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Prenatal stress produces anxiety prone female offspring and impaired maternal behaviour in the domestic pig.

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Abstract

Numerous studies have shown that prenatal stress (PNS) can have profound effects on postnatal well-being. Here, the domestic pig (*Sus scrofa*) was used to investigate PNS effects owing to the direct relevance for farm animal welfare and the developing status of the pig as a large animal model in translational research. Pregnant primiparous sows were exposed, in mid-gestation, to either a social stressor (mixing with unfamiliar conspecifics) or were kept in stable social groups. The ratio of levels of mRNAs for corticotropin releasing hormone (CRH) receptors 1 and 2 in the amygdala, measured for the first time in the pig, was substantially increased in 10-week-old female, but not male, PNS progeny indicating a neurobiological propensity for anxiety-related behaviour. Mature female offspring were observed at parturition in either a behaviourally restrictive crate or open pen. Such PNS sows showed abnormal maternal behaviour in either environment, following the birth of their first piglet. They spent more time lying ventrally, more time standing and showed a higher frequency of posture changes. They were also more reactive towards their piglets, and spent longer visually attending to their piglets compared to controls. Associated with this abnormal maternal care, piglet mortality was increased in the open pen environment, where protection for piglets is reduced. Overall, these data indicate that PNS females have their brain development shifted towards a pro-anxiety phenotype and that PNS can be causally related to subsequent impaired maternal behaviour in adult female offspring.

Keywords: amygdala; CRH receptors; domestic pig; maternal behavior; prenatal stress.

1. Introduction

It is increasingly evident that early life events can result in long-term changes in biological function. Interest in early life effects has been stimulated by human epidemiological evidence indicating the important influence that the fetal environment can exert on disease susceptibility in later life [1]. In particular, stress experienced by pregnant mothers has been shown to have wide-ranging and important effects on their offspring's later physiology and behaviour. Prenatal effects may occur due to pathological alterations to normal development, or represent instances where fetal biological adjustments to cope with a challenge have long-term effects [2].

Alternatively, in some instances such effects may have an adaptive basis but may produce maladaptive outcomes when there is a mismatch between the predicted environment and the reality of the actual postnatal environment [3]. This may be particularly true for captive animals where the environment experienced during postnatal life is often highly artificial. Moreover, some data indicate that domestication may have resulted in increased sensitivity to prenatal effects [4, 5].

Overall, the implication for captive domesticated species is that variation in the conditions for development provided by the reproductive tract or egg, for instance by altered nutritional supply or hormonal milieu, may explain a large degree of variation in many aspects of biology, some of which may impact on health and welfare [6].

Indeed, research across a wide range of farmed species has shown negative impacts of prenatal stress [7]. Prenatal stress studies in pigs have also shown many diverse outcomes in the offspring of stressed mothers [8, 9, 10, 11, 12, 13, 14, 15]. There is clear evidence that aspects of stress physiology can be affected in the offspring of stressed mothers. For example, social stress experienced by primiparous sows results in a state of stress hyper-reactivity in the female offspring, with disturbed behaviours,

increased corticotropin-releasing hormone (CRH) mRNA expression in the amygdala (a key brain region involved in mediating behavioural responses to stress, including anxiety and fear responses [16]) and in the paraventricular nucleus (PVN) of the hypothalamus (the brain region that mediates the neuroendocrine response to stress [17]) [10]. Haussmann and colleagues [8] also found evidence of increased stress reactivity in prenatally stressed pigs. However, other studies [9, 11, 12, 13] have found no such effect. These differences may reflect different animal genotypes, maternal stress models, offspring stressors, outcomes measurements and animal ages.

CRH receptor-1 (CRH-R1) and -2 (CRH-R2) in the amygdala play a critical, and largely opposing, role in regulating emotionality and stress responses in vertebrate species [18, 19]. Broadly, activation of CRH-R1 by its principal ligand CRH increases behavioural indications of fear/anxiety and physiological stress responding, whilst activation of CRH-R2 by its main ligands (urocortins II and III) has the opposite effect [19]. Here we investigated the hypothesis that an altered balance in the relative expression of mRNAs for CRH-R1 and CRH-R2 in the amygdala, measured for the first time in the pig, may underlie the prenatal stress phenotype seen previously [10]. We also investigated the separate and interacting effects on these measures of a postnatal painful challenge, using tail-docking, which is a common commercial practice within pig farming.

We furthermore sought to examine the hypotheses that prenatal stress (PNS) exposure would impact adversely on subsequent maternal behaviour in the adult female offspring and that this effect would be more substantial in a restrictive environment. Maternal behaviour was a particular focus of the study given our previous findings indicating that prenatal stress increases the likelihood of primiparous sows showing piglet-directed aggression [10] and rodent studies

demonstrating impaired maternal behaviour in females exposed to prenatal stress [20]. Also, the pig has been suggested as a possible large animal model for harmful human maternal behaviour [21, 22] and the role of early life experiences in later maternal behaviour deserves consideration. This is particularly important as social stress during pregnancy represents a relevant paradigm for comparison with the experiences of human females [23].

2. Materials and methods

2.1 Experimental over-view

The work detailed here consisted of two phases (Figure 1). Phase one involved pregnant sows being exposed to a social stressor during pregnancy. In phase two, individual male and female prenatally stressed and control offspring were euthanized, at around nine weeks of age, and brain sections were collected for measurement of CRH-R1 and CRH-R2 mRNA levels in the amygdala. In the second stage of phase two, female offspring from PNS or control litters were kept to maturity and observed when they themselves gave birth. Other data from this experiment have been reported separately [14].

All work was carried out in compliance with EC Directive 86/609/EEC, under UK Home office licence where appropriate, and following ethical approval by the Animal Experiments Committee at Scotland's Rural College (SRUC).

2.2 Phase 1: Prenatal stress treatment

Thirty-six primiparous sows (Pig Improvement Company (PIC), Camborough-23) were kept in groups of six under normal commercial sow housing conditions.

Gestation pens consisted of six individual feeder spaces (0.5m wide, 1.8m long), a passageway (3.6m by 1.95m), and a bed area of straw covered concrete (3.6m by 2.5m). Straw was replenished as necessary. Sows were fed once a day (~2.2Kg, pelleted standard sow diet) at 07:30h and had free access to drinking water. Sows were artificially inseminated (PIC, GP1020 Large White semen) and oestrus was not artificially synchronized within a group. Sow age and weight at insemination were balanced across treatment groups and there was no difference between treatments in these measures. Successive groups of six sows were inseminated at approximately monthly intervals, apart from between groups four and five (see below) when there was an interval of two months. All sows were pregnancy checked via ultrasound, 32 to 37 days after insemination. Of the 36 sows that were inseminated, 27 became pregnant (16 pre-allocated to stress treatment, 11 pre-allocated to control treatment). To avoid altering group social dynamics the two non-pregnant sows in the stressed group were kept in their original assigned groups and were exposed to the mixing treatment (described below) along with pregnant group-mates. Non-pregnant controls were also kept in their initial groups throughout their group-mates' gestation period for the same reason.

