to this PhD project stating how the present thesis primarily aims at looking at some basic aspects of the raised hypotheses. Then, it presents a short explanation of the Hypothalamic-Pituitary-Adrenocortical (HPA) axis and its regulation as well as a brief on the neonatal stress sensitivity period. Information on social recognition (SR) process is then introduced. At last, a short summary of the aim and outline of this thesis is presented.

1. Pigs living in (semi-)natural conditions

Wild and domestic pigs are highly social, gregarious animals that use olfactory, auditory, and visual signals to communicate with conspecifics (von Klingholz and Meynhardt, 1979; Graves, 1984). Under free-ranging conditions, pigs tend to form subgroups and maintain closer social bonds to certain individuals than others, resulting in animals sleeping in separate nests (Stolba and Wood-Gush, 1984). Non-member sows are usually not allowed to integrate into a stable group (Stolba and Wood-Gush, 1989).

The basic social unit is commonly a maternal group of 2-6 sows, their most recent litters, and pre-pubertal gilts (Graves, 1984). The maternal group is characterized by long-lasting associations among members (Newberry and Wood-Gush, 1986; Petersen et al., 1989; Stolba and Wood-Gush, 1989). Males leave the social group when they reach 7-8 months of age (Fradrich, 1974). When boars mature, they generally live in seclusion (Graves, 1984; Mauget, 1981), but temporarily reunite with maternal groups when the sows are in oestrus (Graves, 1984).

In the wild, pigs have opportunity to freely interact with conspecifics and naturally develop social behavioral patterns leading to the formation of dominance hierarchy and group stability. Social behavior is extremely developed in young pigs. Within hours, newborn piglets start forming social dominance relationships among littermates that will lead to the formation of a stable social hierarchy. Juveniles also maintain a clear hierarchy that is stable over time. In stable social groups, sows are dominant to all other members and a linear

hierarchy is observed between sows (Mauget, 1981). In general, dominance is determined by age and strength, and the social hierarchy is maintained through the avoidance of fighting by subordinate animals rather than enforcement by the dominants (Jensen and Wood-Gush, 1984), thus preventing unnecessary distress. Only during the mating season the social hierarchy changes, when a boar joins the group and assumes dominance (Schnebel and Griswold, 1983). At this time, the group consists only of the breeding sows and boar. Non-breeding females and pre-pubertal animals stay apart from the main group (Mauget, 1981; Blasetti et al., 1988).

In maternal groups, newborn offspring integrate into the maternal group over a period of around 7 weeks (Petersen et al., 1989). Pregnant sows separate from their group approximately 10 days prior to parturition (Graves, 1984). This separation seems to have evolved to give the mother and young enough time to learn specific cues for mutual recognition, contributing to the formation of a social bond between sow and litter and among littermates (Mauget, 1981). When piglets reach 10-14 days of age they start following the sow and by doing so gradually integrate into the maternal group (Jensen and Redbo, 1987; Stangel and Jensen, 1991). From about one week post-partum up to weaning, non-littermates slowly mingle. This process is usually non-aggressive, and social interactions are often playful (Petersen et al., 1989). As the pigs grow older, non-littermates are more often seen together on long excursions, without the presence of the mother (Petersen et al., 1989). However, the social bond between littermates remains strong (Stolba and Wood-Gush, 1981; Jensen,

1986; Newberry et al., 1988; Petersen et al., 1989). At 8 weeks of age, social interactions between non-littermates are seen more frequently than between littermates (Petersen et al., 1989). The amount of social interactions then stabilizes and the animals are totally integrated into the maternal group (Petersen et al., 1989).

Under natural conditions weaning is a gradual process. The length of the lactation period is dependent on the nutritional state of the sow and the amount of food available in the environment (Jensen and Recén, 1989). Usually, milk let-down gradually decreases when piglets are 6 weeks-old (Newberry and Wood-Gush, 1985, 1986) and Petersen (1994) reported a marked increase in solid food intake around 5 and 8 weeks of age, indicating a transition from a complete dependence on milk to nutritional independence from the sow. The nutritional independence also contributes to the independence of piglets from the sow and a broader integration within the maternal group (Jensen, 1986). Weaning hardly ever includes aggressive behavior (Jensen, 1986). Instead, the sow makes suckling more costly for the piglets by terminating nursing (Jensen and Recén, 1989). If weaning is defined as the time when nursing of the litter is entirely ceased, then natural weaning is not complete until 12-19 weeks post-partum (Stolba and Wood-Gush, 1989; Newberry and Wood-Gush, 1985; Jensen and Recén, 1989; Petersen, 1994).

2. Domestication

Pigs were domesticated nearly 9,000 years ago and presumably derive from the Eurasian wild boar (*Sus scrofa*) that is common in Europe, Asia, and North Africa (Clutton-Brock, 1999). Due to profound phenotypic differences between domestic pigs from those regions, Darwin recognized a possibility of distinct origins (Darwin, 1868), although this suggestion was only later confirmed by genetic studies showing clear differences between European and Asian domestic pigs (Watanabe et al., 1986; Okumura et al., 1996; Larson et al., 2005). Recently, by means of a more comprehensive molecular comparison using mitochondrial DNA and nuclear genes of wild and domestic pigs from Europe and Asia, the time of divergence of the two was estimated at around 500,000 years ago (Giuffra et al., 2000), much earlier than the time of domestication. It is therefore suggested that instead of importing domesticated pigs around the world, people from several distinct areas domesticated the existing wild animals themselves for meat production. Among others, the pig its ability to procreate in captivity and prolificy contributed to the domestication process and may explain at least in part why pigs, rather than more local meat sources that may have been more difficult to domesticate, were chosen for meat production in various distinct areas.

One can assume that at the time of early domestication, pigs were kept under fairly natural conditions, slaughtering young pigs when meat was needed, but further causing little disturbance to the animals. Over the course of domestication, however, animals with better genetically based behavioral

features for living in captivity were preferred (selected) and through selection, domestication has had profound effects on the anatomy (e.g. higher meat production) and physiology (e.g. diminished stress response) of the pig (Mormède, 1995; Price, 1997; Désautés et al. 2002; O'Regan and Kitchener, 2005). On one hand, selection has enabled farmers to increase production efficiency and meet the desires of the consumer. The development of genetic maps in livestock has allowed the detection of genomic regions that influence growth, body composition, meat quality, or reproduction (Bidanel et al., 2001; Malek et al., 2001a,b), behavior and stress neuroendocrine responses (Fuji et al., 1991; McGlone et al., 1998; Mormède et al., 2002). On the other hand, selection for high production efficiency may also lead to a number of undesirable effects (Breuer et al., 2005; for a review see Rauw et al., 1998). In pig production, highly lean pigs have been reported to demonstrate leg problems due to a decrease in leg strength (Webb et al., 1983; Sather, 1987) and also more excitable temperaments (during handling: Grandin, 1994; increased anxiety in a open field test: Shea-Moore, 1998; more aggressive: Busse and Shea-Moore, 1999). Thus far, little scientific evidence have shown the effects of lean growth lines on behavioral traits.

In spite of selection, the basic species-specific behavioral repertoire (e.g. nest-building) of domestic pigs resembles that of their ancestors (Stolba and Wood-Gush, 1984; Jensen, 1986; Špinka et al., 2000; Edwards, 2003). It seems that the behavioral differences between wild and domestic pigs are more quantitative rather than qualitative in character (Stolba and Wood-Gush, 1984;

Graves, 1984; Jensen, 1986), and can be better explained by differences in response threshold (Price, 1999). The reduced responsiveness (i.e. sensitivity) towards environmental changes has been pointed at as being the most prominent difference, resulting from adaptation of living in a biologically 'safe' environment (Price, 1999). In an environment that is characterized by limited opportunities for perceptual and locomotive stimulation, constant space intrusion by humans, and limited opportunity to avoid confrontation with dominants, pigs have adapted to be less reactive towards environmental changes (e.g. novel objects and humans) than wild animals.

In the past decades, however, living conditions for domestic pigs have changed drastically. Producers strive for higher production, economic benefits, and labor efficiency, and this has led to the development of intensive husbandry systems. These systems largely differ from the natural conditions in which wild pigs evolved and have been domesticated. While wild pigs live in the forest, living in small and stable social groups and spend most of their time foraging and eating, in commercial husbandry systems, domestic pigs are now housed in barren pens, with limited space allowance, and in highly unstable social groups, since they are frequently mixed with unfamiliar pigs. The barren environment offers little opportunity for exploration, and pigs spent most of the time lying inactive.

In sum, the conditions in which pigs live in 'nature' are characterized by a vast physical environment with abundant substrate for rooting and exploration. The animals live in fairly stable and heterogeneous social groups. During the first

days of life the young stay in close contact with the closest family members and are slowly introduced to the maternal group. This gradual social experience seems to contribute to the normal development of social behavior which may also involve social learning experience, perhaps preparing the individual for social events. Weaning is also a gradual process in which the individual slowly adapt to different sources of food and by increasing social interactions with other members of the group the separation from the mother occurs naturally.

3. Pigs living in intensive husbandry systems

Social grouping in husbandry systems occurs in different social (e.g. group size, composition, instability) and physical (e.g. restricted space, absence of substrate) conditions from what animals usually encounter in more natural environments. Although pigs are very social animals, high levels of aggression in social groups are often seen in modern farms. If living conditions are not offering the opportunity for the animals to develop appropriate social communication and behavior, this could interfere with group formation and stability.

The group structure in farm conditions, as in natural circumstances, is also based upon dominance hierarchy (Meese and Ewbank, 1973). However, due to the practice of regrouping (mixing) to obtain uniform cohorts, unfamiliar pigs that are brought together often engage in vigorous fight that will end once the dominance hierarchy is settled (Puppe and Tuchscherer, 1994). Different from more natural enclosures, from the first weeks of life, confined piglets only have social contact with littermates and sow. Furthermore, piglets are usually abruptly

mixed after weaning at 3-5 weeks post-partum, whereas under semi-natural conditions piglets meet non-littermates for the first time after about 2 weeks. Mixing of unfamiliar pigs disrupts group stability and increases aggression (Algers et al., 1990; Fraser et al., 1998).

Pigs may benefit from early social experience that reduces aggressive behavior (Weary et al., 1999). The first weeks after birth are crucial for developing social skills, as indicated by increases in social interaction, such as play and play-fight behavior (Petersen et al., 1989; Blackshaw et al., 1997). On the other hand, if piglets are hampered in social skills at this age, they show improper agonistic behavior at later ages (Schouten, 1986). The absence of gradual social experience with other litters seeing in husbandry systems may explain at least in part the high occurrence of aggressive behavior at regrouping.

One-week old pigs fight less during a second meeting, indicating that they use the information of experiences acquired from earlier interactions (Jensen, 1994). However, it was also suggested that they may be less motivated to continue fighting, because at this age the animal has only a very limited experience in defending resources (teat). The shorter fighting time in young pigs may therefore be a sign of a lesser interest in potential resources rather than better assessment capacity.

In order to reduce the incidence of fights and injuries caused by mixing, researchers have investigated the possibility of socializing litters prior to weaning. Several methods involving socializing and/or mixing piglets during this period have demonstranted positive results in minimizing aggression (Pitts et al., 2000;

Weary et al., 2002; D'Eath, 2005; Parratt et al., in press). A recent study by D'Eath (2005) revealed that young pigs that were socialized (mixed with unfamiliar litter) before weaning start fighting more quickly and form a stable social hierarchy more rapidly than the controls pigs (kept in littermate groups). However, contrary to what had been observed in free-ranging systems, socialization of litters in confinement does not seem to be playful in nature and can cause skin lesions primarily as a consequence of fights between unfamiliar pigs (Wattanakul et al., 1997).

In commercial farms, weaning is a multifactorial stressor for the piglets as it simultaneously involves abrupt separation from the sow, changes in diet, and transfer to a novel environment (Varley et al., 1985; Dybkjær, 1992; Weary and Fraser, 1997; Fraser et al., 1998). This is in contrast with (semi-)natural conditions where weaning is a gradual process occurring at a later age (e.g. Newberry and Wood-Gush, 1985). Aiming at enhancing productivity and producing high health status breeding stock, the North American swine industry has adopted practices in which the animals are weaned within a few days after birth (e.g. Medicated Early Weaning: at 5-6 days of age; Segregated Early Weaning: at 5-10 days of age). The latest report of the NAHMS (2001) revealed that in the USA piglets are weaned at 19.3 days-old on average, with 63.9% of the pigs being weaned between 16 and 20 days of age and 15% under 16 days-old. However, these figures vary according to the size of the production sites (for more detailed information see Table 1).

Studies have shown that weaning at early stages of life causes distress in pigs, with prolonged vocalization, restlessness, and long-term behavioral changes (Metz and Gonyou, 1990; Dybkjær, 1992; Hohenshell et al., 2000; Patience et al., 1997; Weary et al., 1999; Worobec et al., 1999; Orgeur et al., 2001), and enhanced cortisol concentration in plasma (Blecha et al., 1985, Mason et al., 2003) and in urine (Hay et al., 2001).

Table 1- Distribution of weaning age by size of site in pig production in the USA

	Size of Sites (Sow and Gilt inventory)					
	Small (<250)		Medium (250 – 499)		Large (≥ 500)	
Weaning Age (Days)	**%**	**SE**	**%**	**SE**	**%**	**SE**
< 16	2.3	1.0	8.8	2.4	25.5	4.6
16 – 20	11.2	1.7	65.3	4.0	67.0	4.4
21 – 27	30.1	2.7	20.7	3.3	6.3	1.3
28 – 34	22.3	2.4	3.3	1.0	0.6	0.3
≥ 35	34.1	2.9	1.9	0.8	0.6	0.4

Adapted from the National Animal Health Monitoring System of the USA (NAHMS, 2001).

A recent study conducted in our lab (Yuan et al., 2004) demonstrated that when they were mixed at 9 weeks of age, piglets that were weaned between 9 and 12 days of age (EW) fought longer and initiated more fights that they did not subsequently win than piglets that were weaned between 21 and 23 days of age (CW). The authors hypothesized that a possible impairment in social skills may be at the heart of these findings, but also proposed that the stress of mixing may have affected cognitive processes in EW pigs, since CW animals did not seem to

be affected. As deficits in discrimination of conspecifics may be a triggering point for initiating aggression (Ewbank and Meese, 1971), studies on discrimination and social recognition (SR) and the effects of stressors on it could improve our understanding of at least some of the underlying mechanisms of aggression in pig production. As mentioned previously, the first weeks after birth are crucial for developing social skills of a pig seen by an augment in social interactions (Petersen et al., 1989; Blackshaw et al., 1997), and if piglets are hampered in social skills at this age, they show inappropriate agonistic behavior at later ages (Schouten, 1986), sustaining Yuan et al.'s (2004) first hypothesis. Relatively little is known, however, about the influence of acute stressors on SR abilities of neonatal pigs, and therefore, the research in this thesis focused on the neonatal period. In commercial pig production, several standard management practices that neonatal pigs are subjected to such as castration and weaning, induce biological changes that are indicative of stress (Hay et al., 2001; Mason et al., 2003; Puppe et al., 2005).

