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Neonatal oxytocin administration and supplemental milk ameliorate the weaning transition and alter hormonal expression in the gastrointestinal tract in pigs

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Abstract

The aim of this study was to investigate the influences of milk supplementation during lactation, over 1 wk after weaning, and oxytocin administration for the first 14 d of life on the pigs' response to weaning. Pigs from 20 litters were allocated to each of these 3 treatments in a randomized factorial design. Oxytocin was administered subcutaneously daily from 0 to 14 d of age at a rate of 10 I.U. per kg. The milk supplement consisted of a mixture of 25% skim milk powder offered either during lactation between 10 and 20 d of age or for the first wk after weaning as a transitional diet along with dry pellets. Pigs were weaned at 21 d of age. Growth rate was measured from birth to slaughter at 140 d of age and feed intake of supplemental milk or feed from 10 to 56 d of age. Organ weights (heart, liver, stomach, kidneys) and the gene expression of ghrelin, leptin, and glucagon-like peptides (GLP-1 and GLP-2) were measured in the stomach, ileum, and duodenum at 10, 21 and 28 d of age. Milk supplementation after weaning resulted in immediate feed intake and partially alleviated the depression in growth rate over the first 7 d post-weaning ($P < 0.001$), but milk supplementation during lactation had no effects ($P > 0.1$). However, effects were only transient and disappeared once the milk liquid diet was removed. Neonatal oxytocin administration reduced weight loss over the first 2 d after weaning ($P = 0.03$), without affecting feed intake ($P > 0.1$), hence possibly reducing weaning stress. Seven d after weaning, oxytocin-treated pigs had greater stomach ghrelin and leptin expression (both $P = 0.02$), and pigs supplemented with milk after weaning had greater stomach leptin and GLP-2 expression ($P = 0.02$ and $P = 0.05$, respectively). Hence, neonatal oxytocin administration or post-weaning milk supplementation are both effective means of enhancing gastric leptin expression and reducing weight loss at weaning, likely improving gut health during this critical period.

35

36 **Keywords:** ghrelin; GLP; gut health; leptin; stress; *sus scrofa*.

37

1. Introduction

Neonatal mammals rely on their dam for survival, through protection and the provision of food. Weaning occurs gradually in natural conditions. For feral pigs, weaning takes between 8 and 19 wk to complete [1]. This time allows the young pig to mature in its digestive and absorptive capacities and in its ability to cope with environmental challenges. However, in commercial pig production, weaning usually occurs abruptly between 3 and 4 wk of age, and the separation of piglets from the dam is usually associated with changes in their diet, physical and social environments. These result in nutritional, thermal, immunological and psychological challenges [2,3]. Weaning is therefore a multi-factorial stressor for pigs, and is generally associated with weight loss and increased morbidity and mortality reflective of the pigs' difficulty in coping with this challenge [4].

A variety of strategies have been attempted to facilitate the weaning transition on the pig, mostly aimed at alleviating the post-weaning growth lag. Dry pelleted feed is the standard diet form given to newly-weaned pigs on most farms, and represents an abrupt change from the highly digestible and palatable nutrients piglets' receive from milk. Studies have shown that specific feeding regimes, such as supplementing piglets with milk during lactation [5-7], or into the early post-weaning period while gradually introducing a dry pelleted feed [6,8-10], can alleviate weight loss and improve indices of gastrointestinal tract structure and function in the post-weaning period.

Hormonal interventions, such as oxytocin, a mammalian peptide, could also facilitate weaning. In rats, repeated oxytocin administration induces long-lasting metabolic and physiologic changes such as increased growth [11,12], decreased corticosterone concentrations and levels of plasma gastrin, cholecystokinin and insulin [13,14]. In addition, oxytocin may

61 reduce the stress response to weaning by reducing the psychological attachment to the dam and
62 favouring self-oriented behaviours [15], although the precise mechanism by which oxytocin
63 exerts its effect remains unclear [16]. The release of oxytocin can be stimulated by touch,
64 warmth and the ingestion of food [17], which are common daily occurrences for the suckling pig
65 but become suddenly sporadic in the post-weaning period.

66 This experiment aimed to determine the influence of milk supplementation during
67 lactation, over 1 wk after weaning, and of oxytocin administration for the first 14 d life on the
68 pigs' feed intake, growth rate, organ weights and the gene expression of hormones released from
69 the gastrointestinal tract, with an emphasis on ghrelin, leptin, glucagon-like peptide-1 (GLP-1)
70 and glucagon-like peptide-2 (GLP-2). The hypothesis of this study was that pigs supplemented
71 with milk, during lactation or after weaning, or pigs administered with exogenous oxytocin
72 would show greater feed intake in the first 7 d after weaning, greater growth rate and greater
73 expression of these gastrointestinal hormones.

74

75 **2. Materials and methods**

76 The project was approved by the Victorian Department of Primary Industries Ethics
77 Committee in accordance with the Australian Code of Practice for the Care and Use of Animals
78 for Scientific Purposes.

79

80 *2.1. Animals and milk supplementation during lactation*

81 Pigs were allocated to treatments in a 2 × 2 × 2 × 2 randomised factorial design with the
82 respective factors being sex (male vs. female), injection (oxytocin vs. saline), pre-weaning

dietary treatment (supplemented with milk during lactation vs. unsupplemented) and post-weaning dietary treatment (pellets vs. pellets plus milk).

Twenty Large White x Landrace multiparous sows of mixed parities suckling 10-12 pigs were randomly allocated to 1 of 2 treatments: pigs were either supplemented with milk or were unsupplemented and relied solely on suckling the sow. The liquid supplement consisted of a mixture of 25 % skim milk powder (SMP) and water. The SMP was available from day 10 of lactation to weaning (day 21). The SMP was reconstituted by adding 1 part powdered SMP (Murray Goulbourn, Melbourne, VIC, Australia) to 4 parts warm tap water; 20 mL of a live probiotic (Yakult Australia Pty Ltd, Dandenong, VIC, Australia) was added per litre as a source of *Lactobacillus casei* Shirota strain to prevent diarrhoea. The SMP mixture was stored at 4 ºC and used within 2 d. The supplement was delivered by a gravity feed system that was designed to minimise spillage and contamination by faeces and urine [6].