Eighteen of the sows (three groups of six, including two non-pregnant pigs) were exposed to a social stress treatment (Fig. 1). For this, each socially stressed group of six was split into sub-groups of three, each of which was mixed with three older multiparous sows, for two separate 7-day periods in the second third of gestation. Social stress treatment sows were exposed to different groups of older sows during each 7-day mix period. The social mixing procedure has previously been shown to produce an increase in sow salivary cortisol concentration [14]. Mixed sows also showed decreased growth rate over the mix period, an increased count of skin lesions

(an indicator of aggression) and behavioural signs of submission [14]. As oestrous cycles were not artificially synchronised, social mix dates for individuals varied but all mixes took place in the second third of gestation. For the period between the two social mixes, and after the end of the second mix the sub-groups of three were returned to their original group of six. Five days prior to their expected parturition dates the 27 sows were moved to standard individual parturition crates (2.25m x 0.45m x 1.05m), provisioned with straw and wood shavings.

2.3 Phase 2: Progeny housing and measures/observations

Litter size and piglet birth weight did not differ between the mixed and control treatments [14] (litter size (Mean \pm SEM): mixed = 12.1 \pm 0.8, control = 11.5 \pm 1.0; birth weight (Mean \pm SEM, Kg): mixed = 1.45 \pm 0.04, control = 1.49 \pm 0.07). All piglets were left with their own mother, and there was no cross-fostering, castration or teeth resection of piglets. As part of normal husbandry, all piglets received oral iron supplementation on postpartum day one, when they were also weighed and ear-tagged. At three days of age all piglets within any one litter were tail-docked or sham-docked (balanced across prenatal treatment) without provision of any anaesthesia or analgesia, as in normal farming practice. For tail- or sham-docking, piglets were removed one at a time from the sow and placed in a plastic box (48 x 64 cm) in the same room. Tail-docked piglets had approximately half of their tail cut off using a pair of clean surgical cutters. Sham-docked piglets were similarly handled but did not have their tail cut. Both docked and sham handled piglets were left in the box for one-minute after docking for recording of their behavioural reactions. Details of the behavioural responses to tail-docking in these piglets have been published separately [14]. Records were kept of all piglets that died during the pre-weaning period. Piglets

were kept in the parturition crate environment until weaning at around 28 days of age (Mean±SEM, age in days at weaning: 28.3±0.4), at which point the litters were reduced to 8 piglets (or left intact for litters with 8 or fewer piglets) and moved to pens (2.85m x 1.85m) with straw bedding. All pigs had *ad libitum* access to food and water from weaning onwards.

2.4 Phase 2: Measurement of CRH-R1 and CRH-R2 mRNA in the amygdala

At around nine weeks of age (Mean±SEM, age in days at euthanasia: 65.8±0.5) one male and one female pig were selected at random from each litter and euthanized for quantification, by *in situ* hybridisation (ISH), of mRNAs for CRH-R1 and CRH-R2 in the amygdala. Pigs were given a sedative injection (5mg/kg Ketamine hydrochloride, 2mg/kg Azaperone, i.m.) in their home pen and were then moved to a quiet isolation area, where they were given an i.v. lethal overdose of pentobarbital sodium (Euthatal). Following confirmation of death, brain tissue was collected. Brains were blocked, frozen on dry ice and stored at -80°C until subsequent ISH. For this, tissue blocks containing amygdala (unilateral) were sectioned coronally on a cryostat at 15µm and thaw-mounted onto Polysine® slides. For each probe, the brains from the four treatment groups were processed in the same hybridisation reaction; however, owing to the large number of slides and the practical limit on number that can be processed together, tissue from male and female pigs were processed separately. ³⁵S-UTP labelled cRNA sense and antisense probes were synthesised from the linearised pBluescript II-SK vector expressing a 1.3Kb cDNA fragment from the coding region of rat CRH-R1[24] (generously provided by Dr. Nicholas Justice and Prof. Wylie Vale, The Salk Institute, La Jolla, California, USA). The plasmid was linearised with HindIII and BamHI, and transcribed using T3 and T7 polymerase (Promega UK Ltd.,

Southampton, UK), for the sense and antisense riboprobes, respectively. To detect CRH-R2mRNA, ³⁵S-UTP labelled sense and antisense riboprobes were generated from the linearised pBluescript-SK vector expressing a 1.0Kb cDNA fragment encoding rat CRH receptor type 2 [25] (provided by Dr. Nicholas Justice and Prof. Wylie Vale, The Salk Institute, La Jolla, California, USA). The plasmid was linearised with XbaI and HindIII, and sense and antisense cRNAs incorporating ³⁵S-UTP were transcribed from the T7 and T3 promoters, respectively. ISH was performed as previously described in detail elsewhere [26]. For both probes, sections were hybridised at 57°C for 18-19h. Sections of rat and pig pituitary gland treated as above were included as positive controls. Some brain and pituitary sections from rat and pig were hybridised with ³⁵S-UTP labelled cRNA sense probes to serve as negative controls and to ensure probe specificity. The hybridisation signal over tissue hybridised with the sense probe was not different from background. Following hybridisation the slides were rinsed in 2X saline sodium citrate (SSC) and then incubated with RNase A (15µg/ml) for 60 minutes at 37°C. Sections were then rinsed in 2X SSC at room temperature before three stringent 60minute washes in 0.1X SSC at 60°C. Next, tissue was dehydrated, air-dried, dipped in autoradiographic emulsion (Ilford K5, Knutsford, Cheshire, UK) and exposed at 4°C for four weeks. Slides were developed (Kodak D-19, Sigma), fixed (Hypam rapid fixer, Ilford, Knutsford, Cheshire, UK) and counterstained with haematoxylin and eosin. Anatomical identification of brain structures was based on the stereotaxic atlas of the pig brain [27], and of the morphology of neurons in chosen structures of the pig amygdala, as described during development [28]. Autoradiographs were photographed using an optical microscope with digital camera at magnification X20 (objective) and X5 (objective). For each probe (i.e. CRH-R1 and CRH-R2), the number of silver grains

were automatically counted in six separate regions of the amygdala (Figure 2) from two sections/pig in digitized images of the sections and the silver grain density per area was measured within the regions of interest using a computer-aided image analysis system with Microimage™ Image Analysis software (version 4.0 for Windows, Olympus, USA). For each individual pig the arithmetic average of all measurements was calculated, which was then subjected to statistical analysis. For all ISH measurements average values (Integrated Optical Density: IOD) per pig were used to calculate group means \pm SEM.

