INVESTIGATION OF THE ROLE OF INSULIN-LIKE GROWTH FACTOR (IGF)-I AND IGF BINDING PROTEINS IN INTESTINAL AND SOMATIC GROWTH REGULATION IN PIGS

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon

By

Min Tang

Spring 1999

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment

of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

Min Tang

Department of Animal and Poultry Science
University of Saskatchewan

Spring 1999

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Investigation of the Role of Insulin-like Growth Factor (IGF)-I and IGF Binding Proteins in Intestinal and Somatic Growth Regulation in Pigs

Insulin-like growth factor (IGF)-I mediates the growth promoting effects of growth hormone and is bound to one of six high affinity IGF binding proteins (IGFBPs) that control its biological action. The importance of IGFBPs and IGF-I in the regulation of somatic and intestinal growth was studied in pigs weaned into a reduced infection pressure (SEW) vs. conventional (CON) rearing environment. SEW pigs grew faster ($P<0.01$) than CON pigs. Weaning resulted in a large decrease in the levels of IGF-I and IGFBP-3 and an increase in IGFBP-1 in the circulation for both treatments. Weaning decreased hepatic IGF-I and IGFBP-3 mRNA, increased intestinal IGF-I, but did not change IGFBP-3 mRNA. Hepatic IGF-I and IGFBP-3 mRNA abundance was uncoupled from levels of IGF-I and IGFBP-3 in plasma. Higher levels of plasma IGF-I and IGFBP-3 were associated with improved growth in SEW pigs. Increased hepatic IGFBP-2 mRNA expression along with elevated plasma IGFBP-2 levels were associated with attenuated growth in CON pigs, suggesting that IGFBP-2 is a negative regulator in somatic growth.

Weaning was associated with increased IGF-I mRNA and decreased IGF-I receptor mRNA coincident with a reduction in the number of IGF-I receptors in intestine. The intestinal IGF-I (31%, $P < 0.01$) and IGFBP-5 (24%) mRNA level were higher in SEW than in CON pigs at 3 d post-weaning. In contrast, SEW pigs showed lower abundance of IGF-I (15%) and IGFBP-5 (40%, $P < 0.05$) mRNA than CON pigs at 34 d of age, probably reflecting the effect of lower infectious pressure. Scanning electron microscopy and AB/PAS staining revealed a thick mucus coating over the gut epithelium in CON

pigs, which was not present in SEW pigs. reflecting an increased pathogen load in the gut of CON pigs. Furthermore, SEW pigs showed higher activities of brush border enzymes, ratios of mucosal protein to DNA and villus height to crypt depth compared with CON pigs at 34 d of age.

The results demonstrate that: (1) weaning and weaning environment alter expression of intestinal IGF-I and IGFBPs; (2) SEW induces advanced post-weaning gut maturation; (3) the concentrations of plasma IGFBPs, particularly that of IGFBP-2, play an important role in somatic growth regulation.

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CERTIFICATION OF THESIS WORK

We, the undersigned, certify that **Min TANG,** candidate for the degree of Doctor of Philosophy has presented a thesis with the following title: **"Investigation of the Role of Insulin-Like Growth Factor (IGF)-I and IGF Binding Proteins in Intestinal and Somatic Growth Regulation in Pigs".** We consider that the thesis is acceptable in form and content, and that the candidate through an oral examination held on March 23, 1999, demonstrated a satisfactory knowledge of the field covered by the thesis.

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ABSTRACT

Insulin-like growth factor (IGF)-I mediates the growth promoting effects of growth hormone and is bound to one of six high affinity IGF binding proteins (IGFBPs) that control its biological action. The importance of IGFBPs and IGF-I in the regulation of somatic and intestinal growth was studied in pigs weaned into a reduced infection pressure (SEW) vs. conventional (CON) rearing environment. SEW pigs grew faster ($P<0.01$) than CON pigs. Weaning resulted in a large decrease in the levels of IGF-I and IGFBP-3 and an increase in IGFBP-1 in the circulation for both groups of pigs. Weaning decreased hepatic IGF-I and IGFBP-3 mRNA, increased intestinal IGF-I, but did not change intestinal IGFBP-3 mRNA. Hepatic IGF-I and IGFBP-3 mRNA abundance was uncoupled from levels of IGF-I and IGFBP-3 in plasma. Higher levels of plasma IGF-I and IGFBP-3 were associated with improved growth in SEW pigs. Increased hepatic IGFBP-2 mRNA expression along with elevated plasma IGFBP-2 levels were associated with attenuated growth in CON pigs, suggesting that IGFBP-2 is a negative regulator in somatic growth.

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The results demonstrate that: (1) weaning and weaning environment alter expression of intestinal IGF-I and IGFBPs; (2) SEW induces advanced post-weaning gut maturation; (3) the concentrations of plasma IGFBP-2 may play a negative role in somatic growth regulation, whereas IGFBP-3 and IGF-I plasma level may be a positive regulator of somatic growth.

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LIST OF ABBREVIATIONS

a.a.	amino acid
AGD	Arg-Gly-Asp
AB/PAS stain	alcian blue and periodic acid schiff staining
ALP	alkaline phosphatase
ALS	acid-labile subunit
bp	base pairs
BB enzymes	brush border enzymes
BSA	bovine serum albumin
cDNA	complementary DNA
CON	conventional
cv	coefficient of variation
EGF	epidermal growth factor
GH	growth hormone
GHRH	growth hormone release hormone
GI	gastrointestine
HE stain	hematoxylin and eosin stain
IGF-I	insulin like growth factor-I
IGF-II	insulin like growth factor-II
IGFBPs	insulin like growth factor binding proteins
IGF-IR	type I insulin like growth factor receptor
kDa	kilo Dalton
K_D	disassociation constant
mRNA	messenger RNA
P	probability (for tests of least significant difference)
PEPCK	phosphoenolpyruvate carboxykinase
PG-PS	peptidoglycan polysaccharidase
PK-A	protein kinase A
PK-C	protein kinase C
RIA	radioimmunoassay
SRIF	somatotropin release inhibiting factor
stdev	standard deviation
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEM	standard error of the mean
SEW	segregated early weaning
SI	small intestine
TPN	total parenteral nutrition
UTR	untranslated region
VIP	vasoactive intestinal peptide

1 INTRODUCTION

Animal growth is regulated through a complex interaction of genetic, nutritional, hormonal and environmental factors. Therefore, differences in growth rates of individual animals are due, in part, to a divergence in the secretion and biological effects of hormones and growth factors in response to nutritional and environmental factors.

Evidence strongly supports that insulin-like growth factor (IGF)-I is an anabolic, mitogenic peptide and an important regulator of postnatal growth. IGF-I is produced by most body tissues and is abundant in the blood circulation. IGF-I exerts its anabolic or mitogenic effects via endocrine as well as autocrine and/or paracrine mechanisms (Cohick and Clemmons 1993). IGF-I is bound to high affinity IGF binding proteins (IGFBPs), which stimulate or inhibit the biological availability of IGF-I via endocrine, autocrine and/or paracrine actions. The dynamic interaction between IGF-I and IGFBPs thus determines the biological action of IGF-I. Recent evidence also shows that IGFBPs directly and independently affect cellular functions by binding to specific cell surface receptors (Rechler 1997). IGFBP-3 is able to directly inhibit growth without blocking access of IGF-I to its receptor (Rechler 1997) and IGFBP-1 stimulates cell migration by interaction with the fibronectin receptor (Jones and Clemmons 1995). Therefore, the concentration of IGFBPs in body fluids has a significant impact on the biological activity of circulating IGF-I and on growth itself.

Most data showing the anabolic, mitogenic and growth-promoting properties of IGF-I were derived from in vitro studies or were obtained in vivo through exogenous IGF-I administration, transgenic models or nutritional trials (Jones and Clemmons 1995). Similarly, information on the stimulatory or inhibitory effects of IGFBPs on IGF-I action was obtained in vitro, including the use of transfected cell lines, and in vivo through mutation of targeted genes, in transgenic models and through nutrient deprivation or restriction (Rechler 1993; Clemmons 1997).

It is well known that weaning results in malnutrition associated with adaptation to a solid diet, social and/or environmental stresses and immunological challenge through dietary antigens and altered microbial colonization. Weaning also induces a precocious maturation of the gut associated with marked acceleration in epithelial cell migration and an increase in mitotic activity (Smith 1985).

Knowledge of the responses of intestinal IGF-I and IGFBPs to weaning and weaning environment in relation to the gut growth and function is lacking. Little is known about the relationship between hepatic gene expression of IGF-I and IGFBPs and their respective protein concentrations in the circulation during normal and suppressed growth in animals.

Segregated early weaning (SEW) offers significant performance advantages in growth and feed efficiency compared with conventional (CON) on-site weaning and it has been adopted widely by the North American swine industry (Van Kessel et al. 1997, Patience 1997). The main objective of the SEW system is to prevent vertical pathogen transfer from the breeding herd to the offspring at weaning, when they are most

susceptible to infectious disease. In the SEW system pigs are weaned at 12 to 18 d of age and moved to a site distant from the breeding herd under strict measures of biosecurity, ensuring a lower pathogen load.

In the current study SEW was used as a model to investigate the effects of weaning and post-weaning environment on gut development and growth. Furthermore, this study measured associated changes in hepatic and intestinal expression of IGF-I, IGFBPs and associated proteins as well as appearance of some of these protein products in the circulation.

1.1 Objectives

The research in this thesis was designed to address the following objectives:

1. To examine the effects of SEW or CON weaning treatment on post-weaning small intestinal development in pigs.
2. To elucidate the effect of early weaning and weaning environment on the expression of intestinal IGF-I, IGFBPs and IGF-I receptor and relate to differences in post-weaning gut growth and development between SEW and CON pigs.
3. To reveal the relative differences between hepatic expression and plasma levels of IGF-I and IGFBPs (1-6) in SEW and CON pigs displaying differing post-weaning somatic growth.

2 LITERATURE REVIEW

2.1 Brief Overview of the Role of the Growth Hormone-IGF Axis in the Regulation of Animal Growth

Pituitary growth hormone (GH) secretion is regulated through a complex interaction between the hypothalamic hormones growth hormone releasing hormone (GHRH) and somatotropin-release inhibiting factor (SRIF) (Millard 1989) and peripheral feedback signals including hormones, growth factors and metabolites (Scanlon et al. 1996). GHRH stimulates and SRIF inhibits the secretion of GH. Age, gender and nutritional status affect the magnitude and pulsatility of GH secretion from the anterior pituitary (Millard 1989). GH controls tissue growth and body size by exerting regulatory effects on a wide range of biological processes, including direct or indirect stimulation of proliferation of several cell types and metabolic processes in skeletal and adipose tissues (Hart and Johnsson 1986; McBride et al. 1988). GH also plays a critical role in adaptation to malnutrition or nutrient deficiency. The somatotrophic effects, including increased long bone growth and protein accretion with a reduction of fat deposition, have been demonstrated in animals through chronic administration of GH or GH transgenesis (Boyd and Bauman 1989).

Salmon and Daughaday (1957) discovered the "serum factor " that stimulates cartilage sulfation and responsible for the "sulfation factor activity" of GH. Subsequent studies revealed that the serum factor showed non-suppressive insulin

activity (Froesch et al. 1963) and multiplication-stimulating activity (Pierson and Temin 1972), including stimulation of DNA (Daughaday and Reeder 1966), proteoglycan (Hall and Uthne 1971) and protein synthesis (Salmon Jr and Duvall 1970). The serum factor was termed somatomedin C and later was renamed insulin-like growth factor-I (IGF-I) based on its similarity to insulin in structure and biological actions (Rindernecht and Humble 1978a). Subsequently, another insulin-like factor with similarity to IGF-I in structure and biological activities was termed insulin-like growth factor-II (IGF-II) (Rinderknecht and Humble 1978b). The importance of IGF-I and IGF-II (IGFs) in fetal and postnatal growth and development is well documented (Cohick and Clemmons 1993). Exogenous IGF-I administration promotes nitrogen retention and studies in transgenic mice over-expressing GH and IGF-I have shown that most of the growth promoting activities of GH are mediated by IGF-I. As reviewed by Jones and Clemmons (1995) and Stewart and Rotwein (1996) IGFs elicit diverse effects on a variety of biological processes to stimulate growth and to mediate the growth-promoting effects of GH. The interaction between GH and IGF-I is known as the GH-IGF or pituitary-hepatic axis regulation of animal growth. GH stimulates synthesis of IGF-I in the liver and peripheral tissues, resulting in raised concentrations of IGF-I in the local tissues and circulation. The negative feedback of IGF-I on GH secretion has been studied extensively (Berelowitz et al. 1981; Morita et al. 1987; Soto et al. 1995). In addition, accumulated evidence shows that IGFs also play a role in tissue repair and regeneration (Lowe Jr. 1991).

Nutritional restriction retards growth through a reduction in plasma IGF-I level in the presence of increased GH secretion from the anterior pituitary gland. This phenomenon is termed the uncoupling of the GH-IGF-I axis. GH stimulation of IGF-1 secretion is impaired because of a fasting or protein restriction induced reduction in the number of hepatic GH receptors and in hepatic synthesis of IGF-I. Nutritional restriction also decreases IGF binding protein-3, the major carrier of IGFs in serum, resulting in a reduced half-life of IGF-I in the circulation and decreasing serum IGF-I concentration (Clemmons and Underwood 1991). These data support the theory that biosynthesis of IGF-I plays a key role in the nutritional regulation of animal growth.

In tissues and biological fluids at least six high affinity IGF binding proteins (IGFBPs) bind IGF-I or IGF-II. IGFBPs bind more than 90% of IGFs in the circulation. The IGFBPs have a major influence on the biological action of IGFs by prolonging the half-life of IGFs in the circulation, controlling the release of IGFs from the vasculature, localizing IGFs to specific tissues and cell types and modulating IGF interaction with its receptors (Rechler and Brown 1992). Recent studies showed that the IGFBPs have direct effects on cellular functions by binding to specific cell surface receptors. The biological actions of IGFBPs, independent of those of IGF-I or II, have been reviewed (Kelly et al. 1996; Rechler 1997). Therefore, the concentration of IGFBPs in biological fluid has a significant impact on the biological actions of IGF-I or IGF-II and thus on growth itself.

2.2 Overview of IGF-I, IGF-I Receptor and IGF Binding Proteins

This review will briefly summarize recent studies on the regulation by IGF-I of somatic and intestinal growth, on the role of IGFBPs in mediating the biological action of IGF-I and on the importance of IGFBPs in growth regulation.

2.2.1 IGF-I: Structure and Regulation of Expression

2.2.1.1 IGF-I Precursor and Gene Structure

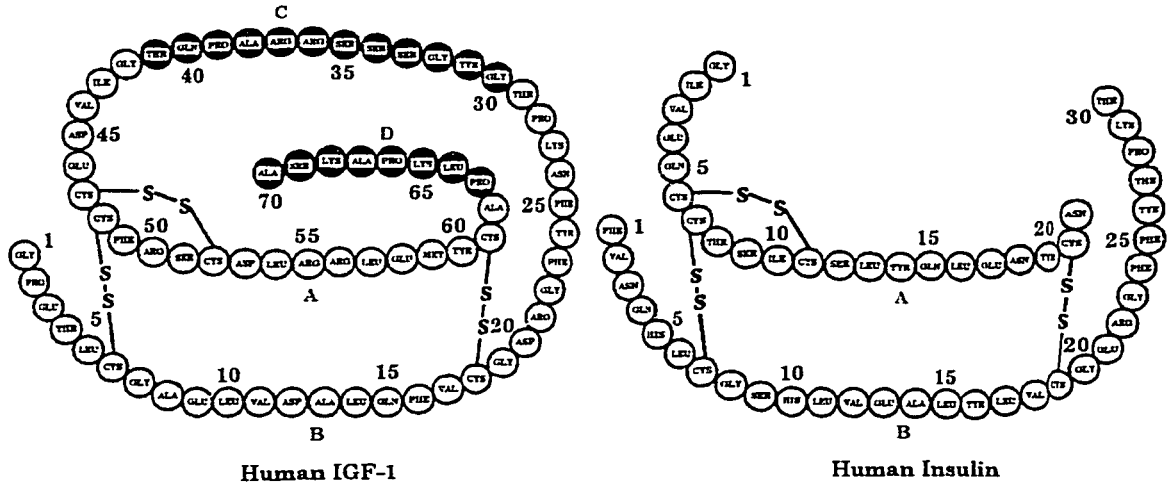


Figure 2.1 Structure of human IGF-I and insulin (Slieker et al. 1993).

IGF-I is a single chain polypeptide with a molecular weight of approximately 7.6 kDa and 6 domains in pro-IGF-I (Blundell et al. 1983). The IGF-I precursor is composed of an amino-terminal B domain of 29 amino acids (aa) and a C domain of 8 residues, an A domain of 21 aa, and a carboxy-terminal D domain of 8 residues (Rotwein 1991). The B and A domains are approximately 40% homologous to the B

and A chains of insulin and approximately 60% homologous to the corresponding domains of IGF-II. The C domain connects the A and B domains and is shorter than the C domain of insulin. The carboxy-terminal D domain exhibits no homologous region to insulin (Foyt and Robert Jr 1991). In addition, the IGF prohormones contain carboxy-terminal E peptides (EIa and EIb) which are not present in the mature IGF-I molecule.

The gene encoding IGF-I has been characterized in the human, rat, chicken and salmon and cDNAs have been isolated from these species and others, including mouse, pig and cow (Foyt and Roberts Jr 1991). The gene structure and pattern of expression of IGF-I are much more complicated compared to the peptide structure of IGF-I itself. In the rat the IGF-I gene spans 80 kb and consists of six exons and five introns (Rotwein 1991). Two promoters, one adjacent to exon 1 and the other 5' to exon 2, control IGF-I gene transcription at multiple initiation sites. Exons 1 and 2 encode alternate 5'untranslated regions (UTRs) and AUG translation initiation codons and probably encode divergent signal peptides. The remainder of the signal peptide and part of the B domain are encoded by exon 3. Exon 4 encodes the remainder of the B domain, the C, A, and D domains, and the amino-terminal of the E domain. The portion of the E domain was determined in exon 5. Different splicing in the E peptide coding region of rat and human has been demonstrated (Foyt and Roberts 1991). The polyadenylation and the carboxy-terminus sites of the E peptide located in exon 6 determine the length of the 3'UTR and the different sizes of IGF-I mRNA. It has been demonstrated that although differences in 5'UTR , the E peptide-coding region length of poly A tail make small

contribution to IGF-I mRNA length. IGF-I mRNA size is predominantly determined by the length of the 3'UTR, that is itself controlled by different polyadenylation site usage of IGF-I.

Studies of synthetic hybrid molecules replacing the A or B domain of IGF-I with the B or A domain of insulin (Joshi et al. 1985 a & b; DeVroede et al. 1985) and site-directed mutagenesis showed that the amino-terminal region of the B domain of IGF-I contributes to interactions with IGFBPs (Cascieri et al. 1988), whereas the carboxy-terminal region of B domain, the C region, and the amino-terminal region of A domain are involved in the affinity for the IGF-I receptor in contrast to the IGF-II receptor (Bayne et al. 1989).The C and D domains are likely to influence the binding affinity for the IGF-I receptor as opposed to the insulin receptor (Bayne et al. 1988).

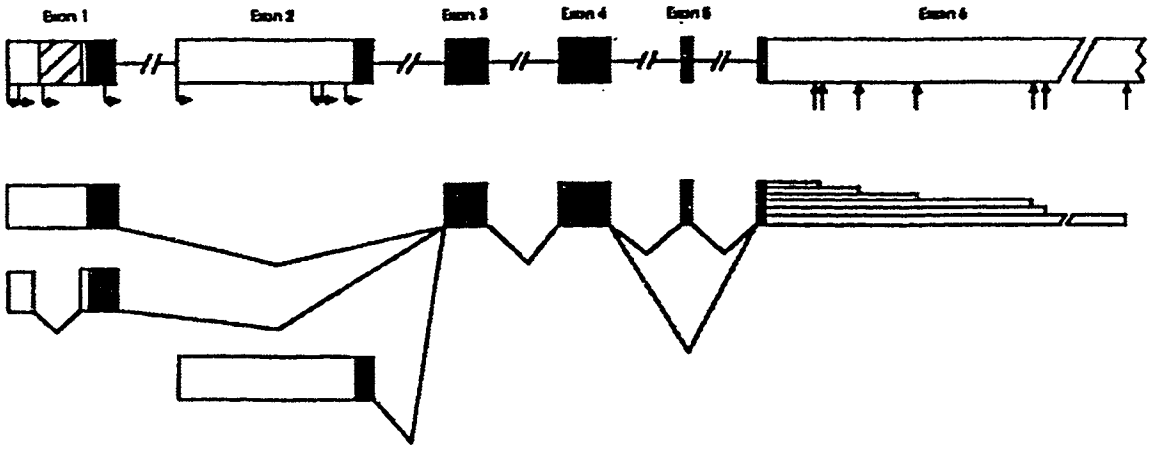


Figure 2.2 Schematic organization of the rat IGF-I gene. Exons (boxes) are to scale, introns (lines) are not. Horizontal arrows below exons 1 and 2 represent transcription start sites. Vertical arrows below exon 6 represent polyadenylation sites. Patterns of differential leader exon usage, splicing, and polyadenylation site usage in different IGF-I mRNA are diagrammed in the lower portion of the figure. (LeRoith and Roberts Jr 1993).

2.2.1.2 Regulation of IGF-I Expression

IGF-I is a highly conserved peptide produced in many tissues with multiple biological effects and is an essential regulator of normal postnatal growth (Jones and Clemmons 1995). Hormonal, nutritional, tissue-specific and developmental factors regulate biosynthesis of IGF-I. GH interacts with its receptors to stimulate synthesis and secretion of IGF-I in both hepatic (Norstedt and Moller 1987) and non-hepatic tissues (Adamo et al. 1991). Administration of bovine somatotropin (ST) into Dorset/Suffolk crossbred sheep showed dose-dependent increases in circulating IGF-I (McLaughlin et al. 1991). A positive relationship (r=0.74) was established between serum IGF-I and body weight of calves (Kerr et al. 1989). Daily injection of porcine ST in pigs increased plasma IGF-I (Azain et al. 1992). GH induces IGF-I synthesis by increasing IGF-I gene transcription. Other hormones exhibit more specific effects on IGF-I expression in their respective target tissues. Estrogens stimulate IGF-I mRNA in the uterus and in ovarian granulosa cells (Murphy et al. 1987) and glucocorticoids decrease hepatic and extra-hepatic IGF-I mRNA in rats (Luo and Murphy 1989).

Nutrition overrides the regulatory effects of GH on the IGF-I concentration in circulation and in multiple tissues (Clemmons and Underwood 1991). The regulatory effect of GH on IGF-I was abolished in fasted rats (O'Sullivan et al. 1989) and in malnourished children (Soliman et al. 1986) as demonstrated by high plasma GH concentration and very low circulating IGF-I. Breier et al. (1988) observed a completely abolished IGF-I response to bovine ST administration in growing steers under severe restriction of intake. Rats fed inadequate protein or energy showed

reduced levels of IGF-I in plasma (Straus and Takemotor 1990 & 1991). Re-feeding fasted pigs increased IGF-I plasma level (Morovat et al. 1994) and re-feeding increased hepatic IGF-I mRNA level and serum IGF-I in fasted pre-pubertal gilts (Charlton et al. 1993). Furthermore, fasting in rats decreased levels of IGF-I mRNA in various tissues, such as an 80% decrease in IGF-I mRNA in the liver and lung, 60% in kidney and muscle and 30-40% in stomach, brain and testes (Lowe et al. 1989). Administration of IGF-I *in vivo* enhanced body growth and showed selective action on different organs (Guler et al. 1988). These results indicate tissue specificity in the IGF-I response to IGF-I treatment and altered nutrition.

Developmental factors also enhance IGF-I gene expression. In rats, hepatic IGF-I mRNA increases progressively during the first two weeks of post-natal life, accompanying a remarkable rise in serum IGF-I (Rotwein et al. 1993). Domestic animals, such as the pig, also showed progressive increases in plasma IGF-I concentration during postnatal development (Owens et al. 1991; Lee et al. 1991). It is clear that the IGF-I gene expression is regulated by multiple factors.

2.2.2 The IGF-I Receptor: Structure, Expression and Regulation

2.2.2.1 Structure of IGF-I Receptor

IGF-I exerts its biological action by interacting with cell surface receptor. The IGF-I receptor (IGF-IR) shows homology with the insulin receptor and is a heterotetrameric structure ($\alpha_2\beta_2$) with a tyrosine kinase domain in the cytoplasmic portion of the β-subunit. IGF-IR shows very high affinity for IGF-I and lower affinity

(100-1000 fold) for insulin (Steele Perkins et al. 1988; Frattali and Pessin 1993). Insulin receptors illustrate high affinity for insulin and a 100-fold lower affinity for IGF-I. IGF-II is able to bind to the IGF-I receptor, but the affinity is 2-15 fold lower than that for IGF-I (Rutanen and Pekonen 1990). Hybrid receptors that are composed of one αβ IGF-IR heterodimer and one αβ insulin receptor heterodimer (Treadway et al. 1991, 1992) are found in several tissues and cell types (Moxham et al. 1989). The hybrid receptors show high affinity for IGF-I and much lower affinity for insulin (Soos et al. 1993).

The human IGF-IR gene is comprised of 21 exons and 15 introns spanning approximately 100 kb (Abbott et al. 1992). The α-subunits are entirely extracellular and the cysteine-rich region of the α-subunits is critical for high affinity IGF binding. The cysteine-rich region of the α-subunits differs from that in the insulin receptor (De Meyts et al. 1994). The β-subunits are composed of a small membrane-spanning segment and a large intracytoplasmic region containing intrinsic tyrosine kinase activity (Frattali and Pessin 1993). IGF-I binding to the α-subunit of IGF-I receptor triggers activation of the intracellular tyrosine kinase domain on the β-subunits. Autophosphorylation of the tyrosine residues causes a signaling cascade, which plays a critical role in mitogenic and metabolic responses (Jones and Clemmons 1995).

