Project 2B 102
Final Report – part 2

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Neonatal oxytocin treatment and milk feeding of pigs before and after weaning influences the expression of gastrointestinal hormones regulating feed intake throughout the gastrointestinal tract of the piglet

4.1 Introduction

In Experiment 2, it was established that the provision of supplemental milk throughout the lactation period correlated to a 5% increase in market slaughter weight. In addition the continuation of supplemental milk provision into the initial post-weaning period aided in reducing the magnitude of the post-weaning growth lag, through immediate feed intake. Piglets receiving supplemental milk post-weaning were heavier, had a greater average daily gain and daily feed intake for the entire first week post-weaning. Independently of post-weaning milk supplementation, the administration of oxytocin to piglets for the first 14 days of life lead to reduced magnitude of the post-weaning growth lag.

There is some evidence in animals, humans and to a much lesser extent pigs, that feed intake is regulated by the endocrine system along with nervous mechanisms. The stomach and intestine are important organs involved in the regulation of feed intake with hormones being secreted into the blood in order to signal information from the stomach or intestine to the brain (Zhang et al., 2006). These hormones regulate feed intake through interactions that either lead to enhanced feed intake or alternatively the induction of satiety. However, it is known that some nutrients can influence mRNA expression and secretion of hormones in the stomach intestine and other organs as well as influence feed intake through cooperation with the nervous system (Zhang et al., 2006). While the specific role of gastrointestinal hormones and their roles in the regulation of feed intake have been extensively explored, it is important to establish their functional roles in pigs and the specific alterations in hormone levels that occur in association with the weaning transition and different periods of growth. The enhanced growth rates of piglets receiving supplemental milk both during lactation and in the initial post-weaning period may be associated with alterations in the expression of particular GI hormones. The establishment of the influence of feeding regime and the expression of these hormones is important in order to understand the piglets ability to effectively utilise diets and consequently to use feeding regimes that could optimise growth potential at critical times in a piglet's life such as weaning. It may also be beneficial to determine sex related differences in the expression of GI hormones, as it may aid in explaining why sex related alterations in growth rate commonly occur around weaning.

The aim of the current study was to determine if supplemental milk provision both pre- and post-weaning beneficially enhanced the expression of gastrointestinal hormones leading to enhanced feed intake. It is also thought that exploring the expression of gastrointestinal hormones may assist in explaining the beneficial effects of oxytocin on partially ameliorating the post-weaning lag in growth as oxytocin administration to rats has been associated with altered expression of leptin and cholecystokinin (Uvnas-Moberg et al., 1998).

4.2 Methodology

4.2.1 *Experimental design and animals*

The experiment used 83 piglets obtained at birth, which suckled 20 sows (Large White x Landrace). The piglets were allocated to treatments in a 2 x 2 x 2 x 2 randomised block design. The respective factors were sex (male v female), injection (oxytocin v saline), pre-weaning dietary treatment (supplemented with skim milk during lactation vs. unsupplemented) and post-weaning dietary treatment (gruel v pellet). These piglets were euthanased at 10 days of age (n=15) or weaning (n=38). Some piglets carried over into the post-weaning period were also subjected to euthanasia (n=30).

Injections were administered to piglets daily from birth to 14 days of age. Injections were given at a rate of 1mg/kg and involved either oxytocin (Ilium Syntocin, Troy Laboratories) or saline (Baxter). From 10 days of age, piglets from 10 of the litters in addition to suckling the sow were introduced to supplemental liquid skim milk (250g/L) which was available *ad libitum* until weaning. Skim milk powder was reconstituted by adding powdered skim milk (Murray Goulbourn, Melbourne) and 200ml of Yakult (probiotic) to warm tap water followed by mixing. Yakult was added to the skim milk as scours have been observed in early-weaned piglets receiving liquid milk diets. The skim milk was delivered by a gravity feed system that was designed to minimise spillage and contamination by faeces and urine (see Experiment 2).

Pigs not euthanased prior to weaning were weaned at 21 days of age into individual weaner cots in an experimental weaner shed. Half of the pigs were weaned onto Ultrawean 75 (Ridley) whereas the other half were weaned onto the same 25% liquid skim milk powder mix as well as having *ad libitum* access to the pelleted diet. For the first two days after weaning those pigs weaned onto the skim milk powder diet were fed 1L of liquid skim milk. On the third day after weaning, 200g of Ultrawean 75 were added and the amount of pelleted diet increased by 100 g/day until day seven, while the amount of skim milk decreased proportionately.