2.2. Oxytocin administration

Within each litter, pigs were allocated at birth into pairs of the same sex based on similar live weight. Within each litter, 2 female and 2 male pigs of each pair (n = 40 per sex) were injected subcutaneously with oxytocin (Ilium Syntocin, Troy Laboratories, Glendenning, NSW, Australia) daily from 0 to 14 d of age at a rate of 10 I.U. per kg of BW (equivalent to 20 μg per kg of BW). The other 2 female and 2 male pigs of the pairs (n = 40 per sex) were injected subcutaneously with 0.9 % saline in the same manner and same quantity as the control. These pigs were used for live measurements. Any additional pig in the litter also received injections of either oxytocin or saline and 117 pigs were later euthanized for the collection of tissue samples and measurements of gene expression.

106

107 *2.3. Weaning and milk supplementation post-weaning*

108 Pigs were weaned at 21 d of age into individual weaner crates in order to measure their
109 feed intake. Forty pigs were fed ad libitum a high quality weaner pelleted diet, Ultrawean 75
110 (digestible energy (DE): 16 MJ/kg, crude protein (CP): 24 %, total Lysine: 1.0 %; Ridley
111 AgriProducts, Pakenham, VIC, Australia), whereas 40 other pigs were fed the same diet but also
112 supplemented with the same SMP mixture as a transitional diet. Over the 7 d post-weaning, the
113 dry matter (DM) content of the SMP mixture was gradually increased by the inclusion of less
114 milk and more dry pelleted feed (Ultrawean 75) until pigs were completely offered the dry
115 pelleted diet. Accordingly, for the first 2 d post-weaning, pigs were offered 1 L of SMP mixture.
116 On the third day post-weaning, 200 g of Ultrawean 75 was added and the amount of pelleted diet
117 increased by 100 g/d until day 7 while the amount of the SMP mixture decreased
118 proportionately. From the end of the first wk after weaning, all pigs were fed *ad libitum*
119 Ultrawean 100 (DE: 16 MJ/kg, CP: 24 %, Lysine content: 0.9 %; Ridley AgriProducts,
120 Pakenham, VIC, Australia) for another week before being changed to an *ad libitum* high quality
121 weaner mash from the third week to 56 d of age (DE: 14.5 mj/kg, CP: 23 %, total Lysine: 1.0 %;
122 Riverbank Stockfeeds, Leongatha, VIC, Australia). At 56 d of age, pigs were returned to the
123 herd, housed in 1 shed in groups of 10 in 3.0 × 3.4 m pens, and fed conventional grower (DE:
124 14.3 MJ/kg, CP: 18.7 %, total Lysine: 0.6 %; Riverbank Stockfeeds, Leongatha, VIC, Australia)
125 and finisher diets (DE: 13.0 MJ/kg, CP: 18.2 %, total Lysine: 0.5 %; Riverbank Stockfeeds,
126 Leongatha, VIC, Australia) until slaughter.

127

128 *2.4. Animal measurements*

Live weight of the pigs was determined at birth and then at 10, 14, 21, 23, 25, 28, 35, 42, 49 and 56 d of age. Supplemental milk intake was determined for each litter daily from day 10 to 20 of lactation. All feed refusals post-weaning were weighed for each pig daily between 21 and 27 d of age and weekly at 35, 42, 49 and 56 d of age to determine individual feed intake. Feed intake was only measured until 56 d of age because this study focused on feed intake around weaning, and therefore by 56 d of age all pigs are expected to have adapted to solid feed. From day 56, the pigs were returned to the herd and housed in group pens and so further observations on individual growth performance could not be made. However, live weight and back fat depth (P_2) were measured on all individual pigs at 140 d of age (market slaughter weight). Ultrasonic back fat depth (P_2) was measured 65 mm from the mid line at the level of the last rib, using a backfat scanner (Renco Lean-Meater Minneapolis, MN, USA).

2.5. Euthanasia and tissue sample collection

For organ weights and gene expression, pigs were euthanized at 10 (n = 15), 21 (weaning; n = 39), 28 (n = 31) or 35 (n = 32) d of age. Pigs were euthanized via a lethal injection of pentobarbital (Lethobarb®, Virbac, Milperra, New South Wales, Australia) intracardially at a dose of 0.3 mL/kg. Immediately following death, a ventral incision was made from the sternum to the pubis and the gastrointestinal tract removed. The gastrointestinal tract was tract removed. Stomach, small intestine, large intestine, liver, kidneys and heart were individually weighed. Following flushing with cold 0.9 % physiological saline solution, a mucosal sample from the fundic region of the stomach and mucosal samples from the proximal duodenum and ileum were collected for subsequent gene expression analysis at 10, 21 and 28 d of age. Samples collected

for gene expression analysis were wrapped in aluminium foil and snap frozen in liquid nitrogen. Samples were later transferred to the -80 ºC freezer and stored until analysis.

2.6. Real-Time polymerase chain reaction (RT-PCR) analysis of gene expression

The RNA was extracted from tissue samples using Trizol® Reagent (Invitrogen, Life Technologies, Mulgrave, VIC, Australia) according to the protocol of the manufacturer. The RNA quality and quantity was determined using an Experion automated electrophoresis station (Bio-Rad Laboratories Inc, Hercules, California, USA) along with the Experion StdSens Analysis Kit (Bio-Rad Laboratories Inc, Hercules, California, USA) according to the protocol of the manufacturer. Electropherograms for each sample were analysed for the concentration of RNA and the ratio of *28S* to *18S* and samples with 2 clean peaks and a ratio close to 1.8 were accepted. Samples with background 'noise' and/or low ratios were not accepted and the original tissue sample was re-extracted.