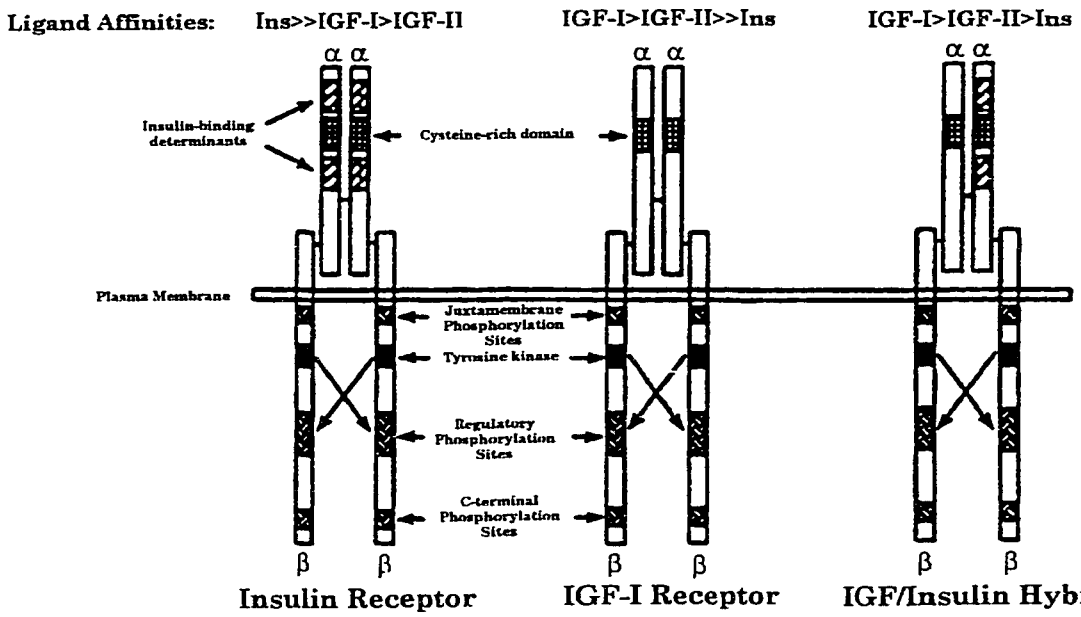


Figure 2.3 Structure characteristics of the receptors for insulin, IGF-I and insulin/IGF-I hybrid receptors. All three receptors consist of two extracellular α-subunits and two transmembrane β-subunits (Jones and Clemmons 1995).

2.2.2.2 Regulation of IGF-I Receptor Expression

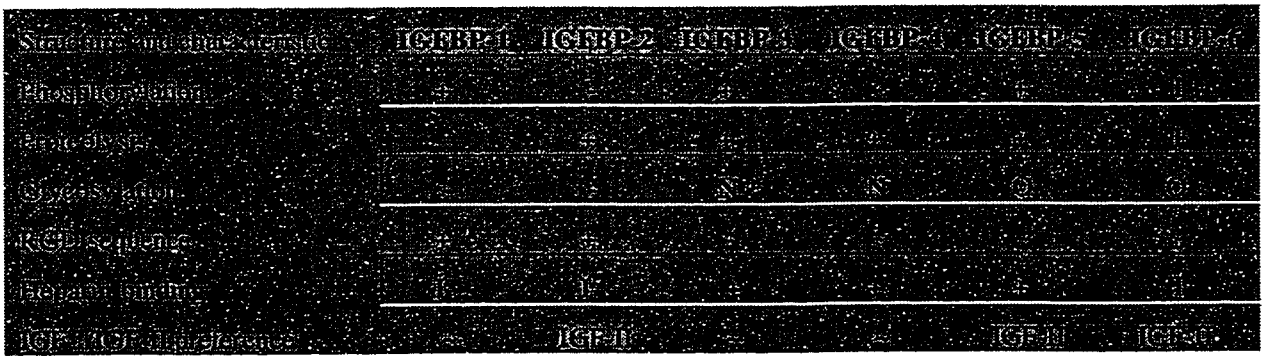
The expression of the IGF-I receptor is regulated by IGF-I, insulin-related peptides, hormones, development and malignancy. A reduction in the number of IGF-IR with increasing IGF-I concentration (down-regulation) has been shown in IM-9 lymphoid endothelial cells (Werner et al. 1991). In the ovary insulin is likely to down-regulate the IGF-IR at high concentrations, and up-regulate the IGF-IR at low concentrations. Up-regulation of IGF-IR numbers in IM-9 cells by IGF-II was also observed (Werther et al. 1989). Human chorionic gonadotropin increases IGF-IR numbers without changing the receptor affinity in cultured rat Leydig cells (Lin et al. 1988). Postnatal development results in a progressive reduction of rat IGF-IR mRNA

abundance in a number of tissues, especially in the liver (Werner et al. 1991). Differentiated adipocytes contain a low number of IGF receptors (Shimizu et al. 1986). Many studies show that the number of IGF-IR is increased in human breast cancer cell lines (Cullen et al. 1990; Peyrat et al. 1989), suggesting that IGF-IR may have oncogenic potential.

2.2.3 IGF Binding Proteins: Structure and Regulation of Expression

The six IGF binding proteins (IGFBPs) belong to a family of soluble proteins that specifically bind IGF-I or -II with high affinity, but which do not bind insulin. The complementary DNA clones of the six IGFBPs have been isolated and protein sequences based on deduced nucleotide sequence have been determined (Rechler and Brown 1992). The proteins share some common structural features and properties.

Table 2.1 IGFBP Structure and Characteristics (Jones and Clemmons 1995).



I indicates that data regarding this characteristic are incomplete and inconclusive.
N, N-linked glycosylation; O, O-linked glycosylation.

2.2.3.1 Structure of IGF Binding Proteins

2.2.3.1.1 IGFBP-1

IGFBP-1 is a single chain peptide consisting of 259 aa with a molecular weight of 25 kDa. The human IGFBP-1 cDNA sequence has been identified (Brinkman et al. 1988a) and the gene is a single copy gene with a length of 5.2 kb containing four exons (Brinkman et al. 1988b). 1.5 kb IGFBP-1 mRNA is present in the liver, kidney, lung, decidua and muscle (Murphy et al. 1990), but is not detectable in spleen, intestine, testes and stomach in rats (Takenaka et al. 1991). The presence of an Arg-Gly-Asp (AGD) sequence at the COOH-terminus may enable IGFBP-1 to bind integrin receptors for many extracellular matrix (ECM) proteins (Jones et al. 1991).

2.2.3.1.2 IGFBP-2

The molecular weight of IGFBP-2 is approximately 32 kDa containing 289 aa (Rechler 1993). Complementary DNAs have been isolated from rat BRL-3A cells (Brown et al. 1989), human fetal liver (Binkert et al. 1989), bovine MDBK cells (Upton et al. 1990) and pig uterus (Song et al. 1996). High homology of the IGFBP-2 aa sequence among human, rat and bovine was reported by Rechler (1993). Simmen et al (1998) also reported that the greatest degree of homology of IGFBP-2 exists between the human and the pig. IGFBP-2 is similar to IGFBP-1 as it has a RGD sequence at its COOH-terminus and no N-glycosylation site. The IGFBP-2 gene spans 32 to 40 kb and is organized into four exons. The exon 2 of the IGFBP-2 gene may have a unique ligand-independent function because of its marked conservation in

vertebrates, whereas this is not conserved between IGFBP-1 and IGFBP-6 (Simmen et al. 1998). Approximately 1.4 kb IGFBP-2 mRNA is abundant in the liver, stomach, kidney and lung, and less abundant in intestine, muscle, heart and skin in rats (Ooi et al. 1990).

2.2.3.1.3 IGFBP-3

IGFBP-3 is a N-glycosylated protein and its molecular mass varies from 45-34 kDa due to N-glycanase reduction. (Clemmons 1991). IGFBP-3 cDNA was isolated from a porcine ovary library (Shimasaki et al. 1990a) and the protein gene is 8.9 kb in size with five exons. The first four exons are homologous to those of IGFBP-1 and -2 and the fifth exon is a 3'UTR. No RGD sequence is present near the COOH terminus. There are three or four potential glycosylation sites in exons 1 and 2 (Rechler 1993), which are important for association with the acid-labile subunit and which are not necessary for IGFBP-3 binding to IGF-I (Conover 1991). IGFBP-3 mRNA (2.5 kb) was analyzed by Northern blot in multiple fetal and neonatal tissues.

2.2.3.1.4 IGFBP-4

In the rat mature IGFBP-4 contains 235 residues with a 24 kDa mass and two cysteine residues in the non-conserved region. Glycosylated and non-glycosylated forms of IGFBP-4 have been described in human (Kiefer et al. 1991) and porcine serum (Coleman et al. 1991a). Complementary DNAs encoding IGFBP-4 were isolated from rat liver and human placenta (Shimasaki et al. 1990b) and shown to contain no RGD sequence. IGFBP-4 mRNA of about 2.5 kb was detected in many tissues.

2.2.3.1.5 IGFBP-5

Mature rat and human IGFBP-5 consists of 252 amino acids with a mass of about 28.5 kDa. Human IGFBP-5 cDNA was cloned from a placenta library. There are no N-glycosylation sites or RGD sequences in the protein. Northern blotting revealed that IGFBP-5 mRNA (~ 6 kb) is present in rat tissues with the most abundance in kidney and the least in liver (Rechler and Brown 1992). IGFBP-5 shows 10-fold higher affinity for IGF-II than for IGF-I (Bautista et al. 1991).

2.2.3.1.6 IGFBP-6

Mature IGFBP-6 in the human contains approximately 210 residues and has a molecular mass 22.8 kDa. Human IGFBP-6 cDNA was isolated from a placenta library (Shimasaki et al. 1991). Differences in the mass of human IGFBP-6 measured by ligand blot may reflect the presence of O-linked polysaccharides. Rat IGFBP-6 mRNA is about 1.3 kb and is widely expressed in tissues (Rechler and Brown 1992). Human IGFBP-6 has a 60-70 fold higher affinity for IGF-II relative to IGF-I.

2.2.3.2 Regulation of IGFBPs Expression

IGFBPs secretion is controlled by developmental and nutritional factors, GH and other hormones or growth factors. The concentration of several IGFBPs in serum changes during fetal and postnatal development, indicating that development is an important regulator of IGFBPs. Serum IGFBP-3 is considered a principal carrier of IGFs in the circulation. However, the concentration of IGFBP-3 in fetal serum is minimal and increases remarkably after birth. Postnatal decreases in IGFBP-2 and

increases in IGFBP-3 concentration in the circulation in porcine were demonstrated (Lee et al. 1991; Peng et al. 1996). Age, nutrition and hormones (Rechler and Nissley 1993) control the concentration of IGFBP-3. GH is a primary and IGF-I is a secondary factor in the control of IGFBP-3 synthesis (Rechler 1993). The IGFBP-3:IGF complex allows the third protein, acid labile subunit (ALS), to form a ternary complex IGF:IGFBP-3:ALS in the circulation (Baxter and Martin 1989). The major function of the ternary complex is to prolong the half-life of IGFs. The synthesis of ALS is dependent upon GH and is not increased by IGF-I. However, high levels of IGF-I reduce GH secretion, and indirectly reduce ALS synthesis and increase IGFBP-3 clearance. A reduced availability of ALS leads to reduced formation of the ternary complex of ALS:IGFBP-3:IGF-I in the circulation and increases free IGFBP-3. IGFBP-3 level in plasma will be reduced, however, because its half-life (1-2 h) is substantially lower than that of the ternary complex (16 h) (Clemmons 1997). There is concomitant increase in IGFBP-3 when IGF-I synthesis is increased by GH, reflecting a coordinate regulation of IGF-I and IGFBP-3 concentrations to maintain an adequate reservoir of IGF-I in the vasculature. The coordinate regulation of IGF-I and IGFBP-3 biosynthesis is further evident from the decrease in plasma IGF-I and IGFBP-3 concentrations during fasting and their level increase after re-feeding (Underwood et al. 1994).

IGFBP-2 is the major IGFBP in fetal serum in the pig (McCusker et al. 1991; Lee et al. 1991), human (Zapf et al. 1990) and rat (Donovan et al. 1989) and its concentration declines markedly after birth. The parallel changes in hepatic IGFBP-2

mRNA abundance and its protein in the circulation were shown in the rat (Brown et al. 1989), rhesus monkey (Liu et al. 1991) and pig (Lee et al., 1993). Hepatic IGFBP-2 mRNA expression is suppressed by administration of insulin (Boni-Schnetzler et al. 1989) and plasma IGFBP-2 is reduced by GH administration in growing pigs (Coleman et al. 1991b) and in cows (Cohick et al. 1992; Vicini et al. 1991). A significant increase in hepatic IGFBP-2 mRNA occurs after 48 h fasting, which is decreased 50% after 6 h of re-feeding and completely reversed after 2 d of re-feeding in rats (Ooi et al. 1990; Tseng et al. 1992). IGFBP-2 mRNA expressed in kidney and brain shows little increase after fasting, reflecting tissue specific regulation of IGFBP-2 gene expression (Tseng et al. 1992).

Hepatic synthesis of IGFBP-1 is primarily controlled by insulin, which is a major negative regulator of hepatic IGFBP-1 mRNA abundance (Garofalo and Rosen 1989). The regulation of the IGFBP-1 gene is similar to those for enzymes in the gluconeogenic pathway, such as the phosphoenolpyruvate carboxykinase (PEPCK) gene. The gene expression of PEPCK and IGFBP-1 is repressed by insulin and stimulated by glucocorticoids and cAMP (O'Brien et al. 1990; Luo and Murphy 1989). Nutrition plays a role in controlling IGFBP-1 transcription. Hepatic IGFBP-1 mRNA increased 10-fold after one or two days of food restriction in the rat and was reduced after 1 h of re-feeding (Murphy et al. 1991). The fasting-induced increase in IGFBP-1 in plasma was also demonstrated in newborn pigs fasted for 48 h (McCusker et al. 1991).

The concentrations of IGFBP-4 in the blood circulation are very low in rats (Clemmons 1997) and pigs (Lee et al. 1991). IGFBP-4 and its mRNA in cultured human bone cells were increased 1.4 and 1.7 fold, respectively, when incubated with 10^{-8} M parathyroid hormone (La Tour et al. 1990). Information regarding hormonal and nutritional regulation of IGFBP-5 and -6 plasma level is limited.

2.3 IGF Binding Proteins Mediating Actions of IGF-I

Secreted IGFs are not stored in a specific organ, but circulate bound to IGFBPs that control the bioavailability of IGFs. IGFBPs bind the majority (more than 95%) of circulating IGFs and serve as a plasma reservoir for IGF-I by prolonging the half-life of IGF-I in the circulation. IGFBPs may also protect the host from the acute insulin-like effect of free IGF-I by controlling the efflux of IGF-I from the vascular space. In addition, IGFBPs transport IGF-I across the vascular endothelium and directly modulate interaction of IGF-I with its receptors. In tissues, IGFBPs in the interstitial compartment are in soluble form and have greater affinity for IGF-I than does the IGF-I receptor. IGFBPs have been shown *in vitro* and *in vivo* to both inhibit and stimulate the bioactivity of IGFs. Recently, biological functions of IGFBPs independent of those involving IGF-I have been reported (Rechler 1997).

2.3.1 IGFBP-1

IGFBP-1 inhibits biological action of IGF-I through its high ratio to IGF-I. Most studies using transformed cell lines and primary cell cultures show that the addition of exogenous IGFBP-1 results in inhibited differentiation of cell function and repressed cell

growth, especially when IGFBP-1 is in molar excess to IGF-I. The inhibitory effects of IGFBP-1 have been attributed to interference with IGF-I-receptor interactions (Jones and Clemmons 1995). Actions of IGF-I such as stimulation of ^{3}H thymidine incorporation into porcine aortic smooth cells and fibroblasts was inhibited by IGFBP-1 purified from amniotic fluid, when IGFBP-1 was added in molar excess to IGF-I (Busby et al. 1988). Administration of a molar excess of IGFBP-1 to IGF-I in hypophysectomized rats showed some attenuation of IGF-I action in stimulating growth and sulfate incorporation into cartilage (Cox et al. 1994).

A high ratio of IGF-I to IGFBP-1 potentiates the biological action of IGF-I. Significant (5.5 times) enhancement of the effect of IGF-I on DNA synthesis was observed by adding purified IGFBP-1 with a molar excess of IGF-I to porcine aortic smooth cells or human fibroblast cultures (Busby et al. 1988). An IGF-I mutant that bound poorly to IGFBP-1 did not show stimulation of DNA synthesis compared to the wild type IGF-I (Clemmons et al. 1990). IGF-I to IGFBP-1 molar ratios between 2:1 to 25:1 resulted in increased ^{3}H-thymidine incorporation (Koistinen et al. 1990). Equimolar or higher ratios of IGF-I to IGFBP-1 increased the response of keratinocytes to IGF-I, whereas IGFBP-1 alone had no effect (Kratz et al. 1992). Under these conditions, degradation and clearance of IGF-I can be inhibited and reduced affinity of IGFBP-1 may allow IGF to remain in prolonged equilibrium with its receptor.

IGFBPs may have to be associated with cells in order to potentiate the action of IGF-I. The presence of an Arg-Gly-Asp (RGD) at the COOH terminus of IGFBP-1 is an integrin receptor recognition sequence (Kelley et al. 1996), which allows RGD specific

binding to the integrin receptor on the cell surface (Hynes 1992; Jones et al. 1993a). The binding of IGFBP-1 to the integrin receptor results in a reservoir of IGF localized on the cell surface. This could enhance IGF-mediated action by providing a high level of local IGF to stimulate IGF-I receptors. The functional significance of the RGD sequence was shown in a mutant of IGFBP-1, with an arginine substitution for tryptophan, which was unable to mediate cell migration (Clemmons et al. 1993). This indicates that the effect of IGFBP-1 on cell migration is independent of IGF-I or IGF-II (Jones et al. 1993b). The IGFBP-1 has a few regions rich in serine where phosphorylation and de-phosphorylation may regulate biological action of IGFBP-1. De-phosphorylation reduces the affinity of the protein for IGF-I, promoting IGFBP-1 release from the IGFBP-1:IGF-I complex and enabling IGF-I to exert biological actions (Jones et al. 1991).

IGFBP-1 gene expression is strongly stimulated under catabolic conditions. Glucagon (Hilding et al. 1993) and cortisol (Conover et al. 1993) stimulate and insulin (Kelley et al. 1996) inhibits IGFBP-1 mRNA expression. The level of IGFBP-1 in plasma is very low and may provide most of the freely available IGF binding sites under conditions of malnutrition or fasting. Serum IGFBP-1 changes acutely in response to rapid changes in metabolism. These data suggest that role of plasma IGFBP-1 may be to limit hypoglycaemia and the growth-promoting potential of IGF-I under insufficient nutrition.

2.3.2 IGFBP-2

The inhibition by IGFBP-2 of the growth promoting effects of IGF-1 *in vitro* has been well documented. IGFBP-2 has been shown to inhibit DNA synthesis in chick

embryo fibroblasts (Frauman et al. 1989) and rat astroglial cells (Knauer and Smith 1980). IGFBP-2 inhibits IGF-I or IGF-II binding to the cell surface (Reeve et al. 1993). IGFBP-2 binding to IGF-I or IGF-II is essential for it to exert inhibitory action. IGFBP-2 reduces the inhibitory effects of IGFs on protein degradation in MDBK cells (Ross et al. 1989). Cloned IGFBP-2 is a potent inhibitor of DNA synthesis in cultured fibroblasts in serum-free medium and inhibits the mitogenic function of IGFs (Schwander et al. 1989). Marked accumulation of IGFBP-2 in culture medium was associated with inhibition of cell proliferation in rat type 2 cells treated with glucocorticoids (Mouhieddine et al. 1996). Mouhieddine et al. (1996) also demonstrated that enhanced production of IGFBP-2 was associated with increased IGFBP-2 mRNA expression in the cell without changes in IGFBP-2 mRNA stability, and that IGFBP-2 expression was induced during cell growth arrest.

A few studies showed that IGFBP-2 is capable of stimulating the biological action of IGF-I. Bar et al. (1989) reported that IGFBP-2 slightly enhanced the effect of IGF-I on glucose uptake in microvascular cells in serum free medium. Gene knockout mice with deletion of the IGFBP-2 gene had small spleens compared with controls, suggesting that the well known effect of IGF-I on enlarging the spleen in animals may, at least partially, be enhanced by expression of IGFBP-2 (Pintar et al. 1995). Specific binding of IGFBP-2 to the integrin receptor has not been established, although there is the presence of an Arg-Gly-Asp (RGD) sequence at the COOH terminus of IGFBP-2. However, IGF-I potentiating activity of IGFBP-2 was lost when the RGD sequence was modified (Delhanty et al. 1993).

IGFBP-2 is a predominant IGFBP in the fetus and the second most abundant IGFBP in the circulation after birth. Neonates show high concentrations of IGFBP-2 in plasma, which may regulate the availability of IGF under conditions where the amount of IGFBP-3 is not great enough to carry all IGFs in the circulation (Baxter 1991). IGFBP-2 does not fluctuate rapidly in response to changes in metabolism compared to IGFBP-1. Nutrition is a weaker regulator of IGFBP-2 (Clemmons 1997), although expression of hepatic IGFBP-2 mRNA is increased in fasted rats (Underwood et al. 1994). Elsasser et al. (1995) showed that administration of endotoxin resulted in reduction in the circulating concentrations of IGF-I and IGFBP-2 in Angus x Hereford steers independent of changes in nutritional intake. Observations of Elsasser et al. (1995) suggested that altered pattern of IGFBPs in the circulation may influence partitioning of IGF-I to target tissues and modify anabolic character of IGF-I during acute disease stress. Recent studies show that IGFBP-2 levels in the circulation are increased in tumor patients (Mohnike et al. 1995; Pintar et al. 1995), in children with chronic renal failure (Tonshoff et al. 1995) and in rats with experimental uremia (Tonshoff et al. 1997), suggesting that IGFBP-2 may play a role in pathogenesis. The role of IGFBP-2 in the regulation of somatic growth in healthy rats or domestic animals is poorly understood.

2.3.3 IGFBP-3

IGFBP-3 is a predominant IGFBP in the circulation in postnatal life. IGFBP-3 is associated with IGF and the acid labile subunit (ALS) to form a ternary complex that prolongs the half-life of IGF. High levels of IGF-I and IGFBP-3 in plasma facilitate

binding to ALS to form the ternary complex (Baxter and Martin 1989). Improved body weight gain associated with increased levels of the ternary complex in the circulation has been demonstrated in hypox rats (Fielder et al. 1996), in dwarf mice (Stewart et al. 1993) and in children with renal failure (Powell et al. 1996). The concentrations of IGFBP-3 in the circulation influence the bioavailability of IGF-I and somatic growth.

IGFBP-3 consistently inhibited the action of IGF in DNA synthesis in a few cell cultures, such as chick embryo fibroblasts, when IGFBP-3 was in 3 to 4 fold molar excess of IGF-I (Blat et al. 1989). IGFBP-3 inhibited the stimulation by IGF-I of cAMP generation and DNA synthesis in rats granulosa cells (Bicsak 1990) and glycogenolysis and glucose oxidation in porcine fat cells (Walton et al. 1989). BALB/c3T3 fibroblasts transfected with an IGFBP-3 cDNA and showing constitutive expression of IGFBP-3 showed inhibition of the stimulatory effects of IGF-I on cell growth (Cohen et al. 1993). The mechanism of inhibition may be through the blocking of IGF binding to IGF-I receptors through formation of IGF:IGFBP-3 complexes (Clemmons et al. 1986 & 1987).

Independent growth inhibition by IGFBP-3, without binding IGF-I to block IGF-I access to its receptors, has been reviewed (Rechler 1997). Liu et al. (1992) showed that rat IGFBP-3 inhibited stimulation of chick embryo fibroblast DNA synthesis in the absence of IGFs. Exogenous IGFBP-3 inhibited DNA synthesis in a human breast cancer cell line (Oh et al. 1993). A 16 kDa fragment of IGFBP-3 had negligible binding affinity for IGF-I, did not bind insulin, and inhibited the stimulation by IGF-I and insulin of DNA synthesis in chick embryo fibroblasts (Lalou et al. 1996). These data suggest that

the inhibitory effect of IGFBP-3 may be independent of its capability to bind to IGF-I. The strongest evidence for a growth inhibitory action of IGFBP-3 independent of IGF-I was obtained in a fibroblast cell line developed from mice that lacked the IGF-I receptor (R^- cells) (Sell et al. 1993). A putative IGFBP-3 receptor on the cell surface may mediate the independent growth inhibitory action of IGFBP-3 and which does not block IGF-I binding to the IGF-I receptors (Smith et al. 1994). Yang et al. (1996) proposed that the interaction of IGFBP-3 with specific cell receptors may be essential for growth inhibition to occur.

The potentiating action of IGFBP-3 on IGF-I has been shown in several cell types. IGFBP-3 caused dose-dependent increases in aminoisobutyric acid (AIB) uptake when co-incubated with increasing concentrations of IGF-I (Conover 1992). Down-regulation of the IGF-I receptor was prevented through pre-incubation with IGFBP-3, and may represent one of mechanisms through which IGFBP-3 enhances the biological actions of IGF-I (Conover 1992). In a rat osteoblast culture, increased accumulation of IGFBP-3 in the medium showed enhanced effects of IGF-I on cell proliferation (Ernst and Rodan 1990). Potentiation of IGF action by IGFBP-3 appears to require its association with the cell resulting in a 10-fold reduction in affinity of IGFBP-3 for IGF-I (Conover 1991). A truncated IGFBP-3 (28-30 kDa) potentiates, but intact IGFBP-3 inhibits IGF-I action in rat osteoblasts (Schmid et al. 1991). Conover (1991) suggested that IGFBP-3, when adhered to the cell surface, is processed to a lower molecular weight form of IGFBP-3. Therefore, proteolysis of IGFBP-3 may be important for IGFBP-3 to be able to potentiate the effects of IGF-I. Lower molecular weight IGFBP-3, truncated IGFBP-3 or cleaved

IGFBP-3 displayed either reduced affinity for IGF-I by cell-associated IGFBP-3 (Conover 1991) or potentiated the growth response to IGF-I. However, in which the cellular activity of IGFBP-3 that may be markedly altered by its cell association and/or its local proteolysis to potential IGF-I actions is unclear.

2.3.4 IGFBP-4

IGFBP-4 has been shown to consistently inhibit biological action of IGF-I *in vitro* and not adhere to the cell surface (Clemmons 1997). Increasing exogenous IGF-I in chick pelvic cartilage reversed the inhibitory action of IGFBP-4 at low concentrations, demonstrating a dose dependency and a saturation effect of IGF-I on the inhibitory actions of IGFBP-4 (Mohan et al. 1989). Purified IGFBP-4 inhibited steroidogenesis in the presence of FSH stimulation (Ui et al. 1989) and inhibited growth of a colon carcinoma cell line (Culouscou and Shoyab 1991). IGF-I stimulation of thymidine incorporation in rat B104 neuroblastoma cells (Cheung et al. 1991) and synthesis of DNA and glycogen in human osteosarcoma cells (Kiefer et al. 1992) was inhibited by IGFBP-4. Exogenous IGFBP-4 inhibited IGF-I-induced DNA synthesis and proliferation in vascular smooth muscle cells (Duan and Clemmons 1998). The mechanism of inhibition of IGF-I by IGFBP-4 may be through formation of an IGFBP-4:IGF-I complex that prevents the interaction of the IGFs with their receptors on the cell surface.

Transgenic mice expressing a higher level of IGFBP-4 mRNA in smooth muscle cell (SMC), with no changes in levels of IGFBP-4 in plasma, had a smaller bladder, aorta, intestine, uterus and stomach compared with controls. This suggests that IGFBP-4

is a functional antagonist of IGF-I action in SMC and over-expressed IGFBP-4 in SMC exerts an entirely paracrine action (Wang et al. 1998).