2.5 Phase 2: Housing and Behavioural Observations at Parturition

Remaining offspring were kept in their pens until approximately ten weeks of age when two females from each litter were selected (at random, apart from selection by good health and similar size) for the second part of the study. These females (n=50) were mixed into new groups of four or six pigs (from the same prenatal treatment group) and kept in these groups from this point onwards. As they approached reproductive maturity these groups were moved to sow accommodation, where they were subsequently artificially inseminated. Due to problems with lameness during the rearing period, six pigs were removed from the study, so only 44 pigs were inseminated. Of these, 38 pigs (16 littermate pairs and 6 singles) became pregnant and were used for parturition observations.

Approximately five days prior to their predicted parturition date individual sows (daughters of the original stressed or control females) were moved to their assigned housing. As a consequence of the reduced sample size (see above) the number of pigs allocated to different treatments was uneven. Females were allocated to give birth in either a standard crate (as above) (PNS: n=14; CONTROL: n=7) or open pen (3m x

2.23m) (PNS: n=13; CONTROL: n=4). The 16 pairs of females from each litter were split such that one gave birth in a pen and one gave birth in a crate. There was no difference in age (Mean±SEM: 410±20 days) or body weight (Mean±SEM: 249.1±17.9kg) at parturition between control and PNS females or between pigs allocated to crates or pens. Video recordings of sow behaviour were made continuously in the lead up to parturition and for 24h after the start of parturition. Behavioural observations (Table 1 shows ethogram) were subsequently carried out on these video recordings for the 24h before and after the birth of the first piglet. Observers were blind to sow treatment. Continuous observations of sow postures and behaviours were made during these periods. More detailed observations on sow behaviours directed towards their piglets were made for the first six hours following the birth of the first piglet.

2.6 Statistical analysis

Analysis was carried out using Residual Maximum Likelihood (REML) in Genstat (11th Edition, VSN International Ltd, Hemel Hempstead, U.K.). All data were checked for normality prior to analysis. For analysis of CRH receptor mRNA data potential confounding factors (pig weight, pig age, and litter size) were examined in initial fixed effect models and if found to be non-significant were discarded. Male and female data were analysed separately, and not statistically compared, as the ISH procedure for each sex was carried out separately. Litter was fitted as a random effect, prenatal stress history and tail status (docked or intact) were fitted as fixed effects. Initial models examined all possible interactions but interaction terms were removed if non-significant.

For analysis of maternal behaviour, stress status and environment were fitted as fixed effects, and group (i.e. pen) and maternal identity were fitted as random effects. The frequency of attacks directed towards piglets was not normally distributed so was analysed (with the same model structure) via a Generalized Linear Mixed Model, with a Poisson distribution and a logarithmic link function. Data are presented, in the text and Tables, as adjusted means for the statistical models from REML with standard error of difference (SED) values. To control for variable levels of piglet approach to the sow's head (i.e. between pen and crate due to differences in space) a responsiveness index [29] was calculated, where responsiveness = (response – no response) / (response + no response). This produced a value (for each sow) that varied between 1 and -1, where 1 indicated that the sow always responded to piglet approaches and -1 indicated that the sow never responded to piglet approaches. Principal Components Analysis (PCA; Genstat) was applied to the post-parturition behaviour (observations from 36 mothers) using a correlation matrix approach. Interpretation was limited to (unrotated) components with an Eigen value above 2 and component loadings greater than 0.4.

3. Results

3.1 Phase 1: Amygdala CRH receptor mRNA expression

As the level of CRH-R2 mRNA expression was positively related to age (Females: $p=0.028$; Males: $p=0.068$), pig age at death was fitted as a co-variate (confounding factor) for CRH-R2 mRNA expression. Other possible confounding factors were found to be non-significant and were excluded from the final statistical models.

3.1.1 Effects of prenatal stress

CRH-R1 mRNA expression was greater in females that had been exposed to PNS compared with controls (IOD: PNS=1.462, CON=1.081, SED=0.136, Wald=7.83, $p=0.01$; Figure 2, Figure 3a). There was no significant difference in CRH-R2 mRNA between the control and PNS females (IOD: PNS=0.880, CON=0.905, SED=0.047, Wald=0.27, $p=0.61$; Figure 3b). However the CRH-R1: CRH-R2 mRNA ratio was significantly greater in PNS females compared with control females (PNS=1.664, CON=1.192, SED=0.1355, Wald=12.29, $p=0.002$; Figure 3c). Neither the expression of CRH-R1 (Integrated Optical Density (IOD): PNS=1.879, CON=2.053, SED=0.218, Wald=0.64, $p=0.43$) nor CRH-R2 mRNA (IOD: PNS=0.592, CON=0.611, SED=0.025, Wald=0.58 $p=0.45$), nor their ratio (PNS=3.206, CON=3.392, SED=0.363, Wald=0.26, $p=0.61$) was significantly affected by PNS in males.

3.1.2. Effects of tail-docking

Tail docking had some minor, but significant, effects on receptor mRNA expression in the amygdala in both the males and females. Docking increased CRH-R1 mRNA (IOD: DOCKED=1.458, INTACT=1.084, SED=0.134, Wald=7.83, $p=0.01$) and CRH-R2 mRNA expression (IOD: DOCKED=0.950, INTACT=0.835, SED=0.048, Wald=5.71, $p=0.026$) in females, but did not significantly affect the ratio between the two receptors (DOCKED=1.523, INTACT=1.333, SED=0.132, Wald=2.08, $p=0.16$). Docking increased CRH-R2 mRNA (IOD: DOCKED=0.632, INTACT=0.571, SED=0.025, Wald=5.86, $p=0.024$) expression in males, but did not have any significant effect on CRH-R1 mRNA (IOD: DOCKED=1.847, INTACT=2.084, SED=0.215, Wald=1.21, $p=0.28$) or the ratio of the two receptors (DOCKED=2.941, INTACT=3.657, SED=0.357, Wald=4.02, $p=0.06$). There were no significant

interactions between prenatal stress and docking status for either CRH-R1 mRNA (Females: Wald = 1.30, p=0.27; Males: Wald = 0.09, p=0.77) or CRH-R2 mRNA expression (Females: Wald=1.5, p=0.24; Males: Wald =1.34, p=0.26) or their ratio (Females: Wald=3.20, p=0.09; Males: Wald =0.70, p=0.41).