The RNA of suitable quality was then reverse-transcribed into cDNA in triplicate using the SuperScript™ III First Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies, Mulgrave, VIC, USA), random hexamers method. Briefly, 8 µL of RNA from each sample was aliquoted in triplicate to a 96 well plate and combined with 1 µL 50 ng/µl random hexamer and 1 µL 10 mM dNTP mix. The plate was incubated at 65 ^{o}C for 5 min using the Corbett Palm Cycler PCR (Corbett Research, Mortlake, New South Wales, Australia). The following cDNA synthesis mix was prepared per sample in the following order: – 2 µL 10x RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M DTT, 1 µL 40 U/µL RnaseOUT, 1 µL 200 U/µL SuperScript III RT. To each of the sample wells, 10 µL of this cDNA synthesis mix was added and the plate centrifuged briefly (Eppendorf, Hamburg, Germany). The plate was then incubated as follows: 10 min at 25 ^{o}C, 50

min at 50 °C, 5 min at 85 °C. The samples were then chilled on ice for 2 min before the addition of 1 µL RNase H to each of the sample wells and the final incubation at 37 °C for 20 min. For each tissue type, 2 µL from each of the sample wells was combined and aliquoted into 10 µl lots for later use as a positive control for polymerase chain reaction (PCR) investigations. These positive control samples were also used to optimise primer conditions before PCR analyses of the experimental samples. All cDNA was stored frozen at –80 °C until required for polymerase chain reaction (PCR) analysis.

Primers for the genes of interest were designed using the pig (*Sus scrofa*) genome database. The messenger RNA sequence of the gene of interest was obtained using the National Center for Biotechnology Information (NCBI) nucleotide database. This sequence was then copied into Invitrogen's OligoPerfect™ Designer web page. The oligoPerfect Designer finds primer sequences from the given mRNA sequence that meets individual specifications of size, annealing temperature, GC content, region of analysis, product size, salt concentration and primer concentration. Potential forward and reverse primer sequences were then transferred to Premier Biosoft International's NetPrimer program to determine if the primers had any of the following adverse characteristics: hairpins, dimers, palindromes or repeats. The 2 forward and reverse primer pairs with the least number of these adverse characteristics were selected (Table 1). These custom primers were ordered through GeneWorks Pty Ltd (Hindmarsh, South Australia, Australia).

The cDNA samples were analysed via RT-PCR using primer sets for *18s* rRNA as the housekeeper gene, together with ghrelin, leptin, GLP-1 and GLP-2 at optimised temperatures and concentrations (Table 1). Primers for each gene of interest were optimised to determine the appropriate annealing temperature and the most efficient concentration of primer to include in

197 the reaction. Temperature optimisation of each primer was undertaken using a temperature
198 gradient between 50 to 65 ^{o}C depending on the primer set. The temperature at which the product
199 amplified with good repeatability above 10 threshold cycles (Ct), and that had a clean melt curve
200 with no evidence of primer-dimer formation was chosen. All PCR reactions were undertaken in
201 real time (RT-PCR) on a BioRad MyIQ Single Colour Real Time PCR Detection System
202 (BioRad Laboratories Inc,, Hercules, California, USA). Reactions were made up in 0.2 mL
203 iCycler 96 well PCR plates (BioRad Laboratories Inc, Hercules, California, USA) and sealed
204 with iCycler iQ optical tape (BioRad Laboratories Inc, Hercules, California, USA). Each PCR
205 reaction mix consisted of 12.5 µL Sybr Green Supermix (BioRad Laboratories Inc, Hercules,
206 California, USA), 1-3 µL forward primer (depending on optimised concentration), 1–3 µL
207 reverse primer (depending on optimised concentration), 2 µL cDNA from the appropriate tissue,
208 with the remaining volume made up to 25 µL with RNase free H_2O. Each 96 well plate also
209 contained 2 wells as a positive control (cDNA used in these wells was from the control batch to
210 ensure consistent results across plates), 2 wells as a blank (containing only 25 µL of RNase free
211 H_2O) and 2 wells as a negative control (the 2 µL of cDNA was replaced in these wells by 2 µL
212 of RNase free H_2O). The cDNA amplification program included step 1: 95 °C for 3 min followed
213 by 60 cycles; step 2: 90 °C for 10 sec and 55 to 63 °C for 45 sec (temperature dependent upon
214 primer, see Table 1); step 3: 95 °C for 60 sec; step 4: 90 °C for 60 sec; step 5: 55 °C for 60 sec;
215 and finally 60 cycles of step 6: 55 °C for 10 sec. A melt curve was produced for each reaction
216 run in order to detect if any primer-dimers were produced. All samples had a Ct value for both
217 the gene of interest and the housekeeper gene, with the difference between the two Ct values
218 evaluated as the Δ Ct. When using RT-PCR to evaluate gene expression in samples obtained
219 from multi-factorial experiments, the Δ Ct method is required to statistically analyze the data

arising from such experiments [18]. Using this method, a lower Δ Ct value indicates an earlier amplification of the product and hence greater gene expression, whereas the reverse is true for a greater Δ Ct value.

2.7. Statistical analysis

Data was checked for normality and homogeneity of variance and statistically analysed using ANOVA with Genstat software 13th edition (VSN International Ltd, Hemel Hempstead, United Kingdom). The experimental unit was the pig and sow was used as the blocking factor. The model was used for performance, feed intake, and gene expression data and included sex, oxytocin administration, pre- and post- weaning milk supplementation as the main factors and all interaction between these factors. As there were no significant interactions (all $P > 0.05$), the data are presented as main effects.

3. Results

3.1. Pre-weaning growth performance and supplemental milk intake

Oxytocin- and saline-administered pigs did not differ in birth weight at the onset of the experiment (1.7 kg vs. 1.7 kg, SED = 0.1 kg, $P = 0.33$), nor was there any effect of sex on birth weight (1.7 vs. 1.7 kg, SED = 0.1 kg, $P = 0.96$). Between birth and 10 d of age, females had a lower average weight gain than males (214 g/d vs. 231 g/d, SED = 8.6 g/d, $P = 0.05$). Supplemental milk intake during lactation increased steadily from 848 g/d to 2516 g/d per litter over the 10 d of supplementation, or the equivalent of ≈ 85 to 252 g/d per pig (Figure 1). Pigs supplemented with milk from day 10 of lactation had greater average weight gain between day 10 and weaning than non-supplemented pigs (303 g/d vs. 262 g/d, SED =17.5 g/d, $P = 0.03$), and

this tended to be greater in females than males (291 g/d vs. 275 g/d, SED = 9.6 g/d, $P = 0.09$). The administration of oxytocin had no effect on average weight gain between birth and weaning, and did not differ between males and females ($P > 0.1$).