4. HPA-axis

The hypothalamic-pituitary-adrenocortical (HPA) axis is assumed to play a critical role in adaptation and homeostasis in times of stress. The end products of the HPA axis activation are glucocorticoid (GC) hormones (e.g. corticosterone in rats, cortisol in humans and pigs). The activation of the HPA axis occurs via the neurosecretory neurons in the medial parvocellular zone of the paraventricular hypothalamic nucleus (PVN). These neurons synthesize and realease

corticotropin-releasing hormone (CRH) and neuropeptides (e.g. arginine vasopressin, AVP) into the pituitary. The corticotropes of the pituitary then releases adrenocorticotropic hormone (ACTH) into the bloodstream (Whitnall, 1993). This mechanism is crucial for adaptation (Dallman et al., 1992) of the individual with its environment, enabling a coping response. The adrenal gland responds to the increase in ACTH by synthesizing and secreting GCs. By binding to GC receptors within the brain, GCs also inhibit the further secretion of CRH from the hypothalamus and ACTH from the pituitary (negative feedback).

During the stress response, GCs perform various functions. Most importantly, they provide the body with the energy needed after the initial activation of the sympathetic nervous system in response to an acute stressor. GCs facilitate the release of glucose and fatty acids into the bloodstream (providing energy for the muscles) and contribute to trigger a redistribution of lymphocytes (preparing the immune system for a possible defense reaction) (Miller et al., 1994; Anderson et al., 1999). Furthermore, they inhibit systems not necessary for immediate survival (such as the reproductive and digestive system).

Acute stress responses facilitate the readjustment of behavioral and metabolic priorities and are usually adaptive, i.e. through a learning process they allow an individual to respond more adequately in future similar stressful situations (Moberg, 1985; McEwen, 2001). However, if the animals have problems in coping with constant and severe stressors (i.e. chronic stress) a sustained activation of the stress response systems will cause a variety of

behavioral and physiological problems, and increase the susceptibility to diseases (e.g. Wiepkema and Koolhaas, 1993).

GCs affect not only bodily functions, but also brain processes (for a review see de Kloet, 2000). GC receptors, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) can be found in various brain structures and their occupation by GCs affect brain functioning, such as learning and memory (Fenoglio et al., 2005). MRs have high affinity for GCs and are believed to modulate the circadian rhythm of GCs secretion, specially during trough. In contrast, the GRs have lower affinity for GCs and are primarily responsible for reactive negative feedback during circadian peak and following an acute stressor (de Kloet et al., 2004, for a review). The severity of the effects of GCs on the brain is dependent on the central concentrations and sensitive period of the individual.

5. Stress in early stages of life

Stress of both physical and social/psychological origin can have profound consequences for various neurophysiological, cognitive, and behavioral functions (e.g. Washburn and Rumbaugh, 1991; de Kloet, 2000; Blanchard et al., 2001). Stress sensitivity may change over the course of development of the individual, however, and it has been suggested that stress during early developmental periods may sensitize animals to the effects of stressors later in life (Graham et al., 1999; Parker et al., 2004; Yuan et al., 2004; Sandstrom and Hart, 2005). During early developmental periods, the brain experiences a "growth spurt" –

defined as the transient period of development when the brain is growing most rapidly. This period may occur prenatally and/or postnatally, depending on the species (guinea pig: Dickerson and Dobbing, 1967; rats: van den Hove et al., 2006; sheep: Richardson and Hebert, 1978; primates: Leigh, 2004). Like in humans, this developmental growth spurt extends from the late prenatal to early postnatal period in pigs (Dickerson and Dobbing, 1967; Dobbing, 1974; Dobbing and Sands, 1979).

During the first few weeks after birth, porcine brain maturation is characterized by the continued proliferation of neuronal cells and development of neural pathways (Dobbing, 1974; Brust et al., 2004) and during this period the brain is sensitive to alterations (Weaver et al., 2000). Interestingly, during this period, levels of circulating cortisol are usually low (Daniel et al., 1999; Carroll et al., 1998). It is therefore possible that during this period, increases in circulating cortisol may negatively affect porcine brain development. There is growing evidence that stress during this sensitive period may result in long-term alteration of emotional reactivity (Weiss et al., 2004), behavioral responses (Schouten, 1986; de Jonge et al., 1996), immune response (Tuchscherer et al., 2004; Kanitz et al., 2004), in addition to dysregulation of the HPA axis (Weaver et al., 2000), but studies examining the effects on brain development and functioning in pigs are limited (Tuchscherer et al., 2004; Poletto et al., 2005; Schwerin et al., 2005). Thus, this thesis is based on the hypothesis that, aside from possible deficits in social competence in EW pigs, the increase in post-mixing aggression reported

by Yuan et al. (2004) is caused by impairments in SR due to an increased susceptibility to the effects of acute stressors.

6. Social recognition

The ability of animals to identify the nature of conspecifics increases their chances of survival. Animals can use this information for their own benefit and develop context appropriate behavior minimizing social conflicts, optimizing energy use through cooperation, defending against predators, and protecting resources. Animals that chose to live in small groups, the formation and maintenance of social relationships is an indication that they are able to recognize other individuals.

Sows and litters can benefit from mutual recognition. From a sow's perspective, it ensures investment of the resources towards the proper offspring. Sows seem to be able to recognize their piglets by using whole body odors within a day post-partum (Horrell and Hodgson, 1992a) or using only vocalizations (Illmann et al., 2002), and usually react aggressively towards alien pigs (Wattanakul et al., 1997). As piglets can easily be separated from the sow when she is foraging (Gundlach, 1968), auditory recognition (e.g. Maletinska and Špinka, 2001) may help the sow to attend to distressing calls (i.e. presence of predators, starvation, hypothermia) from her litter. From a litter's perspective, recognition enables them to approach the sow when in search of milk and protection. Piglets are able to distinguish their own mother and also identify several odor cues and/or auditory stimuli derived from her (Horrell and Hodgson,

1992b). In systems where sows and litters are kept in communal areas it is probably advantageous for the piglets to discriminate their dam and stay close, since other sows could recognize them as aliens and attack during attempts to suckle (Newberry and Wood-Gush, 1985).

7. Social recognition terminology and assessment

Social discrimination is the individual's perception of a difference between conspecifics. This is a pre-requisite for SR as it allows individuals to establish mental representations of social categories. SR however, is the individual's perception that the presented conspecific belongs to a known class of individuals (e.g. species, familiarity, kinship, social hierarchy, sex, and age), whereas individual recognition is the ability to identify others as being a particular and unique individual (for reviews see Gheusi et al., 1994; Zayan and Vauchair, 1998). In animal behavioral studies, the term SR memory has been widely used to indicate changes in behavioral responses towards a previously encountered stimulus (e.g. Thor and Holloway, 1982).

Tests to assess SR ability are based on the animal's natural tendency to intensely investigate novel conspecifics. Most of the protocols to study SR are primarily designed for rodents. Rodents are highly social animals (Barnett, 1958; Lore and Flannelly, 1977) and usually engage in spontaneous investigatory behavior as a way of learning the nature of conspecifics or their signatures (e.g. urine). In the laboratory, it is possible to make use of spontaneous (e.g. in preference tests, habituation-dishabituation test) or learned behavioral responses

(operant conditioning systems). Tests involving spontaneous responses measure the response of the subject to a social stimulus, whereas tests involving learned responses involve the association of a stimulus and a proper response using rewards to motivate the subjects. The subjects can be tested either in its home environment or not (e.g. in an apparatus). As in the present dissertation SR was assessed using spontaneous response, learned behavioral methods will not be further mentioned.

The advantage of using spontaneous behavior is that it does not require high motivation of the subject nor numerous trials for learning, which could be ideal for assessing SR in very young animals. The tests based on the assumption that the subject in study has innate interest or is motivated enough to develop a specific response in the presence of the stimulus or stimuli. In addition, it better resembles what occurs in more natural conditions. The response of the subject towards a social stimulus is usually assessed by measuring latency of approaches towards the stimulus, duration or frequency of social investigation, aggressive behavior, and others. Different responses towards different stimuli indicate that social discrimination has occurred. In preference tests, no learning is required and social discrimination is demonstrated by comparing exploratory behavior of the subject two or more stimuli (pigs: Kristensen et al., 2001; lambs: Ligout and Porter, 2003; hens: Dawkins and Woodington, 1997; rats: Levy et al., 2003). The main disadvantage of using preference tests is that negative results cannot be interpreted as inability to discriminate. It is possible that although the subject perceives the difference between stimuli it does not repond differently, for

example, because the subject has different motivation (aggression towards strangers and curiosity torwards group-members). As an example, Hauser and Huber-Eicher (2004) demonstrated that, in hens, variations in testing experience can cause negative results in a preference test.

SR can also be assessed by using the habituation-dishabituation test. Habituation is a form of learning that is defined as a progressive decrease in responsiveness resulting from the repeated presentation of a particular stimulus (Thompson and Spencer, 1966). It enables individuals to behave efficiently (Hinde, 1970), once an animal becomes acquainted with a stimulus, less investigation is needed. The research investigating the nature of SR using habituation was originally described by Thor and Holloway who proposed a social memory test using mature male laboratory rats as a model (Thor and Holloway, 1982). Juvenile male rats were used as stimuli to eliminate possible confounding effects of aggression and sexual behavior during the testing exposures. The test consisted of repeatedly presenting adult rats to the same juvenile resulting in a decrease of social investigation, which is a reliable index of SR. The habituation is considered to reflect the presence of a memory for the presented individual. However, this decrease in social investigation after repeated exposures to the same stimulus could also be interpreted as sensory adaptation or social fatigue, which lead to another experiment where the subject was habituated to a stimulus and subsequently exposed to either the same or to a novel stimulus. The results demonstrated an increase in social investigation only towards the different

stimulus at levels comparable to the first encounter (Thor and Holloway, 1982), indicating SR rather social fatigue.

A combination of habituation and dishabituation procedures in one single experiment was later presented (Dluzen and Kreutzberg, 1993; Winslow and Camacho, 1995). Further adaptation of the habituation-dishabituation paradigm is presented by Engelmann et al., (1995) in which during the testing phase the previously exposed individual and a novel individual are presented simultaneously. SR is assessed by comparing the differences between the time spent investigating familiar and unfamiliar conspecifics. This adaptation of the original SR test can be completed in two trials only and is suitable for testing sexually mature males as well as females.

The habituation-dishabituation procedure and its variations have been described as being easy to implement and offer the opportunity to test a large number of animals in a short-period of time (Gheusi et al., 1994; Ferguson et al., 2002), ideal for pharmacological studies. Winslow and Camacho found that ovariectomized females can be used as an alternative for stimuli and can be repeatedly used over months (Winslow and Camacho, 1995). Studies have reported SR in individually housed rodents to last no longer than 2 hours (Thor and Holloway, 1982; Bluthe and Dantzer, 1990; Ferguson et al., 2001). Recently, Kogan and colleagues demonstrated that individually housed mice were able to only form short-term SR (with delay of 30 minutes), whereas group housed mice formed a more robust memory that lasts for at least 7 days (Kogan et al., 2000). The later study demonstrates that in rodents the habituation-dishabituation

procedure can be used in short- and long-term SR memory studies, however, the housing condition should be taken into account in the experimental design, at least in rodents.

8. Aim and outline of the dissertation

SR is essential for a broad range of social behaviors and is an element of learning and memory that, in many species, is sensitive of interference by stressors. It has been suggested that failure to remember a previously encountered individual can increase aggression in social groups. This PhD project aims at gaining insight in the modulating effects of acute stressors on SR of neonatal pigs. For that purpose, the objective of the research was three-fold. Firstly, to develop a protocol to assess SR that is suitable for using in non-weaned piglets. Secondly, to examine whether the protocol designed could be used to assess short- and long-term SR memory. Thirdly, to investigate whether acute stressors interfere with SR ability of neonatal pigs.

Chapter 2 describes a study in which the 'habituation-dishabituation' paradigm is used to test SR in 11 day-old pigs. This protocol has been widely used for assessing SR in weaned animals; therefore, an adaptation for use in non-weaned pigs was necessary. This study also investigates whether acute increases of cortisol impair short-term SR. Constraints associated with the protocol such as distress of handling and fighting between animals lead to the subsequent experiment.

Chapter 3 presents a different method to assess SR abilities in 12 day-old pigs primarily aimed at minimizing problems associated with the previous protocol. This method proved to be suited for use in non-weaned pigs and will the used in the subsequent studies.

Chapter 4 reports an experiment in which short- and long-term SR memory of 21-day-old pigs were assessed. For short-term memory the animals were tested at 4h after the last familiarization procedure whereas for the long-term memory the animals were tested 24 hours after familiarization.

Chapter 5 describes a study designed to investigate whether early weaned (11 days of age) pigs are more susceptible than conventionally weaned (21 days of age) animals to SR disturbance by an acute stressor (15 minutes of social isolation). This information is fundamental in the context of the body of evidence produced in our lab showing that these animals seem be more susceptible to acute stressors.

Chapter 6 reports a summary of the major findings of chapters 2-5 and further discusses the main findings. It subsequent presents the final conclusions and proposes future studies.

CHAPTER 2

THE FIRST EXAMINATION OF THE ROLE OF CORTISOL IN SOCIAL RECOGNITION IN NEONATAL PIGS

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Submitted

Abstract

The ability of pigs to acquire and retrieve information regarding previously encountered conspecifics may be impaired if circulating cortisol levels are acutely elevated. The main objective of this experiment was to investigate whether acute increases of cortisol impair short-term social recognition (SR) in 11-day-old female piglets. The habituation-dishabituation procedure, originally designed to assess SR in adult rodents, was adapted for use in neonatal pigs. Twenty-four female piglets were randomly assigned to an intramuscular injection of 1 mg/kg BW of hydrocortisone sodium succinate (SOLU-CORTEF) or vehicle (saline). Salivary samples were collected 15 minutes before injection (baseline) and at 15, 35, and 55 minutes after the treatment. Fifteen minutes after injection, the female subjects were exposed to a male piglet (stimulus) for four one-minute periods separated by 10-minute intervals (habituation phase), after which a different stimulus male was presented (dishabituation phase). The results show that female piglets decreased the amount of time spent investigating a repeatedly presented male piglet (familiar) and increased investigation when exposed to a novel male in the subsequent exposure (unfamiliar). Salivary cortisol levels in 11-day-old piglets significantly increased after the cortisol injection. There was no evidence that the cortisol treatment affected their ability to recognize a familiar individual. The habituation-dishabituation method for assessing SR can be further used in neonatal pigs.