3.2 Phase 2: Peri-parturient sow behaviour, litter characteristics, and piglet mortality

3.2.1 Pre-parturition behaviour

In the 24h prior to the birth of the first piglet, behaviour was significantly affected by environment but not by prenatal stress (Table 2). Sows in pens showed more fixture/substrate-directed behaviour than those housed in crates and also spent more time standing and less time lying during the pre-parturition period compared to crated animals. However, sows in crates showed more posture changes.

3.2.2 Post-parturition behaviour

In the 24h after the birth of the first piglet, although there was no overall difference in lying time, PNS sows spent more time ventral lying than control sows. PNS sows were also more restless, showing an increased frequency of posture changes (Table 2). PNS sows spent more time focussing attention to their piglets and were also more likely to react when piglets approached their head. However, treatment did not significantly affect how often sows attacked their piglets. Maternal PNS did not significantly impact on how likely piglets were to approach their mother's head. Environment did not significantly impact on the piglet focussed behaviours or piglet approach response even though piglets were more likely to approach the sow's head

in the pen. Sows in pens also spent more time lying ventrally, less time lying laterally, stood up for longer and changed posture more often.

A PCA of all behavioural measures taken in the post-parturition observations found two dimensions with Eigen values greater than 2 that accounted for 46% and 26% of the total variance respectively (Table 3). The first dimension can be interpreted as udder accessibility and relates largely to maternal posture varying from high levels of lateral lying, to alternatively ventral lying or standing, and to a lesser extent restlessness and focussing attention to piglets. The second dimension related to behaviour towards piglets (focussing attention towards the floor/fixtures versus attacking piglets and having a high piglet response index). Both prenatal stress (Wald=12.28, $p<0.001$) and type of environment (Wald=22.21, $p<0.001$) affected the udder accessibility dimension. The second, piglet-directed, behaviour dimension was affected by prenatal stress (Wald=5.29, $p=0.039$) but not by environment (Wald=0.14, $p=0.72$) (Figure 4).

3.2.3 Piglet mortality

There was an interaction between maternal prenatal stress and parturition environment (Wald=4.69, $p=0.038$) in total piglet pre-weaning mortality: within the control litters mortality did not differ between pen and crate (Mean \pm SEM: PEN = 10.8 \pm 2.4%; CRATE = 14.9 \pm 6.6%), however, within litters from PNS sows mortality was greatly increased in the pen environment compared to the crate (Mean \pm SEM: PEN = 32.0 \pm 6.4%, CRATE = 11.0 \pm 2.6%). There was no significant effect of maternal mixing stress on gestation length, duration of parturition, litter size, piglet birth weight or litter sex ratio (Table 2).

4. Discussion

An alteration to the balance of mRNA for CRH receptors 1 and 2 in the pig amygdala was seen as a consequence of PNS. Furthermore, as predicted, PNS had an adverse effect on sow maternal behaviour and consequently on piglet survival, although contrary to predictions the altered behavioural profile was seen in either an open or restrictive parturition environment.

4.1 CRH receptor mRNA expression in the amygdala

We set out to examine possible impacts of prenatal stress on selected aspects of brain and behavioural development in domestic pigs. Specifically, we hypothesised that prenatal stress generated by maternal social stress would impact upon the ratio of CRH-R1: CRH-R2 mRNA expression in the amygdala. As hypothesised, a substantial increase in the ratio of CRH-R1: CRH-R2 mRNA expression in the amygdala of female PNS pigs indicative of an anxiety-prone phenotype was seen. This effect was largely due to greater CRH-R1 mRNA expression. CRH-R1 is the selective target for CRH and mediates stress and anxiety-related actions of CRH [19, 30, 31]. CRH-R2 has lower affinity for CRH, but is the selective target for urocortins II and III [32, 33], which are considered to have actions opposing those of CRH on stress and emotionality [19, 34]. The amygdala, as part of the limbic system, is a brain area that is central to processing emotional information and organisation of behavioural and physiological reactions to threatening events. The observed increase in the CRH-R1: CRH-R2 mRNA ratio, seen here as a consequence of prenatal stress, thus indicates a more fear/anxiety prone neurobiological phenotype. No effect of prenatal stress on the ratio of CRH-R1: CRH-R2 mRNA expression in the amygdala of male pigs was seen.

We have previously shown that PNS increased expression of CRH mRNA expression in both the amygdala and PVN of the hypothalamus and this was associated with increased peripheral concentrations of cortisol as a consequence of acute social stress in female pigs [10]. The current data on CRH receptor mRNA expression are consistent with these changes, and indicate a combination of increased CRH and CRH-R1 availability in the amygdala in the PNS females. Studies in mice have shown that CRH-R1 gene deletion reduces anxious behaviour and attenuates peripheral stress responses [30, 35], whereas CRH-R2 knockouts show increased stress responsiveness [36]. In a rat model, prenatal stress altered CRH-R1 and CRH-R2 mRNA expression in the amygdala and PVN, along with an increase in CRH expression in the PVN of female offspring [37]. Specifically, in female offspring PNS decreased CRH-R2 expression in the amygdala with no effect on CRH-R1, and these changes, which would also alter the receptor ratio in the same direction as seen in the present study in pigs, were also associated with increased anxiety in an elevated-plus-maze test. Moreover, a recent study using a rodent model of prenatal social stress reported an increase in the ratio of CRH-R1 to CRH-R2 mRNA in the amygdaloid complex of male prenatally stressed offspring, which exhibit an anxiety-phenotype, with no change in the female offspring, which do not [38].

4.2 Maternal behaviour of prenatally stressed female pigs

Female offspring born to pigs exposed to social stress during pregnancy were also shown in this study to have impaired maternal behaviour when they themselves became mothers. In the 24h after the birth of the first piglet, abnormal maternal behaviour in PNS sows was indicated by more ventral lying and increased restlessness. PNS sows spent more than twice as much time as control sows lying on

their front during the first 24 hours after the birth of the first piglet. Ventral lying (seen here more often in PNS sows), is increased in sows that crush their offspring [39] and prevents access to the udder, reducing the ability of piglets to feed at a time when colostrum intake is particularly critical for piglet health [40]. Increased restlessness is positively associated with a higher risk of piglet crushing [39, 41, 42], with sow aggression towards piglets [29, 43], and with sow stress reactivity [44].