4.2.2 *Euthanasia and tissue sample collection*

Pigs were euthanased via a lethal injection of Lethobarb® (Virbac, Australia) at a dose of 0.3ml/kg at 10 days of age (n=15), weaning (n=38) or at 28 days of age (n=30). Immediately following slaughter an incision was made into the peritoneal cavity. Samples were collected from the proximal duodenum and ileum and the intestines removed. The stomach was then removed flushed with saline and a sample collected from the fundic region. The skull of the piglets was opened and the entire hypothalamus removed from the brain. All samples were immediately cleaned with saline upon removal, wrapped in aluminium foil and snap frozen in liquid nitrogen. Samples were later transferred to the -80ºC freezer and stored until analysis.

4.2.3 *Real-time polymerase chain reaction analysis of gene expression in tissue samples*

RNA was extracted from tissue samples using Trizol® Reagent (Invitrogen, Australia) as per Section 2.1.6.1. RNA quality was determined using the Experion™ (Bio-Rad, USA), as per Section 2.1.6.2. RNA of suitable quality was reverse transcribed into cDNA in triplicate using the SuperScript™ III First Strand Synthesis System for RT-PCR (Invitrogen, USA), random hexamers method as per section 2.1.6.3. cDNA samples were analysed via RT-PCR using primer sets for 18s rRNA (housekeeper), ghrelin, leptin, NPY, GLP-1 and GLP-2 at optimised temperatures. The PCR protocol used was the optimal temperatures for the primer sets in cycle 2, step 2.

4.2.4 *Statistical Analysis*

PCR results were determined using the ΔCt method. ΔCt is derived from subtracting the ct cycle of the housekeeping gene from that of the target gene. For ΔCt data, the larger the value, the lower the amplification of the target gene. All ΔCts were statistically analysed using residual maximum likelihood (REML) using GENSTAT for Windows Version 7.0 (Payne *et al.* 1993). Gene was used as the Y-variate, fixed model was injection, sex, pre-weaning dietary treatment and post-weaning dietary treatment. Sow was used as the random model. Statistical significance was accepted at $P \leq 0.005$.

4.3 Outcomes

4.3.1. *Observed alterations in gene expression at ten days of age.*

All gene expression data for piglets slaughtered at 10 days of age are outlined in Table 4.1. At 10 days of age no alterations in gene expression were observed in the hypothalamus or duodenum of piglets. Leptin expression was higher in the stomach of oxytocin treated piglets then those receiving injections of saline (9.35 v 15.04, $P=0.028$). In the ileum the expression of GLP-2 was higher in females then males (3.35 v 7.46, $P<0.001$).

Table 4.1. RT-PCR ΔCT ratios for pigs slaughtered at day 10.

	Injection		Sex		s.e.d.	I	S
	Oxytocin	Saline	Female	Male			
Hypothalamus							
Ghrelin	6.59	8.62	9.57	5.64	2.54	0.148	0.126
Leptin	-1.03	0.014	-0.07	-0.81	2.10	0.434	0.727
NPY	3.72	4.86	3.83	4.76	1.81	0.560	0.517
GLP-1	8.61	10.12	9.83	8.91	1.48	0.162	0.604
GLP-2	3.82	5.40	4.08	5.12	1.20	0.221	0.290
Stomach							
Ghrelin	6.68	7.38	8.31	5.75	2.37	0.528	0.236
Leptin	9.35	15.04	14.02	10.37	3.30	0.028	0.235
Duodenum							
Leptin	1.31	0.89	0.49	1.71	3.14	0.784	0.679
GLP-1	0.60	2.83	2.20	1.24	3.24	0.417	0.727
GLP-2	3.19	2.60	4.13	1.67	2.51	0.906	0.331
Ileum							
Leptin	9.58	9.09	8.99	9.66	2.01	0.656	0.828
GLP-1	6.57	8.54	7.02	8.09	1.42	0.206	0.376
GLP-2	4.59	6.22	3.35	7.46	0.99	0.826	<0.001

Alterations in gene expression at 21 days of age

All gene expression data for piglets slaughtered at 21 days of age is outlined in Table 4.2. In the hypothalamus, GLP-2 expression was higher in males then females (1.45 v 4.24, P=0.04). No alterations in expression were observed in either the stomach or duodenum. In the ileum, GLP-2 expression was higher in females then males (7.40 v 10.56, P=0.037). The expression of leptin in the ileum was higher in piglets that had not received supplemental milk in comparison to those that had been provided with supplemental milk from ten days of lactation (4.27 v 9.42, P=0.043).