Recently, *in vivo* studies showed that IGFBP-4 might potentiate IGF-I action. Colonic IGFBP-4 mRNA abundance increased in association with increased colonic growth and function (Mantell et al. 1995) and IGFBP-4 mRNA in jejunum and ileum increased following bowel resection in rats (Ziegler et al. 1998). Increased expression of IGFBP-4 was observed in inflamed colon in rats with colitis (Zeeh et al. 1995) and in cultured colonic smooth muscle cells (Zeeh et al. 1997). These actions indicate that IGFBP-4 may be involved in modulating IGF-I effects in response to intestinal resection or inflammation.

The concentrations of IGFBP-4 in plasma are very low and show little change during postnatal development (Lee et al. 1991). The role of IGFBP-4 in the circulation is poorly understood.

2.3.5 IGFBP-5

Molar excess of IGFBP-5 inhibited stimulation by IGF-I of DNA and glycogen synthesis in human osteosarcoma cells (Kiefer et al. 1992) and steroidogenesis in granulosa cells (Ling et al. 1993).

Most studies show that IGFBP-5 is capable of potentiating the action of IGF-I on cell proliferation and growth. A unique property of IGFBP-5 is to adhere tightly to the extracellular matrix (ECM) of fibroblasts and to potentiate the growth promoting effect of IGF-I (Jones et al. 1993b). The mechanism may involve a decreased affinity of

IGFBP-5 for IGF-I when IGFBP-5 is associated with the ECM, allowing matrix-associated IGF-I to be released to its cell surface receptor.

A 22 kDa IGFBP-5 fragment generated by proteolysis directly stimulates DNA synthesis in osteoblasts, suggesting that truncated IGFBP-5 may stimulate growth through a mechanism independent of that involving IGF-I (Andress et al. 1993). It is unclear if the truncated IGFBP-5 fragment binds to a specific cell surface receptor, but it was observed that the fragment bound to a protein with a molecular weight of 300 kDa present on the surface of osteoblasts (Andress 1995). The IGFBP-5 fragment incubated with IGF-I in mouse osteoblast cultures enhanced mitogenic effects of IGF-I when compared with IGF-I alone (Andress and Brinbaum 1992).

Evidence shows that IGF-I up-regulates the expression of IGFBP-5 and that IGFBP-5 plays an important role in augmenting the stimulatory action of IGF-I *in vivo*. Ye and D'ercole (1998) demonstrated in transgenic mice that IGF-I up-regulates IGFBP-5 expression at both mRNA and protein levels in brain and that increased IGFBP-5 enhances the stimulatory action of IGF-I on the central nervous system development. Increased IGF-I mRNA transcripts in inflamed bowel induced IGFBP-5 gene expression in rats with experimental enterocolitis (Zimmermann et al. 1997). Exogenous IGF-I stimulated IGFBP-5 mRNA expression in the jejunum that augmented the selective growth promotion by IGF-I in surgically stressed rats (Yang et al. 1997).

2.3.6 IGFBP-6

Information regarding the physiological role of IGFBP-6 *in vitro* or *in vivo* is limited. IGFBP-6 was shown to inhibit the growth promoting effects of IGF-I in

neuroblastoma cells (Babajko et al. 1997) and in L6 myoblast cells (Ewton and Florini 1995). Increased IGFBP-6 production in neuroblastoma cells was associated with the arrest of cell growth (Grellier et al. 1998). It is likely that the role of IGFBP-6 may be to inhibit biological activities of IGFs.

2.4 Biological Actions of IGF-I

IGFs are anabolic, mitogenic and differentiating factors, which are capable of exerting insulin-like metabolic effects. IGFs are produced by most tissues of the body and are abundant in the blood circulation. Thus, the IGFs can have endocrine as well as paracrine and/or autocrine effects. IGFs are bound to one of the six high affinity IGFBPs that are produced by almost all tissues and which mediate the biological actions of IGFs (Cohick and Clemmons 1993).

2.4.1 Endocrine Function of IGF-I in Somatic Growth

The important role of IGF-I in regulating postnatal growth and in mediating the growth promoting effects of GH in multiple ways has been reviewed (Jones and Clemmons 1995; Stewart and Rotwein, 1996). There is considerable evidence that circulating IGF-I regulates the growth and metabolism of animals. Plasma IGF-I level is positively correlated with body weight of dogs (Eigenmann et al. 1988), sheep (Roberts et al. 1990), cattle (Bishop et al. 1989) and pigs (Owens 1991). The concentrations of IGF-I in the circulation increase during postnatal development in young pigs (Buonomo et al. 1987; Scanes et al. 1987; Lee et al. 1991; Peng et al. 1996). Progressive increases

in the IGF-I level in plasma are observed in calves until weaning (Breier et al. 1988). In chickens and turkeys serum IGF-I concentrations increase 2 to 4 fold from 4 to 6 weeks of age (McMurtry et al. 1994).

Administration of exogenous IGF-I into animals has confirmed the stimulatory effects of IGF-I on growth. Infusion of IGF-I into hypophysectomized rats increases growth in a dose-dependent manner (Schoenle et al. 1982; Guler et al. 1988). Administration of IGF-I through continuous infusion or daily injection increases body weight in rats compared to controls (Schoenle et al. 1982, 1985) providing evidence that IGF-I mediates linear growth. Continuous infusion of IGF-I causes a selective increase in kidney, spleen, and thymus weight (Guler et al. 1988), while GH treatment results in quantitative effects on total body gain and growth (Glasscock et al. 1992). These differences may be a result of obvious changes in serum IGFBP profile by the two different treatments. GH directly stimulates IGFBP-3 and ALS secretion. Synthesis of ALS is dependent on GH, but is not increased by IGF-I. The ternary complex could be present in GH-treated animals, but not in IGF-I-treated animals, although IGF-I does stimulate IGFBP-3 secretion. These results reflect direct end-organ effects of GH and of IGF-I in local tissues, suggesting that for growth promotion GH is required for IGF-I to be effective. The high levels of the ternary complex in the circulation enhance bioavailability of IGF-I and improve body weight gain in sheep (Stewart et al. 1993) and in children with renal failure (Powell et al. 1996). The long-term growth promoting effects of IGF-I and GH on body weight and circulating IGFBPs in hypox rats were investigated by Fielder et al. (1996). They observed that greater growth promoting and

anabolic activity was associated with the combination treatment of GH and IGF-I, when compared with either GH or IGF-I alone. They also demonstrated that the restoration of normal concentrations of the IGF-I:IGFBP-3:ALS complex in serum plays an important role in growth regulation, which further confirms that IGFBPs modulate the growth promoting effect of IGF-I.

Insertion of the human IGF-I gene in transgenic mice increased IGF-I levels in plasma by 50%, and was associated with an increased body weight when compared with controls (Mathews et al. 1988). This further confirms that IGF-I has a profound influence on body growth. The higher levels of IGF-I in the transgenic mice were associated with increased weight in several tissues including liver, pancreas, lung, kidney and brain. Crossing IGF-I transgenic mice with GH-deficient mice increased both body weight and size in offspring to the same levels as in normal litter mates. This suggests that IGF-I can mediate almost all of the effects of GH on somatic growth (Behringer et al. 1990).

Metabolic effects of IGF-I were tested comparing continuous infusion of IGF-I with infusion of insulin in rats (Jacob et al. 1989). The results confirmed the anabolic effects of IGF-I including increases in peripheral glucose uptake, glycogen synthesis and decreases in plasma amino acid level and in the rate of protein degradation. No change was observed in the level of free fatty acids in plasma. The anabolic effect of IGF-I on protein synthesis and nitrogen balance was shown to counteract the catabolic effects of dexamethasone treatment in rats (Tomas et al. 1992). Administration of IGF-I stimulated wound healing induced by corticosteroids in rats (Suh et al. 1992) and increased total body protein accretion in dexamethasone treated rats (Tomas et al. 1992). Increased plasma IGF-I through

administration of exogenous IGF-I enhanced the small intestinal weight and length in normal rats (Steeb et al. 1994) and improved nutrient absorption and reduced body weight loss after 60% bowel resection in rats (Mantell et al. 1995). These results together show that IGF-I enables the growth-promoting effects of GH by stimulating metabolism and growth in a wide variety of tissues and cell types, as well as more specialized functions in specific cell types or tissues.

Growth promoting and anabolic effects of IGF-I have been well demonstrated through exogenous IGF-I treatment, in transgenic animals, in GH deficiency models and in nutritional trials. Information regarding the relationship between expression of hepatic IGF-I and IGFBPs and the levels of their respective proteins in the circulation in relation to growth performance in normal animals is limited. Knowledge of stimulatory or inhibitory effects of the individual IGFBP in the circulation on effects of IGF-I in growth in normal and healthy animals is lacking. In addition, the effects of weaning on serum IGF-I and IGFBPs profiles in relation to post-weaning growth retardation have not been elucidated. It is well known that weaning results in reduced feed intake associated with adaptation to a solid diet and other considerable stresses such as social and/or environmental stress (Gonyou et al. 1998).

2.4.2 Paracrine and/or Autocrine Action of IGF-I

IGF-I is synthesized and secreted by multiple cell types in the animal and IGF-I receptors are present in a variety of cell types. Local production of IGF-I is thought to be important in the paracrine and/or autocrine regulation of cell proliferation and

differentiation. To date, there is no clear evidence regarding the distinct endocrine as opposed to paracrine and/or autocrine actions of IGF-I in the intestine or other organs. The following review will briefly summarize recent studies on the biological action of IGF-I in the intestinal tract.

2.4.2.1 IGF-I Action in Neonatal Gut Growth

IGF-I and -II receptors have been identified in the intestinal epithelium of mammals including the human, pig, rabbit, rat and mouse (Schober et al. 1990; Laburthe et al. 1988; Termanini et al. 1990; Young et al. 1990). Dietary insulin-like growth factor-I increases intestinal growth and alters the population of intestinal IGF-I receptors in the neonatal calf (Baumrucker et al. 1994). Orally administered IGF-I is capable of stimulating cell proliferation in the gastrointestinal tract and mucosal growth in newborn pigs (Burrin et al. 1996; Xu et al. 1994) suggesting that IGF-I may enhance intestinal growth in newborn pigs. Oral gavage or intraperitoneal injection of IGF-I in newborn rats stimulates jejunal brush border enzymes including maltase, lactase, alkaline phosphatase and aminopeptidase, with no effect on sucrase (Young et al. 1990). Oral administration of IGF-I in piglets increases disaccharidase activity in the brush border and intestinal weight (Houle et al. 1997) confirming the stimulatory effect of IGF-I on proliferation and differentiation in neonatal intestine. Prolonged administration of IGF-I by osmotic minipumps enhances the small intestinal weight and length in normal rats when compared with controls (Steeb et al. 1994). Furthermore, IGF-I exerts trophic

effects in jejunal epithelial structure and function in the absence of luminal nutrition during total parenteral nutrition in rats (Peterson et al. 1996).

The local expression of IGF-I mRNA in the intestine confirms that IGF-I exerts paracrine and/or autocrine actions to control gut growth (Lund et al. 1986). Significant decreases in ^{125}I-IGF-I binding to the mucosal membranes in pigs at 3 d of age reflect a major reduction in the number of IGF-I receptors present, while at same time the mucosal content of IGF-I is significantly increased (Schober et al. 1990). The number of IGF-I receptors in porcine intestine declines dramatically during the suckling period and is negatively correlated with maltase and sucrase activities (Morgan et al. 1996). These data suggest that interaction between IGF-I and IGF-I receptors may regulate mucosal receptors or protein synthesis and/or degradation during postnatal development. Evidence presented here indicates that both increased plasma IGF-I and enhanced intestinal IGF-I expression exert trophic effects on the gut morphology and function as well as on intestinal growth in neonates.

Gene expression of IGFBP-2 declines in rat intestine post-natally (Orlowski et al. 1990). However, the relationship between intestinal IGFBPs and post-natal gut development in normal animals is poorly understood. The gastrointestinal tract is the fastest growing organ after birth. Studies of gene expression of the intestinal IGFBPs during post-natal development may provide insight regarding the roles of IGFBPs and IGF-I in postnatal gut growth regulation.

2.4.2.2 IGF-I Action in Intestinal Adaptive Growth

It is well established that IGF-I regulates adaptive growth of the small intestine after large bowel resection. Systemic administration of IGF-I increases the mass of the small intestine in rats after proximal small bowel resection (Lemmey et al. 1991). IGF-I treatment greatly increased colonic mucosal weight, absorption and reduced body weight loss in rats after 60% small intestine resection and cecal resection (Mantell et al. 1995). Exogenous IGF-I increased IGF-I mRNA level in the jejunum, enhanced body weight in transplanted rats and greatly increased the level of jejunal IGFBP-3 mRNA, but not intestinal IGFBP-4 mRNA. This suggests that both exogenous and endogenous IGF-I may stimulate adaptive growth of transplanted intestine in rats (Zhang et al. 1995). Exogenous IGF-I induced IGFBP-5 mRNA expression in the jejunum that augmented anabolic effects of IGF-I in rats on total parenteral nutrition (TPN) (Yang et al. 1997). Yang and coworkers (1997) suggest that changes in intestinal IGF-I mRNA expression are unlikely to account for decreases in local intestinal growth. They base their conclusion on the very low level of jejunal IGF-I mRNA that was not changed by TPN, GH or IGF-I treatment in surgically stressed rats. Winesett et al. (1995) showed that jejunal IGF-I mRNA levels were declined in fasted rats but did not recover after 24 hours refeeding either intravenously or orally. Read et al. (1992) demonstrated that administration of IGF-I and its analogues induced marked gut growth in rats treated with dexamethasone. Increasing evidence supports that IGF-I promotes mucosal growth and exerts mitogenic effects on the intestinal epithelial cells via endocrine pathways (Park et

al. 1990, 1992; Simmons et al. 1995). Elevated IGF-I expression in the intestine in response to bowel resection suggests a role of local IGF-I in the regulation of adaptive growth (Albiston et al. 1992). Ziegler et al. (1998) showed that local increased expression of IGF-I mRNA in the jejunum (183%) and ileum (249%) might regulate adaptive growth in rat following bowel resection. Marked increases in the expression of IGFBP-4 mRNA in the jejunum and ileum and decreases in IGFBP-3 mRNA in the ileum after bowel resection indicate that the intestinal IGFBPs may be also involved in the regulation of adaptive growth (Ziegler et al. 1998).

It is well known that early weaning of rats induces precocious maturation of brush border enzymes and is associated with marked acceleration in cell migration and an increase in mitotic activity (Smith 1985). In the pig, villus atrophy and crypt hypertrophy and alteration in brush border enzymes activities associated with weaning have been long demonstrated (Nabuurs 1995). Weaning also introduces immunological challenges associated with dietary antigens and altered microbial colonization as well as social and/or environmental stresses. Little is known about the role of the local IGF-I and IGFBPs in the adaptive processes occurring in the intestine in response to weaning.

2.4.2.3 IGF-I Action in Intestinal Inflammation

IGF-I plays a role in the protection against mucosal cell damage or restoration of the normal intestinal architecture during gut inflammation (Lund and Zimmermann 1996). Recent evidence suggests that IGF-I may be up regulated locally in the gut of animals with experimental enterocolitis. Zimmermann et al. (1993) observed

dramatically increased expression of IGF-1 in the bowel of rats in response to intramural injection of peptidoglycan polysaccharidase (PG-PS), which causes chronic intestinal inflammation. Zhee et al. (1995) observed increased IGF-I expression in the lamina propria and in the smooth muscle layers of rats with chronic inflammation induced by intracolonic instillation of ethanol or trinitrobenzene sulphonic acid. Rats with experimental colitis showed increased IGF-I binding sites as well as a 2-3 fold increased IGFBP-4 and IGFBP-5 mRNA expression in inflamed colon, suggesting that IGFBPs play an important role in modulating IGF-I effects during inflammation and tissue repair (Zeeh et al. 1995). Zeeh et al. (1997) showed expression of IGF-I receptors and IGFBPs in cultured colonic smooth muscle cells, suggesting that the trophic effect of IGF-I is effected through its receptor and that altered IGFBPs expression may influence the response to induced IGF-I expression during chronic intestinal inflammation. The PG-PS model showed up-regulated IGF-I expression in smooth muscle cells during development of chronic inflammation, which in turn induced synthesis of IGFBP-5 (Zimmermann et al. 1997). Septic rats treated with exogenous IGF-I showed reduced gut atrophy, enhanced intestinal architecture and improved gut metabolism (Chen et al. 1995). IGF-I stimulated mucosal DNA and protein synthesis and decreased the incidence of bacterial translocation after severe burn injury in rats (Huang et al. 1993). The evidence suggests that the trophic effects of IGF-I may improve gut mucosal function and promote gut tissue repair during the gut inflammation. Knowledge of the effect of rearing environment, including pathogen load, on the intestinal IGF-I and IGFBPs

mRNA expression related to gut morphology and function in normal animals has not been investigated.

2.5 Summary

IGF-I, IGF-IR and IGFBPs are ubiquitously distributed and it is not surprising that IGF-I actions can be identified among several tissues and cell types. Growth promoting and insulin-like anabolic effects of IGF-I have been documented. IGFBPs are important modulators of IGF-I action. IGFBPs control the half-life of IGF-I in the circulation and IGF-I distribution among tissues, as well as play a critical role in controlling IGF-I. IGFBPs have many variable structures that can alter the affinity of IGFBPs for the IGFs. However, the effects of the IGFBPs in the circulation on somatic growth of normal and weaned animals are still unclear. The intestinal IGFBPs mediate IGF-I expression in the intestine and functions of IGFBPs related to early post-weaning gut growth also remain to be defined. Investigation of IGFBPs in somatic and intestinal growth regulation is very important for animal production since the unique function of the small intestine is to digest and absorb nutrients.

3 EFFECT OF SEGREGATED EARLY WEANING ON POST-WEANING SMALL INTESTINAL DEVELOPMENT IN PIGS

3.1 Abstract

The effect of segregated early weaning (SEW) on postweaning small intestinal development was investigated in SEW and control (CON) pigs. Small intestines were collected from a total of 15 pigs killed at 11 (pre-weaning), 15 (3 d post-weaning) and 34 d of age. At 3 d postweaning, both SEW and CON pigs showed shorter villi ($P < 0.01$), deeper crypts ($P < 0.01$), and reduced ($P < 0.01$) ratios of villus height to crypt depth (V/C) compared with pre-weaning. Weaning also reduced specific activities of lactase ($P< 0.01$) in the duodenum and ileum and alkaline phosphatase (ALP) ($P< 0.05$) in the duodenum and jejunum. Sucrase activity in the three regions of the small intestine marginally decreased in both groups at 3 d post-weaning. The ratio of mucosal protein to DNA in the duodenum and jejunum increased ($P < 0.05$) in SEW and CON pigs at 3 d postweaning compared to preweaning pigs. SEW and CON treatments resulted in major differences in post-weaning gut development. At 15 d of age, SEW pigs showed 20, 25.5 ($P < 0.05$), and 1.5% lower ratios of mucosal protein to DNA in the duodenum, jejunum and ileum, respectively, when compared with CON pigs. However, at 34 d these ratios in the duodenum, jejunum and ileum were 43.5 ($P < 0.05$), 24.3, and 32.9% ($P < 0.05$) higher, respectively, in SEW pigs than in CON pigs. Longer villi, shorter crypts ($P < 0.01$) and higher V/C ratio ($P < 0.01$) in the jejunum

and ileum were observed in SEW pigs vs CON pigs at 34 d of age. The specific activities of lactase in the duodenum ($P < 0.01$) and jejunum ($P < 0.05$) and ALP in the duodenum ($P < 0.01$) were consistently higher in SEW pigs, and sucrase activity in the duodenum, jejunum and ileum was 21.7, 46.3 ($P < 0.05$), and 11.2% greater in SEW than in CON pigs at 34 d of age. These results demonstrate significant differences in post-weaning gut development between SEW and CON pigs. Furthermore, the number of intraepithelial lymphocytes in the jejunum was higher ($P < 0.001$) in 34 d old SEW pigs compared with CON pigs. Scanning electron microscopy and AB/PAS staining revealed a thick mucus coating over epithelial cells in the ileum of 34 d old CON pigs, which was not apparent in the SEW pigs. These observations are consistent with reduced pathogen exposure associated with SEW. We conclude that segregated early weaning advances post-weaning gut maturation, which is consistent with improved growth and feed efficiency observed in SEW pigs.

3.2 Introduction

The mucosal epithelium of the small intestine serves as a delicate interface between external and internal environments in the gastrointestinal tract, and its primary function is to digest and absorb dietary nutrients. This epithelium also provides a physical and immunological barrier to prevent penetration of potential harmful materials including bacteria, viruses, parasites, and allergenic macromolecules (Kato and Owen 1994). The mucosal epithelium of the small intestine is anatomically and functionally immature in neonatal pigs (Gaskins and Kelley 1995). Postnatal gut development is important for the digestion and absorption of nutrients to support body

growth as well as for defense against enteric infection. A number of exogenous factors, including diet, microbial colonization, stress and rearing environment can significantly affect postnatal gut development (Henning 1987, Lebenthal 1989).

The system of segregated early weaning (SEW) offers significant performance advantages in growth and feed efficiency in swine and is being adopted widely by the North American swine industry (Patience et al. 1997, Wilson 1995). The most widely held hypothesis for the growth advantage observed in the SEW system is that, for young pigs, the sow herd is a primary source of infectious organisms. Reduced transmission of disease from the sow to her offspring results in a more efficient pig which spends less energy on immune system activity (White 1995). However, the precise mechanisms responsible for improved performance of pigs raised in the SEW system are still unclear.

The objectives of this study were to (1) analyze the effect of segregated early weaning on post-weaning gut development and maturation; (2) reveal factors affecting changes in morphological and functional development of the small intestine. In addition, we report general information on changes in morphology and function of the small intestine in very early weaned (12 d of age) pigs.

3.3 Materials and Methods

Eight litters (PIC C15X Canabrid) from sows farrowed at Prairie Swine Centre Inc. (PSCI, Saskatoon, SK) with at least 10 pigs per litter were weaned at 12 d ± 2 d of age and weighed. Four litters were moved into a segregated nursery (SEW) 16 km

distant from the sow herd and the remaining litters were moved to an all-in-all-out on-site (CON) nursery room located in the same building as the sow herd. Pig environment, nutrition, and management were kept as consistent as possible between the two nurseries. Piglets were fed diets I, II, III and IV from 12-18, 19-23, 24-39 and 40-56 d of age, repectively. The composition of the four diets is listed in Table 3.1. Weight and feed intake of piglets in each nursery were recorded at regular intervals. Piglets (n=3) were injected with euthanyl forte (MTC Pharmaceutical, Cambridge, ON) and killed by terminal bleeding at 11 (pre-weaning), 15 (3 d post-weaning) and 34 d of age. The protocol of this study was approved by the Animal Protocol Review Committee of the University of Saskatchewan in accordance with the guidelines and regulations of the Canadian Council on Animal Care.

Tissue Collection

The small intestine was dissected free of its mesentery and placed on ice immediately. The segment from the pylorus to the ligament of Trietz was considered as duodenum, the proximal segment of the rest of the small intestine was considered the jejunum, and a distal segment 10 cm proximal to ileocecal junction as ileum. The three segments, duodenum, jejunum and ileum, were rinsed thoroughly with ice-cold physiological saline solution. The mucosa was scraped off with a blunt spatula, placed in a sterile tube and immediately frozen in liquid nitrogen. Samples of mucosa were stored at -80°C until assayed.

Brush Border Enzymes

Intestinal mucosa was homogenized in ice-cold deionized water, using a ratio 1:5 (wt/vol.), then centrifuged at 2200 x g for 30 minutes at 4°C and the supernatant was harvested. The supernatant was diluted (1:25) with deionized water for measurement of lactase and sucrase activities. The substrates, β-lactose and sucrose (Sigma Chemical Co. St. Louis. MO), concentration and incubation were used as described by Kidder and Manner (1980). The liberated glucose was determined using a glucose-oxidase kit (Sigma) and measured with a microplate reader (Molecular Devices, Menlo Park, CA). The mucosal supernatant was diluted 1:5 with deionized water for examination of alkaline phosphatase (ALP) activity (245-20, Sigma) using a 96-well flat bottom micro-plate instead of cuvets.

Mucosal Protein and DNA

Protein concentration of mucosal homogenates was determined by the method of Bradford (1976) using bovine albumin as standard. Mucosal DNA was estimated by the fluorometric assay using the bisbenzimidazole fluorescent dye and calf-thymus DNA as standard according to the method of Labaca and Paigen (1980). The mucosa samples

Table 3.1 Composition of diets I, II, III and IV.

Ingredients	**Diet I**	**Diet II**	**Diet III**	**Diet IV**
Wheat	35.48	37.49	47.99	65.94
Whey	25.00	25.00	15.00	-
Soybean meal	15.00	25.00	25.00	25.00
Lactose	5.00	-	-	-
Plasma proteins	6.00	2.50	-	-
Red blood cells	1.50	1.25	2.50	-
Menhaden fish meal	2.27	-	-	-
Limestone	1.10	1.40	1.35	1.50
Dicalcium phosphate	0.65	1.20	1.50	1.60
Salt	0.2	0.25	0.35	0.40
Mineral premix	0.50	0.50	0.50	0.50
Vitamin premix	0.50	0.50	0.50	0.50
Choline chloride	0.05	0.05	0.05	0.05
Zinc oxide	0.35	-	-	-
Carbadox	0.10	0.10	0.10	0.10
L-Lysine	-	-	-	0.22
Threonine	0.02	-	0.03	0.09
Methionine	0.08	0.07	0.04	-
Tallow	2.00	2.25	5.00	4.00
Canola oil	4.00	2.25	-	-
Pellet Binder	0.20	0.20	0.10	0.10
Diet form	Crumble	Crumble	Crumble	Pellet

were homogenized in ice-cold TNE-buffer (10 mM Tris; 1 mM EDTA and 0.2 M NaCl, pH 7.4), using a ratio of 1:5 (wt/vol.). Mucosal protein to DNA ratio was expressed as μg protein per ng DNA.

Histology

Each sample from the small intestine was cut longitudinally at the antimesenteric attachment. Samples for light microscopy were immediately fixed in 10% neutral buffered formalin and embedded in paraffin. Four to six μm sections were cut, mounted on poly-lysine coated slides and stained with hematoxylin and eosin (HE), or alcian blue and periodic acid schiff (AB/PAS), respectively. Villus height and crypt depth were evaluated without knowledge of treatment using the Northern Eclipse image analysis program (Hamamatsu, Japan). Villus height and crypt depth were determined by the method of Jaeger et al. (1990) and Nunez et al. (1996). Reported mean value for each animal was based on ten measurements. Intraepithelial lymphocytes (IEL) were identified morphometrically from HE stained sections. The number of IEL per 100 enterocytes was recorded using light microscopy.

Samples for scanning electron microscopy were fixed immediately in phosphate-buffered 3% glutaraldehyde and osmium tetroxide. Samples were further treated using the method of Vogelweid and Elmore (1983). Specimens were viewed with a scanning electron microscope (SEM 505, Philips, The Netherlands) and photographed.