1. Introduction

Glucocorticoids (GCs), mainly corticosterone in rodents and cortisol in other mammals, are released in response to stressful situations. GCs are lipophilic and can freely cross the blood-brain barrier to act directly on the brain (Dallman, 1993; Wolf, 2003), affecting areas involved in learning and memory (for review, see Lupien and Lepage, 2001).

The modulating effects of circulating GCs on learning and memory depend on a multitude of factors, including concentration in the body (Pavlides et al., 1993), length of the exposure (acute or chronic, e.g. Shors, 2001; Fuchs et al., 2001), moment of the exposure (i.e. during memory acquisition, consolidation, or retrieval, e.g. de Quervain et al., 2000; Roozendaal, 2002, 2003; Elzinga et al., 2005), time of the day (i.e. levels of basal GCs according to circadian rhythm, Lupien et al., 2002ab), sex of the animal (Szuran et al., 2000; Bowman et al., 2004; Shors et al., 2004), and age of the individual (Frisone et al., 2002; Driscoll et al., 2005).

Circulating GCs affect cognition by binding with two GC receptor subtypes that are present throughout the brain, but are specifically abundant in the hippocampus, an area that plays an important role in learning and memory (for reviews, see McEwen and Sapolsky, 1995; de Kloet, 2000). The mineralocorticoid receptor (MR or Type I) has a 10-fold higher affinity for GCs than the glucocorticoid receptor (GR or Type II) and thus at low GC concentrations primarily MRs are activated. In contrast, high levels of endogenous or exogenous GCs saturate MRs and activate the GRs in the

hippocampus (Reul and de Kloet, 1985). MR activation in the hippocampus enhances memory formation (Sandi and Rose, 1994, 1997) while GR activation hampers memory formation (Wolkowitz et al., 1990). In line with these findings, Pfaff and collaborators demonstrated that an acute exposure to high concentrations of GCs (corticosterone injection of 1 mg/kg BW) decreases hippocampal activity with a delay of approximately 30 minutes (Pfaff et al., 1971), possibly caused by the activation of GRs (Joëls, 2001).

The hippocampus has been shown to play a role in social recognition (SR) (Terranova et al., 1994; Maaswinkel et al., 1996; Kogan et al., 2000). In the present study, SR is used to refer to the ability to discriminate a previously encountered individual from a novel individual conspecific. Most of the procedures to assess SR rely on spontaneous behavioral responses; the natural tendency of animals (e.g. rodents, pigs) to intensely investigate novel conspecifics. Therefore, decreases in the duration of social investigation after successive exposures to the same individual can be used as an indication of SR ability (Thor and Holloway, 1982).

The most common technique to test the ability of animals to recognize (remember) pre-exposed (familiar) individuals is the habituation-dishabituation, in which an animal is presented with the same conspecific over multiple exposures and then presented with a novel stimulus conspecific (Dluzen and Kreutzberg, 1993; Winslow and Camacho, 1995). The decline in time spent on social investigation between exposures from the first stimulus is inferred to reflect habituation, indicating SR of the presented individual. After a novel and

unfamiliar stimulus animal is presented, dishabituation, indicated by an increase in social investigation, should occur, serving to rule out the possibility of habituation being a product of a generalized social fatigue rather than SR. The habituation-dishabituation procedure has been described as being easy to implement and offers the opportunity to test a wide range of animals in a short-period of time (for a review, see Gheusi et al., 1994).

It has been demonstrated that pigs are capable of recognizing conspecifics (Kristensen et al., 2001; McLeman et al., 2005; Souza et al., 2006). Despite the vast literature investigating the impact of hormones released in response to an acute stressor on learning and memory processes, little is known about the effects of short-term increases of exogenous cortisol on SR of animals. We have shown that SR in early-weaned pigs can be disrupted by an acute social stressor (Souza and Zanella, submitted), but the role of GCs in this disruption is still unclear. In the present study our goal therefore was to assess the role of an acute increase in circulating cortisol levels on short-term SR of 11-day-old non-weaned female piglets.

For that reason, we adapted the habituation-dishabituation SR paradigm for use in non-weaned piglets. Based on preliminary studies (Zanella, unpublished), we anticipated that a single injection of 1 mg/kg BW of hydrocortisone (cortisol) sodium succinate prior to testing would significantly increase salivary cortisol concentrations throughout the test time-period and that SR would be impaired at these concentrations.

2. Material and methods

The study was carried out at the Swine Teaching and Research Center (STRC) of Michigan State University (MSU), USA. All procedures used in this study were reviewed and approved by the All-University Committee for Animal Use and Care of MSU.

2.1 Animals and housing

Six unweaned litters (Yorkshire x Duroc) were used in this experiment. Within 36 hours post farrowing, litters were standardized to 10 piglets by cross-fostering (balanced for sex) and all animals were weighed following the standard operating procedures of the STRC. Sows and litters were housed in standard commercial farrowing crates (1.8 x 2.3 m) throughout the experiment.

The testing arena (91.4 x 71.1 x 60.9 cm) consisted of a metal bar crate with two doors. The doors allowed two neighboring litters to access the arena at the same time. A solid plastic barrier dividing the arena in two halves prevented mixing of animals from neighboring litters (Figure 1).

Water was available ad libitum and sows were fed standard commercial feed twice a day. Environmental temperature was controlled (20oC ± 1) and artificial lights were provided from 08:00 to 17:00 throughout the experiment.

2.2 Acclimatization: apparatus and experimental protocol

All litters were acclimatized to the testing arena and experimental protocol. From day 4 until day 11 after birth, the doors to access the arena were

continuously opened allowing all litters to freely explore the testing arena. A solid plastic barrier divided the arena in half, allowing two litters to be acclimatized at the same time.

The piglets were tested at 11 days of age. In the four days prior to testing (7-10 days of age), all piglets were acclimatized to the testing procedure by holding each piglet for 10 seconds and subsequently placing them individually in the arena for four 1-minute periods with 10-minute intervals. While the piglets showed signs of distress (intense vocalization and attempts to escape) during the first exposure to the arena, they were noticeably calmer on the last day, indicating acclimatization to the experimental procedure.

2.3 Cortisol injection

A total of 24 female piglets (subjects) were selected (4/litter) and within each litter, two piglets were randomly assigned to either vehicle (0.2 ml saline, n = 12) or cortisol (hydrocortisone sodium succinate (SOLU-CORTEF; the Upjohn Co., Kalamazoo, Mich.), 1 mg/kg BW diluted in 0.2 ml of saline, n = 12) treatment. All subjects were given an intramuscular injection behind the right ear in the side of the neck 15 minutes prior to the SR test. After injection the subjects returned to their farrowing crate and stayed with the sow and littermates until testing. All animals were injected between 13:00 and 16:00 hours.

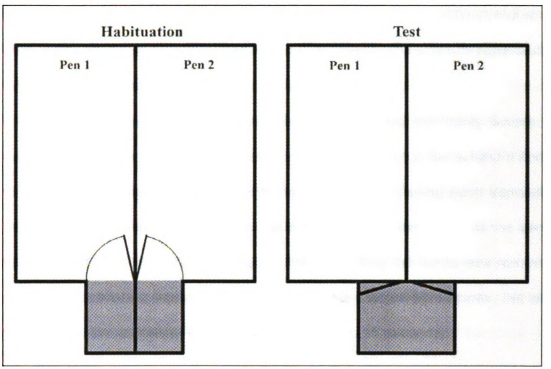

Figure 1- Design of the farrowing environment (white, Pen 1 and Pen 2) and the arenas (grey). During acclimatization of the piglets to the arena, a solid barrier dividing the space in two halves prevented mixing of litters and the door-ways were open so the animals could freely access the arena. During the habituation-dishabituation test the doors to access the arena were closed.

2.4 Social recognition testing

The SR test was conducted in two phases. In phase 1 (habituation), the subject was exposed to an unfamiliar castrated male (stimulus) for four successive sessions of 60 seconds, each separated by a 10-minute interval, during which both animals were placed back in their farrowing crate. In phase 2 (dishabituation), the subject was exposed to a different stimulus male for 60

seconds. The selected stimulus animals were unfamiliar to the subject and were 20% lighter to minimize possible agonistic interactions. The subjects were tested only once, at 11 days of age.

During testing, the doors by which the litters could voluntarily access or exit the arena were closed. The test was always conducted in the subject's home environment (the arena attached to its farrowing crate). During each exposure, subject and stimulus were placed on opposite sides of the arena at the same time. After 30 seconds, the solid plastic barrier dividing the arena was removed so the animals could freely interact. Each exposure lasted 60 seconds, but was terminated if the animals were fighting for more than 15 seconds.

2.5 Behavioral observations

Behavior during the test was videotaped and subsequently observed using "The Observer" behavioral recording software (Noldus Information Technology, Wageningen, The Netherlands). Observations were done by an observer unaware of the treatments. We measured the percentage of time that the subject spent investigating the stimulus, defined as sniffing or touching (with the nose or mouth) any part of the stimulus its head and/or body, or following within approximately 10 cm.

2.6 Saliva sampling and cortisol analysis

Saliva was collected from the female subjects 15 minutes before injection (baseline) and 15, 35, and 55 minutes after injection. For saliva collection, piglets

were allowed to chew on a cotton bud tied with dental floss. When thoroughly moist, the samples were stored in a tube and kept on ice until centrifugation (5 minutes, 3,000 x G, 4°C). After centrifugation, the saliva was transferred to 1 ml eppendorf containers and frozen at –20°C until further analysis.

Salivary cortisol concentrations were determined using the ACTIVE Cortisol Enzyme Immunoassay (EIA) kit (Diagnostic Systems Laboratories, Inc.; DSL-10-67100) modified for piglet salivary cortisol. According to the manufacturer, cross reactivity of the assay for other components (cortisone, 11-deoxycortisol, 21-deoxycortisol, 17α-hydroxycortisol) was 7.0, 5.7, 1.9, and 0.9% respectively. Assay sensitivity was 0.072 µg/dL to a maximum concentration of 10µg/dL. Intra- and inter-assay coefficients of variance were 5.75% and 1.46%, respectively.

2.7 Statistical analysis

The distribution of all variables was tested for normality and homogeneity of variance, and appropriately transformed if not normally distributed. Subsequently, the cortisol data was log-transformed. Data were analyzed using SAS® 9.1 mixed model (PROC MIXED) software (SAS Institute inc., Cary, NC, 2003). The linear mixed model (LMM) included the effects of the treatments (cortisol, saline), times for saliva collection (-T15, T15, T35, T55) or habituation (habituation, dishabituation) and their interaction. Furthermore, random litter effects were specified as well as serial correlation between subsequent measurements within piglet over time. Hence, a repeated measures analysis was

used to analyze effects of treatment on salivary cortisol levels and effects of cortisol treatment on the amount of social investigation, correcting for repetition effects of subject and litter. Key comparisons in the analysis for the social investigation data were differences between the first and fourth exposures (habituation phase) and between first and fifth exposures (habituation and dishabituation).

A total of 30 salivary cortisol samples were omitted from the data set when the subject had fought in a previous exposure or when cortisol levels were higher than 10 µg/dl. All data are presented as means ± S.E.M. Differences between the experimental groups were considered to be significant at a Type I error rate of 5%.

3. Results

3.1 Salivary cortisol

Salivary cortisol concentrations before and after cortisol or saline injection are presented in Figure 2a. Salivary cortisol levels were affected by treatment and time (LMM, $F_{3,37.1} = 2.45$, $P = 0.078$). Post-hoc analysis revealed that baseline salivary cortisol concentrations did not significantly differ between treatment groups ($P = 0.90$). An increase in cortisol levels was observed only after cortisol injection (LMM, $F_{1,18} = 5.64$, $P < 0.05$) and it changed over time (LMM, $F_{3,37.1} = 5.38$, $P < 0.01$). In the cortisol treated animals, salivary concentrations increased over three-fold to a peak level 15 minutes after injection ($P < 0.001$). Cortisol levels were still higher 35 minutes after injection compared

to the baseline levels in subjects injected with cortisol ($P < 0.001$) validating the efficacy of the pharmacological treatment. At 55 minutes after injection of cortisol, salivary concentrations declined but were still higher than baseline values ($P = 0.073$). Contrary to cortisol treatment, salivary cortisol levels in piglets injected with saline did not significantly alter after 15, 35, and 55 minutes after injection ($P = 0.446$, $P = 0.152$, and $P = 0.106$, respectively)

3.2 Investigative behavior

Social investigative behavior of subjects is presented in Figure 2b. Repeated encounters to the same conspecific decreased social investigation in cortisol and saline treatment groups across the four habituation sessions (LMM, $F_{4,60.2} = 20.73$, $P < 0.0001$). The subjects spent more time investigating the stimulus pigs during the first than the second, third, and fourth habituation exposures ($P < 0.05$, $P < 0.0001$, $P < 0.0001$, respectively). To rule out the possibility that the above habituation was due to generalized social fatigue, the last habituation (4^{th}) exposure was compared with the dishabituation (unfamiliar stimulus pig). Social investigation of the unfamiliar stimuli pigs was greater than of the familiar one (DF = 58.2; $t = -8.04$; $P = 0.0001$). The amount of social investigation during the dishabituation phase was comparable to that during the first habituation subject (DF = 60.4; $t = -0.87$; $P = 0.389$). No effect of cortisol administration was found on the amount of social investigation of the stimulus pig during the habituation-dishabituation test (LMM, $F_{1,8.52} = 0.04$, $P = 0.849$) or interaction between treatment and exposures (LMM, $F_{4,60.2} = 0.55$, $P = 0.698$).

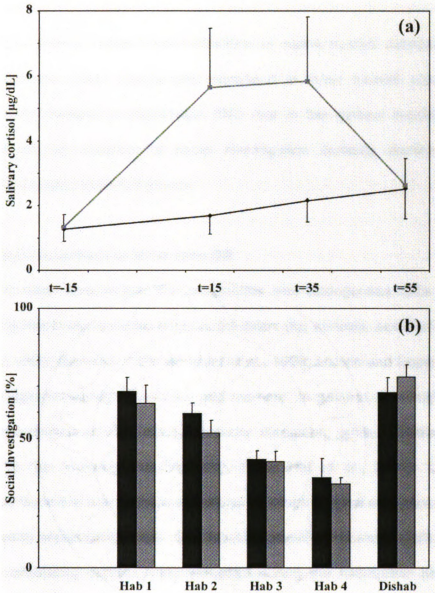

Figure 2- Salivary cortisol concentration (a) and amounts of social investigative behavior (b) by treatment during the SR test in 11-day-old female pigs. The grey line and bars indicate cortisol treatment and the black line and bars indicate vehicle (saline). Hydrocortisone and saline were injected 15 minutes before SR test started. Data are presented as ± S.E.M. For significant differences see results.

4. Discussion

While salivary cortisol concentrations of saline treated subjects did not significantly alter, they considerably increased in those treated with cortisol. However, no changes in habituation (SR) due to the cortisol treatment were observed, as the duration of social investigation similarly declined during habituation in both treatment groups.