Alterations in gene expression at 28 days of age

All gene expression data for piglets at 28 days of age are outlined in Table 4.3. In the hypothalamus no alteration in gene expression was observed at 28 days of age. In the stomach, ghrelin expression was higher in oxytocin treated piglets then saline treated piglets (3.22 v 8.10, P=0.016). Leptin expression in the stomach was also higher in oxytocin treated piglets then those treated with saline (6.17 v 8.89, P=0.017). Leptin expression in the stomach was higher in piglets that were weaned onto the gruel-based diet then those weaned onto the pelleted diet (5.86 v 9.21, P=0.018). GLP-1 expression in the duodenum was higher in males then females (6.06 v 8.93, P=0.048). GLP-2 expression in the ileum was higher in piglets weaned onto the dry pelleted diet then those weaned onto the gruel based diet (7.11 v 9.14, P=0.048).

Table 4.2. RT-PCR ΔCT of pigs slaughtered at weaning.

	Injection		Sex		Pre-weaning		s.e.d.	I	S	W
	Oxytocin	Saline	Female	Male	Milk	Unsup				
Hypothalamus										
Ghrelin	2.26	5.25	3.60	3.91	2.38	5.12	2.02	0.215	0.961	0.165
Leptin	1.18	0.18	1.86	-0.51	0.13	1.22	1.6	0.434	0.109	0.607
NPY	3.43	2.69	3.85	2.27	2.99	3.13	1.79	0.626	0.109	0.607
GLP-1	2.78	2.86	3.71	1.93	2.67	2.97	1.45	0.745	0.158	0.781
GLP-2	3.38	2.31	4.24	1.45	2.54	3.15	1.57	0.391	0.04	0.591
Stomach										
Ghrelin	3.57	4.06	3.86	3.77	3.31	4.32	1.91	0.93	0.981	0.712
Leptin	8.49	6.34	5.87	8.96	10.21	4.62	2.20	0.547	0.142	0.088
Duodenum										
Leptin	3.59	3.47	2.26	4.79	3.76	3.3	1.85	0.971	0.225	0.874
GLP-1	9.86	9.84	10.84	8.86	9.23	10.47	1.38	0.857	0.160	0.627
GLP-2	7.87	6.01	5.91	7.96	8.15	5.73	1.33	0.348	0.105	0.220
Ileum										
Leptin	5.40	8.29	6.35	7.34	9.42	4.27	2.17	0.106	0.623	0.043
GLP-1	4.77	6.15	4.10	6.82	4.63	6.28	1.44	0.164	0.131	0.297
GLP-2	8.47	9.73	7.40	10.56	9.08	9.12	0.02	0.318	0.037	0.881

Table 4.3. RT-PCR ΔCT of pigs slaughtered at d28.

	Injection		Sex		Pre-weaning		Post-weaning		s.e.d.	I	S	L	W
	Oxytocin	Saline	Female	Male	Milk	Unsup	Dry	Gruel					
Hypothalamus													
Ghrelin	10.64	7.28	9.18	8.73	8.00	9.92	9.04	8.87	2.48	0.114	0.667	0.339	0.784
Leptin	7.04	5.02	8.15	3.91	6.08	5.98	6.19	5.86	2.72	0.480	0.130	0.970	0.779
NPY	10.78	8.57	10.41	8.94	9.58	9.76	9.61	9.74	1.53	0.164	0.229	0.992	0.734
GLP-1	8.62	7.11	8.20	7.53	8.33	7.40	8.19	7.54	1.24	0.084	0.454	0.666	0.606
GLP-2	5.07	4.27	4.52	5.82	4.71	4.63	4.91	4.43	1.31	0.062	0.895	0.790	0.210
Stomach													
Ghrelin	3.22	8.10	5.06	6.26	5.71	5.61	5.52	5.81	3.25	0.016	0.311	0.354	0.895
Leptin	6.17	8.89	7.24	7.82	8.35	6.72	9.21	5.86	1.49	0.017	0.932	0.200	0.018
Duodenum													
Leptin	3.91	5.58	4.85	4.64	5.44	4.04	4.89	4.60	1.48	0.609	0.729	0.432	0.944
GLP-1	7.00	7.99	8.93	6.06	7.91	7.08	6.36	8.63	1.5	0.355	0.026	0.552	0.078
GLP-2	5.02	6.99	5.78	6.27	6.22	5.79	5.61	6.41	1.02	0.063	0.501	0.978	0.511
Ileum													
Leptin	5.77	4.63	5.46	4.94	6.23	4.16	4.38	6.02	1.37	0.492	0.776	0.082	0.243
GLP-1	5.13	3.17	5.46	2.83	4.74	3.56	4.25	4.05	2.91	0.098	0.515	0.636	0.306
GlP-2	9.14	7.11	8.18	8.07	9.12	7.13	7.11	9.14	1.25	0.082	0.824	0.067	0.048