3.2. Post-weaning growth performance and feed intake over the first week post-weaning (21-28 d of age)

The DM feed intake was over 10 times greater in the first 2 d after weaning in pigs weaned onto the gruel diet consisting of pellets supplemented with milk compared with pigs weaned on pellets alone ($P < 0.001$; Table 2). This greater feed intake continued for 7 d after weaning during which time these pigs had their feed gradually changed from the SMP mixture to a solid diet ($P < 0.001$). Pigs weaned onto the SMP mixture plus the dry pelleted feed lost 2.5 times less weight over the initial 2 d post-weaning than those weaned onto pellets alone ($P < 0.001$), and started regaining weight faster during the first 7 d after weaning (at 4 d and 7 d post-weaning; both $P < 0.001$). However, the feed conversion efficiency was never affected ($P > 0.10$).

Supplemental milk offered during lactation did not affect feed intake, live weight, average weight gain nor feed conversion efficiency over the first 7 d after weaning ($P > 0.10$).

The administration of oxytocin for the first 2 wk of life had no effect on feed intake for the first 7 d after weaning ($P > 0.10$), but resulted in the pigs losing less weight over the first 2 d post-weaning ($P = 0.03$). There was no effect of oxytocin on the feed conversion efficiency ($P > 0.10$).

Females seemed to better adapt to their new diet better than males, eating about 14 % more over the first 7 d after weaning ($P < 0.05$). However, this greater feed intake in females did

not translate into immediate differences in terms of average weight gain nor feed conversion efficiency ($P > 0.10$).

3.3. Growth performance and feed intake from week 2 post-weaning to market slaughter weight (28-140 d of age)

The pigs fed the post-weaning transitional diet (SMP mixture with the pellets) during the first 7 d after weaning showed a lower feed intake in the second wk post-weaning ($P = 0.04$; Table 3). The pigs fed the pellets plus milk diet were heavier than the pigs fed pellets alone 7 d after weaning ($P = 0.02$), but this difference disappeared once all pigs were offered dry pelleted feed ($P > 0.10$).

Surprisingly, pigs that received supplemental milk during lactation showed heavier body weight than non-supplemented pigs at 49 and 56 d of age ($P = 0.02$ and $P = 0.05$ respectively). However, they did not differ in terms of feed intake, average weight gain nor feed conversion efficiency ($P > 0.10$).

The administration of oxytocin during the first 2 wk of life resulted in heavier pigs at 49 d of age (P = 0.04), with a greater average weight gain from 42 to 49 d of age ($P = 0.01$) and a better feed conversion efficiency ($P = 0.03$). This increase in growth rate was associated with a h greater feed intake observed in the following wk from 49 to 56 d of age in pigs given oxytocin ($P = 0.04$).

Females maintained an almost 15 % greater feed intake compared with males in the second wk after weaning ($P = 0.01$), and had heavier body weight than males from 35 to 49 d of age ($P < 0.05$). However, they did not show differences in terms of feed conversion efficiency ($P > 0.10$).

289 The greater live weight observed in pigs supplemented with milk during lactation seen at
290 49 and 56 d of age was still present at 140 d of age compared with pigs that were not
291 supplemented (P = 0.05). No differences were observed in P2 back fat across treatments (P >
292 0.10).
293
294 *3.4. Organ weights*
295 For the euthanized pigs, administering oxytocin from 0 to 14 d of age caused a lower
296 body weight at 10 d of age compared with saline-administered pigs (3.1 kg vs. 3.3 kg, SED = 0.2
297 kg; P = 0.004); however, this was likely an artifact of the small sample size at that time point (n
298 = 15: 7 oxytocin- and 8 saline-administered pigs) as this result did not appear for the pigs
299 followed up throughout the experiment (n = 160).
300 Oxytocin administration, milk supplementation during lactation or after weaning, or sex
301 had no effect on any of the organ weights at any time point (P > 0.10).
302
303 *3.5. Gene expression*
304 At 10 d of age, leptin gene expression was greater in the stomach of oxytocin-treated
305 pigs than those receiving saline during that period (9.4 Δ Ct vs. 15.0 Δ Ct; SED = 3.3 Δ Ct, P =
306 0.03). GLP-2 gene expression was greater in the ileum of female compared with male pigs (3.4 Δ
307 Ct vs. 7.5 Δ Ct, SED = 1.0 Δ Ct, P < 0.001). No alterations in gene expression were observed in
308 the duodenum (leptin, GLP-1, GLP-2) or for ghrelin in the stomach or leptin and GLP-1 in the
309 ileum (P > 0.10).
310 At 21 d of age, on the day of weaning, leptin gene expression was greater in the ileum of
311 pigs that had not received supplemental milk in comparison to those that had been provided with

supplemental milk from day 10 of lactation (4.3 Δ Ct vs. 9.4 Δ Ct, SED = 2.2 Δ Ct, $P = 0.04$). GLP-2 gene expression was still greater in the ileum of female compared with male pigs (7.4 Δ Ct vs. 10.6 Δ Ct, SED = 0.1 Δ Ct, $P < 0.001$). No alterations in gene expression were observed in either the stomach or duodenum ($P > 0.10$).

At 28 d of age, 1 wk after weaning, both ghrelin and leptin gene expression were greater in the stomach of oxytocin-treated pigs compared with saline-treated pigs (both $P = 0.02$; Table 4). Leptin gene expression was also greater in the stomach of pigs that were weaned onto the pelleted diet supplemented with milk rather than onto pellets alone ($P = 0.02$), and GLP-2 gene expression was greater in the ileum ($P = 0.05$). The expression of GLP-1 gene was greater in the duodenum of male compared with female pigs ($P = 0.03$).