4.1 The role of cortisol in short-term SR

It is well documented that exogenous and endogenous GCs not only circulate in the body, but also enter in the brain (for reviews, see McEwen and Sapolsky, 1995; Bremner 1999; de Kloet et al., 1999; Lupien and Lepage, 2001) acting in regions related to learning and memory. In general, very high or very low concentrations of GCs cause memory disruption, while moderate levels enhance it (for reviews, see Sapolsky, 1999; Het et al., 2005). Our initial hypothesis therefore was that administration of a high dose of exogenous cortisol would lead to impairments in SR. Our results, however, indicate that although the levels of circulating cortisol were increased during the habituation phase, the ability of the subjects to acquire and recall information concerning previously presented conspecifics was not significantly affected by the cortisol treatment.

Animal and human studies have reported conflicting results on the effects of GC administration on learning and memory (for reviews, see Mendl, 1999; Het et al., 2005). According to Mendl, methodological variations may underlie the contradictory findings. It is likely that different routes of administration may

produce a different outcome and that pharmacological doses might not be biologically relevant. In line with this suggestion, a noteworthy finding by Krugers et al. (1997) suggests that while prolonged psychosocial stress caused spatial learning deficits, artificial elevation of corticosterone to comparable levels did not. It may therefore be that cortisol or corticosterone is not the only or even the major mechanism by which stressors affect cognitive processes. During the stress response, other hormones, such as catecholamines, are released at the same time. There is evidence suggesting that these may interact with GCs in affecting cognition (Roozendaal, 2000). Additionally, artificial manipulations of single hormones are likely to result in feedback and cascade effects on other hormones, thus questioning the precise mechanism of their interference with cognitive processes (Mendl, 1999).

A review by Het et al., (2005), states that difference in methodology may account for some of the conflicting results in studies on the effects of GCs on cognition. In studies where cortisol was administered before learning, detrimental effects on cognition were only found in those studies conducted in the morning (high circulating cortisol), whereas those conducted in the afternoon (low circulating cortisol) found enhancement or no effect (see also Maheu et al., 2005). In our study, the subjects received a hydrocortisone injection in the afternoon, which in that line may have obscured any potential effect of GCs on SR. However, the effects of time of the day on cognition are likely caused by the occurrence of a circadian rhythm in the levels of circulating cortisol. In humans, these levels are higher in the morning (see Lupien et al., 2005 for a review),

leading to full occupation of MRs, and lower levels in the afternoon, resulting in a lower occupation of MRs (de Kloet et al., 1998). This creates a 'buffer' of unoccupied MRs to protect the brain from the detrimental effect of GR occupation. Although it is possible that in our experiment this may have accounted for our results, it is unlikely. In young piglets, as in human babies, a circadian rhythm does not seem to be present (humans: de Weerth et al., 2003; piglets: Evans et al., 1988; Kattesh et al., 1990; Klemcke and Pond, 1991; Ekkel et al., 1996) and thus the time of injection should not have affected short-term SR.

Unfortunately, we cannot determine with certainty whether the ineffectiveness of exogenous GCs to affect cognition was due to methodological constraints (e.g. injection effect, hydrocortisone dose) or whether exogenous GCs do not alter short-term SR, as measured using the habituation-dishabituation paradigm. Based on the results by Krugers et al. (1997), we propose that exogenous GCs are not as potent as real-life stressors in affecting SR in piglets, but stress the need for further research. For a better understanding of the role of exogenous cortisol on short-term SR further studies on the dose response curve are needed.

4.2 Habituation-dishabituation protocol

In the present study we have adapted a habituation-dishabituation procedure previously used in adult mice (Dluzen and Kreutzberg, 1993; Winslow and Camacho, 1995) to study the effects of GCs on SR in non-weaned piglets.

The protocol relies on the natural tendency of pigs to investigate novel and unfamiliar conspecifics rather than familiar ones. As we anticipated, during habituation, female 11-day-old pigs showed a decrease in social investigation towards the same male stimulus pig, indicating recognition of the stimulus pig. To rule out the possibility that this decrease was a result of social fatigue or exhaustion instead of recognition of an encountered conspecific, we exposed the subjects to a last exposure to a novel stimulus male. The presence of the novel stimulus resulted in vigorous investigation, ruling out social exhaustion. The habituation-dishabituation paradigm can therefore be used to test SR ability in neonatal pigs.

In our study, we aimed to assess the effects of an acute exposure of cortisol followed by increases in it on SR and thus needed to minimize stress caused by the testing procedure itself. If testing conditions are stressful to the subject, it may cause deficits in learning and memory functions. Indeed, Burman and Mendl (2000) demonstrated that handling and moving rats to a different environment may hamper SR, and it has been suggested that cognition in pigs is also susceptible to disruption by acute stressors (spatial memory in pigs: Mendl et al., 1997; social memory in weaned pigs: Souza and Zanella, submitted). Therefore the subjects of the present study were tested in their home environment and all animals were habituated to handling.

Interestingly, our results show that although the subject interacted with the same stimulus pig for four trials, social investigation already significantly decreased after the first exposure, indicating that a single 60-second exposure

was sufficient to acquire information concerning the identity of the stimulus. This may be an important finding for future studies testing the effects of drugs with a short half-life or studies requiring a minimum of handling (such as studies on the effects of stressor in SR), since the number of learning trials can be reduced to one.

5. Conclusions

In this experiment, a hydrocortisone injection and the resulting increase in cortisol levels did not interfere with the ability of 11-day-old female piglets to acquire and retrieve information about a male conspecific after repeated exposures. It indicates that the injection of 1 mg/kgBW of cortisol does not induce impairments in acquisition and/or immediate recall of SR. However, future studies are needed to clarify the role of cortisol on acquisition, consolidation, and retrieval. This experiment does indicate that the habituation-dishabituation paradigm can be adapted to investigate SR in non-weaned piglets. This paradigm can be further used in very young pigs to address the effects of stressors on SR.

Acknowledgments

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CHAPTER 3

A NOVEL METHOD FOR TESTING SOCIAL RECOGNITION IN YOUNG PIGS AND THE MODULATING EFFECTS OF RELOCATION

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Abstract

Mixing litters of pigs often leads to short- or long-lasting aggression. As social recognition (SR) is important for the development and maintenance of stable social groups, a lack or disruption of SR could prolong agonistic interactions and reduce welfare. Investigations of SR abilities are therefore of considerable importance. However, experimental assessment of SR generally involves moving subjects to unfamiliar environments, or excessive handling, or aggression during the familiarisation period (in which subjects become acquainted prior to testing their ability to recognise each other), which in turn may change the outcome of the test. In this study, we tested a novel method for assessing SR in young pigs aiming to minimise these problems. For the familiarisation procedure, flexible netting was placed between farrowing pens allowing physical contact between litters but preventing unwanted mixing and fighting. We also investigated whether recognition of a familiar individual is affected by relocation to a novel environment. Forty-eight piglets from 12 litters (4 piglets/litter) were tested in a split plot design with litters as the experimental units for comparing two familiarity (familiar/unfamiliar) and three relocation (no relocation/relocation to pens with the same spatial orientation/relocation to pens with a different spatial orientation) treatments. Results indicated that piglets exposed to unfamiliar animals spent more time on social investigation than those exposed to familiar ones, suggesting that SR can be successfully tested using this novel approach. The relocation treatment did not influence the amount of time spent on social investigation, but did affect duration on exploration of the

arena. Piglets meeting familiar individuals were more susceptible to this effect after relocation than piglets meeting unfamiliar conspecifics.

1. Introduction

Recognising familiar conspecifics enables pigs to form and maintain stable social groups, thereby reducing social stress. In free-ranging systems, pigs tend to form subgroups and maintain closer social bonds to certain individuals than others, resulting in pigs sleeping in separate nests (Stolba and Wood-Gush, 1984). After forced mixing of different groups, the original bonds persist and are distinguishable for over 190 days (Stolba and Wood-Gush, 1984). The low level of aggression observed in free-ranging systems can be accounted for by the availability of space and familiarity between animals (Jensen and Wood-Gush, 1984). In general, it is well documented that pigs usually react more aggressively towards unfamiliar than familiar conspecifics (Arey and Franklin, 1995; Puppe, 1998; Turner et al., 2001). In intensive husbandry systems, social problems may arise from disrupted bonding and restricted possibilities for group formation. Fighting between pigs in stable social groups is rare, in contrast with groups of unfamiliar pigs, which show a significantly higher incidence of aggression (growing pigs: Ewbank and Meese, 1971, gilts: Spoolder et al., 1996, sows: Arey, 1999).

Many studies have investigated factors that affect and control aggression among pigs such as familiarity and relatedness (Puppe, 1998; Stookey and Gonyou, 1998), individual variation in aggressiveness (Erhard et al., 1997;

Bolhuis et al., 2005), visual cues (Ewbank et al., 1974; Friend et al., 1983), body weight (Andersen et al., 2000), age (Jensen, 1994; Pitts et al., 2000), group size (Turner et al., 2001; Morrison et al., 2003; Schmolke et al., 2004), pre-exposure (Jensen and Yngvesson, 1998; D'Eath, 2005), familiarity with the environment (Wattanakul et al., 1998), enrichment (Morgan et al., 1998) and space allowance (Wiegand et al., 1994; Turner et al., 2000), but while a potential role for social recognition (SR) in moderating the levels of aggression observed has been suggested (Yuan et al., 2004), it has not yet been thoroughly addressed.

Under commercial pig farming conditions, common husbandry practices such as mixing of animals or even reintroduction of a previously familiar animal contribute to the instability of social groups, leading to an increase in aggression that may be partly explained by disruptions in SR memory (Puppe, 1998; Yuan et al., 2004), particularly when the reintroduced animal is of lower rank in the social hierarchy (Ewbank and Meese, 1971). If social memory is essential for distinguishing familiar from unfamiliar individuals and thus for the maintenance of stable social groups, then memory interference or impairments could increase aggression leading to welfare problems (Mendl et al., 2001). However, there have been limited studies on the role of social recognition memory – used here to refer to the ability to discriminate a previously encountered individual from a novel individual – in moderating aggression between pigs, and the effects of changes in the environment on SR. Information on the effects of relocation on SR is important as pigs are often moved to novel crates when weaned and mixed (i.e. Graves et al., 1978).

A common method for assessing SR memory in rodents involves the habituation - dishabituation technique, which consists of repeatedly exposing the same subject to a stimulus animal (Thor and Holloway, 1982) and then, in a test phase, exposing the same subject to either the same animal, or an unfamiliar animal (Dluzen and Kreutzberg, 1993; Winslow and Camacho, 1995). Social recognition is usually measured by a decrease (habituation) in social investigation during successive exposures to the same conspecific, but an increase (dishabituation) in social investigation when the unfamiliar animal is presented. However, behavioural responses of subjects during SR tests are not always easy to interpret. Agonistic interactions between subject and stimulus animal can occur during the habituation period (e.g. hamsters: Johnston and Jernigan, 1994, pigs: Souza and Zanella, in preparation), which in turn can influence behaviour in subsequent encounters, interfering with the outcome of the test. Also, considerable handling and moving of animals between different environments during the repeated exposures of the habituation period can distress and change the experience of individual animals. It has been demonstrated that when rats are handled or placed in a different cage, these procedures may cause interference with social memory (Burman and Mendl, 2000) which may be dependent in part on contextual cues from the environment (Burman and Mendl, 1999).

In the current experiment, our main goal was therefore to test a novel protocol for assessing SR memory in young piglets that minimised handling and reduced the occurrence of aggression during familiarisation (habituation). The

familiarisation occurred in arenas situated between neighbouring crates that were separated by flexible netting allowing interaction between litters but preventing full physical contact and mixing, hence minimising handling, and aggression. By using the netting we prevented some of the problems demonstrated in previous studies where agonistic interactions between piglets and aggression of sows towards foreign pigs were observed after mixing (i.e. Weary et al., 1999; D'Eath, 2005). Our second goal was to investigate whether recognition of familiar individuals is affected by relocation to a novel but similar farrowing pen to clarify potential effects of relocation on SR.

2. Materials and methods

This study was carried out at the Swine Teaching and Research Center (STRC) at Michigan State University (MSU), USA. All procedures used in this study were reviewed and approved by the All-University Committee for Animal Use and Care of MSU.

2.1. Animals and housing

A total of 12 unweaned litters (Yorkshire × Duroc) were used in this study. Within 36 h post-farrowing, piglets were cross-fostered so that litters totalled 10–11 piglets (balanced for sex) following the standard operating procedures of the STRC. The animals had ad libitum access to water and sows were fed according to National Research Council (NRC, 1998). Sows and litters were housed in standard farrowing pens (1.8 m × 2.3 m) with slatted floors throughout the

experiment. Two similar oval arenas (1 m × 1.5 m) were located between the farrowing pens (Figure 1), enabling piglets to access the arena without handling when the doors to the arena were opened. The temperature of the room was controlled (20 ± 1°C) and artificial light was provided from 08:00 to 18:00 h throughout the experiment.

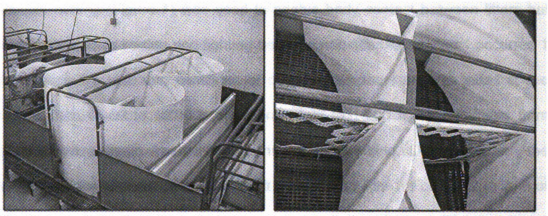

Figure 1- Side view (left) and top view (right, showing the netting used for familiarisation) of the arenas.

2.2 Habituation and familiarisation of piglets

All piglets were habituated to human presence and contact from birth until 12 days of age by the researcher holding each piglet in their home pen for 30 s every 2 days. This procedure aimed to habituate piglets to handling needed for the test protocol, where animals would be carried and placed in an arena. Piglets were also habituated to the layout of the arenas from 08:00 to 18:00 h, every 2 days, by opening the doors between the farrowing pen and the arena. Litters

from neighbouring pens were habituated to the arenas on alternate days and the arenas were cleaned daily to prevent odour transfer. We considered all litters to be unfamiliar with each other prior to the familiarisation procedure.

The familiarisation procedure consisted of two familiarisation phases: exposures between litters (Fam1) and exposures between pairs of siblings (Fam2, see Table 1). Both familiarisation phases were carried out in the arenas. Flexible netting (Figure 1) was used to enable body contact between litters but prevent unwanted mixing and fighting between non-littermates. Fam1 occurred 1 day before the test, and consisted of opening the doors on both sides of the arenas from 08:00 to 12:00 h and from 14:00 to 18:00 h, so that litters from adjacent pens could freely interact through the netting. Doors between the arenas and the farrowing pens were left open during Fam1. For Fam2, a total of 48 unweaned piglets were randomly selected from the 12 litters (26 females and 22 males, 4 per litter). During Fam2, occurring on the day of testing, pairs of littermate piglets from adjacent pens were placed in the arena for two 10-min periods separated by a 10-min interval. Pigs were familiarised in pairs with their siblings to prevent stress resulting from social isolation (e.g. Kanitz et al., 2004) during the familiarisation procedure. During Fam2, the doors to the farrowing pens were closed to prevent the animals from returning to their home pens and to habituate them to being placed in a closed arena. After these two familiarisation phases, the piglets were considered to be familiar.