Statistical Analysis

All experimental data are presented as mean ± SEM. Data were subjected to an analysis of variance using the GLM procedure of SAS (1985). When a significant F

value was present, Fisher's least significant difference test was used for pre-planned individual comparison of means (SAS, 1985).

3.4 Results

Brush Border Enzyme Activity

Weaning resulted in only a marginal reduction of the sucrase activity in the small intestine, and the activity was not different between 15 d old SEW and CON pigs. The specific activity of sucrase in the duodenum, jejunum and ileum was 21.7 ($P = 0.06$), 46.3 ($P < 0.05$) and 11.2% higher in 34 d old SEW pigs than in CON pigs, respectively (Fig.3.1).

At 3 d post-weaning lactase activity fell ($P < 0.01$) in the duodenum and ileum, but not in the jejunum in both groups relative to pre-weaning. Lactase activity was similar between SEW and CON pigs at 15 d of age, however, SEW pigs showed a higher activity of the enzyme in the duodenum ($P < 0.01$) and jejunum ($P < 0.05$) relative to CON pigs at 34 d of age (Fig. 3.2).

Specific activity of ALP was highest at 11d of age and declined ($P < 0.05$) rapidly in all three regions of the small intestine at 3 d post weaning except in the ileum in 15 d SEW pigs. ALP activity in the duodenum, jejunum and ileum was higher ($P = 0.08$) in SEW pigs than in CON pigs at 15 d of age. At 34 d of age, SEW pigs showed higher ($P < 0.01$) ALP activity in the duodenum only compared with CON pigs (Fig. 3.3).

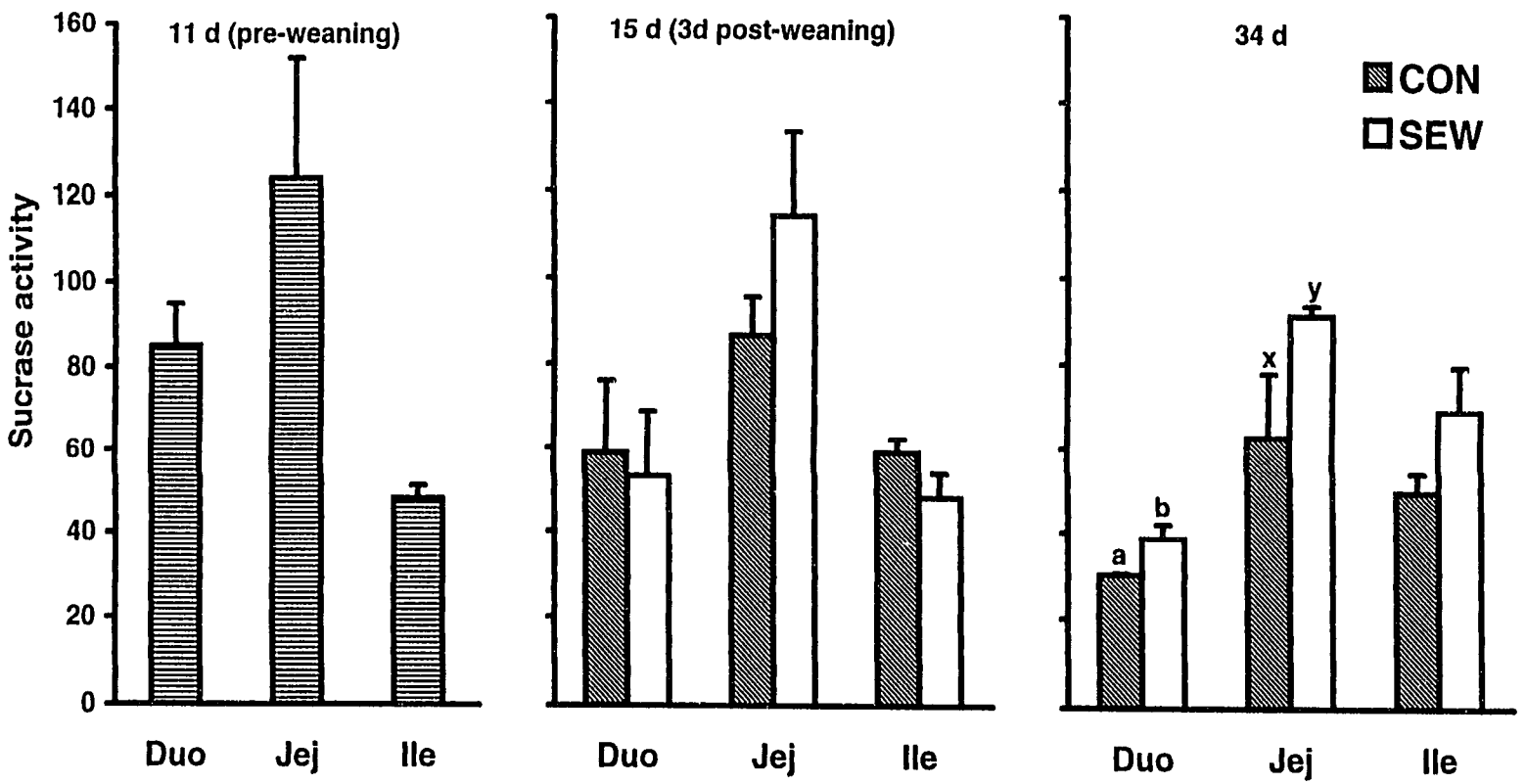


Figure 3.1 Specific activity of sucrase in duodenum, jejunum and ileum in SEW and CON pigs around the time of weaning. Values of sucrase activity are expressed as μmol substrate $minute^{-1}$·g $protein^{-1}$ and means ± SEM (n=3). Values with different letters, a or b and x or y, are different at $P < .1$ and $P < .05$, respectively, between SEW and CON pigs of the same age.

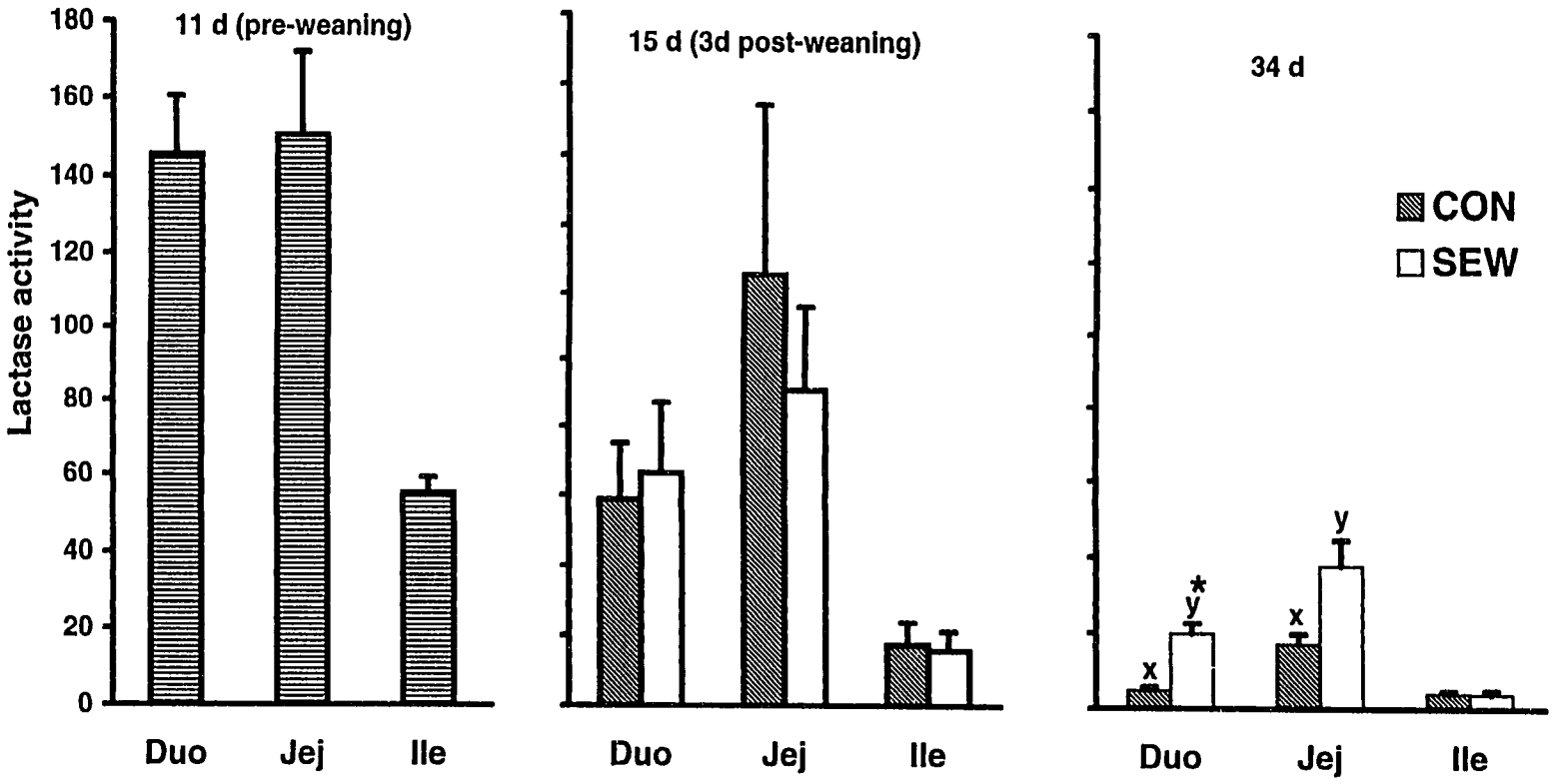


Figure 3.2 Specific activity of lactase in duodenum, jejunum and ileum in SEW and CON pigs around the time of weaning. Values of lactase activity are expressed as μmol substrate minute^{-1}·g protein^{-1} and means ± SEM (n=3). Values with different letters, x or y and letter with *, are different at $P < .05$ and $P < .01$, respectively, between SEW and CON pigs of the same age.

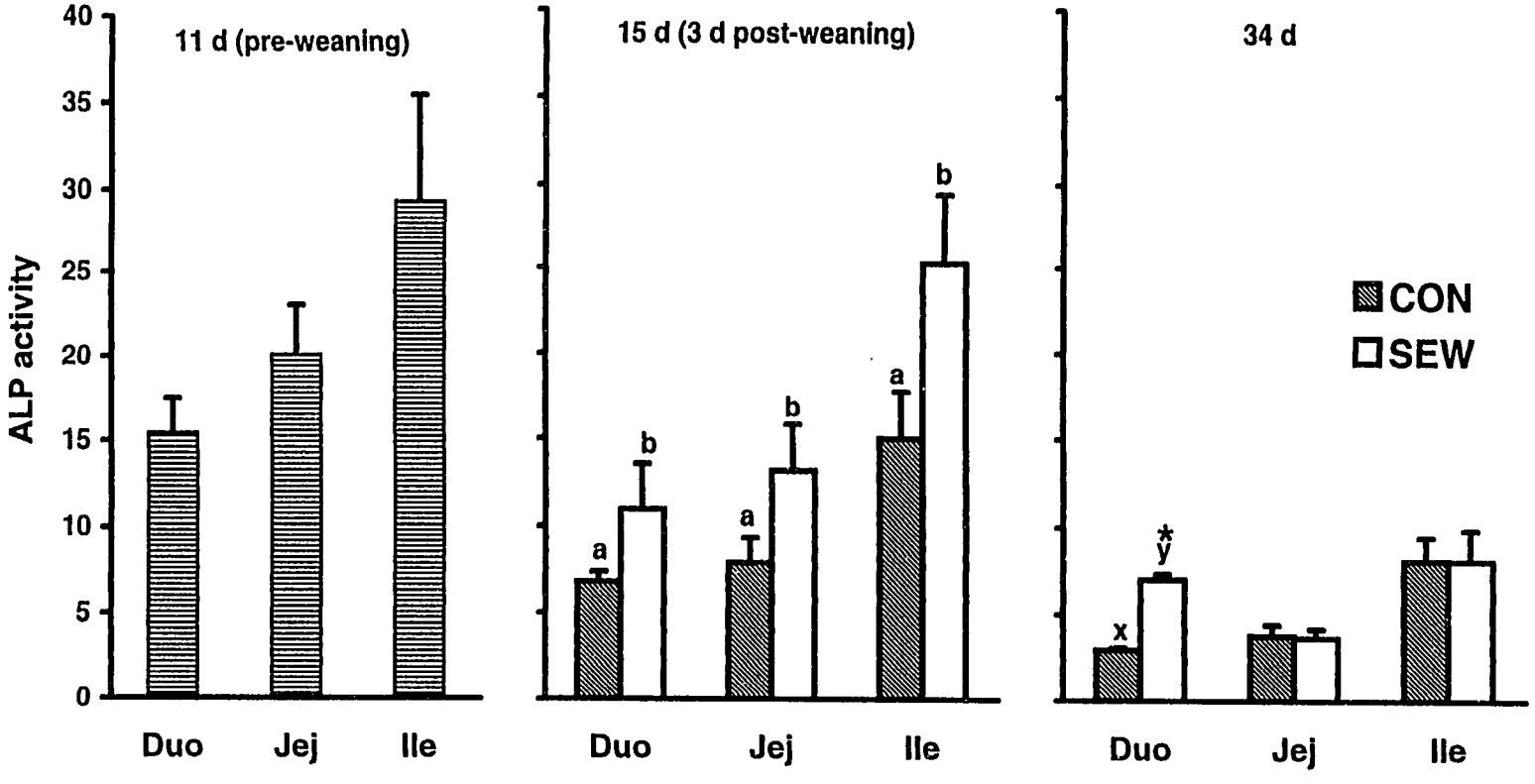


Figure 3.3 Specific activity of alkaline phosphatase in duodenum, jejunum and ileum in SEW and CON pigs around the time of weaning. Values of alkaline phosphatase activity are expressed as mmol substrate $minute^{-1}$ · mg $protein^{-1}$ and means ± SEM (n=3). Values with different letters, a or b and x or y with ✱ , are different at $P < .1$ and $P < .01$, respectively, between SEW and CON pigs of the same age.

Mucosal Protein to DNA Ratios in SEW and CON Pigs

Mucosal protein to DNA ratio in the duodenum and jejunum was increased ($P < 0.05$) in both SEW and CON pigs at 3 d post-weaning, and this ratio in the duodenum, jejunum and ileum was 20, 25.5 ($P < 0.05$) and 1.5% lower in 15 d old SEW pigs vs. CON pigs. In contrast, at 34 d mucosal protein to DNA ratio from the duodenum, jejunum and ileum was 43.5 ($P < 0.05$), 24.3 ($P = 0.06$) and 32.9% ($P < 0.05$) higher in SEW pigs compared to CON pigs (Fig. 3.4).

Villus Height, Crypt Depth and Villus Height to Crypt Depth Ratio

Scanning electron microscopy suggested objectively a weaning-induced reduction in villus height (Fig. 3.5) and microvillus length (Fig. 3.6) in a duodenal section obtained from SEW and CON animals at 11 d of age and 3 d post-weaning (15 d of age).

Weaning resulted in a decrease ($P < .01$) in villus height and an increase ($P < .01$) in crypt depth leading to a dramatic decrease ($P < .01$) in villus height to crypt depth (V/C) ratio. Villus height, crypt depth and V/C ratio were similar between SEW and CON pigs at 15 d of age. At 34 d of age, SEW pigs tended to have longer villi and shallower ($P < .01$) crypts in jejunum and ileum compared with CON pigs. V/C ratio was higher ($P < .01$) in the jejunum and ileum for SEW pigs versus CON pigs at 34 d of age (Table 3.1).

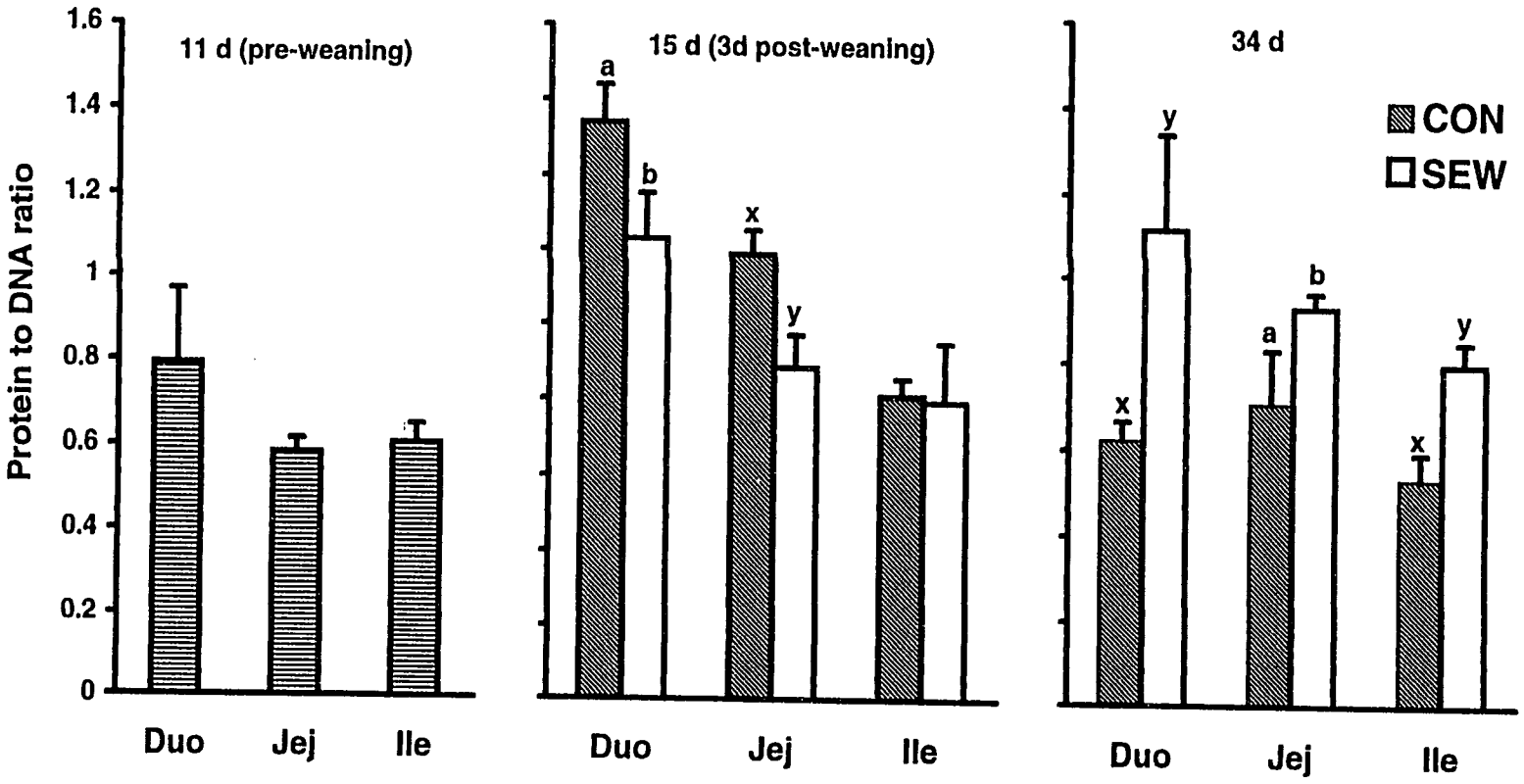


Figure 3.4 Mucosal protein to DNA ratio in duodenum, jejunum and ileum in SEW and CON pigs at 11, 15 and 34 d of age. Values of mucosal protein to DNA ratio are expressed as μg protein ng DNA^{-1} and means ± SEM (n=3). Values with differentletters, a or b ($P < .1$) and x or y ($P < .05$), are different between SEW and CON pigs of the same age.

Table 3.2 Effect of weaning and nursery sites on small intestinal morphology in pigs.

		11d-pigs	15d-CON	15d-SEW	34d-CON	34d-SEW
D	Villus µm	777.4 ± 46.7^{a}	412.1 ± 14.6^{b}	438.7 ± 16.0^{b}	458.2 ± 13.3^{bc}	506.5 ± 12.6^{c}
	Crypt µm	171.7 ± 6.1^{a}	234.1 ± 11.5^{b}	242.1 ± 9.9^{b}	295.9 ± 11.8^{c}	294.9 ± 11.6^{c}
J	Villus µm	746.3 ± 44.3^{a}	405.4 ± 18.5^{b}	385.8 ± 12.1^{b}	473.1 ± 18.6^{c}	522.2 ± 12.6^{c}
	Crypt µm	181.3 ± 7.7^{a}	218.2 ± 11.1^{b}	213.3 ± 8.2^{b}	249.6 ± 12.9^{c}	210.1 ± 9.6^{b}
I	Villus µm	588.2 ± 22.8^{a}	374.6 ± 15.6^{b}	384.7 ± 13.2^{b}	400.6 ± 14.3^{b}	420.0 ± 10.1^{b}
	Crypt µm	154.7 ± 6.5^{a}	197.7 ± 9.4^{b}	221.8 ± 7.1^{b}	259.6 ± 11.4^{c}	204.4 ± 8.7^{b}
v/c	D µm/µm	4.5 ± 0.2^{a}	1.8 ± 0.8^{b}	1.8 ± 0.8^{b}	1.7 ± 0.6^{b}	1.9 ± 0.9^{b}
	J µm/µm	6.0 ± 0.3^{a}	1.9 ± 0.8^{b}	1.8 ± 0.6^{b}	1.5 ± 0.8^{b}	2.6 ± 0.1^{c}
	I µm/µm	3.9 ± 0.2^{a}	1.9 ± 0.7^{b}	1.7 ± 0.5^{b}	1.6 ± 0.7^{d}	2.2 ± 0.1^{c}

D, duodenum; J, jejunum; I, ileum; V/C, villus height to crypt depth ratio.
Results are expressed as mean ± SEM (n=3).
Means with different superscripts are significantly ($P < 0.01$) different.

A

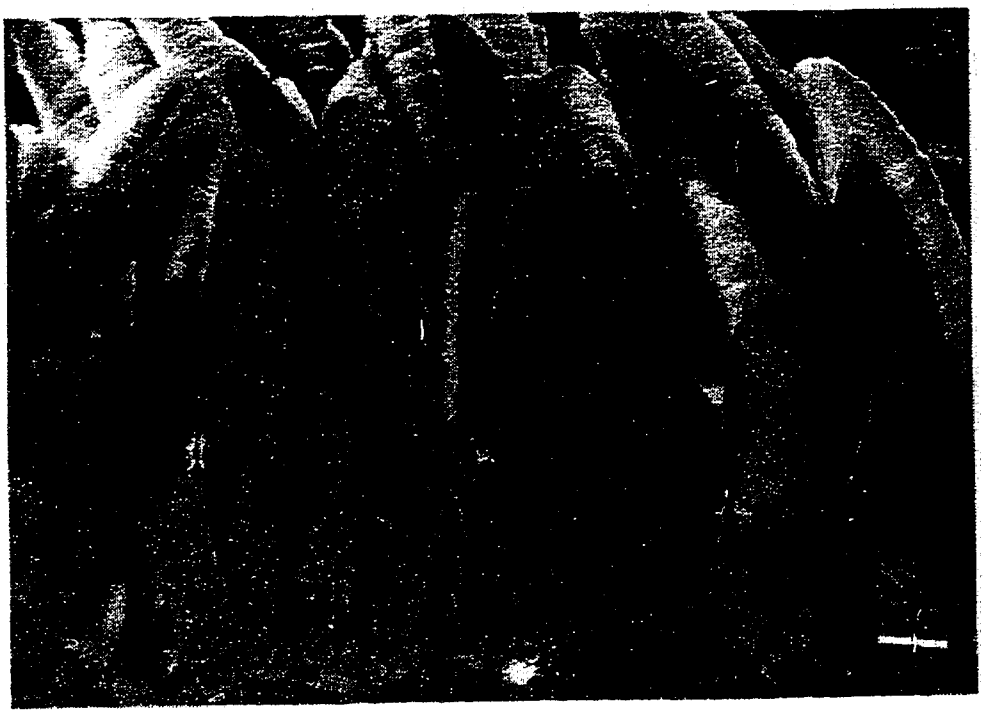
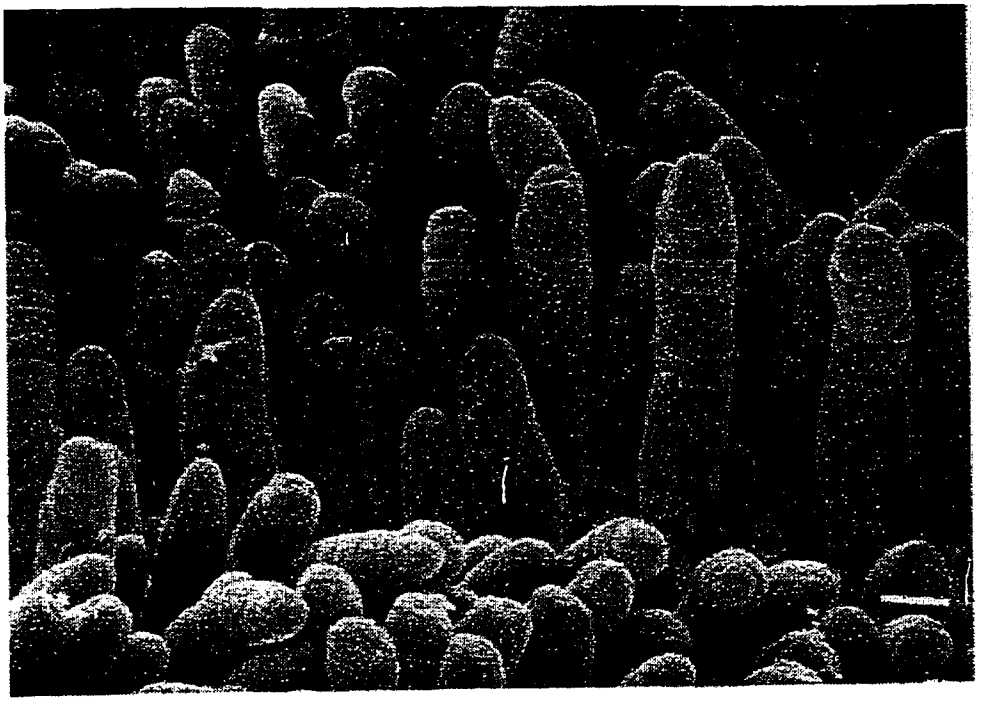


Figure 3.5 Scanning electron micrographs show villus height of intestinal mucosa in the duodenum of a representative pig at 11d of age (pre-weaning; A), a CON pig at 15 d of age (3 d post-weaning; B) and a SEW pig at 15 d of age (3 d post-weaning; C). (Bar = 100 μm).