4. Discussion

Weaning pigs onto a milk-liquid-based diet and through a gradual transition to the dry pelleted diet in the first week after weaning resulted in immediate feed intake and partially alleviated the depression in growth rate commonly observed during this critical period. Weaning is a highly stressful period to the young animal, resulting in nutritional, thermal, immunological and psychological challenges [2,3,19]. Therefore, offering a gradual rather than an abrupt change in the diet after weaning is likely to have the most beneficial effect in reducing stressful effects on growth, possibly assisting in maintaining gut structure and function around that period [4,20]. However, this effect was only transient and disappeared once the milk liquid diet was removed. The pigs not offered any of the SMP mixture compensated in the second week after weaning when all pigs were fed exclusively the dry pelleted diet, which coincided with the pigs

334 previously supplemented with milk for the first week reducing their intake once exclusively fed
335 the dry pellet diet at 28 d of age.

336 Neonatal oxytocin administration given daily from 0 to 14 d of life reduced the extent of
337 the weight loss in the first 2 d after weaning, suggesting a possible adaptive role for this hormone
338 in the immediate post-weaning period. Weaning is a multi-factorial stressor for pigs in
339 commercial settings. Oxytocin has been shown to be involved in the stress response of pigs
340 [16,21], and may reduce the stress response associated with weaning since these pigs lost less
341 weight without showing greater feed intakes. Similarly, repeated administration of oxytocin to
342 rats for the first 14 d of life caused increased weighed gain [12] without changes in feed intake
343 [11]. Other studies have shown that oxytocin administration caused a decrease in plasma
344 corticosterone concentration, and increased circulatory levels of gastric hormones and insulin-
345 like growth factors [12-14]. In the present study, oxytocin administration early in life did not
346 influence live weight until 49 d of age. It has previously been demonstrated that increased weight
347 gain observed in rats does not occur immediately after injection, and instead coincides with the
348 onset of puberty and consequently with rises in circulating levels of growth hormone and steroid
349 hormones [12]. However, the increased live weight gain observed with the pig does not coincide
350 with the onset of puberty and therefore the reason for this delayed effect requires further
351 research.

352 Milk supplementation or oxytocin administration did not generally affect feed conversion
353 efficiency, back fat depth nor organ weights. Hence, the feed supplementations or administration
354 of oxytocin did not appear to cause any metabolic or gross anatomical physiological changes.
355 Notwithstanding, both oxytocin administration and milk supplementation increased leptin gene
356 expression in the stomach 1 wk after weaning. Gastric leptin gene expression has been shown to

be under the influence of feed intake, with gastric leptin mRNA decreasing in fasting conditions and increasing rapidly after a short period of feed intake in rats [22]. Leptin acts on hypothalamic neurons involved in feed intake regulation and energy metabolism. However, little is known about leptin action in the small intestine, but possible roles have been deduced such as intestinal lipid handling or intestinal sugar absorption [23-25]. Our results also showed that, on the day of weaning, leptin was lower in the ileum of pigs that were supplemented with milk during lactation as compared with their counterparts suckling the sow alone. Leptin is secreted from the mammary gland and excreted along with milk and consumed by the young. Indeed, pigs feeding on sows' milk have greater intestinal leptin expression than pigs fed on milk replacer alone [26], explaining why pigs receiving supplemental milk during lactation in our experiment showed lower leptin expression as they may have reduced their sow milk intake by substituting some for supplemental milk. Oxytocin administration also increased ghrelin expression in the stomach 1 wk after weaning. Opposite to leptin expression, ghrelin expression increases in the stomach in response to fasting and reduces within 45 min of re-feeding [27,28].

Milk supplementation after weaning also increased GLP-2 gene expression in the stomach 1 wk after weaning. Enhanced expression of intestinal GLP-2 has been coupled with increases in intestinal mucosal mass, increased villous height, crypt depth and brush border enzyme activity [29,30], which are all intestinal markers of small intestinal maturity and development. The influence of GLP-2 on intestinal growth and health occurs independently of feed intake and theoretically through suppression of proteolysis and apoptosis [29]. The greater intestinal mass induced by GLP-2 suggests that pigs submitted to a gradual diet transition over the first wk after weaning have enhanced digestive and absorptive functional capacity, which may in part explain the greater growth rate of these animals.

Pigs readily consumed a substantial amount of supplemental milk between day 10 and 20 of lactation, from approximately 85 g/d to 252 g/d per pig over the 10 d of supplementation. However, there were no differences in pre-weaning growth performance. Nonetheless, and interestingly, providing supplemental milk to pigs during lactation influenced lifelong growth performance with pigs being heavier at 49 d of age and also upon reaching market slaughter weight. Other authors have observed similar effects [6-8], as the long-term benefits conferred by additional milk early in life may be mediated by specific components contained in the milk.

Females adapted better to weaning than males, with a greater feed intake over the 2 wk post-weaning period and a greater growth rate in subsequent weeks. This phenomenon has previously been demonstrated [31-33]. The better adaptation to weaning by females in our study appeared independent of changes in organ weight or organ development. However, our results show a greater expression of GLP-2 in the ileum of females at 10 d of age and at weaning, which may explain the better ability of females to cope with weaning since GLP-2 improves gastrointestinal tract development and function. Similarly, Pluske *et al.* [4] reported that females have a more developed gastrointestinal tract system, greater pancreatic enzymatic capacity and greater mean villous height than males at 2 and 4 wk of age. Males showed greater GLP-1 gene expression in the duodenum at 1 wk after weaning. Unabsorbed nutrients in the lumen of the small intestine appears to be an important stimulus for GLP-1 secretion [34], and this is thought to induce satiety along with delayed gastric emptying [35]. Therefore, the delayed maturation of the males' gut may explain their lower feed intake after weaning and slower digestion, which ultimately impacts their growth.