Table 1- Familiarisation between litters and pairs of piglets

Familiarisation	Animals in the arena	Doors to access the arena	Netting	Exposure time
Between litters (Fami1)	Up to 11 piglets from each litter	Opened – free access	Present	2 x 4 h (2-h interval)
Between pairs (Fami2)	Only 2 piglets from each litter	Closed – controlled access	Present	2 x 10 min (10-min interval)

2.3 Relocation

After Fam2, the piglets were randomly assigned by litter to one of the three relocation treatments (see Figure 2): no relocation (NR, n = 12 piglets from 3 litters), relocation maintaining the arena orientation relative to the farrowing pen (RM, n = 20 piglets from 5 litters) or relocation changing the arena orientation relative to the farrowing pen (RC, n = 16 piglets from 4 litters). Relocation consisted of moving sows and litters to novel but similar farrowing pens located in the same room as their original pen. Relocation was completed within 40 min. The relocation protocol was arranged so that litters to be tested together would share the same arena (see Figure 2). Immediately after Fam2, litters and sows subjected to the NR treatment remained in their home pens (acting as the control for the relocation treatment). Litters and sows subjected to the RM treatment were moved to novel farrowing pens located on the same side of the arena relative to their original pens, while litters and sows subjected to the RC treatment were moved to novel farrowing pens on the opposite side of the arena.

Allocation of the animals per treatment group is presented in Figure 2. A balanced design comparison of the relocation treatments with the unfamiliar treatment was not possible due to a constraint in size of the room, given that pairs of unfamiliar piglets to be tested together could not be previously housed in adjacent pens.

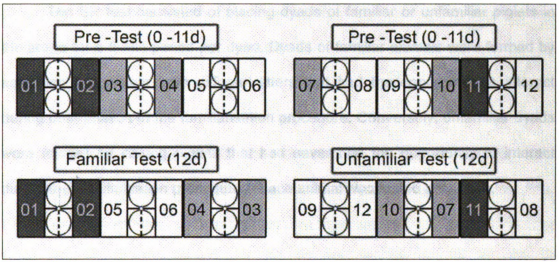

Figure 2- Allocation of the farrowing pens and treatments before (Pre-Test) and after (Test) relocation. Dark pens indicate NR-treatment, grey pens indicate RC-treatment, and white pens indicate RM-treatment. Dashed line indicates netting in arenas between pens. Numbers indicate litters.

2.4 Social recognition test

Summarising the treatment distribution, the subjects were allocated to one of two familiarisation treatments (familiar or unfamiliar (n = 24, for each treatment)) and one of three relocation treatments (NR, RM, and RC). The dyads

were formed by either females and males or male and female (balanced for the total amount of dyads). The piglets were tested at 12 ± 1 days of age. The SR test began approximately 4 h after Fam2. Only the rear arena, out of view of the sow, was used for the SR test to minimise the influence of the sows on piglets' behaviour. Prior to testing, the netting was removed from the arena so that piglets could freely interact.

The SR test consisted of placing dyads of familiar or unfamiliar piglets in the arena for a 5-min period per dyad. Dyads of familiar animals were formed by assigning two piglets from different litters that had the opportunity to interact during both phases of the familiarisation procedure. Conversely, unfamiliar dyads were formed by pairing piglets that had never had the opportunity to interact during the familiarisation procedures. Each animal was tested only once.

2.5 Behavioural observations

We investigated whether during the familiarisation between litters (Fam1) the animals would visit the arena and interact with non-littermates by themselves. During the total of two 4-h periods (= 480 min) the events in the arena were videotaped and later analysed using instantaneous scan sampling performed at 5-min intervals. The amount of time the piglets spent in the arena, with and without the future test pig (for the familiar group), are presented in percentages. Fam2 was not analysed in this way because the test pairs of the familiar group were always placed together in the arena for the two 10-min periods (= 20 min).

The behaviour of individual piglets in the arena during the SR test was also videotaped and subsequently recorded using "The Observer 3.1" behavioural recording software (Noldus Information Technology, Wageningen, The Netherlands). The following behaviours were scored in a continuous focal sampling paradigm: social investigation, defined as investigating the conspecific (sniffing or touching (with the nose or mouth) any part of the other piglet's head and/or body, or following the other piglet within 10 cm) and exploring the arena (rooting or licking the floor, doors or walls of the arena while sniffing).

2.6 Statistical analysis

The distribution of all variables was tested for normality and homogeneity of variance, and appropriately transformed if not normally distributed. After square-root transformations, all variables reasonably met the normality and homogeneity criteria. The data were analysed using the mixed model analysis of variance procedure (PROC MIXED) of SAS 8.2 (SAS Institute Inc., Cary, NC, 2001) to test the effects of familiarity (familiar or unfamiliar), relocation (NR or RM or RC), sex and test mate sex as well as all possible two-way interactions between these four factors. Due to the split plot structure of our experimental design, the experimental unit for relocation effects was defined by sow whereas the experimental unit for familiarity was defined by both sow and dyad, thereby necessitating the use of Satterthwaite's approximation for determining degrees of freedom (Sattherwaite, 1946) as invoked in PROC MIXED. The data are presented as adjusted least-squares means ± S.E.M. with degrees of freedom

determined using Satterthwaite's approximation. Differences between the experimental groups were considered to be significant if $P \leq 0.05$. Sex, test mate sex and their interaction ($P > 0.10$) did not significantly influence any of the response variables and will not be mentioned in Section 3.

3. Results

During Fam1 (480 min), the test piglets spent 46.5% (223.2 min) of the time in the arena. In 7.81% (37.49 min) of the total time, a piglet was in the arena in presence of the future assigned 'familiar' test piglet. All the tested piglets meeting familiar animals thus met their future test mates at least once during the first familiarisation.

During the SR test, unfamiliar piglets spent significantly more time on social investigation (26.59 ± 3.5) than familiar piglets (11.55 ± 3.21) (GLMM, $F_{1,9.6} = 9.96$; $P < 0.01$). Familiarity did not affect the amount of time spent on exploration of the arena (GLMM, $F_{1,10.5} = 0$; $P = 0.96$). The total percentage of investigation and exploration between pairs of familiar and unfamiliar piglets by relocation treatments is presented in Figure 3. There were no significant effects of relocation on the duration of social investigation (GLMM, $F_{2,9.68} = 2.14$; $P = 0.17$). Although relocation did not significantly affect the amount of time spent on exploration of the arena (GLMM, $F_{2,10.3} = 2.21$; $P = 0.15$), the interaction between familiarity and relocation was significant (GLMM, $F_{2,9.75} = 4.50$; $P < 0.05$). Familiar piglets that were relocated to a farrowing pen with changed arena orientation (RC) spent more time exploring the arena than familiar piglets relocated to a

farrowing pen that maintained the arena orientation (RM) ($P < 0.01$), see Figure 3.

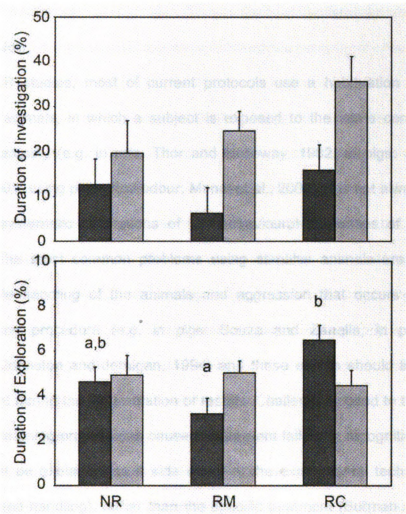

Figure 3- Effects of familiarity and relocation of 12-day-old piglets on time spent on social investigation and exploration of the arena during the SR test. Familiarity is indicated by dark bars (familiar) and grey bars (unfamiliar). NR, RM, and RC indicate relocation treatments. Values represent adjusted means ±S.E.M.

Significant differences ($P < 0.01$) within familiarity are specified with different letters.

4. Discussion

In SR studies, most of current protocols use a habituation method to familiarise animals, in which a subject is exposed to the same conspecific or odour, repeatedly (e.g. in rats: Thor and Holloway, 1982, in pigs: Souza and Zanella, 2001, using pigs urine odour: Mendl et al., 2002). It is not always easy to carry out systematic evaluations of the behavioural responses of the tested subjects. The most common problems using stimulus animals are related to considerable handling of the animals and aggression that occurs during the familiarisation procedure (e.g. in pigs: Souza and Zanella, in preparation; hamsters: Johnston and Jernigan, 1994) and these effects should to be taken into account during the interpretation of results. Challenges posed to the animals during the learning process can cause an apparent failure in recognition and this effect might be explained as a side effect of the experimental technique itself (e.g. repeated handling), rather than the specific treatment (Burman and Mendl, 2000). We therefore designed a novel paradigm for familiarisation using netting barriers which successfully prevented fighting between non-littermate piglets during this period. There was only one pair of non-littermates that engaged in aggressive interaction during familiarisation, but it did not last more than 4 s because both animals went back to their home pen without being injured.

Furthermore, the animals were familiarised and tested in arenas located next to the farrowing pens which seemed to reduce the stress of moving animals to a different environment during the test or other possible confounding factors, such as isolation (e.g. Kanitz et al., 2004). This method for familiarisation appeared to reduce some of the previously mentioned challenges commonly seen during the habituation phase.

Ewbank et al. (1974) suggested that distinction between a previously encountered individual and an unfamiliar one might be improved by processing the information through more than one sensory system. In our study, we increased the chances of piglets to become familiar with individual conspecifics by allowing them to use their olfactory, visual, auditory, and tactile cues during the familiarisation period. By subjecting the piglets to Fam1, we increased their chances of gaining information about non-littermates. The piglets spent almost half of the total 480 min in the arena and more than 30 min with their future test animal. We tested whether this novel familiarisation protocol would successfully familiarise non-littermate piglets by analysing differences in social investigative behaviour between dyads of familiar and unfamiliar 12-day-old piglets during subsequent testing. Unfamiliar piglets spent significantly more time investigating each other than familiar animals, a finding supported by previous results (Kristensen et al., 2001; Souza and Zanella, 2001), suggesting that familiar animals remembered each other from the preceding familiarisation period and indicating successful familiarisation.

Relocation did not affect social investigation, indicating that, in pigs, minor changes in a novel environment do not interfere with social memory, in contrast with findings in rats by Burman and Mendl (2000). However, relocation did affect the overall time the animals spent on the measured behaviours during testing, most notably in familiar dyads. Familiar animals relocated with changes in the arena orientation (RC) spent significantly more time exploring the environment than those relocated with no change in arena orientation (RM). For RC animals, the position of the testing arena had changed relative to the home pen and this difference might have motivated the animals to explore the 'new' surroundings rather than a familiar conspecific more than in RM animals where orientation of the novel arena was the same. Novelty arouses a motivation to explore, likely an adaptive response necessary to gather information (Wood-Gush and Vestergaard, 1991; Wemelsfelder and Birke, 1997). No effects of relocation were found in unfamiliar dyads, suggesting that investigation of an unfamiliar has priority over exploration of a new environment. Conclusive remarks cannot be made, but it is possible that animals prioritise information gathering, e.g. if an animal is familiar, investigation of the environment becomes a priority. Further investigation on this subject is needed to fully understand the role of the environmental context on social memory and clarify the processes in which animals prioritise their information processing.

5. Conclusions

We described a novel method to test social recognition in pigs which avoids fighting and resulting distress during the familiarisation (habituation) phase. This familiarisation methodology resulted in clear differences in time spent on social investigation between familiar and unfamiliar dyads during the testing phase, up to 4 h after familiarisation. Relocation of the animals between the familiarisation and testing phases did not interfere in the outcome of the SR test in terms of time spent on social investigation, but had effects on time spent in exploration of the arena.

Acknowledgments

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CHAPTER 4

LONG LASTING SOCIAL RECOGNITION IN 3 WEEK-OLD FEMALE PIGS

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In preparation for publication

Abstract

While the establishment of social recognition (SR) memory in rodents has been extensively explored, studies that systematically investigate SR in pigs, especially at very young ages, remain sparse. As in rodents, a decrease in spontaneous social investigation is observed when a pig re-encounters a familiar conspecific, and this phenomenon can be used as a valid measure of SR ability. We have recently designed a protocol to test SR in non-weaned pigs and showed that 12-day-old pigs were able to remember members of a familiar litter for at least 4 hours after the last familiarization exposure. In the present study, we aimed at investigating whether the same protocol can be used to assess SR in 21-day-old female pigs up to 24 hours. The results demonstrated that the animals were able to recognize familiar conspecifics at least 24 hours later, as similar amounts in the duration of social investigation were found in piglets meeting familiar conspecific at 4 and 24 hours after being familiarized. We therefore concluded that the used SR protocol allows investigation of a more robust SR memory in 21-day-old female pigs.

1. Introduction

The basis of social relationships in mammals involves at least the ability to discriminate between classes of individuals, such as dominant/subordinate, group-member/non-member, or familiar-unfamiliar. An animal's ability to discriminate between familiar and unfamiliar conspecifics has been demonstrated in a number of species (a.o. pigs: Kristensen et al., 2001; rats: Engelmann et al., 1995; ground-squirrels: Mateo and Johnston, 2000).

In pigs, discrimination probably relies on familiarity gained over a period of mutual association rather than genetic relatedness, as pigs are similarly aggressive towards unfamiliar kin and unfamiliar non-kin (Puppe, 1998; Stookey and Gonyou, 1998). Familiarity, and recognition thereof, may therefore be a key factor in attaining and maintaining stability within a social group, and failure in distinguishing between conspecifics may result in outbreaks of fighting (Ewbank and Meese, 1971; Puppe, 1998). There is evidence that pigs can retain information about certain conspecifics for long-periods of time. As an example, Ewbank and Meese (1971) have shown that top-ranking fattening pigs could be safely returned to their original group even after 25 days of isolation. However, while indirect assessment of SR based on the amount of fighting may indicate SR of high-ranking animals, it does not provide insight in SR of low-ranking animals, since fighting will occur with both low-ranking and unfamiliar animals. Depending on the social rank of individuals, dominant or subordinate, in a social encounter an appropriate behavioral response is usually expected. During interactions between the same individuals in a stable social group, the behavioral

responses tend to be very consistent over time. This consistency may indicate reflect a stability of social relationship which requires that individuals are able to recognize or at least discriminate members of its group. However, this ability may be disturbed, for example, as a result of incorporation of unfamiliar conspecifics, illness or temporary removal.