B



C

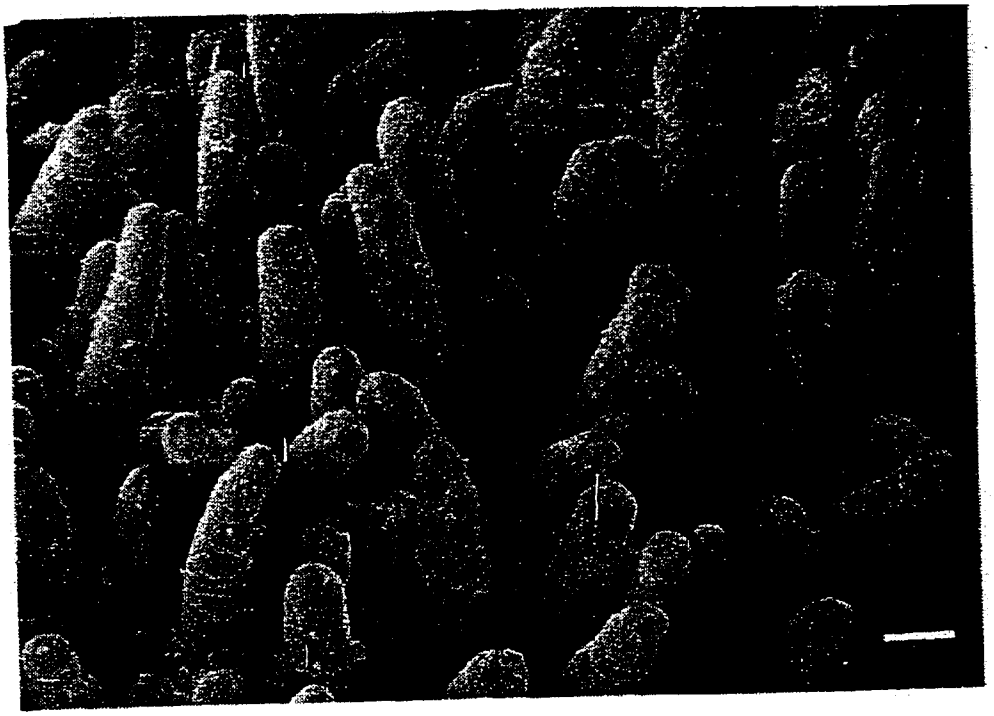
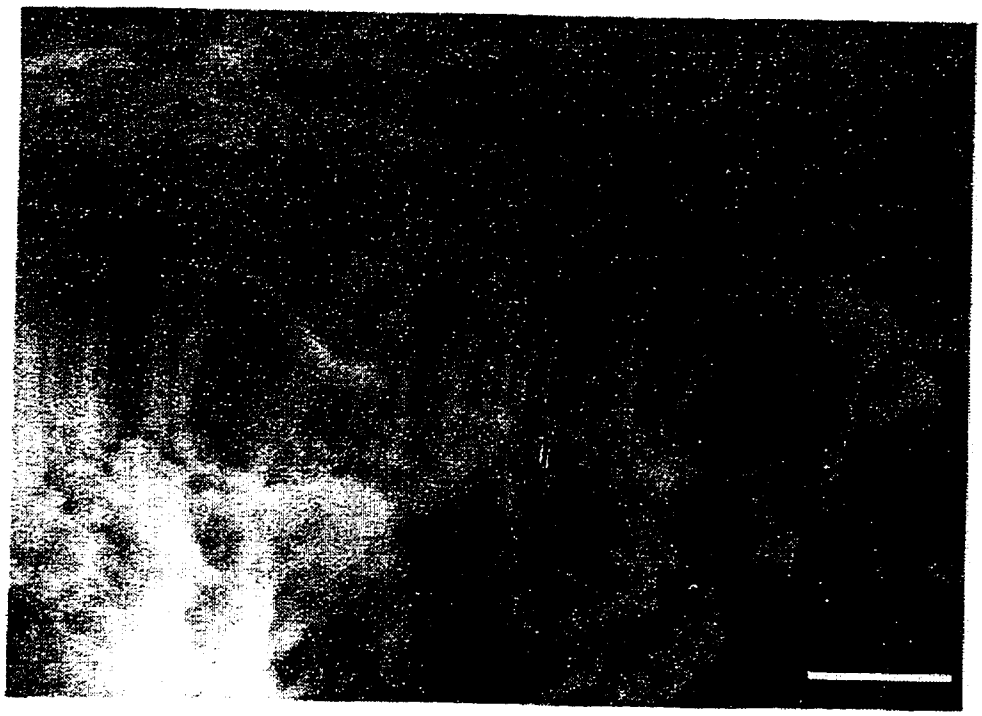


Figure 3.5

A

Figure 3.6 Scanning electron micrographs show micro-villus height of intestinal mucosa in the duodenum of a representative pig at 11d of age (pre-weaning; A), a CON pig at 15 d of age (3 d post-weaning; B) and a SEW pig at 15 d of age (3 d post-weaning; C). (Bar = 1 μm).

B



C

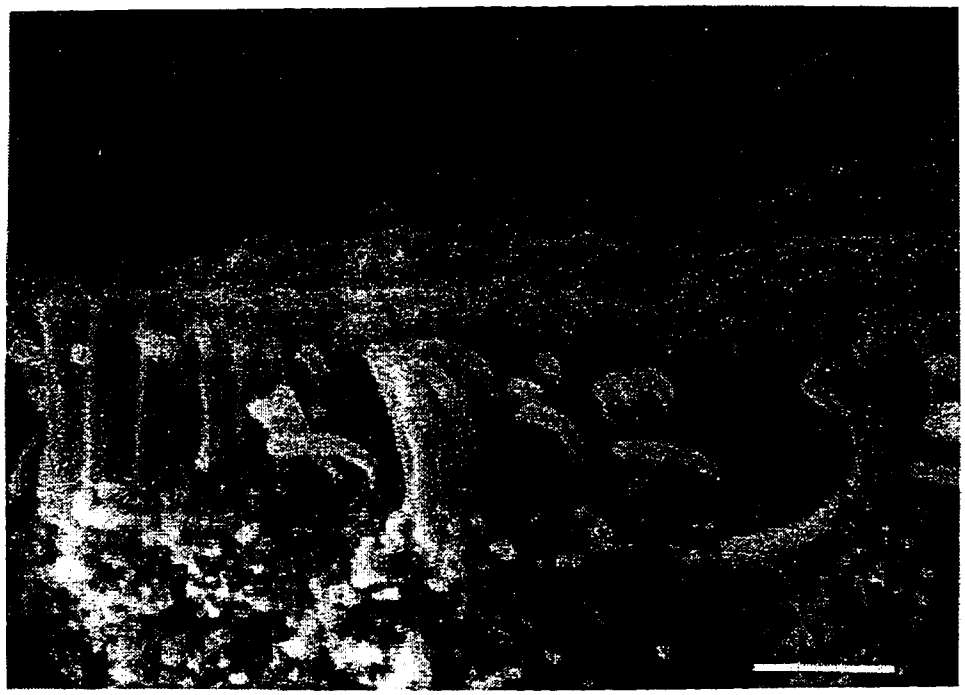


Figure 3.6

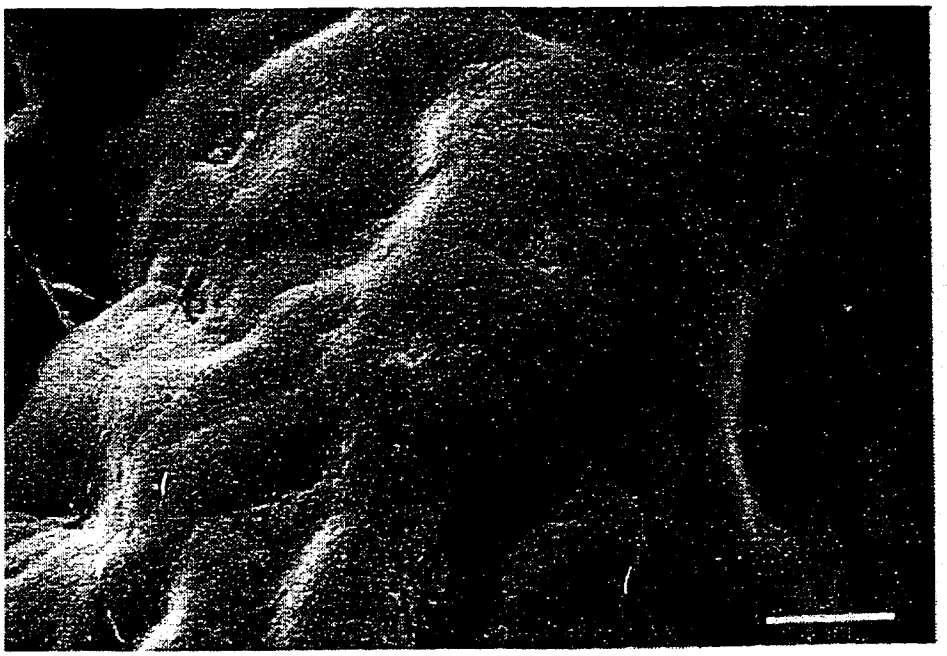
Mucus

A thicker mucus coating over the apical surface of epithelial cells in the ileum in 34 d old CON pigs relative to SEW pigs was revealed by scanning electron microscopy (Fig. 3.7). AB/PAS staining confirmed a thicker mucus layer covering on villi in the ileum of CON pigs compared with SEW pigs at 34 d of age (Fig. 3.8).

Intraepithelial Lymphocytes (IEL)

The number of intraepithelial lymphocytes per 100 enterocytes in the jejunum was greater ($P < 0.001$) in SEW pigs (11.6 ± 0.3) than in CON pigs (3.1 ± 0.2) at 34 d of age (Fig. 6.9).

A



B

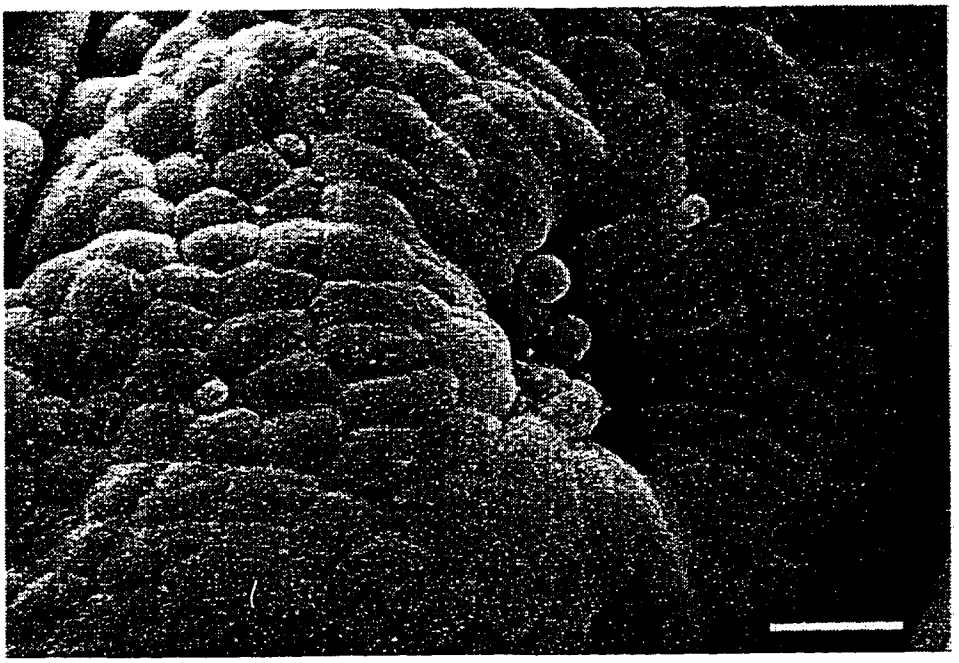
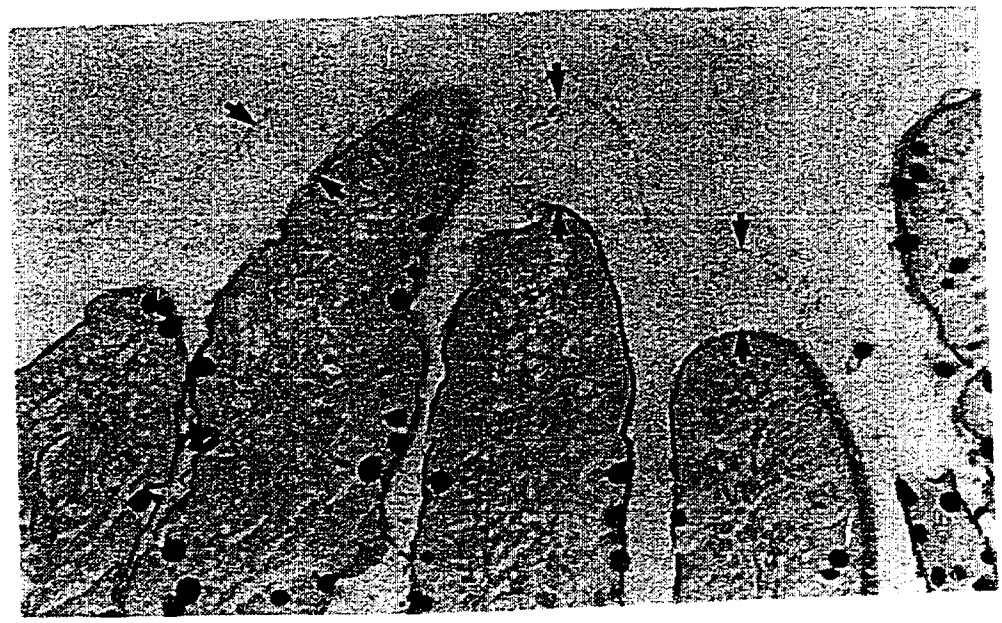


Figure 3.7 Scanning electron micrograph of the ileal lumen for a representative CON (A) and SEW (B) pig showing variation in mucus covering (bar = 10 μm).

A



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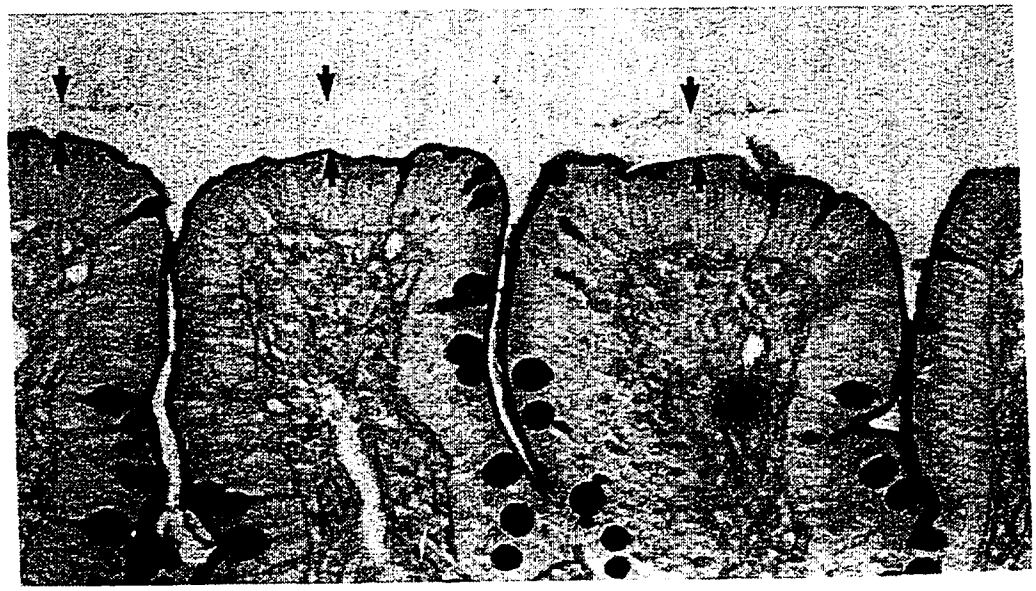

Figure 3.8 Light micrograph following AB/PAS staining of mucosal epithelial in ileum of 34 d old CON (A) and SEW (B) pigs. Thickness of the mucus layer is indicated using arrows (*AB/PAS* x 285).

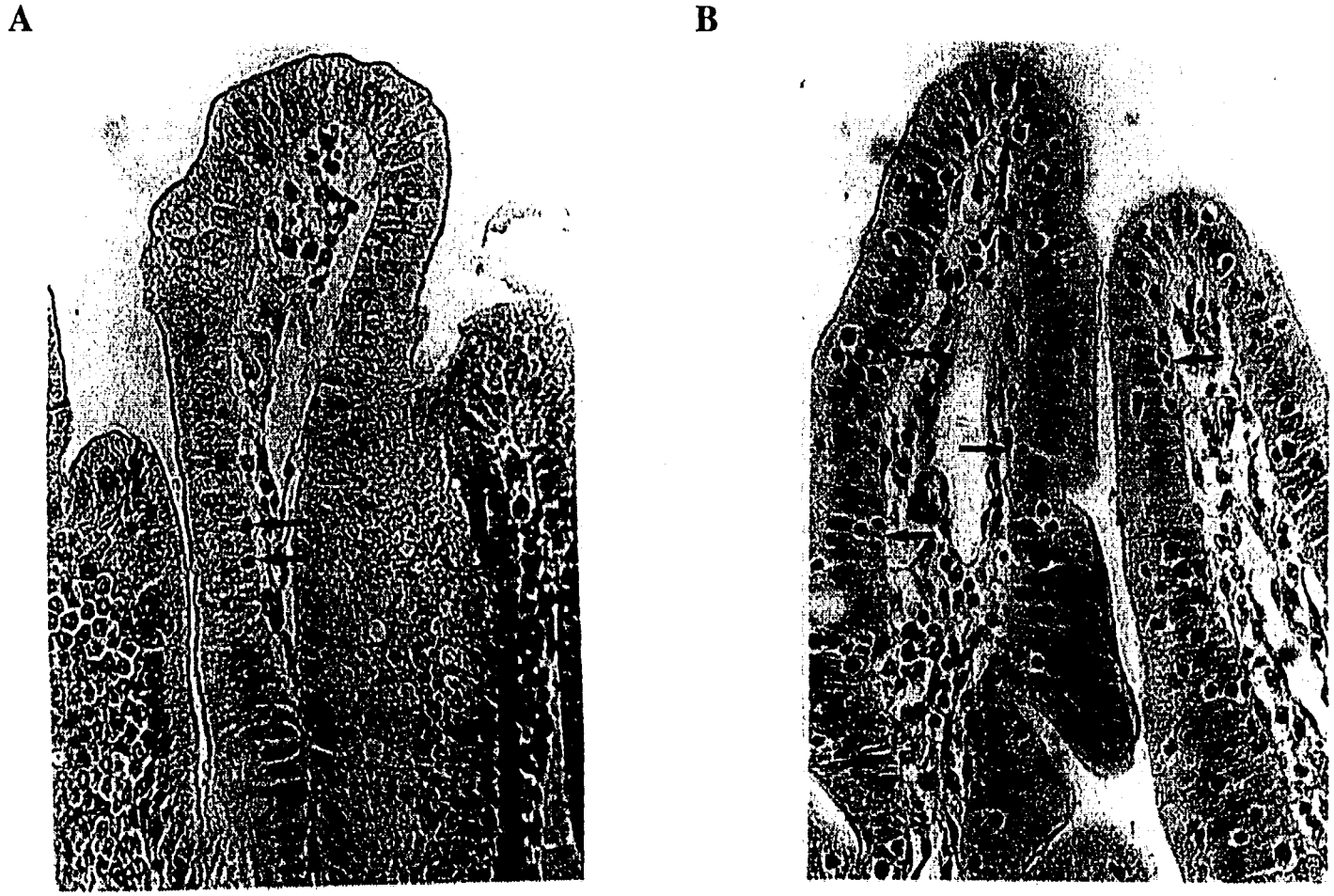

Figure 3.9 Light micrograph of jejunal epithelium in CON (A) and SEW (B) pigs at 34 d of age. Arrows indicate intraepithelial lymphocytes (IEL) located along basolateral membrane of epithelial cells (*H & E* x 400).

3.5 Discussion

The findings of this study demonstrate that rearing environment can exert a significant impact on postweaning gut development, both biochemically and morphologically. Alkaline phosphatase has long been used as an indicator of intestinal maturation in rat (Henning 1981, 1987), whereas lactase and sucrase are predominant brush border proteins in neonatal and weaned pigs, respectively (Hampson 1986). The specific activity of lactase and sucrase was measured to indicate maturity of enterocytes and functional capacity (Hampson and Kidder 1986, Henning 1985). Data of this study showed that the specific activities of lactase, sucrase and alkaline phosphatase were consistently higher in SEW pigs than in CON pigs at 34 d of age, suggesting that enterocytes in villi were more mature with greater synthesis of brush border enzymes. This observation is consistent with a previously reported increase in feed intake and feed efficiency (Patience et al. 1997) and superior performance in SEW pigs compared with CON pigs at 34 d of age (Van Kessel et al. 1997) using this experimental protocol.

Mucosal protein to DNA ratios in all sections of the intestine were lower in SEW pigs than in CON pigs at 3 d post weaning. However, the ratios in the duodenum, jejunum and ileum were significantly greater in SEW pigs compared with CON pigs at 34 d of age. This indicates that mucosal protein synthesis in the brush border was higher in 34 d old SEW pigs than in CON pigs, which is consistent with our finding of increased brush border enzyme activity.

The population of enterocytes is in a dynamic state, constantly being replaced by regeneration of epithelial cells from crypts, and the rate of regeneration matches the

normal loss of villus epithelium. The epithelial cells near the villus tip are the most mature and have the greatest digestive and absorptive capacity (Aitken 1984). In general, measurements of villus height and crypt depth give an indication of the likely maturity and functional capacity of enterocytes (Hampson, 1986). Observations in the current showed similar villus height, crypt depth and V/C ratio between SEW and CON pigs at 15 d of age. In contrast, longer villi, significantly shorter crypts and higher V/C ratios in the jejunum and ileum of the small intestine were shown in SEW pigs compared with CON pigs at 34 d of age. Abrams (1977) demonstrated that shorter villi and deeper crypts were caused by resident bacterial flora when compared with germ-free animals. Increased rate of epithelial renewal in response to invasion of pathogens that damage epithelial cells may play a key defensive role in the intestine (Gaskins 1997). Inadequately developed digestive enzymes and transport of nutrients at the villus surface accompanied an increased rate of epithelial cell migration from crypt to villus and a low proportion of mature enterocytes (Thake et al. 1973). The different morphology in the intestine between SEW and CON pigs at 34 d of age indicates that CON pigs may have a larger proportion of functionally immature epithelial cells due to the more rapid migration of epithelial cells from crypts. This finding is consistent with observations that suggest that SEW pigs experience reduced pathogen exposure relative to control pigs (White 1995).

The function of mucus on the microvilli of the epithelium has been extensively studied in health and disease. Mucus protects epithelial cells from digestive enzymes produced by intestinal flora and provides an extensive barrier to prevent penetration of

potential pathogens into epithelium (Neutra and Forstner 1987). The stimulation of mucus secretion from goblet cells was demonstrated by bacterial infection (Cohen et al. 1983), the resident intestinal flora (Mantle et al. 1989), toxins (Roomi et al. 1984) and parasitic infestation (Miller et al. 1981). Both scanning electron microscopy and AB/PSA staining showed that there was a much thicker mucus covering on the villus surface in the ileum of all CON pigs at 34 d of age, but not in SEW pigs. This finding indicates increased presence of potential pathogens in the gut lumen of CON pigs compared with SEW pigs and is consistent with suggestion that SEW pigs experience reduced pathogen challenge (Boeckman 1996, Van Kessel et al. 1997). A thicker mucus layer on villi in the jejunum in 34 d old CON pigs than in the age matched SEW pigs was also observed (data not shown). Such thicker mucus on the surface of epithelial cells in CON pigs may reduce digestion and absorption of nutrients by attenuating free diffusion of various nutrients, even water, to the apical surface of epithelial cells which contains disaccharidases, peptidases, receptors and transport proteins (Forstner 1978). Therefore, in addition to the observed increase in specific activity of brush border enzymes in SEW pigs, the reduction in mucus thickness may also be a contributing factor in improving feed efficiency as observed in SEW pigs (Patience et al. 1997).

Intraepithelial lymphocytes (IEL) may play a role in suppression of immune response, or in elimination of damaged or infected epithelial cells (Cerf-Bensussan and Guy-Grand 1991). IEL usually do not appear in pigs until 5-7 weeks of age (Stokes et al. 1994) and their development is arrested in germ free animals (Rothkotter et al. 1991). The higher number of IEL in the jejunum of 34 d old SEW pigs compared with

CON pigs may reflect a more adult phenotype gut. The physiological and immunological significance of the numerous IEL in epithelium of SEW pigs awaits further study.

The more rapid post-weaning gut adaptation in SEW pigs may not be solely explained by reduced pathogen load and /or altered intestinal bacterial colonization. Early weaning in rats induces precocious appearance of brush border enzyme activity due to an increase in serum glucocorticoids (Boyle et al. 1980). Forced weaning of pigs at 12 d of age in this study could also elevate plasma glucocorticoids, which has been shown to be a primary signal to trigger premature expression of sucrase-isomaltase in weaned animals (Chapple et al. 1989). The SEW treatment itself may also have imposed increased stress and glucocorticoid levels relative to CON pigs through transportation of SEW pigs to a distant nursery. An acute rise in plasma glucocorticoid could be a beneficial "trigger" to promote the appearance of 'adult-type' intestine (Needham and Fletcher1992), and also through a catabolic effect to decrease synthesis and increase degradation of mucosal protein (Burrin et al. 1998), which could explain the higher sucrase activity in the jejunum and lower mucosal protein to DNA ratio observed in SEW pigs relative to CON pigs at 3 d post weaning.

In summary, the data overall point to enhanced postweaning gut maturation in SEW pigs that was associated with reduced potential pathogen presence in the gut lumen. This would be expected to improve capacity of digestion and absorption of nutrients from the small intestine and thus increase growth performance and feed efficiency.

3.6 Implications

This study analyzed the differences in morphology and function of the small intestine between SEW and CON pigs, and demonstrated that rearing environment influenced postnatal gut maturation. SEW provides benefits through enhanced gut maturation and possibly reduced enteric immune challenge. These findings suggest an important role of pathogen load on the morphological and functional development of the small intestine. It is likely that the intestinal development changes observed here contribute significantly to improved production performance observed with SEW management.

4 INTESTINAL EXPRESSION OF IGF-I AND RELATED PROTEINS IN THE EARLY WEANED PIG

4.1 Abstract

Pigs were weaned early (12 d) into a reduced pathogen (SEW) or conventional (CON) weaning environment. Small intestinal (SI) IGF-I, IGFBPs and IGF-I receptor (IGF-IR) mRNA expression was measured in pigs (n=3) killed at 11 (pre-weaning), 15 (3 d post weaning) and 34 d of age. Weaning resulted in increased IGF-I and decreased IGF-IR mRNA abundance in SI in both groups. Intestinal mRNA encoding for IGFBP-4 increased in CON and decreased in SEW, IGFBP-5 increased in SEW, and IGFBP-6 mRNA decreased in both groups at 3 d post-weaning were observed. Intestinal IGF-I (39%, $P < 0.01$) and IGFBP-5 (24%) mRNA levels were higher in SEW than in CON pigs at 3 d post-weaning. In contrast, intestinal IGF-I (15%) and IGFBP-5 (40%, $P < 0.05$) were lower in SEW relative to CON pigs at 34 d of age. Results showed that weaning and weaning environment altered the expression of IGF-I and related proteins in SI.