Conclusions

Providing pigs with a gradual change in diet after weaning is likely to have the most beneficial effect in reducing stressful effects on growth and preserving gut health, partly though increasing ghrelin and GLP-2 expression in the stomach. Oxytocin administration daily for the first 2 wk of life also conferred advantages by reducing weight loss over the first 2 d after weaning and increasing both ghrelin and leptin. However, the provision of supplemental milk during lactation had no effect on the response to weaning.

References

1 Jensen P, Recen B. When to wean: observations from free-ranging domestic pigs. Appl. Anim. Behav. Sci. 1989;23:49-60.

2 Pluske JR, Williams IH, Aherne FX. Nutrition of the neonatal pig. In: The neonatal pig development and survival, Varley MA (Ed.), CAB International, 1995, pp 187-235.

3 Weary DM, Jasper J, Hotzel MJ. Understanding weaning distress. Appl. Anim. Behav. Sci. 2008;110:24-41.

4 Pluske JR, Hampson DJ, Williams IH. Factors influencing the structure and function of the small intestine in the weaned pig: A review. Livest. Prod. Sci. 1997;51:215-236.

5 Azain MJ, Tomkins T, Sowinski JS, Arentson RA, Jewell DE. Effect of supplemental pig milk replacer on litter performance: Seasonal variation in response. J. Anim. Sci. 1996;74:2195-2202.

6 Dunshea FR, Kerton DJ, Eason PJ, King RH. Supplemental skim milk before and after weaning improves growth performance of pigs. Aust. J. Agr. Res. 1999;50:1165-1170.

7 Wolter BF, Ellis M, Corrigan BP, DeDecker JM. The effect of birth weight and feeding of supplemental milk replacer to piglets during lactation on preweaning and postweaning growth performance and carcass characteristics. J. Anim. Sci. 2002;80:301-308.

8 King RH, Boyce JM, Dunshea FR. Effect of supplemental nutrients on the growth performance of suckling pigs. Aust. J. Agr. Res. 1998;49:883-887.

9 Pluske JR, Williams IH, Aherne FX. Villous height and crypt depth in piglets in response to increases in the intake of cows' milk after weaning. Anim. Sci. 1996;62:145-158.

10 Zijlstra RT, Whang KY, Easter RA, Odle J. Effect of feeding a milk replacer to early-weaned pigs on growth, body composition, and small intestinal morphology, compared with suckled littermates. J. Anim. Sci. 1996;74:2948-2959.

11 Uvnas-Moberg K, Alster P, Petersson M. Dissociation of oxytocin effects on body weight in two variants of female sprague-dawley rats. Int. Physiol. Behav. Sci. 1996;31:44-55.

12 Uvnas-Moberg K, Alster P, Petersson M, Sohlstrom A, Bjorkstrand E. Postnatal oxytocin injections cause sustained weight gain and increased nociceptive thresholds in male and female rats. Pediatr. Res. 1998;43:344-348.

13 Petersson M, Hulting A, Andersson R, Uvnäs-Moberg K. Long-term changes in gastrin, cholecystokinin and insulin in response to oxytocin treatment. Neuroendocrinol. 1999;69:202-208.

14 Petersson M, Hulting A-L, Uvnäs-Moberg K. Oxytocin causes a sustained decrease in plasma levels of corticosterone in rats. Neurosci. Lett. 1999;264:41-44.

15 Kavushansky A, Leshem M. Role of oxytocin and vasopressin in the transitions of weaning in the rat. Dev. Psychobiol. 2004;45:231-238.

16 Rault J-L, Carter CS, Garner JP, Marchant-Forde JN, Richert BT, Lay Jr DC. Repeated intranasal oxytocin administration in early life dysregulates the hpa axis and alters social behavior. Physiol. Behav. 2013;112–113:40-48.

17 Uvnas-Moberg K. Oxytocin may mediate the benefits of positive social interaction and emotions. Psychoneuroendocrinol. 1998; 23:819-835.

18 Yuan J, Reed A, Chen F, Stewart CN. Statistical analysis of real-time pcr data. BMC Bioinformatics 2006;7:85.

19 Carroll JA, Veum TL, Matteri RL. Endocrine responses to weaning and changes in post-weaning diet in the young pig. Domest. Anim. Endocrin 1998;15:183-194.

20 Spreeuwenberg MAM, Verdonk JMAJ, Gaskins HR, Verstegen MWA. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J. Nutr. 2001;131:1520-1527.

21 Rault J-L, Mack LA, Carter CS, Garner JP, Marchant-Forde JN, Richert BT, Lay Jr DC. Prenatal stress puzzle, the oxytocin piece: Prenatal stress alters the behaviour and autonomic regulation in piglets, insights from oxytocin. Appl. Anim. Behav. Sci. 2013;148:99-107.

22 Pico C, Oliver P, Sanchez J, Palou A. Gastric leptin: A putative role in the short-term regulation of food intake. Br. J. Nutr. 2003;90:735-741.

23 Macajova M, Lamosova D, Zeman M. Role of leptin in farm animals: A review. J. Vet. Med. 2004;51:157-166.

24 Morton NM, Emilsson V, Liu YL, Cawthorne MA. Leptin action in intestinal cells. J. Biol. Chem. 1998;273:26194-26201.

25 Lostao MP, Urdaneta E, Martinez-Anso E, Barber A, Martinez JA. Presence of leptin receptors in rat small intestine and leptin effect on sugar absorption. FEBS Lett. 1998;423:302-306.

26 Wolinski J, Biernat M, Guilloteau P, Westrom BR, Zabielski R. Exogenous leptin controls the development of the small intestine in neonatal piglets. J. Endocrinol. 2003;177:215-222.

27 Sanchez J, Oliver P, Palou A, Pico C. The inhibition of gastric ghrelin production by food intake in rats is dependent on the type of macronutrient. Endocrinol. 2004;145:5049-5055.

28 Salfen BE, Carroll JA, Keisler DH, Strauch TA. Effects of exogenous ghrelin on feed intake, weight gain, behavior, and endocrine responses in weanling pigs. J. Anim. Sci. 2004;82:1957-1966.