In an experimental setting, SR may be assessed using the individual's natural 'interest' to intensely investigate novel conspecifics. Investigation is important for acquisition of information and increases familiarity or at least reduces novelty of the stimulus (Todrank et al., 1999), since a lesser amount of investigation is often observed after repeated exposure of a (social) stimulus (Thor and Holloway, 1982; Engelmann et al., 1995; Souza and Zanella, 2001).

Recently, we showed that 11-day-old piglets were able to distinguish between familiar and unfamiliar conspecifics 4 hours after the last exposure time (Souza et al., 2006). McLeman et al. (2005) have provided evidence that 6-week-old pigs are able to discriminate conspecifics sharing similar characteristics 24 hours after the last training session. The required amount of handling and training in McLeman's et al. (2005) experiment, however, may not be ideal for use in very young pigs. Therefore, the present study aimed at testing a protocol to investigate SR in 21-day-old pigs using a method specifically designed for neonatal animals (for details see Souza et al., 2006). In the present study, we investigated a more robust SR of 21-old female pigs using two delay periods, 4 and 24 hours.

2. Materials and methods

This study was carried out at the Swine Teaching and Research Center (STRC) at Michigan State University (MSU), USA. All procedures used in this study were reviewed and approved by the MSU All-University Committee for Animal Use and Care.

2.1 Animals and apparatus

Eight litters of piglets were used in a total of four trials. Within 36 hours of farrowing, piglets were cross-fostered following the standard operating procedures of the STRC so that each experimental litter contained 8 female piglets. Sows and litters were housed in standard farrowing crates (1.8 x 2.3 m) with slatted floors. The animals had ad libitum access to water and sows were fed according to National Research Council (NRC, 1998). The temperature of the room was controlled (20°C ± 1) and artificial light was provided from 08:00 to 18:00 throughout the experiment.

The piglets in this study had been previously used in a spatial learning task. The spatial learning task consisted of an adapted form of the Morris Water Maze (MWM: for details see Laughlin and Zanella, 2003). The animals performed the MWM task at 13 and 14 days of age and were classified for the spatial experiment as 'good performers' or 'poor performers' (n = 24 per group: GP, average latency time ≤ 50 seconds; PP, average latency ≥ 70 seconds; Laughlin and Zanella, 2003). The remaining littermates classified as 'intermediate performers' were kept with the sow and eliminated from this study.

2.2 Habituation and familiarization protocols

Piglets were habituated to human presence and contact from birth until 12 days of age by the researcher holding each piglet in their home pen for 30 seconds every other day. This procedure aimed at accustoming piglets to the handling needed for SR testing, where animals would be carried and placed in the testing arena located between farrowing crates. Piglets were habituated to the testing arenas from 08:00 to 18:00, every other day by opening the doors to the arenas (located in between two neighboring farrowing crates; see Figure 1). Neighboring litters were habituated to the arenas on alternate days and the arenas were cleaned daily to minimize odor transfer between litters. All litters were assumed to be unfamiliar to each other prior to the familiarization procedure.

The familiarization procedure consisted of two phases: exposures between whole litters (Fam1) and exposures between groups of 3 siblings (Fam2). Six piglets/litter, balanced for spatial memory task performance, were used (n = 48). Both familiarization phases were carried out in identical oval arenas (1 m × 1.5 m) located between the farrowing crates (Figure 1). Flexible netting divided each arena in half to enable body contact between litters while preventing mixing and fighting between non-littermates.

Fam1 occurred one day before SR testing, and consisted of opening the doors on each side of an arena from 08:00 to 12:00 and from 14:00 to 18:00, so that two litters from adjacent crates could interact freely through the netting. Each litter was exposed just once to Fam1. During Fam2, which was performed the

day after Fam1, three littermates from adjacent farrowing crates were placed in the arena for two 10-minute periods separated by a 10-minute interval. As social isolation can be highly stressful for pigs (Kanitz et al., 2004), the animals were familiarized in groups with their siblings during the familiarization procedure. During Fam2, the doors to the farrowing crates were closed to prevent the animals from returning to their pens and to accustom them to being placed in a closed arena. After Fam2, neighboring litters that had opportunity to social interact in the arena were considered to be familiar.

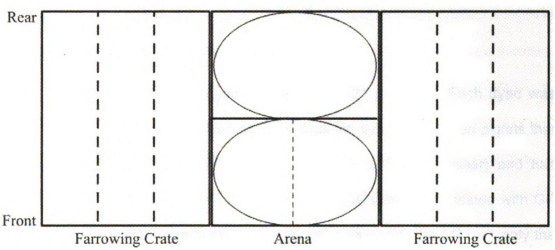

Figure 1- Farrowing crates and testing arenas. Adjacent farrowing crates shared the same arenas. During 'familiarization', both front and rear arenas were used. During 'testing' only the rear arena, out of view of the sow, was used.

2.3 Relocation

After Fam2, all litters and sows were relocated to novel farrowing crates, so that the animals assigned to be tested together (familiar or unfamiliar) were placed in crates adjacent to the testing arenas. We moved the litters prior to testing to minimize disturbance of the animals during testing. Relocation consisted of moving sows and litters to novel but similar farrowing crates, maintaining the orientation or the arena and located in the same room (for details see Souza et al., 2006). Relocation of all animals was completed within 20 minutes.

2.4 Testing

Piglets were tested in dyads at the age of 21 ± 3 days. Each dyad was made up of piglets from two different litters. Half the dyads comprised piglets that had been exposed to each other during Fam1 and Fam2 (familiar) and half comprised piglets that had not (unfamiliar). GP piglets were only tested with GP piglets and PP piglets were tested only with PP piglets. For the SR test, only the rear arena, out of view of the sow, was used for testing, in order to minimize the influence of the sow on piglet behavior. Prior to testing, the doors to access the arena were closed and the netting was removed, so that piglets could freely interact, but could not return to their home crate. Dyads of piglets were placed in the arena for a 3-minute period. Twenty-four animals (6 'familiar' dyads and 6 'unfamiliar' dyads) were assigned to be tested after a delay period of 4 hours

after Fam2 (T4) whereas the remaining 24 were assigned to be tested 24 hours after Fam2 (T24).

2.5 Behavioral observations during SR test

The behavior of individual piglets in the arena during the SR test was videotaped and analyzed using "The Observer 3.1" behavioral recording software (Noldus Information Technology, Wageningen, The Netherlands). Duration (expressed as the percentage of the total testing exposure) of social investigation, defined as sniffing or touching (with the nose or mouth) any part of the conspecific's head and/or body, or following within approximately 10 cm, was scored by continuous focal sampling recording.

2.6 Statistical analysis

The distribution of all variables met the normality and homogeneity criteria. The data were analyzed using the mixed model analysis of variance procedure (PROC MIXED) in SAS 8.2 (SAS Institute inc., Cary, NC, 2001) to test for effects of familiarity (familiar or unfamiliar), performance (GP, PP), and delay period (T4 or T24), as well as all interactions. All effects were randomized for trial within litter and for pair. To establish equivalence between means of T4 and T24 a Bioequivalence testing was used. The data are presented as adjusted means ± S.E.M. Differences between the experimental groups were considered to be significant if $P \leq 0.05$.

3. Results

The duration of social investigation was affected by familiarity (GLMM; $F_{1,37}$= 15.20; P < 0.001); after the familiarization procedure unfamiliar pigs spent significantly more time investigating the testing mate (26.35 ± 1.91) than familiar animals (14.18 ± 1.28). No effect of delay period (T4, T24) was found for the duration of social investigation (GLMM; $F_{1,37}$= 2.6; P > 0.1). Pigs tested 4 hours and 24 hours after being exposed to the familiarization procedure spent similar amounts of time on social investigation (18.45 ± 1.40 and 21.89 ± 2.21, respectively). The duration of social investigation was not significantly affected by performance in the Morris-Water Maze test (GLMM; $F_{1,37}$= 1.11; P > 0.1).

There was no interaction between familiarity, performance, and delay period on the duration of social investigation (Figure 2; GLMM; $F_{1,37}$ = 0.01; P > 0.1). The results on two-way interactions showed no effect between familiarity and performance on the Morris-Water Maze test (GLMM; $F_{1,37}$ = 1.06; P > 0.1), between performance and delay period (GLMM; $F_{1,37}$ = 0.12; P > 0.1), neither between familiarity and delay period (GLMM; $F_{1,37}$ = 15.20; P > 0.05). The testing power of the interaction between familiarity and delay period falls into the 90% rate; therefore bioequivalence testing was applied for comparisons between familiar groups tested at T4 and T24. The results showed that the mean values of T4 and T24 (14.57 ± 1.58 and 13.78 ± 2.05, respectively) are similar (t = 0.23, P = 0.8). Also, comparisons between mean values of unfamiliar groups tested at T4 and T24 (22.33 ± 2.04 and 30.74 ± 3.11) showed that they are not similar (t = -2.48, P = 0.01).

4. Discussion

The present study demonstrated that 21 day-old female pigs are able to remember members of a familiar litter (of the same sex, age, and non-related) for up to 24 hours. The amount of social investigation did not differ between pigs tested after a delay period of 4 hours and those tested after 24 hours, indicating that this testing protocol can be used to assess a relatively long-term memory in non-weaned 21 day-old pigs.

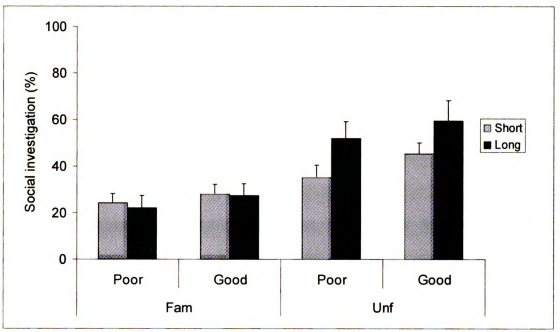

Figure 2- Effects delay period length and spatial learning performance between familiarization and testing on duration of social investigation. Dark bars indicate short-term (4h delay) and light bars indicate long-term (24h delay) SR memory. Values represent adjusted means ± S.E.M.

The familiarization protocol provides a relatively long exposure of the animals to each other (approximately 8½ hours) with minimal distress as fighting was prevented by the initial presence of mesh netting, all of which may favor a more robust memory for members of familiar litters. The relevance of these findings lays in the possibility to clarify the role of pharmacological compounds and acute and chronic stressors on memory in young pigs. Such work may potentially lead to a valid model for SR memory in humans (e.g. by using neonatal pigs as a model more invasive interferences can be used), but can also provide a method for investigating the underlying causes of prolonged aggression in group-housed pigs. The SR familiarization and testing methodology presented here is a good method for conducting such much needed research.

5. Conclusion

The present study demonstrated that the protocol (familiarization and testing procedure) used to assess the ability of neonatal pigs to discriminate members of a familiar litter can be used to assess both short- and long-term memory. Furthermore, this method offers the possibility for further investigation of both short- and long-term memory in a single trial, and with minimal disturbance to the animals.

Acknowledgements

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CHAPTER 5

SOCIAL ISOLATION ELICITS DEFICITS IN THE ABILITY OF NEWLY-WEANED PIGS TO RECOGNIZE FAMILIAR FEMALE CONSPECIFICS

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Submitted

Abstract

In pigs, weaning and social isolation can be highly stressful and may impair social recognition (SR). Using a modification of a previously presented familiarisation protocol, newly-weaned pigs were familiarised within litters. The goal of this study was to assess the ability of the piglets to recall the information about familiar conspecifics and examined how this was affected by weaning age and/or social isolation. A total of forty-eight female piglets were weaned at day 11 (D11, n = 24) or at day 22 (D22, n = 24) of age. To examine whether social isolation impaired SR, 24 pigs were isolated for 15 minutes prior to the SR test, while the control group (n = 24) remained with their littermates in nursery pens. Immediately after social isolation pigs were exposed to a familiar (n = 24) or an unfamiliar (n = 24) conspecific for a 3 minute-period. The animals were tested only once, two days after weaning, i.e at 13 days of age for D11 piglets and 24 days of age for D22 piglets. The results indicated that during familiar interactions, isolated D11 pigs investigated more than non-isolated D11 and D22 pigs, whereas during unfamiliar interactions no differences between the treatment groups were found. Interestingly, the amount of social investigation displayed by familiar isolated D11 pigs was similar to that displayed by unfamiliar animals. Taken together, this indicates that SR is disrupted by 15 minutes of social isolation in D11, but not in D22 pigs. Possible mechanisms for this disruption are discussed.

1. Introduction

In feral and free-ranging conditions pigs are weaned gradually. Weaning begins when the sow leaves the farrowing nest for foraging, but the complete cessation of suckling is normally not seen before 4 months of age, when the young pigs continue to live in social contact with the sow and her social group (Newberry and Wood-Gush, 1985; Jensen, 1986; Jensen and Stangel, 1992; Petersen, 1994). In commercial husbandry conditions, piglets are abruptly weaned at much younger ages. Under these circumstances, weaning is a multifactorial stressor for the piglets as it simultaneously involves separation from the sow, changes in diet, and relocation to a different environment (Varley, 1985; Dybkjær, 1992; Weary and Fraser, 1997). Studies have shown that weaning causes distress in pigs, with prolonged vocalisation, restlessness, and long-term behavioural changes (Week 4: Dybkjær, 1992; Week 2 x Week 4: Weary et al., 1999; Day 6 x Non Weaned : Orgeur et al., 2001; Week 3 x Week 4: Colson et al., 2006), and enhanced cortisol concentration in plasma (Week 2: Blecha et al., 1985; Week 3 X Week 5: Mason et al., 2003) and in urine (Day 6: Hay et al., 2001). These studies show that biological changes are associated with weaning these animals abruptly and at much early stages of life than would occur in a more natural environment, which may in turn compromise pig's welfare. The latest report of the NAHMS (2001) revealed that, in the USA, 63.9% of the pigs are weaned between 16 and 20 days of age and 15% are weaned before 16 days of age.

Social isolation is also stressful for a pig (Herskin and Jensen, 2000; Ruis et al., 2001; Kanitz et al., 2004) and may occur during routine management practices (e.g. tail docking, castration, iron injection, veterinary inspection) or experimental purposes (e.g. metabolism chamber, Moeser et al., 2002). Still, the effects of social isolation for young pigs from the mother and/or siblings are poorly understood.

It is well established that glucocorticoid (GC) hormones, secreted by the adrenal cortex after a stressful event, influence cognitive performance (for a review see Roozendaal, 2002). A recent study conducted in our lab investigated the effects of weaning age and social isolation on a spatial learning task. Laughlin and Zanella (2003) demonstrated that the ability to reach a hidden platform in a Morris Water Maze was hampered if female pigs were weaned at D12 and tested at D14 or D23 of age, but only if they were socially isolated immediately before testing, whereas performance of female pigs weaned at D21 and tested at D23 was not affected by social isolation. No differences in performance were found between non-isolated animals and the animals did not differ in post-isolation salivary GC levels (Laughlin and Zanella, 2002). This finding may indicate a possible cognitive impairment in animals weaned at D12 pigs during heightened HPA axis activity caused by social isolation.