4.2 Introduction

Early weaning of rats induces precocious maturation of brush border enzymes and is associated with a marked acceleration in cell migration and an increase in mitotic activity (Smith 1985). In the pig morphological and functional changes in the small intestine after weaning include rapid shortening of villi, deepening of crypts and

alteration in brush border enzyme activities (Nabuurs 1995). These acute responses to weaning have been attributed to a low post weaning feed intake, adaptation to a solid diet, social and/or environmental stresses and immunological challenge associated with dietary antigens and altered microbial colonization.

The role of local insulin-like growth factor (IGF)-I, IGF binding proteins (IGFBPs) and IGF-I receptor (IGF-IR) in the adaptive processes occurring in the intestine in response to weaning are not well understood. Increasing evidence supports that insulin-like growth factor (IGF)-I regulates growth of the intestine and plays an important role in neonatal gut development (Burrin et al. 1996). IGF-IR mediate the growth promoting actions of IGF-I in various tissues including in the small intestine (Lund 1994) and the number of IGF-IR in porcine intestine decreases with age (Morgan et al. 1996). IGF-I mRNA expression in the jejunum and ileum is increased during adaptive growth following small bowl resection in rats (Ziegler et al. 1998). Bowel resection in rats increased IGFBP-4 mRNA in the jejunum and ileum and decreased IGFBP-3 mRNA in the ileum (Ziegler et al. 1998).

Rearing environment and pathogen pressure also influence gut morphological and functional development in young animals (Miller et al. 1986), but related responses in gut expression of IGF-I and IGFBPs have not been well characterized. Induced IGF-I mRNA expression in inflamed bowel may exert trophic effects of the growth factor potentially limiting mucosal damage or promoting tissue repair (Lund and Zimmermann 1996). Increased IGF-I mRNA abundance has been observed in inflamed intestine in animals with experimental enterocolitis (Zimmermann et al. 1993) and in patients with

Crohn's disease (Cohen et al. 1993). Inflammation of the colon is accompanied by increased expression of IGFBP-4 and -5 (Zeeh et al. 1995). These findings suggest that local expression of IGF-I and IGFBPs may influence adaptive growth of the gut following resection or inflammation.

Segregated early weaning (SEW) is being rapidly adopted to limit pathogen exposure and maximize growth in weaned pigs (Van Kessel et al. 1997). It is obvious that growth in young animals is dependent on an adequate supply of absorbed nutrients and that attenuated growth may be associated with compromised gut development. In a related study (Tang et al. 1999) showed that SEW enhances post-weaning gut development and function in weaned pigs. This study reports on the changes in intestinal IGF-I, IGFBPs and IGF-IR gene expression in response to weaning and different weaning environments. Similarities in the gastrointestinal anatomy and physiology between the human and the pig (Moughan et al. 1992) suggest that this data may also be useful in the study of human neonatal intestinal development.

4.3 Material and Methods

Animals

Ninety-two piglets (PIC C15X Canabrid) reared at Prairie Swine Centre Inc. (PSCI, Saskatoon, SK) were weighed and weaned early at 12 d of age. Forty-six piglets were moved into an off-site nursery with reduced infection pressure (SEW) distant from the breeding herd, and forty-six pigs were moved to an on-site conventional (CON) nursery in close proximity to the breeding herd. Efforts were employed to maintain

environmental temperature and moisture, nutrition and management as consistent as possible between the two nurseries. Weight and feed intake of piglets in each nursery were recorded at regular intervals. The faster growth rate and potentially lower pathogen presence in the gut lumen of SEW pigs compared with CON pigs are reported elsewhere (Van Kessel et al. 1997).

The Animal Protocol Review Committee of the University of Saskatchewan approved the protocol of this study in accordance with the requirements and regulations of the Canadian Council on Animal Care.

Tissue Collection

Piglets were injected with urethanyl forte (MTC Pharmaceutical, Cambridge, ON) and killed (n=3) by terminal bleeding at 11 (pre-weaning), 15 (3 d post-weaning) and 34 d of age.

The small intestine was dissected free of its mesentery and placed on ice immediately. The segment from the pylorus to the ligament of Trietz was considered as duodenum, the proximal segment of the rest of the small intestine was considered the jejunum, and a distal segment 10 cm proximal to the ileocecal junction as ileum. The three segments were rinsed thoroughly with ice-cold physiological saline solution. Mucosal tissue scrapings were obtained from each segment for measurement of the number of IGF-IR. Whole segments of the small intestine used for measurement of IGF-I, IGFBPs and IGF-IR mRNA were immediately frozen in liquid nitrogen and stored at -80°C until assayed.

Radio-Receptor Binding Assay

Microsomal membranes were prepared (Hofig et al. 1991) and stored at -80°C until assayed. The protein concentration of the membranes was determined using the method of Bradford (1976) with bovine albumin as standard. The membranes were incubated with $MgCl_2$ (20 mM final concentration) in binding buffer in order to remove endogenously bound IGF according to the method of Kelly et al. (1979). Recombinant human IGF-I (^{59}Thr, ICN Biomedical, Costa Mesa, CA) was iodinated with ^{125}I (Amersham, Arlington Heights, IL) by the chloramine-T method (Hunter and Greenwood 1962) with a specific activity of 131 μCi/μg. Pretreated membranes were diluted in 200 μl of binding buffer to a final concentration of 350 μg. The membranes were then incubated in a final volume of 500 μl with approximately 0.15 ng (25,000 cpm) of ^{125}I-IGF-I in the presence or absence of unlabelled IGF-I. Radioactivity corresponding to ^{125}I-IGF-I in the pellet was measured in a gamma counter. Specific binding (SB) was determined as the difference between total binding (TB) and non-specific binding (NSB) that was calculated as the radioactivity remaining in the pellet with 300 molar excess of unlabeled IGF-I.

Receptor Specificity

To determine the specificity of ^{125}I-IGF-I binding to IGF-I receptors, 350 μg membrane protein prepared from pooled jejunal mucosa from 5 pigs was diluted in a final volume of 500 μl. The membranes were incubated with approximately 0.15 ng ^{125}I-IGF-I and increasing concentrations from 10^{-12} to 10^{-3} M of IGF-I, IGF-II (Gropep, Adelaide, SA, Australia), porcine insulin (Sigma Chemical Co., St. Louis, MO) and

porcine vasoactive intestinal peptide (VIP) (Bachem, Torrance, CA). Percent specific binding was calculated for each concentration of each peptide and plotted.

Scatchard Analysis

Pooled (n=3) jejunal mucosal membranes (350 μg protein) from 11 d pigs and SEW and CON pigs at 15 and 34 d of age were incubated in a final volume of 500 μl with varying concentrations of ^{125}I-IGF-I (0.15 to 7.5 ng). Affinity and capacity of IGF-I receptors were calculated by Scatchard plot (Scatchard 1949) analysis using the LIGAND computer program (Munson and Rodbard 1980).

Preparation of cDNA Probes

The human type I IGF receptor cDNA corresponding to a 700 bp fragment was purchased from the American Type Culture Collection (Rockville, MD). Porcine (p) IGF-I and IGFBP-2 (gifts from Dr. F. A. Simmen, University of Florida) and pIGFBP-3 (gift from Dr. S. Shimasaki, University of California) cDNA contained 580 bp, 1.4 kb and 595 bp inserts, respectively. Human (h)IGFBP-1, -4, -5 and -6 cDNA containing 350, 506, 317 and 267 bp fragments, respectively, were generously provided by Dr. S. Shimasaki. Individual cDNA were labeled with [^{32}P] dCTP (Mandel, Boston, MA) using an Oligolabelling kit (Pharmacia Biotech Inc., Piscataway, NJ).

Northern Blot Hybridization Analysis

Frozen SI tissue was pulverized in liquid N_2 and total RNA was extracted using TRIzol reagent (Gibco BRL, Burlington, ON). Total RNA was quantified by spectrophotometric absorbance at 260 nm. Twenty μg of total RNA was electrophoresed on 1.2 % agarose containing 2.2 M formaldehyde. Ethidium bromide-stained gels were

photographed to illustrate the 18S and 28S rRNA bands before transfer to a nylon membrane. The procedure for Northern hybridization was based on the GeneScreen Plus protocol (NEN Research Products, Boston, MA) with some modifications. Briefly, pre-hybridization (42°C for at least 4 h or overnight) was performed in 5X SSPE (pH 7.4), 50% deionized formamide, 5X Denhardt's solution (Amersham), 10% dextran sulphate, Na salt, 1% SDS, and 300 µg per ml herring sperm DNA (Boehringer-Mannheim, Germany). The membranes were then hybridized with respective [^{32}P]-labeled dCTP cDNA in the same solution at 42°C overnight. The membrane was then washed twice with 0.2X SSPE, 1% SDS and 0.2X SSPE, 0.1% SDS at 42°C for 15 to 20 min, respectively, followed by autoradiography at - 80°C using an intensifying screen. The membrane was stripped using 0.1X SSPE and 1% SDS and then re-probed with porcine 3-phosphoglyceraldehyde dehydrogenase (pGAD) used as a normalizing or "housekeeping" probe (Gadsby et al. 1996) to quantitate total RNA loading and transfer. The procedure for Northern blotting for pGAD mRNA was the same as for others except that 100 µg per ml herring sperm DNA was incubated in the pre-hybridization solution. The size of RNA transcripts was evaluated according to the positions of 18S and 28S rRNA.

Autoradiographs were quantitated by computer-assisted densitometry. The density of the transcript was expressed in arbitrary units to the relative abundance of pGAD.

Statistical analysis

All results are presented as mean ± SEM and subjected to a one-way analysis of variance with 5 levels (11d, 15d CON, 15d SEW, 34d CON and 34d SEW) using the

GLM procedures of SAS (1985). Least significant means ($P < 0.05$) was used to separate means when the model was significant.

4.4 Results

Gene Expression of IGF-I in the SI

Weaning increased intestinal IGF-I mRNA abundance in SEW (39%, $P< 0.01$) at 15 d of age (3 d post-weaning), with no significant change in CON pigs (Fig. 4.1). IGF-I transcript levels were higher (31%, $P < 0.01$) in SEW than in CON pigs at 15 d of age. During post-weaning development intestinal IGF-I mRNA abundance increased in CON pigs and decreased in SEW pigs such that by 34 d of age IGF-I mRNA was marginally lower (15%) in SEW pigs relative to CON pigs.

Small Intestinal mRNA Encoding for IGF-IR

Weaning resulted in an immediate decrease in SI mRNA encoding for IGF-IR in SEW pigs (34%, $P< 0.05$) and, to a lesser extent, in CON pigs (20%) at 3 d post-weaning (Fig. 4.2). At 34 d SI IGF-IR mRNA abundance was lower in both groups when compared to pre-weaning values.

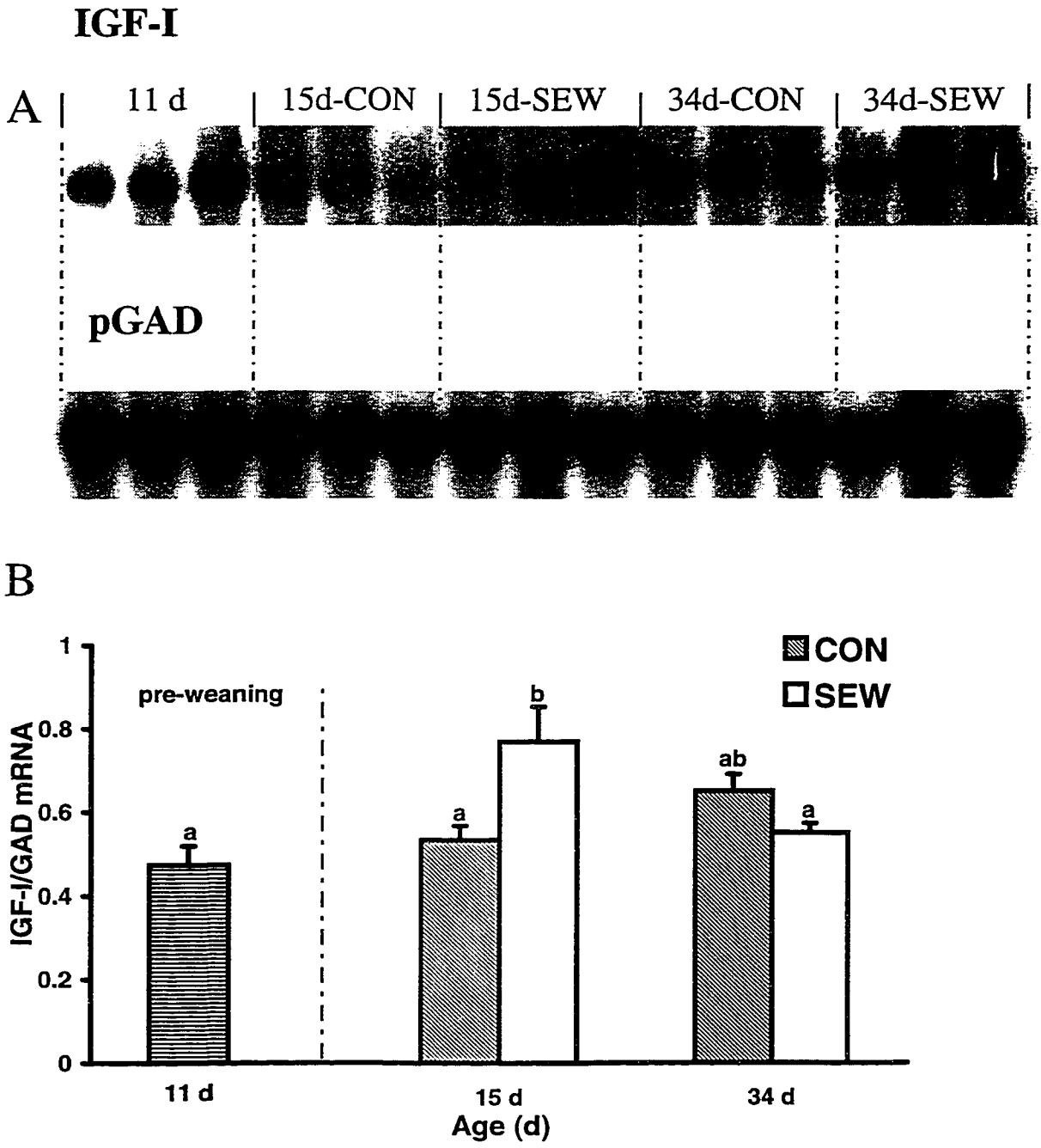

Figure 4.1 Northern blot analysis of IGF-I mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 7.8 kb IGF-1 transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P < 0.05$) different.

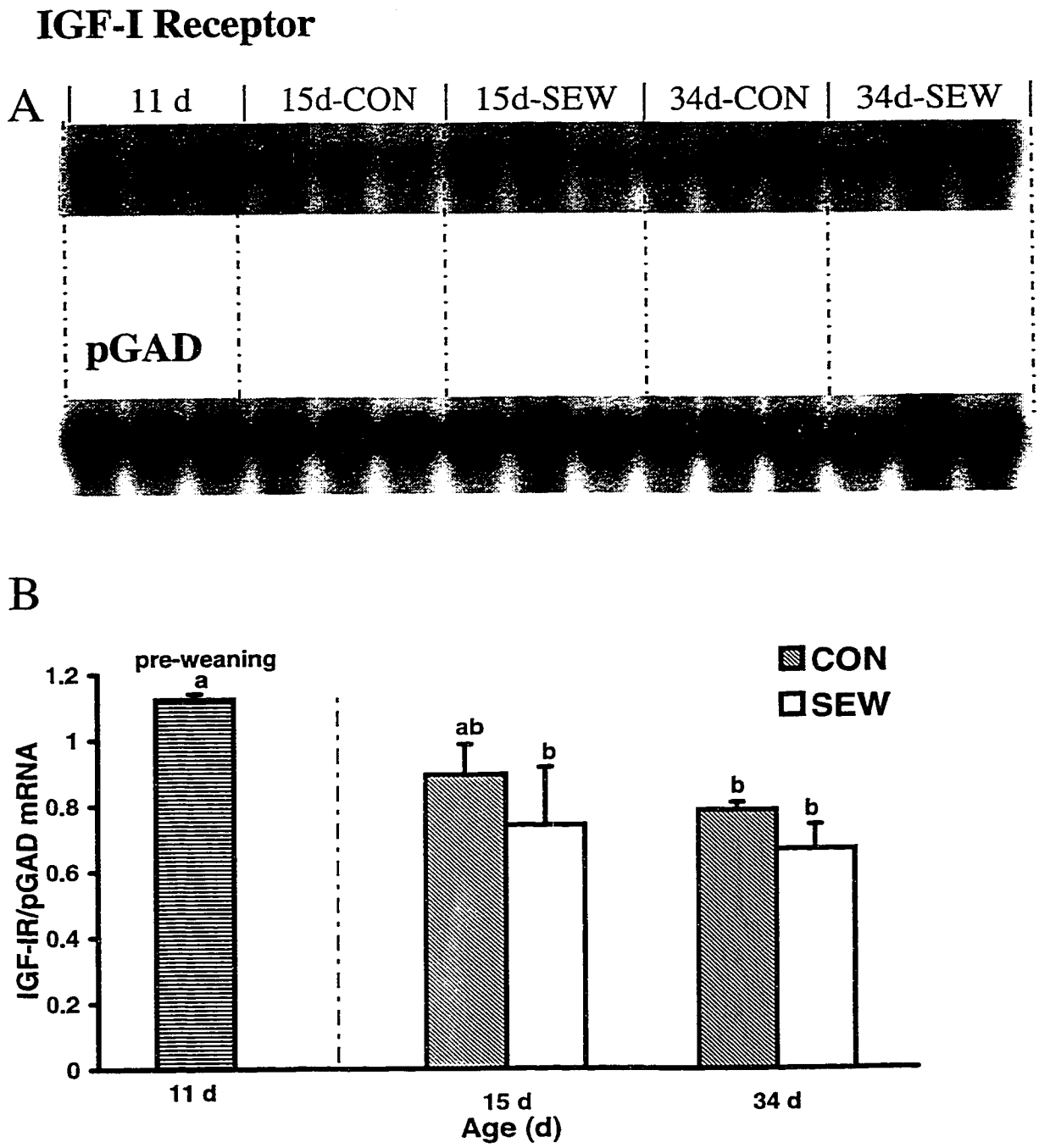

Figure 4.2 Northern blot analysis of IGF-IR mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 7.0 Kb IGF-IR transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel B) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P < 0.05$) different.

IGF-I Receptor Characteristics

Specificity of ^{125}I-IGF-I binding to IGF-IR: Binding of ^{125}I-IGF-I to the jejunal membranes was diminished with increasing amounts of unlabeled IGF-I and IGF-II (Fig. 4.3). However, 50% inhibition of binding occurred at 10^{-7} M for IGF-I and at 5 x 10^{-5} M for IGF-II. Insulin displaced ^{125}I-IGF-I binding only at high concentration (5 x 10^{-3} M), whereas porcine VIP did not displace ^{125}I-IGF-I binding.

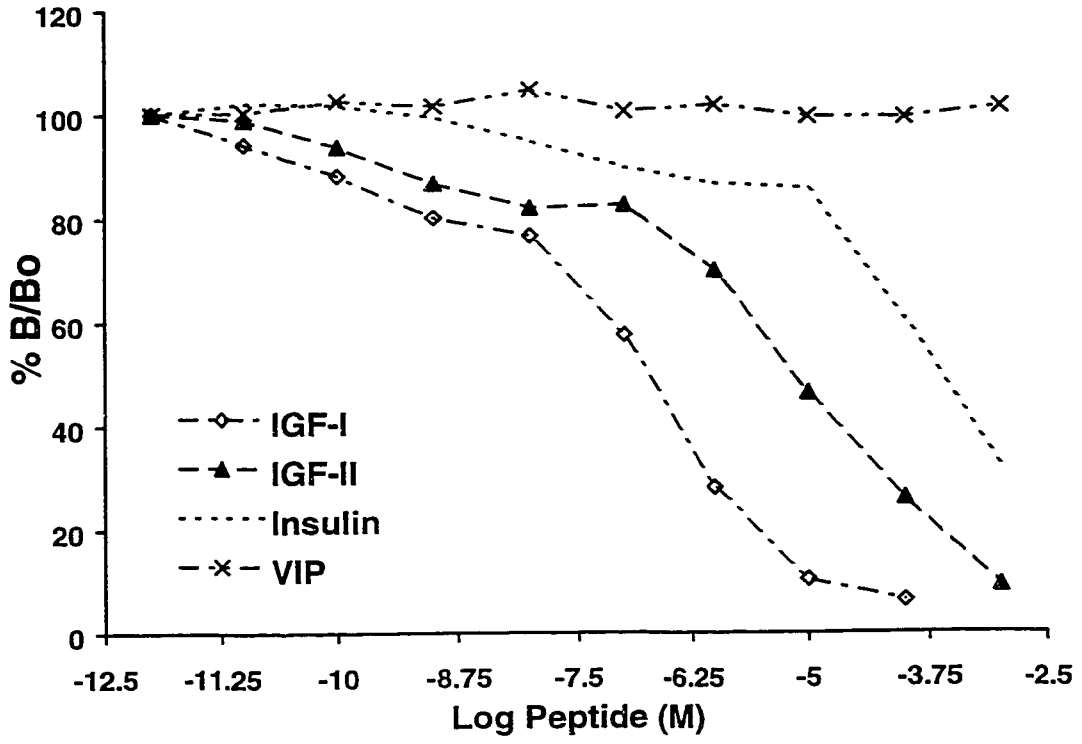

Figure 4.3 Displacement of ^{125}I-IGF-I binding by IGF-I, IGF-II, insulin and VIP to membranes prepared from pooled jejunal mucosa. Membranes were incubated at 4°C with 25,000 cpm ^{125}I-IGF-I with increasing concentrations of hIGF-I, hIGF-II, porcine insulin and porcine vasoactive intestinal polypeptide. Data are means ± SEM of three replicate determinations.

Scatchard analysis: Analysis of equilibrium binding data yielded linear plots and showed a decline in binding capacity (B_{max}) of IGF-I receptors during post-weaning development (Fig. 4.4). A constant affinity (K_a) of 4.8-5.0 ± 0.1-0.5 nM with a single class of binding sites was revealed in both groups of pigs throughout the study (Table 4.1).

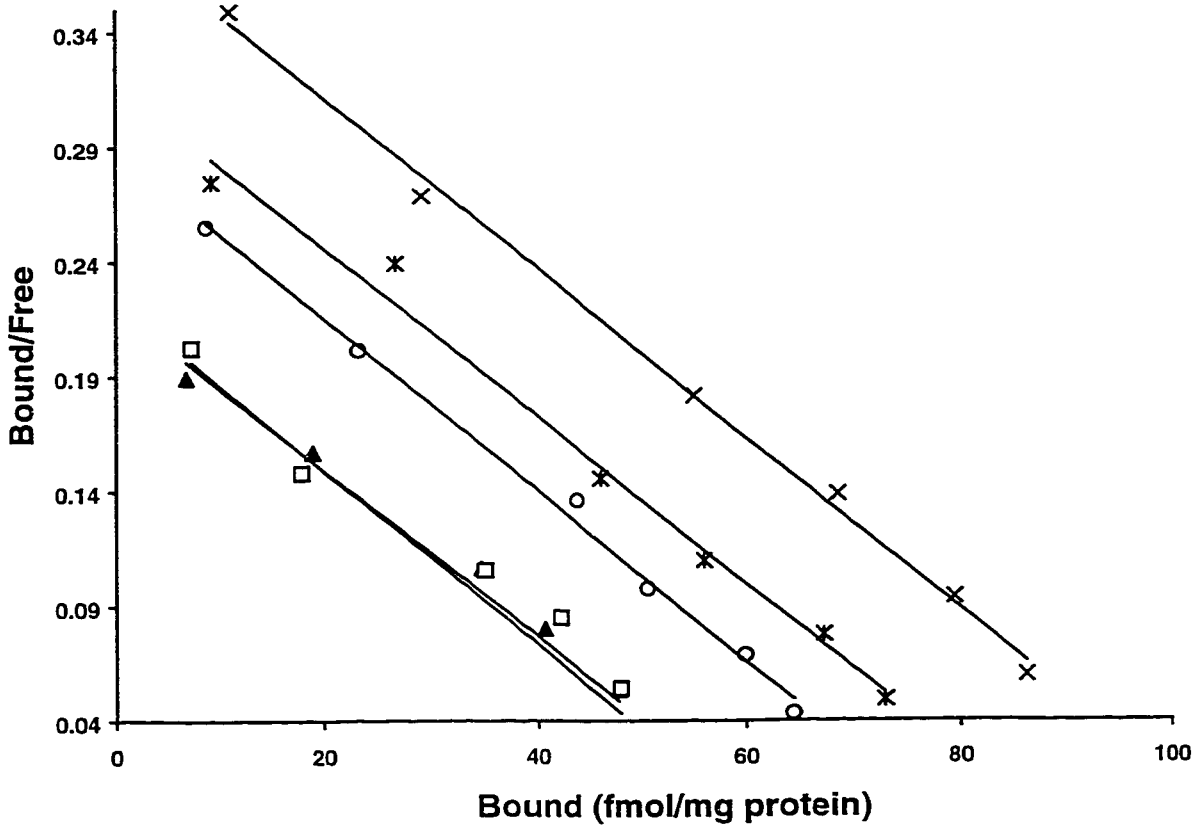

Figure 4.4 Scatchard plots of ^{125}I-labeled hIGF-I to membranes prepared from jejunum of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a off-site nursery, low pathogen segregated from the breeding herd (SEW). 11d pigs (×), 15d-CON pigs (✱), 15d-SEW pigs (O), 34d-CON pigs ( ) and 34d-SEW pigs (▲).

Table 4.1 Affinity (K_a) and capacity (B_{max}) of IGF-I receptors examined in homogenates of jejunal mucosa from 11d pigs, and SEW and CON pigs at 15 and 34 d of age.

	11d	15d-SEW	15d-CON	34d-SEW	34d-CON
K_a (nM)	4.8 ± 0.1	5.0 ± 0.2	4.8± 0.2	4.9 ± 0.5	4.9 ± 0.3
B_{max} (fmol/mg protein)	73.0 ± 0.8[a]	54.2 ± 0.1[b]	61.1± 0.1[a]	43.1 ± 0.2[b]	42.6 ± 0.1[b]

Data represents means ± SEM of three replication determinations.

Means with the different superscripts are significantly different ($P < 0.05$).

IGF-IR : The distribution of IGF-I receptors was not significantly different among the duodenum, jejunum and ileum (Fig. 4.5). Binding of ^{125}I-IGF-I to jejunal membranes was highest in 11 d pigs, and decreased 18% in CON pigs and 34% ($P < 0.01$) in SEW pigs at 3 d post weaning (Fig. 4.6). SEW pigs showed 20% ($P < 0.05$) lower binding of ^{125}I-IGF-I to the jejunal membranes compared with CON pigs at 15 d of age, but this difference disappeared by 34 d of age.