29 Shin ED, Drucker DJ, Brubaker PL. Glucagon-like peptide 2: An update. Curr. Opin. Endocrinol. Diabetes 2005;12:63-71.

30 Drucker DJ. Gut adaption and the glucagon-like peptides. Gut 2002;50:428-435.

31 Power GN, Pluske JR, Campbell RG, Cranwell PD, Kerton DJ, King RH, Dunshea FR. Effect of sex, weight and age on post-weaning growth of pigs. Proc. Nut. Soc. Aust. 1996;20:137.

32 Dunshea FR, Brown WG, Gough CD, Eason PJ. Female pigs better handle weaning then male pigs. Proc. Nut. Soc. Aust. 1998;22:103.

33 Dunshea FR, Kerton DJ, Eason PJ, King RH. Supplemental skim milk before and after weaning improves growth performance of pigs. Aust. J. Agr. Res. 1999;50:1165-1170.

34 Van Ginneken C, Verlinden K, Van Meir F, Sys S, Weyns A. A stereologic evaluation of glucagon-like peptide-1 (glp-1) mucosal cells in the small intestine of the developing pig. Anat. Embryol. 2002;205:153-157.

35 Larsen PJ, Holst JJ. Glucagon-related peptide 1 (glp-1): Hormone and neurotransmitter. Regul. Peptides 2005;128:97-107.

494 **Table 1.** List of primers and optimised conditions to quantify genes of interest.

495

496 **Table 2.** Effect of oxytocin administration (OT), sex (S), and supplemental milk feeding during lactation (L), and pellets
497 supplemented with milk diet post-weaning (W), on growth and dry matter (DM) feed intake from 21 d of age (weaning) to 28 d of age
498 (1 wk after weaning)

499

500 **Table 3.** Effects of oxytocin administration (OT), sex (S), supplemental milk feeding during lactation (L), and pellets supplemented
501 with milk diet post-weaning (W), on growth and dry matter (DM) feed intake from 28 d of age (1 wk after weaning) to 140 d of age
502 (market slaughter weight).

503

504 **Table 4.** Effects of oxytocin administration (OT), sex (S), supplemental milk feeding during lactation (L), and pellets supplemented
505 with milk diet post-weaning (W), on gene expression according to the RT-PCR ΔCT of pigs slaughtered at 28 d of age.

506 **Figure 1.** Liquid skim milk intake of nursing litters offered supplemental milk between 10 and
507 20 d of age (pre-weaning).

Table 1. List of primers and optimised conditions to quantify genes of interest.

Gene (Abbreviation)	Accession number	Primer sequence	Optimum annealing temperature (^{o}C)	Primer concentration (nM)
18s Ribosomal RNA (r18S1)	AY265350	Forward 5' GAA CGC CAC TTG TCC CTC TA 3' Reverse 5' GAC TCA ACA CGG GAA ACC TC 3'	61.2	60
Ghrelin	NM213807	Forward 5' CAC CAG AAA GTG CAG CAG AG 3' Reverse 5' GAA CAG AGG TGG CTG GTC TC 3'	57.0	200
Leptin (Lep1)	NM213840	Forward 5' CCT CTG AAT GGT CTG GGT TG 3' Reverse 5' GGA CTT GGG ACC ATC TGC TA 3'	57.5	400
Proglucagon (GLP-1)	AY242124	Forward 5' ACC ATT TAC TTT GTG GCT GGA 3' Reverse 5' GAG CTG GGA ATG ATC TGG ATT 3'	58.2	200
Glucagon-like peptide 2 (GLP-2)	NM214324	Forward 5' GCT GAC CAG TGA CAA TGA CC 3' Reverse 5' GGC ACC GGA ATC TCC TAG TC 3'	58.5	300

Table 2. Effect of oxytocin administration (OT), sex (S), and supplemental milk feeding during lactation (L), and pellets supplemented with milk diet post-weaning (W), on growth and dry matter (DM) feed intake from 21 d of age (weaning) to 28 d of age (1 wk after weaning)

	Administration		**Sex**		**Pre-weaning**		**Post-weaning**			**Significance (P-values)**			
	Oxytocin	Saline	Female	Male	Milk	Unsuppl-emented	Unsuppl-emented	Pellets + milk	SED	OT	S	L	W
Liveweight (kg)													
d 21	6.5	6.5	6.5	6.5	6.7	6.4	6.5	6.5	0.16	0.92	0.86	0.32	-
d 23	6.2	6.5	6.7	6.5	6.6	6.6	6.3	6.9	0.18	0.49	0.36	0.74	<0.01
d 25	6.9	6.9	7.0	6.8	7.0	6.8	6.7	7.1	0.17	0.88	0.48	0.39	<0.01
d 28	7.4	7.4	7.5	7.3	7.5	7.2	7.2	7.6	0.17	0.94	0.32	0.28	0.02
Average weight gain (g/d)													
Wean to d 23	-214	-293	-236	-272	-301	-207	-373	-135	25.4	0.03	0.39	0.32	<0.01
Wean to d 25	52	45	54	43	48	49	8	89	13.1	0.59	0.45	0.99	<0.01
Wean to d 28	106	94	108	91	104	96	76	124	9.6	0.33	0.16	0.54	<0.01
DM feed intake (g/d)													
Wean to d 23	369	374	397	346	367	376	51	692	16.7	0.70	0.05	0.77	<0.01
Wean to d 25	342	349	371	320	351	341	91	601	12.1	0.59	0.01	0.67	<0.01
Wean to d 28	343	350	369	324	352	341	131	561	11.2	0.58	0.02	0.54	<0.01