Social recognition, here defined as the process by which animals discriminate familiar conspecifics, is of fundamental importance for a wide range of social behaviours and is a component of learning and memory. In rodents, SR is sensitive to disruption by acute stress (Burman and Mendl, 2000; Kogan et al.,

2000). In this study, we therefore aim at providing an insight on the effects of a real life acute stressor on the learning ability of newly weaned female pigs. For that purpose, we investigated whether the ability of D11 and D22 piglets to recognise conspecifics is susceptible to disruption by a single acute stressor (15 minutes of social isolation).

To that end we used a SR protocol developed by Souza et al. (2006), which is based on the social discrimination paradigm, using pigs' natural tendency to investigate novelty (novel environment: Stolba and Wood-Gush, 1980; de Jong et al., 2000; novel conspecifics: Souza and Zanella, 2001). In this protocol, piglets are familiarised with neighbouring animals through flexible netting, which has been shown to prevent mixing and fighting between unfamiliar pigs during familiarisation (for details see Souza et al., 2006). A lack of differences in the duration of social investigation between familiar and unfamiliar encounters indicates impairments in recognising familiar individuals (in rats: Heinrichs, 2003).

2. Material and methods

This study was carried out at the Swine Teaching and Research Center (STRC) and the Intensive Metabolism Unit (IMU) at Michigan State University. All procedures used in this study were reviewed and approved by the All-University Committee for Animal Use and Care of MSU.

2.1 Weaning and isolation treatments

Three factors were studied, weaning age with 2 levels: Day 11 (D11) and Day 22 (D22), isolation with 2 levels: Isolated and Non-Isolated and familiarity with 2 levels: Familiar, Unfamiliar.. A total of 8 treatments were used in this factorial design with 6 replicates per treatment group.

2.2 Animals, housing, and weaning

Eight litters (Yorkshire x Duroc) were selected for this study. Within 36 hours post-farrowing the piglets were cross-fostered so litters were standardised to 10 or 11 females and the males were fostered to non-experimental litters, following the standard operating procedures of the STRC. During the suckling period, sows and their piglets were housed in standard farrowing crates (1.8 x 2.3 m) with access to food and water.

Nine female piglets were selected per litter and balanced for two weaning ages (4-5 piglets/ litter). Half the litter was weaned at 11 days of age (D11) and the other half was weaned at 22 days of age (D22). Weaning was carried out by taking the selected piglets from their farrowing crates and driving them to experimental facility – Intensive Metabolism Unit (IMU) (distance: 1.6 km, time: 5 minutes) where they remained with littermates in nursery pens (1.5 m^2, 4-5 animals/ pen). A heating pad, a nipple drinker and a 5-space feeder were provided in each pen. Circular arenas (diameter, 1.5 m) were located between the nursery pens enabling pigs from adjacent pens to access the common area when the doors to the arena were opened. Flexible netting dividing each arena

into two halves prevented mixing and agonistic interactions between non-penmates (for details see Souza et al., 2006). All animals had ad libitum access to water and were fed according to National Research Council (NRC, 1998). Artificial lights were provided from 06:00 to 23:00 hour. Room temperature was controlled (27°C ± 2°C).

2.3 Familiarisation

The day after weaning, the pigs were subjected to the familiarisation procedure, which was conducted in two phases: exposure between non-penmates (fam1, see Table 1) and exposure between pairs of penmates to pairs of non-penmates (fam2). Fam1 occurred on the day after weaning, when the pigs were habituated to the neighbouring pigs by allowing them to freely access the testing arena for two consecutive days from 08:00 - 12:00 and 14:00 - 18:00. Fam2 was carried out on the subsequent day (testing day), when pairs of pigs from adjacent pens (e.g. 2 pigs of pen-A with 2 pigs of pen-B) were placed in the arena twice for 10 minutes. Both familiarisation phases occurred in the arena with a netting barrier. After the familiarisation procedure pigs from adjacent pens that had previously interacted were considered familiar.

Table 1- Familiarisation between litters and pairs of piglets

Familiarisation	Animals in the arena	Doors to the arena	Netting	Exposure time
Between litters (Fam1)	Up to 5 piglets from each litter	Open – free access	Present	2 x 4 hours (2 hour interval)
Between pairs (Fam2)	Only 2 piglets from each litter	Close – controlled access	Present	2 x 10 minutes (10 minute interval)

2.4 Social recognition test

A total of 48 female pigs were selected for the SR test. As part of the testing protocol, immediately after fam2 all litters were relocated (within approx. 10 minutes) to novel nursery pens. The novel pens were similar to their home pens and were located in the same room. After relocation the pigs assigned to be tested together were housed in adjacent pens and shared the same arena. The spatial orientation of the novel pens, position of the arena, feeder, and drinker did not change. The SR test began 4 hours after fam2.

Prior to testing, the 24 female pigs selected for the isolation treatment were individually taken from their nursery pens placed in a weighing cart and subsequently transported to another room located in the same experimental building. Here every pig was kept alone in the weighing cart for 15 minutes. After isolation each pig was immediately subjected to the SR test. The non-isolated pigs were left undisturbed in their home pens during this time and only placed in the arena for the testing. The SR test consisted of a single 3-minute exposure of

pigs in the arena without the netting, which allowed the animals to have full body contact. The testing exposure was done by exposing pairs of non-penmates (i.e. from different litters). For familiar groups, the pair was made by two pigs that were familiarised together, whereas in the unfamiliar groups the pigs were completely unfamiliar to each other. The pigs were tested two days after weaning; D11 animals were tested at 13 days of age and D22 pigs at 24 days of age. Every pig was tested only once.

2.5 Behavioural observations

Social behaviour of individual pigs in the arena during the SR test was video recorded and subsequently analysed using "The Observer 3.1" behavioural recording software (Noldus Information Technology, Wageningen, The Netherlands). The duration of social investigation was the only behaviour used for SR memory assessment. Social investigation was defined as sniffing or touching (with the nose or mouth) any part of the other piglet's head and/or body, or following the other piglet within approximately 10 cm.

The total amount of general behavioural activity was recorded to detect possible non-specific effects of social isolation, such as changes in activity patterns that could alter social investigative behaviour and was calculated by subtracting the duration of general inactivity from the total duration of the exposure and in the results is presented as a percentage. Inactivity was defined as lying on side or belly without visibly sniffing the arena or the other pig.

2.6 Statistical analysis

The distribution of social investigative behaviour met the criteria for normality and homogeneity. Data were analysed using a mixed model analysis of variance procedure (PROC MIXED) in SAS® 9.1 (SAS Institute inc., Cary, NC, 2003) to test for effects of familiarity, weaning age, and social isolation and all possible interactions between these three factors. All factors were tested against the random effect of litter within familiarity and of pair. Post-hoc analyses were done using Tukey adjustment. Data were collected from individual pigs and are presented as means ± S.E.M. Differences between the experimental groups were considered to be significant if $P \leq 0.05$.

Due to a lack of normality and homogeneity of variance of general behavioural activity, the difference between groups (isolated D11, non-isolated D11, isolated D22, and non-isolated D22) was analyzed using a two-tailed Wilcoxon Two Sample Test.

3. Results

3.1 General behavioural activity

Pos-hoc comparisons between treatment groups were performed and showed that they were not significantly different from each other. Isolated D11 pigs did not significantly differ in amounts of general behavioural activity when compared with non-isolated D11 ($P = 0.31$), isolated D22 ($P = 0.31$), and non-isolated D22 ($P = 0.98$) pigs. No differences were found in general behavioral

atictivity when comparing non-isolated D11 with isolated D22 pigs (P = 0.86) and isolated D22 with non-isolated D22 (P > 0.31).

Table 2 - Percentages of general behavioural activity (means ± S.E.M.) during familiar and unfamiliar exposures in the SR test.

		Familiar	Unfamiliar
Weaning at D11	Isolated	100.00 ± 0.00 (n = 6)	100.00 ± 0.00 (n = 6)
	Non-Isolated	93.55 ± 5.50 (n = 6)	98.53 ± 1.47 (n = 6)
Weaning at D22	Isolated	95.48 ± 4.52 (n = 6)	82.06 ± 11.64 (n = 6)
	Non-Isolated	100.00 ± 0.00 (n = 6)	100.00 ± 0.00 (n = 6)

3.2 Social investigation

The percentage of social investigation in familiar and unfamiliar female pig dyads during the SR test is presented in Figure 1. There was a significant effect of familiarity (GLMM; $F_{1,6} = 9.30$, $P < 0.05$) with familiar dyads spending less time on social investigation (16.70 ± 2.86 %) than unfamiliar ones (29.09 ± 3.71 %). A significant effect of isolation (GLMM; $F_{1,34} = 7.20$, $P = 0.01$) showed that socially isolated animals had in average higher duration of social investigation (28.35 ± 3.59) than non-isolated (17.45 ± 3.14). There was no significant effect of weaning age (GLMM; $F_{1,34} = 0.93$, $P > 0.1$) on social investigation time.

An interaction effect between weaning age and social isolation was found (GLMM; $F_{1,34} = 8.25$, $P < 0.01$), such that D11 pigs that were socially isolated spent a significantly larger percentage of time on social investigative behaviour than non-isolated D11 ($P < 0.001$), isolated D22 ($P = 0.1$), and non-isolated D22

animals (P = 0.01). There was no interaction between isolation and familiarity factors (GLMM; $F_{1,34}$ = 0.55, P > 0.1). A tendency towards significance for an interaction between weaning and familiarity was found (GLMM; $F_{1,34}$ = 3.50, P < 0.07), where familiar groups weaned at D11 (22.46 ± 4.75) spent more time on social investigation then those weaned at D22 (10.95 ± 2.39).

There was no evidence of a tree-way interaction between the factors isolation familiarity and weaning (GLMM; $F_{1,34}$ = 0.73, P > 0.1). Interestingly, the mean values of amount of social investigation between familiar animals that were weaned at D11 and social isolated (36.98 ± 3.29) and unfamiliar animals from the same weaning and isolation treatment group (35.29 ± 4.64) seemed comparable. The bioequivalence test showed that the means are indeed equal (P = 0.8).

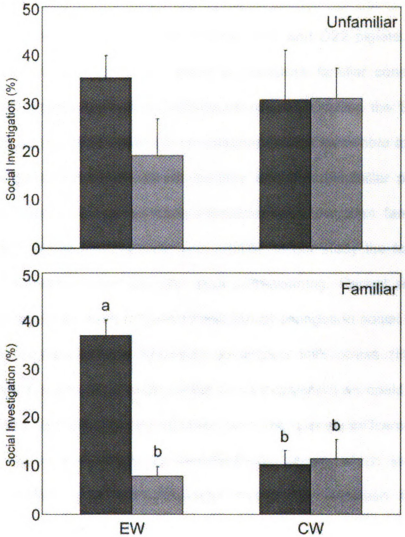

Figure 1- Effects of social isolation and weaning age on the percentage of time spent on social investigation during familiar and unfamiliar exposures in the SR test. Dark bars indicate pre-test social isolation, light bars indicate non-isolation. Values represent adjusted means ± S.E.M. Different letters indicate difference between treatments ($P < 0.001$).

4. Discussion

In this study we investigated whether D11 and D22 piglets, after being socially isolated, differed in their ability to recognise familiar conspecifics, as indicated by differences in their behavioural response during the SR test. We have previously demonstrated that non-weaned piglets were able to distinguish between familiar and unfamiliar conspecifics and that unfamiliar piglets spent significantly more time on social investigative behaviour than familiar piglets (Souza and Zanella, 2001; Souza et al., 2006). In our study the familiarisation procedure occurred during the two days post-weaning. Recent reports have shown that after weaning, piglets manifest abrupt changes in social and feeding behaviours, signs that are frequently associated with stress (Henskin and Jensen, 2000; Orgeur et al., 2001). Prior to our experiment we could not rule out the possibility that weaning-induced stress would hamper social learning ability or motivation to explore during the familiarisation period, which in turn could eliminate possible differences in social investigation between familiar and unfamiliar dyads. Although we did not design this experiment to investigate this hypothesis, the outcome of the test showed that SR learning ability during the two-day period following weaning was not impaired, in contrast with the disrupting effect of social isolation in D11 pigs only. Our results indicated that there is a significant difference in social investigation between familiar and unfamiliar conspecifics, where unfamiliar dyads spent more time on social investigation than familiar ones.

Our results demonstrated that SR memory of D22 pigs was not impaired 4 hours after the familiarisation procedure and that 15 minutes of social isolation prior to testing did not significantly affect the SR retrieval per se. Isolated D11 pigs however showed possible deficits in recognising familiar conspecifics, as indicated by a significant increase in the percentage of time spent on social investigation.

It could be argued that after social isolation the animal was more motivated to socially investigate (see Marin et al., 2001), irrespective of whether the subject recognized an individual or not. If so, we would expect to find an increase in social investigation in animals weaned at D11 and D22 groups. However, as noted only D11 animals were affected by social isolation, and only when exposed to a familiar conspecific. Unfortunately, no conclusive remarks can be made but it is clear that the weaning treatment had a different impact on the social investigation time of familiar dyads which was age dependent. The increase in social investigation may also be a result of altered general behavioural activity caused by the social isolation rather than a deficit in recognising familiar conspecifics. Rearing rats in social isolation post weaning has been shown to significantly increase general locomotor activity (Heidbreder et al., 2000; Paulus et al., 2000), whereas repeated social isolation of piglets during the first days of life caused a decrease in their behavioural activity (Kanitz et al., 2004). However, social isolation had no effect on general behavioural activity regardless of weaning age, in contrast with findings by Kanitz et al.

(2004) and disproving the argument that the decrease in social investigation was a side-effect of general behavioural inactivity.

Interestingly, the amount of social investigation of isolated D11 piglets was comparable to the amounts found when unfamiliar animals were tested. These results strengthen the hypothesis that SR in D11 pigs, but not in D22 pigs, is susceptible to interference by social isolation prior to testing and that the difference in social investigation found in the present study is likely to reflect impairments in short-term SR. From now on we will explore the possibility that weaning at D11 elicits deficits on the ability of neonatal pigs to recognize familiar conspecifics.

Post-learning administration of GCs produces a dose-dependent effect on memory consolidation and retrieval. Acute administration of low doses of GCs post-learning enhances memory consolidation (Pugh et al., 1997; Roozendaal, 1999), but performance is impaired if the subjects are tested under high circulating GC levels (Kirschbaum et al., 1996; Diamond et al., 1999; Kim and Diamond, 2002). High GC levels shortly before testing impair retrieval of previously learned information (de Quervain et al., 1998). In our study, although the acute stressor was applied after the familiarisation (i.e. after acquisition and retention), it did not significantly interfere with SR memory retrieval in D22 pigs, but it negatively affected the ability of D11 pigs to recognise familiar conspecifics. It is unlikely that the impairment in SR was caused by differences in the increase of GC levels; i.e. EW leads to a higher sensitivity of the HPA-axis and an increased glucocorticoid response to social isolation, since Laughlin and Zanella

(2002) found no differences between EW and CW pigs in HPA-reactivity using a similar stress paradigm. To investigate possible mechanisms through which weaning and/or acute social isolation may affect gene expression in the brain of female piglets an experiment was conducted in our lab by Poletto et al. (2005). Their results indicated that early weaning suppressed mRNA expression of stress-related hippocampal genes, while no changes were observed in conventionally-weaned piglets. It was suggested that the decrease in GR mRNA in those younger animals is an indication of difficulties in reacting to increases of GC levels. These deficits in SR memory are therefore possibly caused by a higher sensitivity of EW animals to an increase in GC levels induced by the acute social stressor.