During the first six hours after the start of parturition, PNS sows also spent more time visually attending to piglets and showed an increased responsiveness to piglet approach towards the head, behaviours that have been previously linked to impaired maternal behaviour [29, 45]. Normal maternal behaviour in sows after the initiation of parturition involves lateral lying, low activity levels and a lack of responsiveness to piglets [45]. This profile is mediated by endogenous opioid, as treatment with naloxone caused a similar behavioural profile to that observed here (increased standing, ventral lying and posture changes, and an increased responsiveness to piglets during the parturition period) [45]. Prenatal stress impaired maternal behaviour in the offspring through a combination of reduced udder accessibility and increased piglet directed behaviours. The finding that there were behavioural differences in these parameters but no significant effect on the frequency of attacking piglets could support an interpretation that PNS increases fear levels rather than aggression *per se*. Indeed, piglet-directed aggression has been proposed as a fear reaction towards the newborn piglets [46]. The initial response of many sows to piglets approaching their head in the early stages of parturition has similarly been characterised as defensive [47], with only a subset of disturbed mothers showing overt aggression.

Contrary to expectations, from a previous study [10], our present data indicate that altered maternal behaviour in PNS sows is similarly expressed in either a restrictive

parturition environment (crate), in which primiparous sows show behavioural and physiological indications of stress [48], or in an open pen. Previous research [10] found behavioural evidence indicating that progeny from mothers stressed during their pregnancy were more likely to attack their own offspring when they themselves gave birth in a crate. This was suggested to be a consequence of PNS sows with a stress-reactive phenotype being forced into a stress-inducing (behaviourally restrictive) situation. However, we found that PNS increased negative reactions to the experience of giving birth and piglet contact, irrespective of the degree of behavioural restriction experienced by mothers during the peri-parturient period, which we interpret as a reaction to parturition and piglet exposure *per se* rather than the immediate impact of the environment. The effect of PNS also interacted with environment to substantially increase piglet mortality levels in the open pen, where deficient maternal behaviour is more likely to cause piglet mortality (e.g. through sows crushing piglets). Although, this could be partially due to altered piglet behaviour, our one measure of piglet behaviour, approach to the sow's head, showed no sign of a stress treatment effect. However, as we did not assess other piglet behavioural parameters, we cannot rule out the possibility that the piglets themselves have a role in this mortality, i.e. that the altered maternal behaviour was actually a response to altered piglet behaviour.

One possible interpretation of the abnormal maternal behaviour (e.g. restlessness, less time lateral lying, reactivity and visual focus towards piglets) seen in PNS sows is that it may be a reflection of a heightened propensity for fear/anxiety indicated here by the increased ratio of CRH-R1: CRH-R2 mRNA in the amygdala in littermate females and stress hyper-responsivity reported in an earlier study using the same social mixing model [10]. A relationship between a fearful/anxious behavioural

profile and later impairments of maternal behaviour in pigs is supported by a study [49] that classified primiparous sows on a behavioural ‘shy-bold’ continuum on the basis of their response in a human-approach test conducted during pregnancy. Sows at the ‘shy’ end of the spectrum were more likely to attack their offspring. More general detriments to sow maternal behaviour as a consequence of maternal anxiety were reported by Janczak and colleagues [50] who found associations between behavioural measures of fear and anxiety at around two months of age and later quality of maternal care as reflected by neonatal mortality. Sow neophobia and nervousness towards humans has also been found to be associated with increased prevalence of neonatal piglets being crushed by their mothers [51]. However, the interpretation of maternal behavioural changes seen in this study in terms of emotionality requires further work.

4.3 The effect of tail-docking

The other early life experience investigated was tail-docking. Although tail-docking was not considered as a factor in the parturition studies (owing to the number of pigs that dropped out from the study prior to insemination) there was evidence of an impact of tail-docking on CRH receptor mRNA expression in the amygdala. Tail-docking increased both CRH-R1 and CRH-R2 mRNA expression in female pigs, but had no overall effect on the ratio of the two receptors. In males CRH-R2 mRNA expression in the amygdala was higher in tail-docked pigs. Activation of CRH-R2 generally dampens stress responses [19]; however it is not clear whether the changes in receptor expression in male pigs affects aspects of their behavioural or physiological reactivity, as these aspects have not been explored in this model. That tail-docking has such a long-term effect is intriguing, yet hard to explain. We have

also shown that reproductive development is affected by tail-docking in a separate study [15].

Our previous work has shown that PNS increased the behavioural distress response to tail-docking [14], indicating that pain sensitivity may be increased in PNS offspring. Other work has indicated that thresholds to noxious mechanical stimuli may be increased as a consequence of prenatal stress [52]. However, whether either of these alterations to the nociceptive system could impact on any pain associated with parturition in the pig and therefore underlie some of the negative behaviours seen in parturient PNS primiparous female pigs is uncertain.

4.4 Implications for pig production systems

Mixing sows together during gestation is increasingly common in pig production systems due to legislative changes banning individual stall housing (e.g. since 1999 in the UK, and since the start of 2013 across the EU). Stall housing had been implemented in the pig industry to avoid aggression between sows, but is now widely considered to be harmful to sow welfare. In many countries pregnant sows are therefore now housed in social groups, and may experience social mixing at various times during gestation. Such mixing is often found to induce behavioural signs of subordination, physiological stress states, and reductions in weight gain in mixed animals [10,14,53,54,55,56]. The method used here, based on previous work [10], did not aim to replicate commercial mixing practice (which is highly variable), but does provide an experimental model of how social stress experienced during pregnancy may affect sow offspring. The findings here further emphasise the potential harm to progeny well-being created by maternal stress during gestation. Pig farmers could act on such findings by minimising social mixing, practising a mixing strategy that

minimises aggression, or by using housing systems that allow subordinate sows to escape aggression.

4.5 Conclusion

Overall, our research provides evidence that prenatal stress can affect brain and behavioural development in pigs. We found a shift in the balance between mRNA expression for CRH receptors 1 and 2 in the amygdala of female pigs as a consequence of prenatal stress, and demonstrated that prenatal exposure to stress impairs their subsequent maternal behaviour. These findings add to the recognition that for gestating animals the interaction, during pregnancy, between mother and environment may contribute to how capable her offspring are at coping with their own environmental conditions later in life. Furthermore, the pig may also represent a valuable model for examining prenatal influences on some human conditions including abnormal maternal behaviour [21, 22], given the similarities in brain structure and development at birth [57].