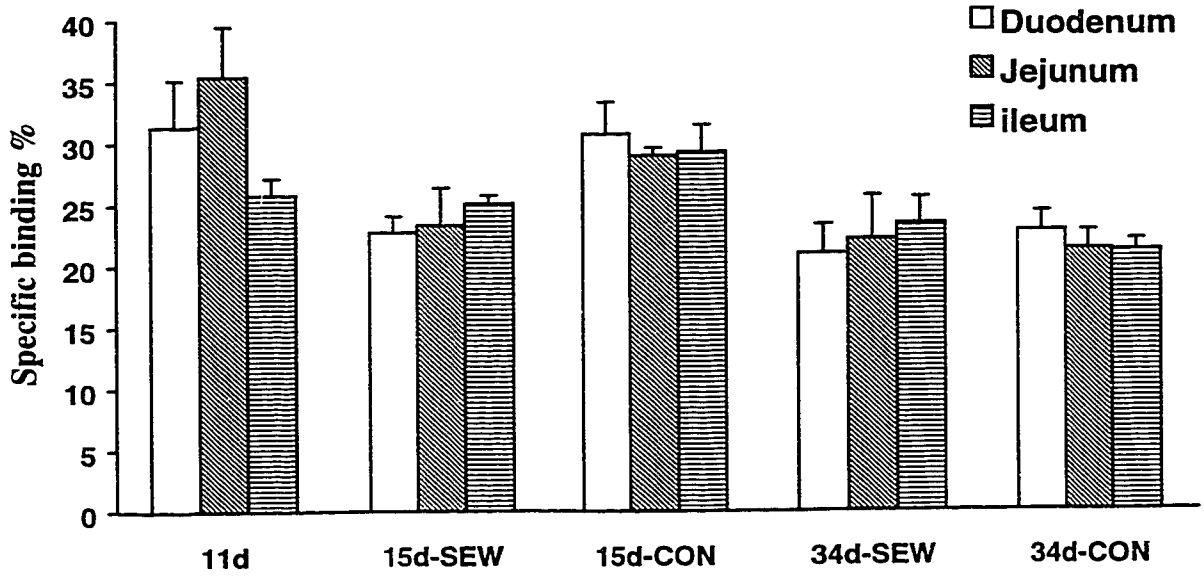

Figure 4.5 Distribution of IGF-IR numbers in the duodenum, jejunum and ileum. Mean ± SEM (n=3) percent specific binding of ^{125}I-IGF-I to membrane preparations obtained from duodenum, jejunum and ileum of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW).

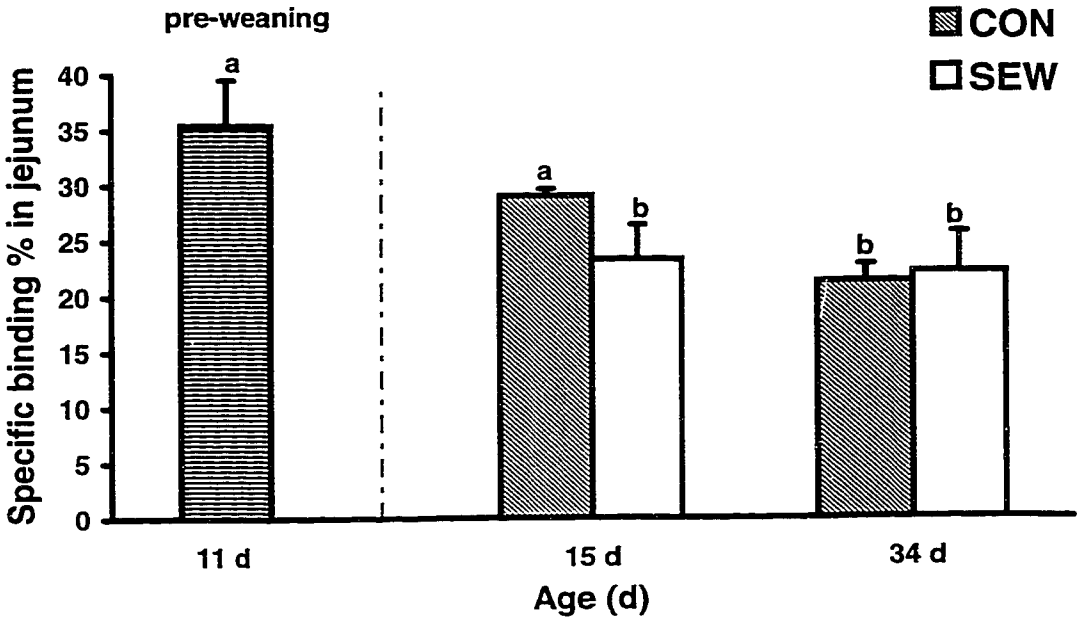

Figure 4.6 The number of IGF-I receptors in the jejunum of pigs with different age and treatment. Mean ± SEM (n=3) percent specific binding of ^{125}I-labeled hIGF-I to membranes prepared from jejunum of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). Bars labeled with different letters are significantly ($P < 0.05$) different.

IGFBPs (1 to 6) mRNA expressed in SI

Prior to weaning (11d) intestinal IGFBP-2 mRNA level showed high variability, which was followed by a large decline ($P < 0.05$) in both groups at 3 d post-weaning. The levels of IGFBP-2 mRNA in SEW and CON pigs at 15 and 34 d of age were low and unaffected by weaning environment (Fig. 4.7).

IGFBP-3 mRNA was not affected by weaning or rearing environment, but tended to be higher in CON pigs relative to SEW pigs at 34 d of age (Fig. 4.8).

IGFBP-4 mRNA transcript decreased 12% in SEW pigs and increased 19% ($P < 0.05$) in CON pigs at 3 d post weaning compared with pre-weaning, such that the mRNA level was 28% higher ($P < 0.01$) in CON pigs than in SEW pigs at 3 d post-weaning (Fig.4.9). By 34 d of age IGFBP-4 mRNA abundance was not different between the two groups.

The level of IGFBP-5 mRNA transcript showed an small increase (24%) in SEW pigs and was unchanged in CON pigs at 3 d post-weaning compared with pre-weaning. At 34 d IGFBP-5 mRNA abundance CON pigs was 40% higher ($P < 0.05$) than in SEW pigs (Fig. 4.10).

Weaning resulted in a decrease ($P < 0.05$) in intestinal IGFBP-6 mRNA levels in both groups of pigs, which became more pronounced (20%, $P < 0.05$) in SEW pigs at 34 d of age (Fig. 4.11)

IGFBP-1 mRNA transcript was not detected in the small intestinal tissue (data not shown).

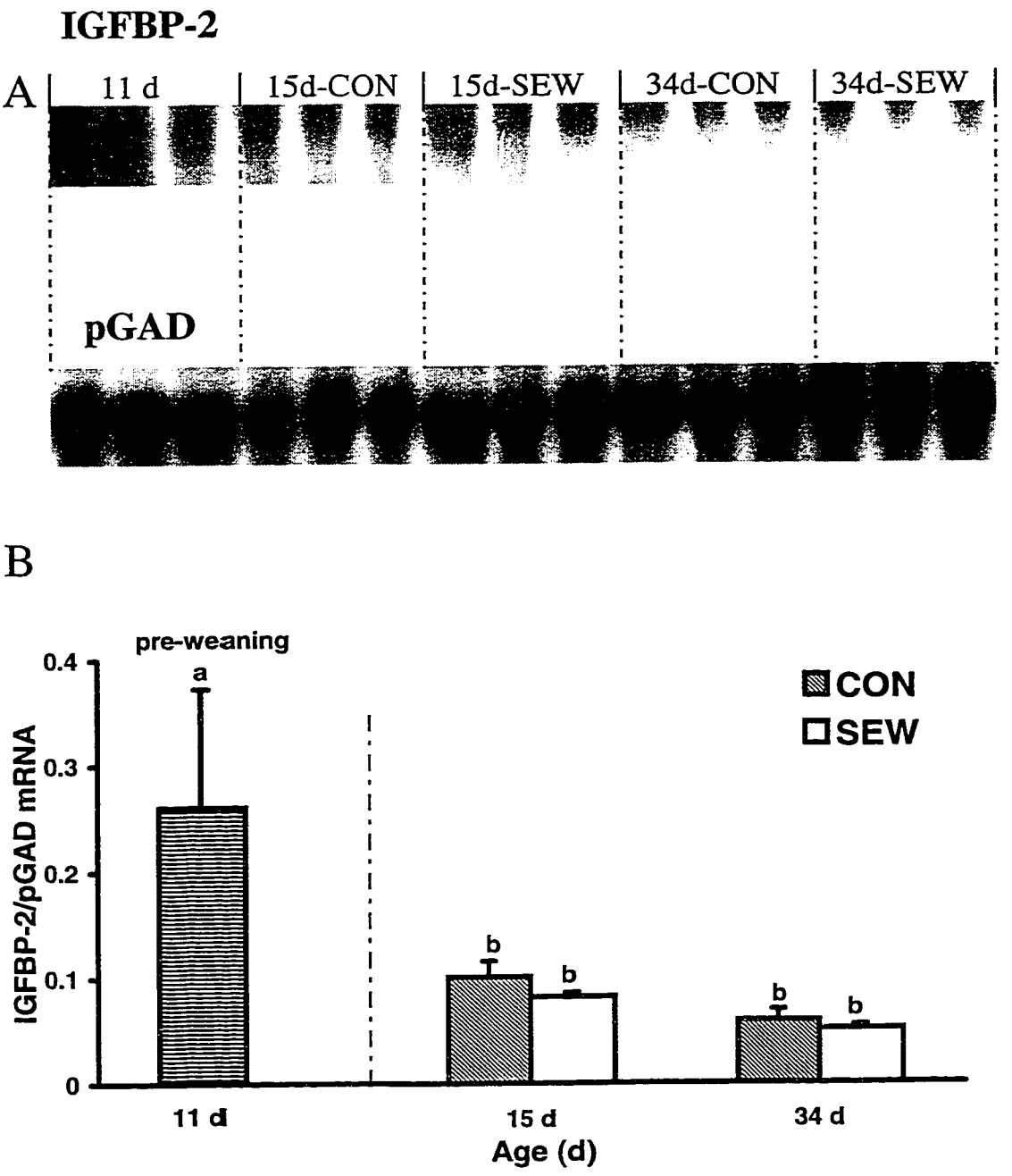

Figure 4.7 Northern blot analysis of IGFBP-2 mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 1.4 kb IGFBP-2 transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel B) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P < 0.05$) different.

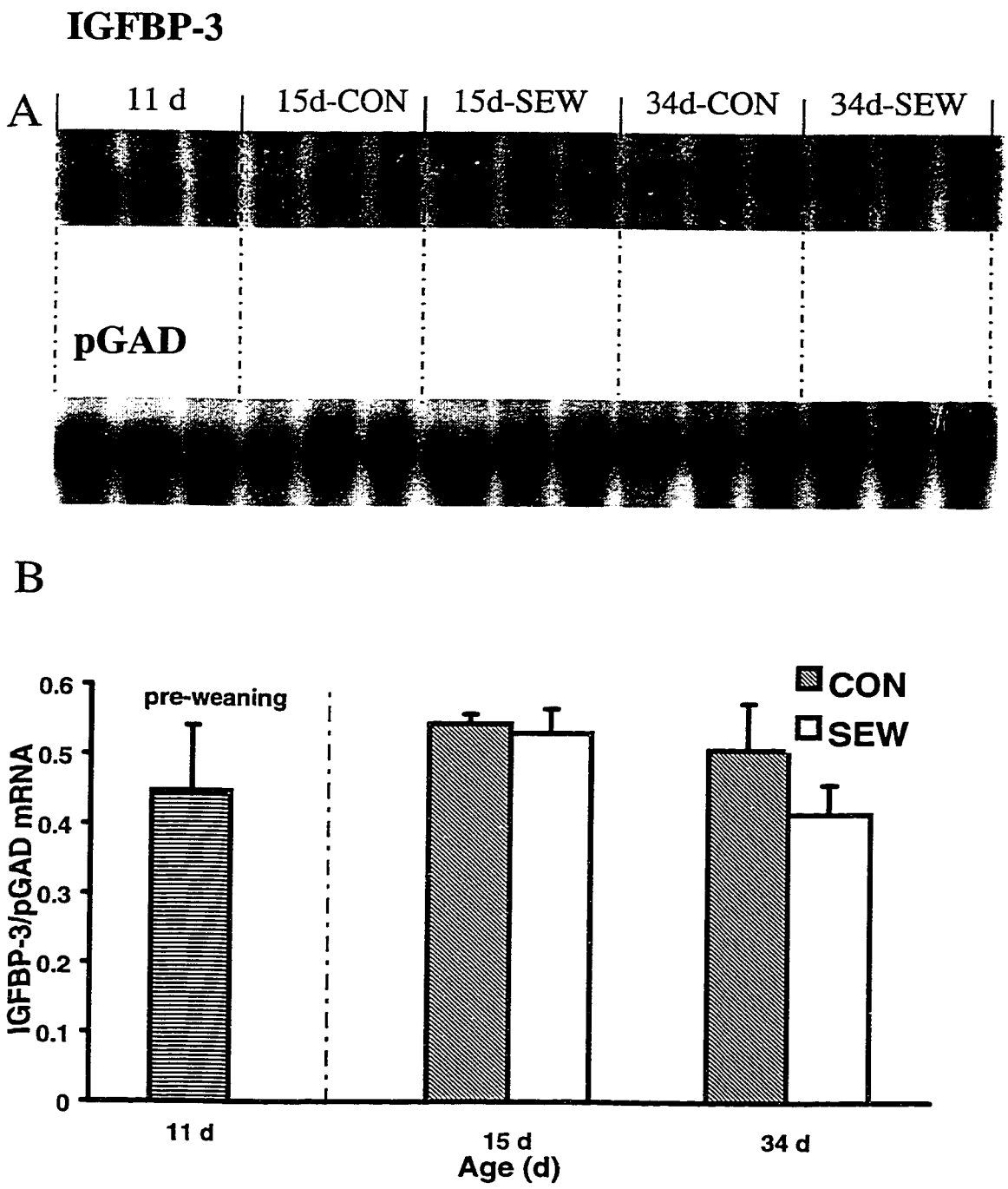

Figure 4.8 Northern blot analysis of IGFBP-3 mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 2.9 kb IGFBP-3 transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P<0.05$) different.

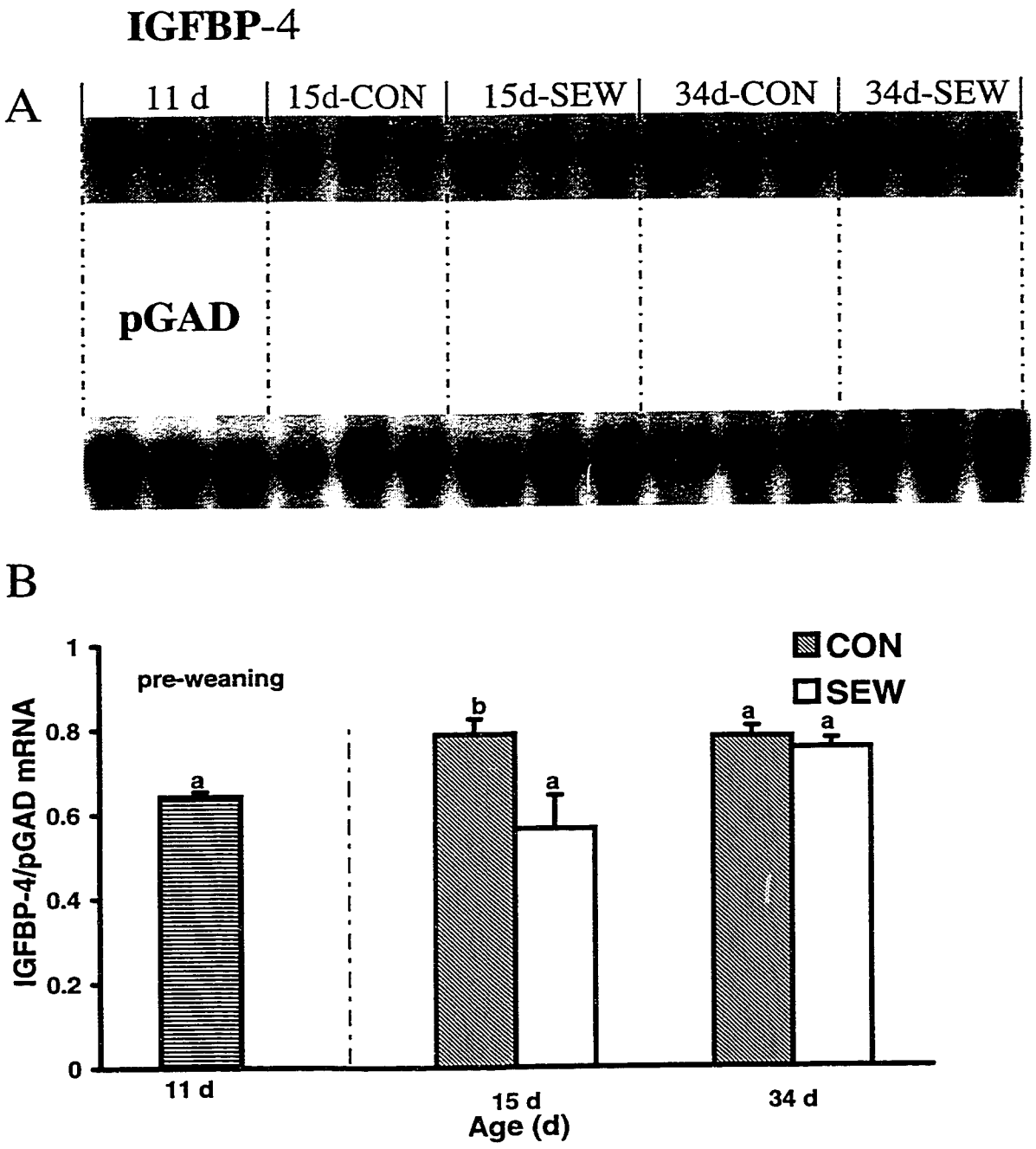

Figure 4.9 Northern blot analysis of IGFBP-4 mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 2.6 kb IGFBP-4 transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P < 0.05$) different.

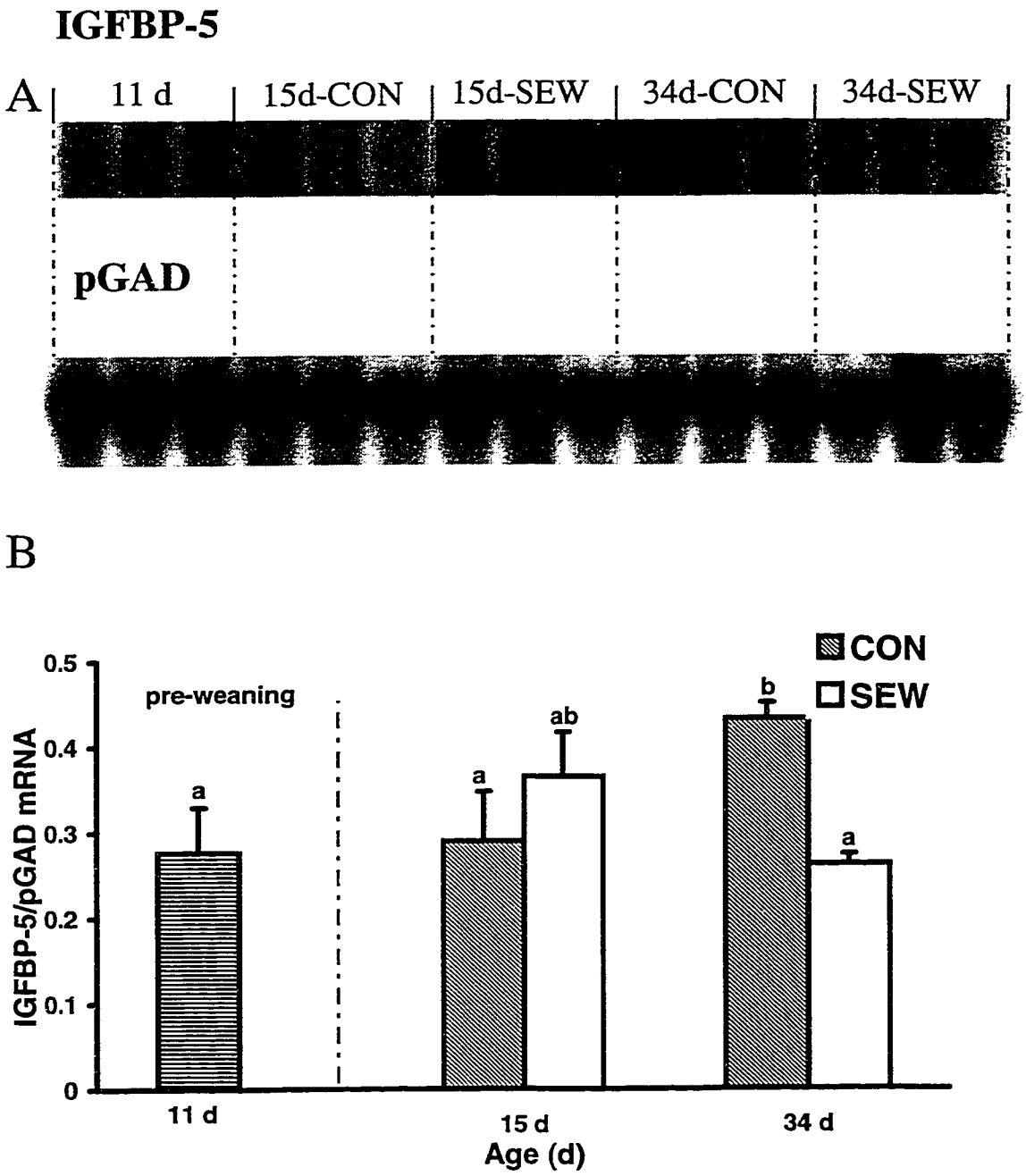

Figure 4.10 Northern blot analysis of IGFBP-5 mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 6.0 kb IGFBP-5 transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel B) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P < 0.05$) different.

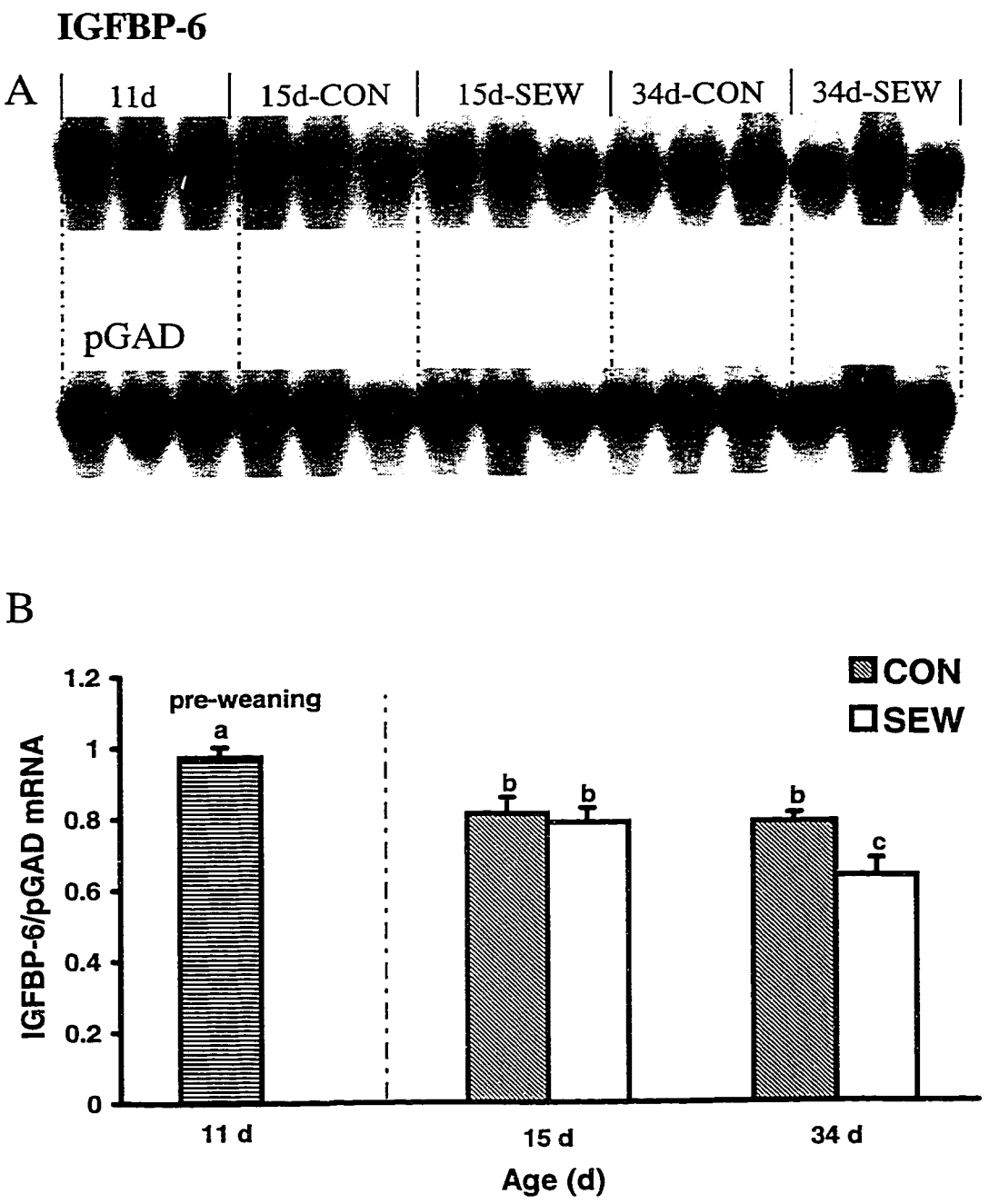

Figure 4.11 Northern blot analysis of IGFBP-6 mRNA abundance in small intestine of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery distant from the sow herd (SEW). A 1.3 kb IGFBP-6 transcript (Upper panel A) was detected and subjected to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel B) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P < 0.05$) different.

4.5 Discussion

Early and abrupt weaning results in marked adaptive changes in the SI associated with reduced feed intake, altered dietary composition, social stress and immunological challenge of dietary and/or microbial origin. Characteristic morphological changes in the intestine include increased epithelial cell mitotic activity and migration out of crypts, villus shortening and deepening of crypts as well as adaptive changes in expression of brush border enzymes (Tang et al. 1999; Nabuurs, 1995). Relative to body weight the small intestinal mass decreases transiently and then increases up to 98% in the 3 weeks following weaning (Makkink et al. 1994; Cranwell, 1995). IGF-I possesses strong mitogenic and differentiating properties and therefore this factor may play a significant role in these adaptive changes in the gut.

The SEW management protocol is designed to limit vertical pathogen transmission in the herd and to lower pathogen pressure in the gut (Van Kessel, 1997). Pig performance responses include increased feed intake, improved feed efficiency and a 13% improvement in average daily gain (Patience et al. 1997). Therefore, differences between treatments in intestinal IGF-I, IGF-IR and IGFBPs mRNA levels observed during post weaning adaptation may not only reflect differences in nutrient supply, but also the degree of enteric infection and immunological challenge.