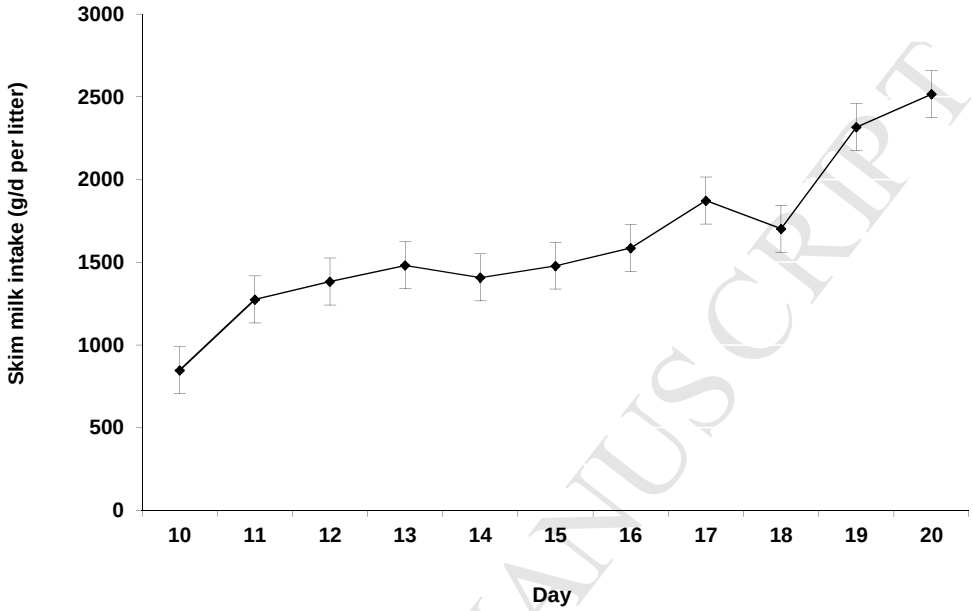
Table 3. Effects of oxytocin administration (OT), sex (S), supplemental milk feeding during lactation (L), and pellets supplemented with milk diet post-weaning (W), on growth and dry matter (DM) feed intake from 28 d of age (1 wk after weaning) to 140 d of age (market slaughter weight).

	Administration		**Sex**		**Pre-weaning**		**Post-weaning**			**Significance (P-values)**			
	Oxytocin	Saline	Female	Male	Milk	Unsupp-lemented	Unsupp-lemented	Pellets + milk	SED	OT	S	L	W
Liveweight (kg)													
d 28	7.4	7.4	7.5	7.3	7.5	7.2	7.2	7.6	0.18	0.94	0.32	0.28	0.02
d 35	9.7	9.4	9.8	9.2	9.9	9.2	9.4	9.7	0.29	0.39	0.04	0.07	0.42
d 42	12.7	12.4	12.9	12.2	13.0	12.1	12.5	12. 6	0.37	0.42	0.04	0.08	0.88
d 49	17.4	16.5	17.4	16.5	17. 6	16.4	17.0	16.9	0.43	0.04	0.04	0.02	0.88
d 56	22.4	21.6	22.4	21. 6	22.6	21.4	21.9	22.1	0.52	0.12	0.08	0.05	0.68
d 140	90.0	87.0	87.9	89.3	86.4	90.5	87.6	89.3	1.72	0.11	0.44	0.05	0.39
Average weight gain (g/d)													
d28 to d35	340	325	350	315	349	316	337	328	23.0	0.54	0.13	0.20	0.63
d35 to d42	441	443	461	423	459	425	449	435	27.3	0.89	0.16	0.35	0.66
d42 to d49	672	593	640	625	655	610	638	627	29.4	0.01	0.57	0.20	0.65
d49 to d56	711	726	724	713	726	711	703	734	32.8	0.72	0.74	0.61	0.26
DM feed intake (g/d)													
d28 to d35	412	389	428	373	416	385	423	378	22.1	0.30	0.01	0.34	0.04
d35 to d42	710	711	728	693	726	694	705	716	32.3	0.92	0.30	0.45	0.70
d42 to d49	1060	1001	1064	997	1043	1018	1044	1017	39.8	0.13	0.09	0.63	0.47
d49 to d56	1284	1200	1257	1227	1277	1207	1217	1267	43.3	0.04	0.46	0.10	0.18

Table 4. Effects of oxytocin administration (OT), sex (S), supplemental milk feeding during lactation (L), and pellets supplemented with milk diet post-weaning (W), on gene expression according to the RT-PCR ΔCt of pigs slaughtered at 28 d of age.

	Administration		**Sex**		**Pre-weaning**		**Post-weaning**			**Significance (P- values)**			
	Oxytocin	Saline	Female	Male	Milk	Unsuppl-emented	Dry	Pellets +milk	SED	OT	S	L	W
Stomach													
Ghrelin	3.22	8.10	5.06	6.26	5.71	5.61	5.52	5.81	3.25	0.02	0.31	0.35	0.90
Leptin	6.17	8.89	7.24	7.82	8.35	6.72	9.21	5.86	1.49	0.02	0.93	0.20	0.02
Duodenum													
Leptin	3.91	5.58	4.85	4.64	5.44	4.04	4.89	4.60	1.48	0.61	0.73	0.43	0.94
GLP-1	7.00	7.99	8.93	6.06	7.91	7.08	6.36	8.63	1.5	0.36	0.03	0.55	0.08
GLP-2	5.02	6.99	5.78	6.27	6.22	5.79	5.61	6.41	1.02	0.06	0.50	0.98	0.51
Ileum													
Leptin	5.77	4.63	5.46	4.94	6.23	4.16	4.38	6.02	1.37	0.49	0.78	0.08	0.24
GLP-1	5.13	3.17	5.46	2.83	4.74	3.56	4.25	4.05	2.91	0.10	0.52	0.64	0.31
GLP-2	9.14	7.11	8.18	8.07	9.12	7.13	7.11	9.14	1.25	0.08	0.82	0.07	0.05

Figure 1. Liquid skim milk intake of nursing litters offered supplemental milk between 10 and 20 d of age (pre-weaning).



Highlights

- Weaning is a multi-factorial stressor associated with a major diet change
- Neonatal oxytocin and milk supplemented post-weaning reduced weight loss
- Neonatal oxytocin increased stomach ghrelin and leptin expression
- Milk supplemented post-weaning increased stomach leptin and GLP-2 expression
- Neonatal oxytocin or post-weaning milk supplementation improve gut health at weaning