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Figure 1: Diagram of experimental timeline. Phase 1: Sow gestation, showing sample sizes and timing of social stress treatment. Phase 2: Offspring measures, including F1 gestation and subsequent observation of maternal behaviour.

Figure 2: Representative in situ hybridisation autoradiographs for corticotropin releasing hormone receptor 1 (CRH-R1) and corticotropin releasing hormone receptor 2 (CRH-R2) mRNAs in the amygdala from a female control/intact pig. a) Toluidine blue stained coronal section of block containing the amygdala from a bisected pig brain; scale bar: 2mm; b) diagram of areas (boxes) in the amygdala sections in which integrated optical density measurements were made. Amyg: amygdala; Cx: external capsule; Cp: piriform cortex; Put: putamen; OT: optic tract; Rh: rhinal sulcus (after [27,28]). Bright field images at c), d) x4 objective magnification and e), f) x10 objective magnification of positively labelled cells in the pig amygdala, hybridised with radio-labelled probes for CRH-R1 (left column) or CRH-R2 (right column) mRNA. The density of CRH-R1 mRNA labelled cells was greater and more uniform than for CRH-R2 mRNA, which was often in cell clusters. Scale bars: 500 μ m (top row), 250 μ m (bottom row).

Figure 3: Expression of corticotropin releasing hormone receptor 1 (CRH-R1, 3a) and corticotropin releasing hormone receptor 2 (CRH-R2, 3b) mRNAs in the amygdala (Integrated Optical Density: IOD), and their ratio (3c) for females (PRENATAL STRESS (PNS)/INTACT n=7, PNS/DOCKED, n=8, CONTROL (CON)/INTACT, n=5, CONTROL/DOCKED n=5) exposed to combinations of PNS and tail-docking. Females that had been exposed to PNS showed an increase

in CRH-R1 mRNA expression (REML, $p=0.01$), no significant change in CRH-R2 mRNA (REML, $p=0.61$) and a highly significant change in the CRH-R1:CRH-R2 mRNA ratio ($p=0.002$). Tail docking increased CRH-R1 mRNA (REML, $p=0.01$) and CRH-R2 mRNA (REML, $p=0.026$) expression but did not affect the ratio between the two receptors (REML, $p=0.16$). There were no significant interactions between stress history and docking status for either CRH-R1mRNA or CRH-R2mRNA or their ratio. Data are expressed as mean \pm S.E.M. * Significant main effect of stress history ($*p<0.05$ and $** p<0.01$). # Significant main effect of tail treatment ($p<0.05$).

Figure 4: Component scores from principal components (PC) analysis of post-farrowing behaviour of control (CON) and prenatally stressed (PNS) sows farrowing in either a crate or pen environment. Vertical dimension (PC1, 46% of variance): poor (up) to good (down) nursing posture; horizontal dimension (PC2, 26% of variance): good (left) to poor (right) piglet-directed maternal behaviour. Prenatal stress (REML, $p<0.001$) and farrowing environment (REML, $p<0.001$) affected PC1 scores. PC2 scores were affected by prenatal stress (REML, $p=0.039$) but not by environment (REML, $p=0.72$).

Table 1: Ethogram used for Sow Behavioural Observations

Behaviour	Definition
Stand	Upright on all four legs
Lateral lying	Lying on side, with one shoulder touching the ground, udder exposed
Ventral lying	Lying on belly, with neither shoulder touching the ground, and udder partly or completely concealed
Posture changes	The total number of transitions between standing, lateral lying and ventral lying.
Piglet focussed	Gaze directed towards one or more piglets
Fixture/Substrate focussed	Touch, scratch, dig, manipulate with foot, nose or mouth (excluding eating and drinking) any substrate (straw, floor, bars, feed trough) except piglet
Attack piglet	Any initiated aggression (bite, attempt to bite, push, root)
Piglet approach	Piglet moves in contact with, or very close to, the sow's head (one piglet body length)
Response to piglet approach	Shows interest toward piglet that is in contact with, or very close to, the sow's head (one piglet body length) including by ears or gaze
No response	No overt response shown to any piglet that is in contact with, or very close to, the sow's head (one piglet body length)

Table 2: Effect of prenatal stress (PNS) and farrowing environment on sow behaviours before and after the start of parturition, piglet mortality and effect of prenatal stress alone on litter characteristics. Sample sizes were: PNS/PEN n=13, PNS/CRATE, n=14, CONTROL/PEN, n=4, CONTROL/CRATE, n=7.

Time	Stress History			Farrowing Environment			Stress x Environment interaction
	PNS	Control	P value (SED, Wald)	Pen	Crate	P value (SED, Wald)	P value (Wald)
Pre-parturition							
<i>Fixture/Substrate directed (time, secs)</i>	13573	14196	0.675 (1485, 0.18)	18574	9194	<0.001 (1539, 37.13)	0.308 (1.04)
<i>Standing (time, secs)</i>	16325	14976	0.514 (2065, 0.43)	22342	8960	<0.001 (2047, 42.74)	0.282 (1.16)
<i>Lateral lying (time, secs)</i>	39285	37907	0.790 (5040, 0.07)	34620	42571	0.027 (3254, 5.97)	0.411 (0.71)
<i>Ventral lying (time, secs)</i>	26507	23625	0.592 (5189, 0.31)	22956	27176	0.206 (3203, 1.74)	0.719 (0.13)
<i>Posture changes (number)</i>	269	317	0.243 (38.4, 1.59)	258	328	0.026 (28.9, 5.87)	0.568 (0.34)
Post-parturition							