4.4 Discussion and Applications of Research

The expression of the gastrointestinal hormones involved in influencing feed intake were seen to have differing responses in response to milk supplementation and oxytocin injection. However, the expression of some of these hormones was also influenced by the sex of the piglet. Gastric leptin expression is under the influence of feed intake (Pico et al., 2003). Pico *et al.* (2002) have demonstrated in rats that the level of gastric leptin mRNA is decreased in fasting conditions and increases rapidly after a short period of feed intake. Therefore the greater expression of leptin observed in the stomach of oxytocin treated piglets at 10 days of age indicates that oxytocin treatment lead to enhanced feed intake at this stage in life. While piglets receiving oxytocin injections did not exhibit a greater live weight at this time it is unknown whether feed intake was in fact greater in these piglets as no information on feed intake throughout this period. However, consistent with this finding it has been shown that in rats feed intake and growth was greater throughout the suckling periods when administered with daily oxytocin injections (Uvnas-Moberg and Petersson, 2005).

Oxytocin has been shown to have central anorexic effects, influences gastric emptying and gastric motility with central administration of oxytocin inhibiting feed intake (Hillebrand et al., 2002). Despite oxytocin being a neuropeptide involved in the inhibition of feed intake, Sohlstrom *et al.* (2000) demonstrated that oxytocin administration throughout the first two weeks of life stimulates post-natal growth particularly in females, decreases blood pressure and plasma levels of corticosterone as well as increases adiposity in adult rats. The result is thought to be that the early administration of oxytocin may permanently alter the endocrine axes resulting in anti-stress and anabolic effects (Sohlstrom et al., 2000; Sohlstrom et al., 2002). The chronic administration of oxytocin has been demonstrated to reduce the stress response to noise as well as anxiety behaviour in rats (Sohlstrom et al., 2000; Windle et al., 1997). Therefore, while oxytocin administration did not lead to greater live weight at 10 days of age, it may have reduced stress leading to greater feed intake and consequently enhanced gastric leptin expression. This enhanced expression of leptin was no longer present at weaning indicating that the anti-stress patterns of oxytocin may be reduced upon cessation of oxytocin administration at fourteen days of age.

However, the administration of oxytocin was observed to enhance gastric leptin expression at 28 days of age indicating that oxytocin administration reduces the stress response to weaning. Conversely the enhanced gastric leptin expression occurred in conjunction with enhanced gastric expression of ghrelin. Ghrelin and leptin in the gastric mucosa have differing fundamental roles. Ghrelin expression rises in the stomach in response to fasting and reduces within 45 minutes of re-feeding (Salfen et al., 2004a; Sanchez et al., 2004). Leptin has the opposite response in that its expression decreases dramatically in response to a long period of fasting and increases rapidly in response to feeding (Pico et al., 2003). The enhanced expression of both ghrelin and leptin in the stomach at 28 days of age in response to oxytocin treatment may assist in explaining the greater average daily gain observed in oxytocin treated piglets over the first two days post-weaning. Oxytocin administration partially ameliorated the post-weaning lag in growth commonly observed post-weaning in piglets. This partial amelioration may have been the result of immediate feed intake in the initial post-weaning period most probably through a reduction in the stress response to weaning reflective upon the enhanced expression of ghrelin and leptin. The elevated expression of both ghrelin and leptin does not put the piglet in a satiated state therefore allowing greater growth rates in oxytocin treated piglets especially throughout the first two days post-weaning.

In the ileum of female piglets, it was seen that the expression of GLP-2 was greater at both 10 days of age and at weaning. Enhanced expression of GLP-2 particularly in the hypothalamus has been associated with increased feed intake although it is expected that enhanced expression of GLP-2 would be correlated with increased expression of leptin and GLP-1 (Burrin et al., 2003). Alternatively it is more likely that the enhanced expression of GLP-2 is associated with an enhanced state of intestinal health or intestinal development. The influence of GLP-2 on intestinal growth and health has been demonstrated to occur independently of feed intake (Shin et al., 2005). 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 (Drucker, 2002; Shin et al., 2005), which are all intestinal markers of small intestinal maturity and development (Pluske et al., 2003a). The effects of GLP-2 on intestinal health are thought to occur through suppression of proteolysis and apoptosis (Shin et al., 2005). The greater intestinal mass induced by GLP-2 exhibits enhanced digestive and absorptive functional capacity.