Dean and Mattews (1999), studying the immature brain of guinea-pigs, found that specifically during the brain growth spurt that occurs at 48-52 days of gestation, increases in GCs resulted in significant changes in mRNA expression of GC receptors in the hippocampus of females. These alterations may influence hippocampal function, which could affect cognition under stress conditions. Whether these changes are transitory or permanent is still unknown. In pigs, the brain is still in development during the first few weeks after birth (Brust et al., 2004). Pond et al. (2000) studied normal growth patterns of the pig brain and found that the developmental peak occurs at two weeks of age. This period of rapid growth is associated with glial cell multiplication, dendritic growth, and synaptic connectivity (Dobbing, 1974). Interestingly, these first two weeks also coincide with a time when the circulating cortisol levels are naturally low (Carroll

et al., 1998; Daniel et al., 1999). It is therefore possible that by weaning pigs as early as 11 days of age the animals are exposed to weaning-induced GC elevations at a time when the brain is in a critical period of development, which, in line with the findings in guinea pigs, may alter GC receptor levels, subsequently increasing the animal' s sensitivity to an acute stressor.

5. Conclusions

Weaning at D11 and social isolation do not impair SR per se, but socially isolated D11 pigs show impairments in the ability to discriminate familiar from unfamiliar conspecifics as indicated by increases in social investigation of familiar animals to amounts comparable to those found in unfamiliar dyads. The SR deficits after social isolation may indicate possible changes in sensitivity to stress (hormones) in D11 pigs and this altered sensitivity to acute stress may be caused by the age at which the developing brain is exposed to increases in stress hormones. Further research is necessary to investigate possible mechanisms through which D11 may affect both brain normal development and stress sensitivity. From a welfare point of view, it should be further investigated whether the increased stress sensitivity is long lasting or if it will compromise pig welfare throughout life.

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CHAPTER 6

GENERAL DISCUSSION

The primary goal of this thesis was to investigate the effects of acute stressors on social recognition (SR) in neonatal pigs in order to determine whether impairments in the SR process could be responsible for the increased post-mixing aggression in early-weaned pigs that was previously reported by our laboratory (Yuan et al., 2004). Because the animals were tested at a very young age (starting at 11 days of age), experimental approaches based on a spontaneous behavioral response, i.e. the amount of social investigation, were chosen, since this does not require extensive training of the animals.

To achieve the main goal, two protocols for assessing SR were tested (chapter 2 and 3). The novel SR paradigm proposed in chapter 3 proved to be appropriate for use in neonatal pigs and was therefore further used to investigate possible interference of an acute stressor on SR of early-weaned (EW) and conventionally-weaned (CW) piglets. If weaning at a very young age has a negative impact on social recognition abilities, it may also lead to deficits in the formation and maintenance of stability in social groups.

In this chapter, the results are briefly described and compared to other results generated in our lab. Subsequently, based on these results, interesting future studies are presented and concluding remarks are drawn.

1. Summary of the results

In the first study (chapter 2), the 'habituation-dishabituation' paradigm was used to assess SR in neonatal pigs. As this method was primarily designed for adult rodents, special attention was given to the acclimatization of the animals to

the apparatus and procedures so that the method would inflict minimal disturbance of the animals. The goal was to examine whether acute increases of cortisol impair short-term SR in 11-day old pigs. The results showed that while an intra-muscular hydrocortisone injection administered 15 minutes prior to testing significantly increased salivary cortisol levels, it did not interfere with the ability of the animals to acquire and retrieve information about a male stimulus pig during successive exposures. Although the results indicated that this paradigm can be further used in pigs (as the subjects were successfully habituated to the stimulus), the protocol caused considerable distress to the experimental animals that could possibly compromise the outcome of the main study focusing on interference of acute stress on SR. If conditions of testing impose stress or fear to the animals it may lead to disregulation of behavioral and cognitive functions and misleading results, aside from the ethical implications for the animals (pigs: Mendl et al., 1997; chicks: Marin et al., 2001; cattle: Grandin et al., 1994; rodents: Kogan et al., 2000). In addition, exogenous GCs did not affect SR as tested in this paradigm. We therefore decided that in future studies, a more natural stressor, such as social isolation, should be used. This was, however, not further investigated, since Laughlin and Zanella (2003) had already shown that 15 minutes of social isolation increased salivary cortisol to levels hampering spatial memory in early weaned piglets.

In the second study (chapter 3), a novel protocol to test SR in pigs was presented. The major accomplishments of this study were that fighting during the familiarization (habituation) phase was prevented, thus reducing distress of the

animals, and that this familiarization procedure resulted in clear differences in time spent on social investigation between familiar and unfamiliar pairs during the testing phase, indicating that neonatal pigs were able to retrieve information about familiar conspecifics up to 4 hours after the familiarization. Additionally, relocation of the animals to similar farrowing crates, between the familiarization and testing phase did not affect SR test in terms of time spent on social investigation. This test therefore proved to be more appropriate for assessing the effects of an acute stressor on SR abilities in neonatal pigs that was the aim of this thesis.

Using the familiarization procedure described in the previous study, another study was designed to assess whether the novel protocol could be used to assess a more robust SR in 20-day-old female pigs (chapter 4). In this experiment, the subjects were testwed at 4 and 24 hours after the last familiarization procedure. The results revealed that the familiarization protocol enabled the animals to successfully remember familiar conspecifics for at least 24 hours after familiarization. This finding is very interesting, since it will allow future studies to further elucidate memory processes in young pigs by using pharmacological agents or various disturbances at different stages of the memory process. In addition, it will also allow for more in depth testing of a relation between familiarization and later aggression, since animals can potentially be familiarized at a very young age and mixed at much later ages (e.g. D'Eath et al., 2005).

Chapter 5 then presented a study investigating whether weaning age (EW at 11d and CW at 21d) and/or acute social isolation (15 minutes) interfered with the ability of female piglets to remember familiar conspecifics. During interactions with familiar animals, isolated EW pigs investigated the conspecific more than non-isolated EW and all CW pigs. Interestingly, the amount of social investigation displayed by familiar isolated EW pigs was similar to that displayed by unfamiliar animals. There was no sign of differences in general locomotion between treatment groups. These findings therefore provide strong evidence that acute social isolation impaired SR ability of EW pigs, providing a second potential cause of the increase in post-mixing aggression in EW pigs reported by Yuan et al. (2004).

The results of all studies have been described and discussed in previous chapters, and at the end of this thesis, I will only aim to integrate these findings with the results of other studies done in our lab. I have worked with other members of the group towards providing a clearer picture of the early weaning process and its effects on young pigs, but do all the results fit or do they contradict? By discussing these findings, I intend to clarify the results of the initial USDA/NRI grant (# 2001-35204-10810), form new ideas on underlying mechanisms and propose hypotheses and future studies aimed at testing these ideas.

2. Stress susceptibility in early-weaned pigs

As previously mentioned in chapter 1, the increasing intensification of husbandry systems has massively changed the natural course of the weaning process for a piglet. In commercial farms, artificial weaning of piglets involves simultaneous separation from both the nutrient supply and social contact with the sow, and it occurs at much younger ages and much more abrupt than in more natural environments.

Modern pig production systems have developed early weaning systems in which pigs are weaned before 21 days of age. It is, therefore, important to integrate the scientific knowledge on the effects of early weaning on different biological functions (neurophysiological, behavioral, and cognitive aspects) of the animal, as was done in our lab. By combining these results, the impact of early weaning for the animal will become clearer, and general hypotheses on the underlying mechanisms, as well as some future studies to test these hypotheses, can be provided.

The research project aimed at investigating how early weaning, as a model for early stress, affected post-mixing aggression, cognitive functions (social and spatial learning), and brain processes (gene expression). The pioneer study was conducted by Yuan et al. (2004), who found that EW pigs fought longer and initiated more fights that they did not subsequently win than CW animals, when they were regrouped at 9 weeks of age. Initially, this was presented as a welfare concern, since pigs at this age are able to inflict serious injuries during fighting, but with the development of a porcine brain library (Nobis

et al., 2003), more central processes could be studied that would be important for both animal welfare and fundamental knowledge on central stress physiology.

Based on Yuan et al.'s (2004) hypothesis, we aimed at investigating whether early weaning would alter stress sensitivity in pigs and how this would reflect in both behavior/cognition and brain physiology. If EW animals were more susceptible to the harmful effects of an acute stressor, this could be tested with cognitive tasks. To this end, the Morris Water Maze (MWM) and the habituation-dishabituation paradigms were adapted for use in neonatal pigs. A study conducted by Laughlin and Zanella (2003) indicated that social isolation of EW pigs impaired their performance in the MWM task at 12 and 23 days of age. Interestingly, this impairment was only observed in EW pigs that were socially isolated 15 minutes prior to testing, whereas performance was not affected in non-isolated EW animals or isolated CW pigs. Furthermore, no differences in the cortisol response to isolation were found, indicating that the differences could not be explained by alterations in HPA-axis functioning, but may instead be the result of changes in central sensitivity to GCs. Since the MWM task is a known hippocampus-dependent learning task, the hippocampus was indicated as a likely candidate for these changes in central sensitivity.

As was presented in this thesis, the results of the SR are in agreement with those of the MWM task. Again, since the hippocampus seems to be involved in SR processes, more support was obtained for a role of the hippocampus in the adverse effects of early weaning on pig behavior. Furthermore, these results provided direct support for the hypothesis that the increase in post-mixing fights

reported by Yuan et al. (2004) was possibly caused by SR deficits in acutely stressed EW animals. More specifically, difficulties in recognizing or at least distinguishing stronger opponents seemed to support their finding that EW animals initiated more fights that they ended up not winning.

Thus, now two cognitive tasks in which the hippocampus might play a role indicated that early weaning in combination with acute stress hampered performance. To investigate possible mechanisms through which weaning and/or acute social isolation may affect gene expression in the brain of female piglets an experiment was conducted by Poletto et al. (2005). Their results indicated that early weaning suppressed mRNA expression of stress-related hippocampal genes, while no changes were observed in conventionally-weaned piglets. It was suggested that the decrease in GR mRNA in early weaned animals is an indication of difficulties in reacting to increases of GC levels. This hypothesis was further explored in our lab in an in situ hybridization study that explored cortisol receptor mRNA in the hippocampus. The hippocampus expresses both MRs and GRs (Veldhuis et al., 1982). Hippocampal MRs bind GCs with a 10-fold higher affinity than GRs (Reul and De Kloet., 1985). As a result, MRs are extensively occupied under basal resting conditions, while substantial GR occupation occurs in circadian peak and following stress (de Kloet et al., 1993). A theory of balance of these receptors was developed by de Kloet, which states that an imbalance in MR- and GR-mediated effects would alter individual-specific vulnerability to stress (de Kloet, 1991). In other words, the ratio of MR:GR in the hippocampus may determine sensitivity to GCs. By subjecting hippocampal slices of early-

weaned and conventionally-weaned piglets to in situ hybridization for GR and MR mRNA, the MR:GR ratio could be calculated. Unfortunately, methodological difficulties have so far hindered this experiment, but the question may in the future be addressed by immunocytochemistry.

Altogether, the experiments on the effects of early weaning on post-mixing aggression, spatial learning, SR, and brain gene expression indicated a possible higher susceptibility of EW pigs to acute stressors (i.e. mixing, social isolation), possibly due to a shift in receptor ratio that alters the optimal GC concentrations for cognitive functioning (as theorized on in the inverted-U-shape models).

Future directions for further exploring the early weaning process seem to be directed in two ways. Firstly, while we have provided a mechanism that might explain why 50-day-old early weaned pigs initiate more fights that they end up not winning, we have not actually tested whether the effect of an acute stressor on the cognitive ability of pigs is actually altered at this age. It would therefore be of interest to further adapt the social recognition protocol for use in 50-day-old animals and test early- and conventionally weaned animals at this age. By familiarizing the animals at a younger age, it may even be investigated whether an acute stressor affects both a relatively short (4h) and long (24h) memory, or whether only short-term memory is affected.

Secondly, while we have hypothesized a neurophysiological mechanism responsible for the cognitive impairments in early-weaned pigs, and have provided a basis for the disruption of this mechanism, we have not actually tested it. The study carried out by Poletto et al. (2005) suggests that early weaning may

cause changes in the brain, but it did not provide a specific location, let alone a biological mechanism. These are issues that should be addressed before any concluding remarks on the effects of early weaning on the brain can be made.

Although the development of a novel SR protocol was only a step towards answering a research question, the results offer very interesting possibilities. In an applied setting, it would be interesting to see whether the familiarization protocol as presented in this thesis would be able to reduce post-mixing aggression at later ages. This is a study that has already been carried out in our lab (Souza, Siegford, Jansen and Zanella, in preparation), but unfortunately could not be presented in this thesis.

In a more fundamental setting, the SR test allows for testing the effects of natural and pharmacological disturbance of various stages of memory formation and allows further testing of the importance of different sensory cues for memory formation. By administering drugs prior to familiarization, the effect of that drug on acquisition can be tested, while isolating the animal immediately after familiarization allows investigating the role of stress on memory consolidation. Furthermore, by replacing the flexible netting with a plastic see-through plate or an opaque plastic barrier with small holes, we could test whether visual or olfactory cues are more important for SR in pigs. Small adaptations in the SR testing protocol may further clarify SR abilities of young piglets; since the familiarization was done between litters, and testing between individuals, it cannot be concluded that the animals recognized one another based on individual cue or on cues shared by its litter litter. If during testing, the subject

would be exposed to an unfamiliar animal from a familiar litter (by, for instance, only subjecting half the litter to Fam1), this question could be answered.

3. Concluding remarks

In conclusion, this thesis indicates that:

1. Short-term SR of neonatal pigs can be assessed using the habituation-dishabituation technique.
2. A relatively short- and long-term SR of neonatal pigs can be assessed with minimal disturbance by using a newly-developed protocol.
3. Early and conventional weaning do not impair the ability of pigs to recognize familiar conspecifics per se, nor does acute social isolation by itself impair the ability of newly weaned pigs to recognize conspecifics.
4. Acute social isolation may hamper the ability of female EW pigs to retrieve information about familiar conspecifics, but does not affect SR in CW pigs.

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CHAPTER 1

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CHAPTER 6

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