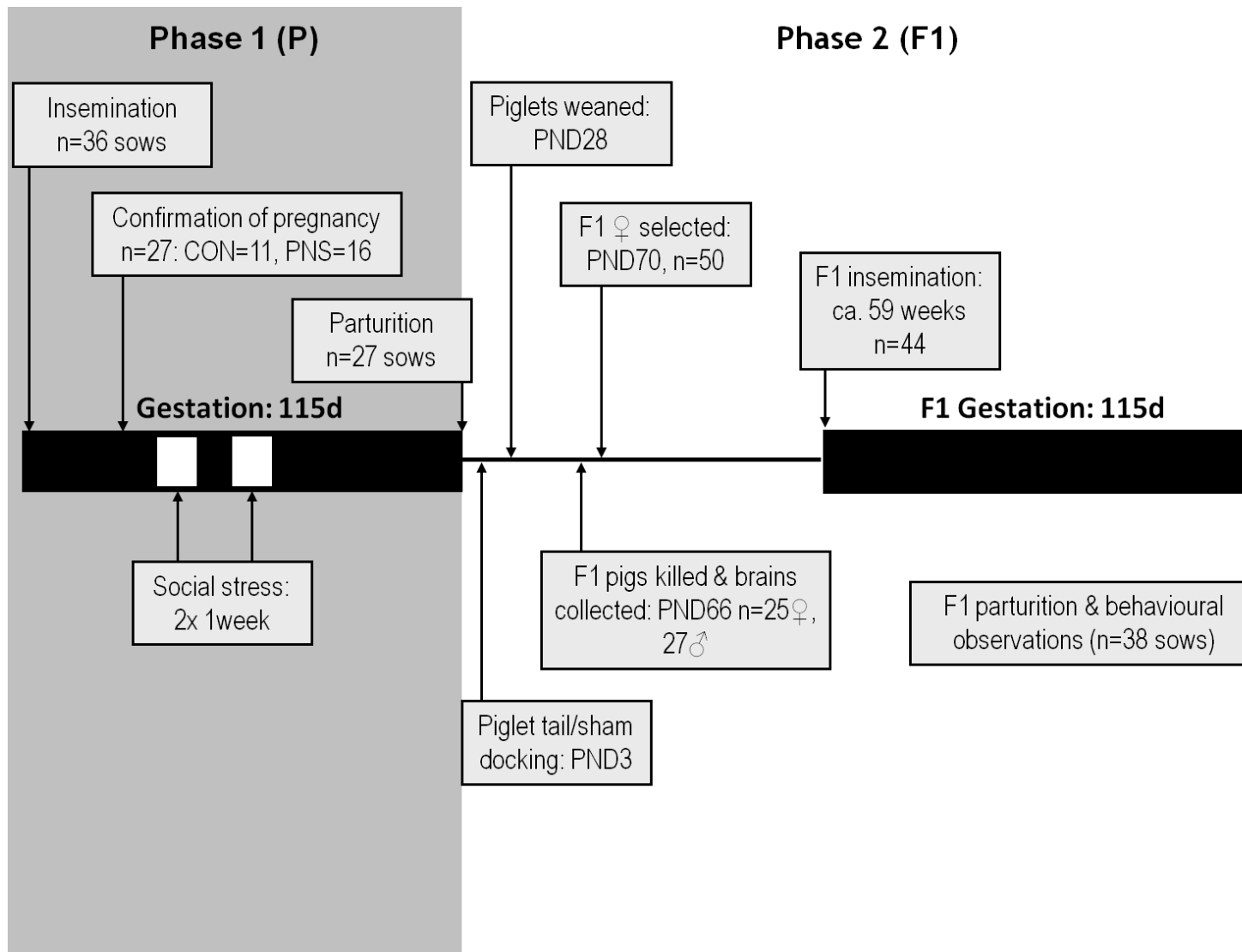
<i>Fixture/Substrate directed (time, secs)</i>	1639	2502	0.211	2393	1748	0.081	0.175
			(657.8, 1.72)			(346.9, 3.46)	(2.02)
<i>Standing (time, secs)</i>	4918	4355	0.669	6674	2599	<0.001	0.88
			(1286, 0.19)			(831.5, 24.02)	(0.02)
<i>Lateral lying (time, secs)</i>	64415	72431	0.076	63181	73665	0.003	0.719
			(3984, 4.05)			(3163, 10.98)	(0.13)
<i>Ventral lying (time, secs)</i>	15539	6892	0.007	14626	7805	0.006	0.796
			(2531, 11.67)			(2186, 9.74)	(0.07)
<i>Posture changes (number)</i>	111.5	69.8	0.005	119.2	62.1	0.001	0.105
			(16.34, 7.77)			(17.48, 10.67)	(2.63)
<i>Attack piglet (number (back transformed mean))</i>	2.1 (8.0)	0.8 (2.2)	0.136	1.4 (4.2)	1.5 (4.3)	0.306	0.429
			(0.75, 2.37)			(0.54, 1.11)	(0.66)
<i>Piglet focussed (time, secs)</i>	6530	2983	0.034	5382	4132	0.331	0.467
			(1492, 5.65)			(1251, 1.00)	(0.55)
<i>Piglet approach (number)</i>	78.2	81.4	0.851	100.0	59.6	0.016	0.138
			(16.88, 0.04)			(15.38, 6.89)	(2.38)
<i>Piglet responsivity index</i>	-0.078	-0.4517	0.04	-0.2361	-0.2937	0.659	0.466
			(0.1545, 5.85)			(0.1282, 0.2)	(0.55)
<i>All Piglet mortality (proportion of all piglets dead before weaning)</i>	0.213	0.137	0.196	0.242	0.108	0.031	0.038
			(0.0527, 2.11)			(0.0593, 5.15)	(4.69)