Previously it has been demonstrated that females eat more, grow more quickly and better adapt to weaning then males (Dunshea, 2001; Dunshea et al., 1998; Power et al., 1996). The enhanced expression of GLP-2 observed in females throughout the pre-weaning period may assist in explaining the better adaptation of females to the weaning process. Cranwell et *al.* (1997) and Pluske *et al.* (1997) stated that females appeared to have a more developed GI system and pancreatic enzymatic capacity then males regardless of whether they were weaned at 14 or 28 days of age. This was associated with greater mean villous height in females then males regardless of weaning age (Pluske et al., 1997). The greater mean villous height is most likely associated with increased GLP-2 expression leading to the better ability of females to adapt to the weaning process.

While GLP-2 was enhanced in the ileum of females at weaning, in males GLP-2 expression was increased in the hypothalamus. GLP-2 expression in the hypothalamus leads to decreased voluntary feed intake. This is a result of inhibiting gastric motility in order to enhance nutrient digestion and absorption (Shin et al., 2005). The enhanced GLP-2 expression seen in the hypothalamus at this time may compromise the ability of males to adapt to weaning.

Research has demonstrated that leptin acts on hypothalamic neurons involved in feed intake regulation and energy metabolism, however little is known about leptin action in the small intestine. Recently possible roles for leptin in the small intestine have been deduced such as intestinal lipid handling and a role in intestinal sugar absorption (Lostao et al., 1998; Macajova et al., 2004; Morton et al., 1998). At weaning it was observed that expression of leptin was higher in the ileum of piglets that had been suckling the sow alone. Leptin is secreted from the mammary gland and excreted along with milk and consumed by the infant. Wolinski *et al.* (2003) suggested that leptin present in colostrum might be important in controlling the development of small intestinal structure and function in neonatal pigs. Consistent with the findings in this study, it has been demonstrated that piglets on sows' milk had higher intestinal leptin expression then those that were on milk replacer alone (Wolinski et al., 2003). While piglets receiving supplemental milk were also suckling the sow in the current study the lower leptin expression may be explained by supplemental milk consumption.

Unabsorbed nutrients in the lumen appears to be an important stimulus for GLP-1 secretion in rats pigs and humans (Van Ginneken et al., 2002). The presence of nutrients in the lumen of the small intestine is thought to have a satiating effect restricting food intake along with the simultaneous release of GLP-1 resulting in delayed gastric emptying (Larsen and Holst, 2005).

The elevated expression of GLP-1 throughout the first week post-weaning, while usually reflective upon enhanced feed intake, may have resulted in delayed gastric emptying consequently reducing feed intake in male piglets as a result of being satiated.

While GLP-2 expression was higher in the ileum of dry-fed piglets, this result was not linked to the differences in feed intake between the two dietary treatments. In this case it most likely linked to intestinal health status. While it has been demonstrated that the provision of whole cows milk after weaning above maintenance requirements in piglets can avoid the post-weaning growth lag and maintain intestinal health (Pluske et al., 1996), it is possible that the alteration in intestinal health in gruel-fed piglets was delayed as by 28 days of age post-weaning feed intake in gruel-fed piglets had decreased as a result of being on a dry pelleted diet. Throughout the first week post-weaning, while the provision of supplemental milk partially ameliorated the post-weaning lag in growth and resulted in immediate post-weaning feed intake, as the week progressed and dry pelleted diet was gradually introduced until they were consuming a completely solid diet, while feed intake was greater then dry-fed piglets, feed intake in gruel-fed piglets gradually decreased. Despite this, intestinal health in the gruel-fed piglets might be greater as a result of dry-fed piglets undergoing a more abrupt weaning, and there is evidence suggesting that it can take over a week for the structure of the small intestine to recover from the harsh weaning process.

4.5 Conclusions and Recommendations

Oxytocin administration to piglets enhanced gastric leptin expression at 10 and 28 days of age, which is associated with enhanced feed intake. This enabled oxytocin to partially ameliorate the post-weaning lag in growth and the possible promotion of immediate feed intake post-weaning. In addition, it was seen that female piglets were better equipped to handle the weaning process as the greater expression of GLP-2 observed in the ileum of this piglets up until weaning indicates that intestinal development in females is more advanced allowing them to better adapt to a change in diet.

4.6 Limitations and Risks

Two experiments now have shown that the use of oxytocin during lactation appears to offer some promise in assisting piglets in overcoming the post-weaning malaise. As mentioned previously, costs associated with its use need to be considered in view of the benefits that could be achieved.

4.7 References

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