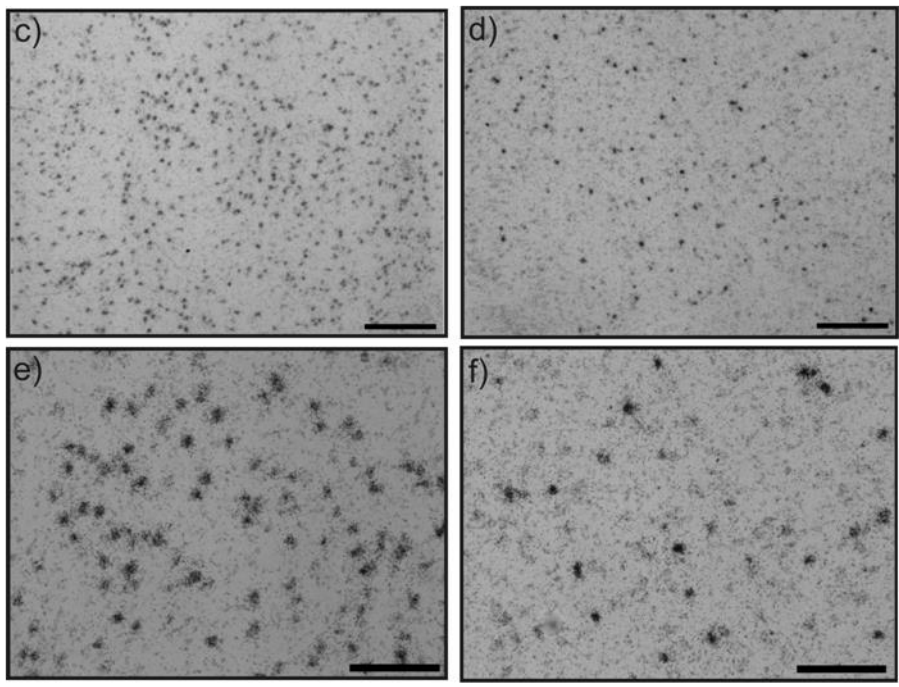
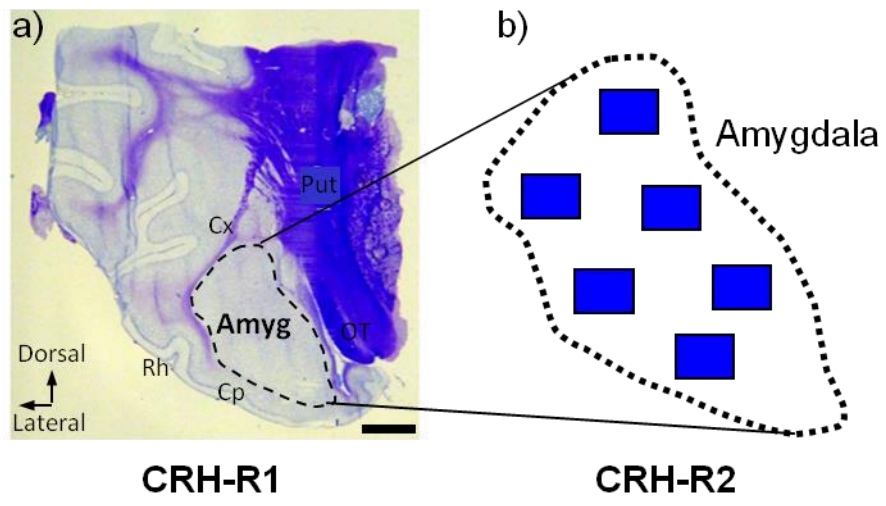
Litter characteristics

<i>Gestation length (days)</i>	116.2	116.1	0.892
			(0.636, 0.02)
<i>Farrowing duration (minutes)</i>	213.4	178.9	0.387
			(37.46, 0.85)
<i>Litter size (number)</i>	14.2	13.6	0.651
			(1.283, 0.21)
<i>Piglet birth weight (Kg)</i>	1.32	1.40	0.202
			(0.060, 1.69)
<i>Litter sex ratio (proportion female)</i>	0.50	0.53	0.505
			(0.047, 0.45)

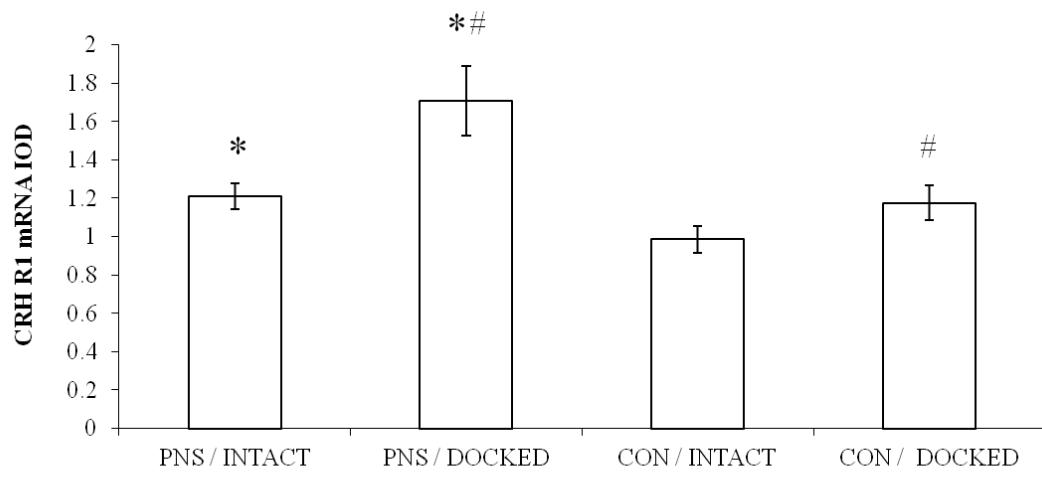
Table 3: Principal component (PC) loadings of post-parturition sow (n=38) behaviours

Behaviour	PC1 “Udder accessibility”	PC 2 “Piglet related behaviour”
Stand	0.422	-0.273
Lateral	-0.506	0.055
Ventral	0.466	0.050
Posture changes	0.382	0.069
Piglet focussed	0.382	0.311
Floor/Fixture focussed	0.177	-0.596
Attack piglet	0.005	0.479
Piglet approach response	0.161	0.482
<i>Eigen value</i>	3.66	2.06
<i>Variation explained</i>	45.7%	25.8%

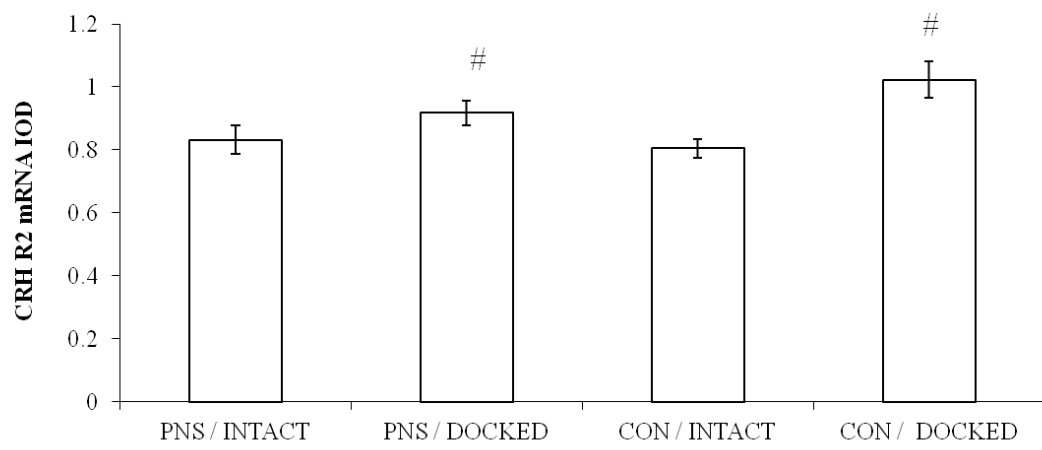




a) **CRH-R1 mRNA**



b) **CRH-R2 mRNA**



c)

CRH R1/R2 mRNA Ratio