Small intestinal IGF-I mRNA abundance remained stable and even increased in the period immediately following weaning as well as at 3 weeks post weaning. This result is surprising in that hepatic expression of IGF-I and circulating IGF-I concentrations are markedly reduced immediately post weaning, related largely to reduced nutrient

intake(Carrol et al. 1998; Tang et al. 1998a). This differential response between liver and SI in local IGF-I expression has been reported previously (Lowe et al. 1989). Variation in local expression of IGF-I may be important to allow for tissue-specific responses to metabolic stimuli.

The increase in small intestinal IGF-I mRNA levels at 3 d post weaning in this study contrast the significant decline in IGF-I expression in SI following fasting in rats (Winesett et al. 1995). However, fasting probably is not a suitable model for the complex adaptive post weaning response. Weaning differs from fasting as it results in adaptive hyperplasia, including increased relative mass and epithelial cell renewal, as well possible immunological challenge, all of which could impact local expression of IGF-I. In support, increased small intestinal expression of IGF-I has been reported during intestinal hyperplasia following small bowel resection (Ziegler et al. 1998) and in inflammatory bowel disease (Lund and Zimmermann, 1996). Up-regulated IGF-I mRNA expression in SI post-weaning therefore may reflect a trophic effect of gut IGF-I in repairing or maintaining gut structure and function (Clark 1997).

Comparison of CON and SEW treatments showed that levels of SI IGF-I mRNA transcripts were higher in SEW pigs at 3 d post-weaning. This appears consistent with the observed increased feed intake and improved gut morphology in SEW animals (Tang et al. 1999), but perhaps not with reduced infectious or inflammatory challenge (Lund and Zimmerman, 1996). On the other hand, reduced inflammatory challenge may have contributed to improved gut adaptation and feed consumption after weaning. The

tendency towards higher IGF-I expression in SI of CON pigs at 34 days of age could reflect increased inflammatory response in the intestine of these pigs.

Changes in specific binding of ^{125}I-IGF-I to intestinal membranes were in good agreement with changes in IGF-IR mRNA expression in SI. An inverse relationship was observed between IGF-I mRNA and IGF-IR numbers and mRNA abundance. This probably reflects receptor desensitization in response to increased local expression of IGF-I. Fasting increases IGF-IR number in many tissues (Lowe et al. 1989; Bornfeldt et al. 1989), although Winesett et al. (1995) failed to observe such an effect on IGF-IR mRNA abundance in small intestine. Our data are in general agreement with Morgan et al. (1996) who observed a decline in IGF-IR number from birth through weaning in pigs.

Gene expression of IGFBP-3 in SI was relatively unaffected by weaning, indicating that IGFBP-3 probably does not play a major role in the adaptive response of the gut to weaning. This finding agrees with Ziegler et al. (1998) who observed that IGFBP-3 mRNA abundance was not changed in the jejunum following bowel resection. However, Winesett et al. (1995) reported a reduction in intestinal IGFBP-3 mRNA transcript in response to fasting.

IGFBP-2 mRNA was highly variable in pre-weaning animals and declined rapidly. These changes agree with Orlowski et al. (1990) who observed a decline in gut IGFBP-2 during post-natal life. Northern analysis failed to show the presence of IGFBP-1mRNA in SI, confirming findings by Takenaka et al. (1991).

Intestinal IGFBP-6 gene expression significantly decreased after weaning in both groups, particularly in SEW pigs at 34 d. Little information is available on the

physiological role of IGFBP-6 in the intestine. Dexamethasone reduces IGFBP-6 and its mRNA abundance in PC 12 cells (Batch et al. 1997) and IGFBP-6 was shown to have autocrine inhibitory activities in the neuroblastoma cells (Babajko et al. 1997). It is possible that decreased expression of IGFBP-6 transcript in the gut may be a response to elevated glucocorticoids associated with weaning, and the reduction in IGFBP-6 may facilitate mitogenic effects of IGF-I on the epithelium in weaned pigs.

The elevated IGFBP-4 mRNA levels in SI of CON pigs at 3 d post weaning are also consistent with the suggestion that adaptive responses occurred more rapidly in SEW pigs and/or SI damage in CON pigs was more severe. Ziegler et al. (1998) has shown that IGFBP-4 mRNA in both jejunum and ileum is markedly elevated during adaptive growth following bowel resection in rats. *In vitro* studies showed that IGFBP-4 strongly inhibits the biological actions of IGF-I, including inhibition of IGF-I-stimulated growth of a human colonic cell line (Singh et al. 1994) and suppression of the response to IGF-I in human hepatic stellate cells (Gentilini et al. 1998).

At 34 d the level of IGFBP-5 mRNA transcript was higher in CON pigs. These differences are generally consistent with effects of increased pathogen pressure. Increases in IGF-I mRNA have been observed in rats with experimental enterocolitis (Zimmermann et al. 1993) and in inflamed rat colon where IGFBP-5 mRNA expression was induced by up-regulated IGF-I mRNA (Zimmermann et al. 1997). In situ hybridization showed that gene expression of IGFBP-5 and IGFBP-4 was increased in experimental colitis (Zeeh et al. 1997). However, in comparison to experimental colitis

and inflammation, the effects of SEW on pathogen status should be considered relatively minor and this may explain the lower responses observed in this study.

In conclusion, we have shown that intestinal expression of IGF-I, IGF-IR and IGFBPs is modified by weaning and by different post-weaning environment that has been shown to affect growth of the gut. Changes in expression of IGF-I, IGF-IR and IGFBPs in the bowel may play an important role in adaptive hyperplasia of the small intestinal mucosa in response to weaning and in protection against mucosal damage during active inflammation. Findings in this study show that trophic effects of IGF-I in regulation of the intestinal growth are inextricably linked with interactions of intestinal endocrinology, physiology and immunology.

5 RELATIVE DIFFERENCE IN HEPATIC EXPRESSION AND PLASMA LEVELS OF IGF-I AND IGFBPS (1-6) IN PIGS WITH DIFFERING POST-WEANING BODY GROWTH

5.1 Abstract

Changes in hepatic mRNA abundance and in plasma levels for IGFBPs and IGF-I were measured in pigs weaned into a reduced infection pressure (SEW) vs. conventional (CON) rearing environment. SEW pigs grew faster ($P<0.01$) than CON pigs. Liver was collected from a total of 15 pigs killed at 11 (pre-weaning), 15 (3d post weaning) and 34 d of age. Hepatic transcripts were readily detectable for IGF-I, IGFBP-1, -2, -3, -4 and -6, but not for IGFBP-5. Western blotting identified four IGFBPs, IGFBP-3, -2, -1 and -4, in the circulation. The levels of hepatic mRNA encoding for IGF-I and IGFBP-3 were not different during post-weaning development from 15 to 34 d of age and between SEW and CON pigs. In contrast, during the same period plasma IGF-I and IGFBP-3 increased in both groups and higher ($P < 0.05$) levels were observed in SEW pigs compared to CON pigs. Parallel changes in levels of hepatic IGFBP-2 mRNA and plasma IGFBP-2 were observed, with markedly lower levels of both in SEW pigs relative to CON pigs. Hepatic IGFBP-1 transcript and plasma IGFBP-1 level surged immediately post-weaning but were otherwise unaffected by treatment. IGFBP-4 level in plasma was low and unaffected by age or treatment. Hepatic IGFBP-4 and -6 mRNA levels were similar between SEW and CON pigs and tended to decrease with age. These results demonstrate

that post-weaning changes in the levels of IGF-I and IGFBP-3 in plasma are uncoupled from changes in hepatic expression of these two proteins. Increased hepatic IGFBP-2 transcript and plasma IGFBP-2 along with low plasma IGF-I and IGFBP-3 accompanied attenuated growth in CON pigs, suggesting that IGFBP-2 may be an important negative regulator of somatic growth.

5.2 Introduction

Insulin-like growth factor (IGF)-I is an anabolic and mitogenic peptide that regulates postnatal growth and mediates the growth promoting effects of growth hormone (GH) in multiple tissues (Jones and Clemmons 1995). Six high affinity IGF binding proteins (IGFBPs) have been identified, which may stimulate or inhibit the biological actions of IGF-I (Kelly et al. 1996). The dynamic interaction between IGF-I and IGFBPs regulates the biological actions of IGF-I. Recent evidence supports that IGFBPs can also exert biological effects independent from their binding function of IGF-I. IGFBP-3 is able to directly inhibit growth without blocking access of IGF-I to its receptor (Rechler 1997) and IGFBP-1 stimulates cell migration by interaction with the fibronectin receptor (Jones and Clemmons 1995). Therefore, the concentration of IGFBPs in body fluids has a significant impact on the biological activity of circulating IGF-I and on growth itself.

IGFBP-3 is a primary carrier of IGF-I in serum and forms the ternary complex: IGF-I:IGFBP-3:acid labile subunit (ALS) in the blood circulation. Higher levels of the ternary complex in serum are associated with improved somatic growth (Stewart et al. 1993, Fielder et al. 1996). Other IGFBPs, such as IGFBP-1, -2 and -4, may transport IGF-I from the

circulation to peripheral tissues (Hossner et al. 1997). IGFBP-2 is the second most abundant IGFBP in serum in postnatal pigs (Lee et al. 1991).

The anabolic, mitogenic and growth-promoting properties of IGF-I were demonstrated in vitro, through administration of exogenous IGF-I in animals, in transgenic models or in nutritional trials (Jones and Clemmons 1995). Similarly, information on the stimulatory or inhibitory effects of IGFBPs on IGF-I action was obtained from different type cell cultures including transfected cell lines, through mutation of targeted genes, in transgenic models and through nutrient deprivation or restriction (Rechler 1993, Clemmons 1997). The relationship between expression of hepatic IGF-I and IGFBPs and the levels of the respective proteins in the circulation in relation to growth performance in normal animals is unclear.

Segregated early weaning (SEW) of pigs limits potential pathogen exposure and offers significant performance advantages in growth and feed efficiency compared with conventional on-site weaning (Van Kessel et al. 1997). When compared to conventional on site weaning, SEW provides a useful model to differentiate post-weaning growth for the study of the relative changes in expression and systemic levels of growth factors and binding proteins.

5.3 Materials and Methods

Animal Preparation and Tissue collection

Ninety-two piglets (PIC C15X Canabrid) reared at Prairie Swine Centre Inc. (PSCI, Saskatoon, SK) were weaned early at 12 d of age and weighed. Forty-six piglets were moved into a segregated nursery (SEW) with reduced infection pressure about 16 km

distant from the breeding herd. The remaining piglets were moved to a conventional nursery room (CON) located on the same site as the breeding herd. Pig environment, nutrition and management were similar between the two nurseries. Weight and feed intake of piglets in each nursery were recorded at regular intervals. Piglets (n=3) were injected with euthanyl forte (MTC Pharmaceutical, Cambridge, ON) and killed by terminal bleeding at 11 (pre-weaning), 15 (3 d post- weaning) and 34 d of age. The liver was rapidly removed, immediately frozen in liquid nitrogen and stored at -80°C until assayed. The Animal Protocol Review Committee of the University of Saskatchewan approved the protocol of this study in accordance with the guidelines and regulations of the Canadian Council on Animal Care.

Blood SampleCollection

Blood samples (5 ml) were obtained by jugular vein puncture around 9:00 AM at 12 (pre-weaning), 14, 17, 19, 21, 23, 26, 29, 33, 36, 40, 42, 46, 50 and 56 d of age. Blood samples were centrifuged (1800 x g, 20 minutes) and the plasma was aliquotted and stored at -20°C until assayed.

Measurement of Plasma IGF-I

Plasma IGF-I was measured by radioimmunoassay (RIA) essentially as described by Kerr et al. (1990). Prior to RIA IGFBPs were extracted from plasma using the acid-ethanol method of Daughaday et al (1980).

Western Ligand Blot:

Ligand blotting was carried out as described by Hossenlopp et al. (1986). Plasma samples were separated by 12% SDS-PAGE under non-reducing conditions and

transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad, Richmond, CA). The methods of blocking non-specific reaction, incubation with ^{125}I-IGF-I and washing of the membrane were those described by Liu et al. (1993).

Preparation of cDNA Probes

Porcine (p) IGF-I and IGFBP-2 (gifts from Dr. F. A. Simmen, University of Florida) and pIGFBP-3 (gift from Dr. S. Shimasaki, University of California) complementary DNA contained 580 bp, 1.4 kb, and 595 bp inserts, respectively. Human (h) IGFBP-1, -4, -5 and -6 cDNA with 350, 506, 317 and 267 bp inserts, respectively, were generously provided by Dr. S. Shimasaki. Individual cDNA were labeled with [^{32}P] dCTP (Mandel, Boston, MA) using the Oligolabelling kit (Pharmacia Biotech Inc., Piscataway, NJ).

Northern Blot Hybridization Analysis

Frozen liver was pulverized in liquid N_2 and total RNA was extracted using TRIzol reagent (Gibco BRL, Burlington, ON). Total RNA (20 μg) was electrophoresed on 1.2 % agarose containing 2.2 M formaldehyde. Ethidium bromide-stained gels were photographed to illustrate the 18S and 28S rRNA bands before transfer to GeneScreen Plus membrane (NEN Research Products, Boston, MA). The procedure for the Northern hybridization was based on the GeneScreen Plus protocol (NEN Research Products) with some modifications. Briefly, pre-hybridization (42°C for at least 4 h or overnight) was performed in 5X SSPE (pH 7.4), 50% deionized formamide, 5X Denhardt's solution (Amersham Life Science Inc., Oakville, ON), 10% dextran sulphate, Na salt (Sigma

Chemical Co., St. Louis, MO), 1% SDS, and 300 μg per ml herring sperm DNA (Boehringer-Mannheim, Germany). The membranes were then hybridized with the respective [^{32}P]-labeled dCTP cDNA in the same solution at 42°C overnight. The membrane was then washed twice with 0.2X SSPE, 1% SDS and 0.2X SSPE, 0.1% SDS at 42°C for 15 to 20 min, respectively, followed by autoradiography at - 80°C using an intensifying screen. The membrane was stripped using 0.1X SSPE and 1% SDS and then re-probed with porcine 3- phosphoglyceraldehyde dehydrogenase (pGAD) used as a normalizing or "housekeeping" probe (Gadsby et al. 1996) to quantitate total RNA loading and transfer. The procedure for Northern blotting for pGAD mRNA was the same except that 100 μg per ml herring sperm DNA was incubated in the pre-hybridization solution. The size of RNA transcripts was evaluated according to the positions of 18S and 28S rRNA.

Autoradiographs (Western and Northern blotting) were quantitated by computer-assisted densitometry. The density of the transcript was expressed in arbitrary units relative to pGAD mRNA abundance.

Statistical Analysis

All the results are presented as mean ± SEM and subjected to one and two-way analysis of variance using the GLM procedures of SAS (1985).

5.4 Results

Body Weight Gain

All piglets demonstrated reduced growth during the period immediately post-weaning (Fig. 5.1). SEW pigs were heavier ($P<0.05$) than CON pigs from 21 through 56 d of age.

IGF-I Levels in Plasma

Plasma IGF-I levels declined ($P < 0.01$) in both SEW and CON pigs at 14 d of age (2 d post-weaning) and then gradually recovered to pre-weaning levels near 29 d in SEW pigs and 40 d of age in CON pigs (Fig. 5.2). SEW pigs showed higher ($P < 0.01$) levels of IGF-I than CON pigs from 33 d of age to the end of the experiment.

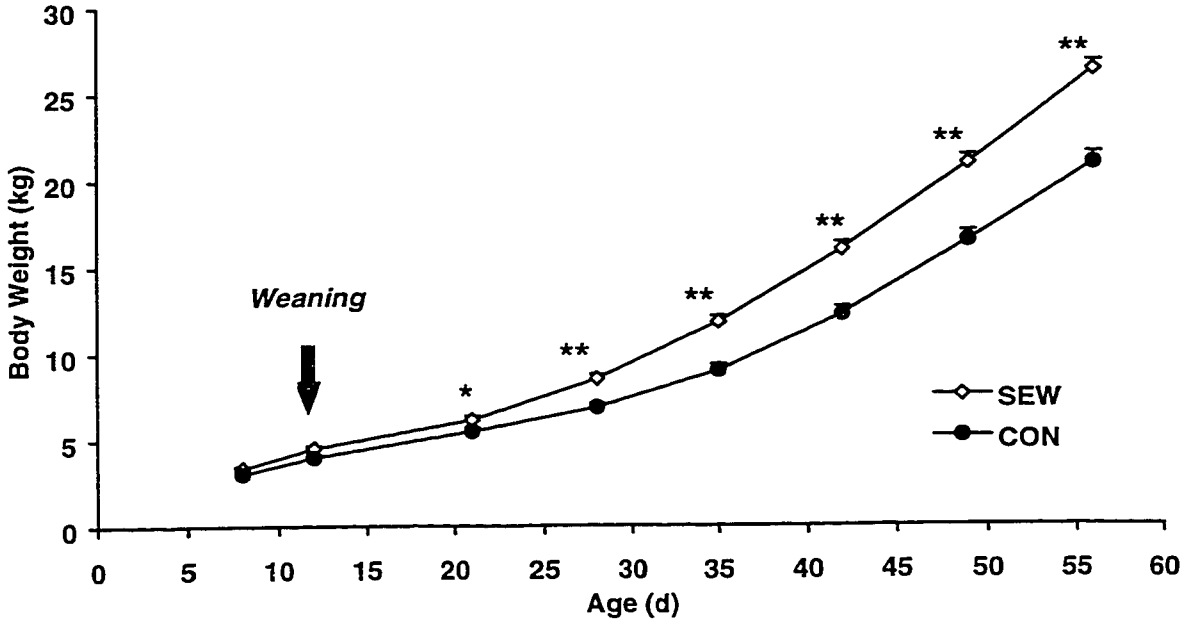

Figure 5.1 Body weight gain in SEW and CON pigs from 8 to 56 d of age. Values are expressed as mean ± SEM (n=10). Values with * or * * are different at $P < 0.05$ and $P < 0.01$, respectively, between SEW and CON pigs of the same age.

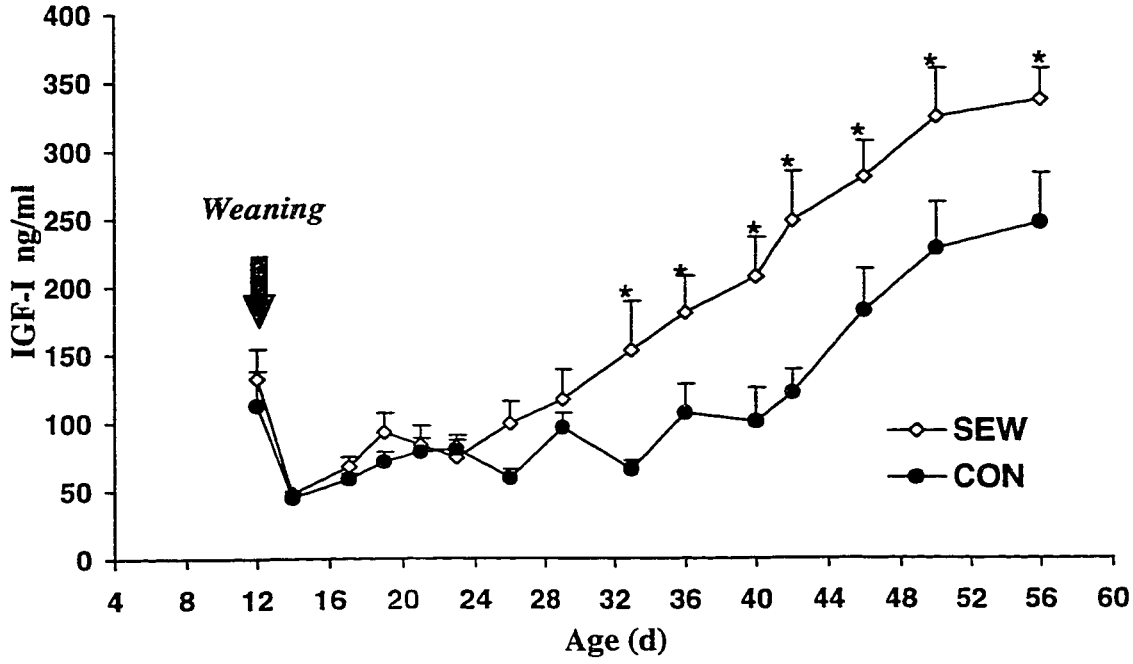

Figure 5.2 Plasma IGF-I concentrations (ng/ml) in SEW and CON pigs from 12 to 56 d of age. Values are expressed as mean ± SEM (n=10). Values with * are different at $P < 0.05$ between SEW and CON pigs of the same age.

Hepatic IGF-I mRNA Transcript Abundance

Abundance of hepatic IGF-I mRNA transcript at 3 d post-weaning declined in SEW (25%) and in CON pigs (41%. $P < 0.05$) when compared to pre-weaning levels and again was slightly decreased in both groups between 15 to 34 d of age (Fig. 5.3). SEW pigs showed more abundance of hepatic IGF-I mRNA than CON pigs at both 15 and 34 d of age, however, the differences were not significant.

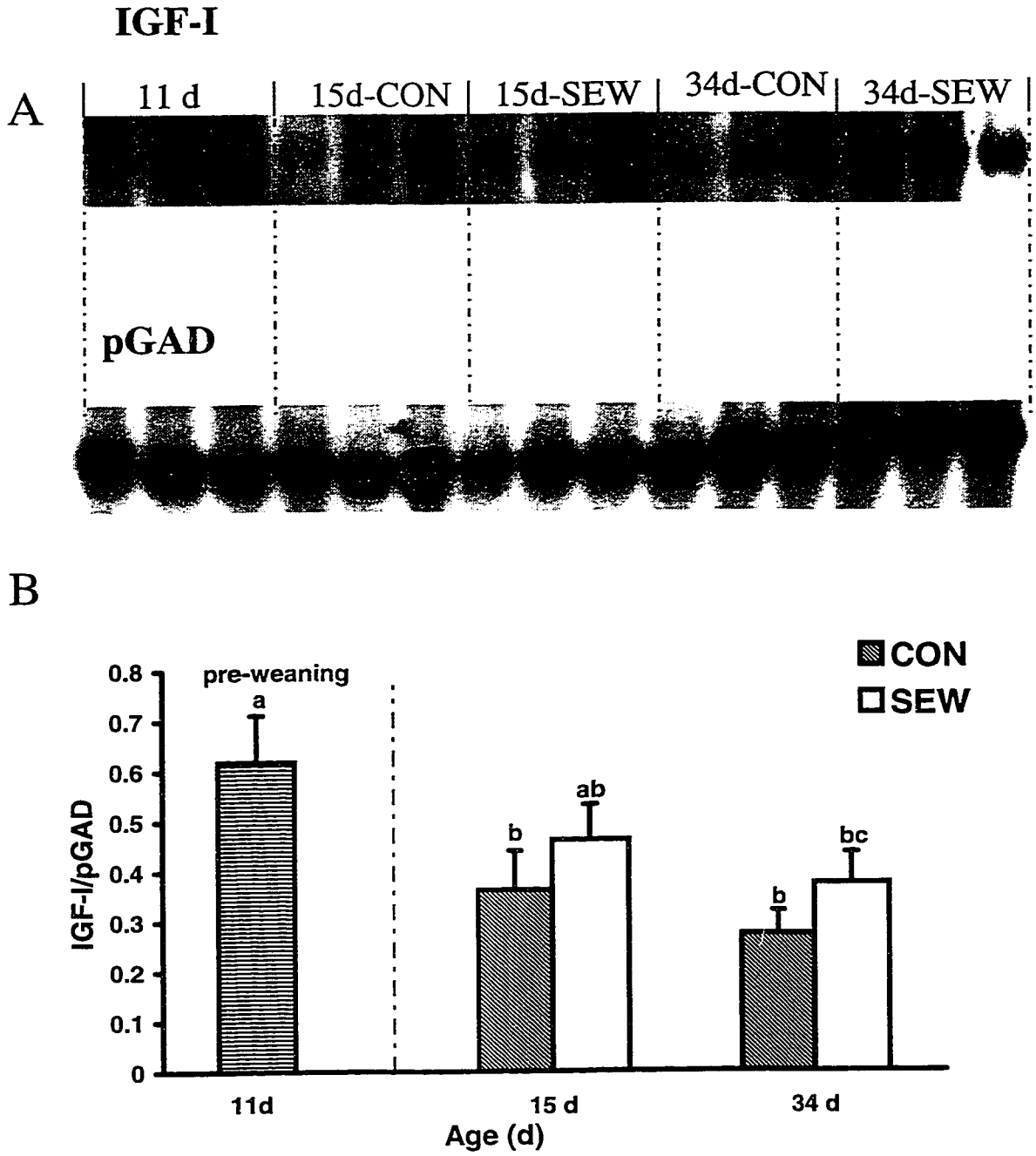

Figure 5.3 Northern blot analysis of IGF-I mRNA abundance in the liver of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery (SEW). A 7.8 kb IGF-1 transcript (Upper panel A) was detected and subjected to densitometric measurement. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P<0.05$) different.

IGFBPs in Plasma

Ligand blot analysis of pig plasma revealed five distinct bands that specifically bound ^{125}I-IGF-I (Fig. 5.4). The electrophoretic mobilities of these bands corresponded to proteins with molecular weights of 43, 40, 31 and 26 kDa that were tentatively identified as IGFBP-3 (43 and 40 kDa), -2, -1 and -4 in accordance with Latimer et al. (1993) and Vinter-Jensen et al. (1996). The 43 and 40 kDa variants of IGF binding protein-3 are due to differentiated glycosylation (Liu et al. 1990).

Weaning reduced ($P < 0.05$) plasma IGFBP-3 levels in both groups of pigs at 14 d of age, followed with a gradual recovery of IGFBP-3 during post-weaning development (Fig. 5.5A). IGFBP-3 plasma levels were increased ($P < 0.05$) in both SEW and CON pigs at 33 d of age when compared to 14 d of age, and SEW pigs showed higher ($P < 0.05$) levels of IGFBP-3 than CON pigs at 33 d of age. IGFBP-3 in plasma was 19 and 11 % higher in SEW pigs than in CON pigs at 46 and 56 d of age, respectively.

At 2 d post-weaning plasma IGFBP-2 levels were not different from pre-weaning values nor were differences observed between SEW and CON treatments (Fig. 5.5B). In contrast, plasma IGFBP-2 tended to be 29 ($P = 0.09$), 33 ($P = 0.08$), and 22% lower in SEW pigs relative to CON pigs at 33, 46 and 56 d of age, respectively. Plasma IGFBP-2 in both groups tended to decrease after 33 d of age.

Plasma IGFBP-1 levels rose in SEW pigs (79%, $P < 0.05$) and CON pigs (57%) at 2 d post-weaning compared to pre-weaning (Fig. 5.5C). The level of IGFBP-1 was higher ($P < 0.05$) in SEW pigs than in CON pigs at 14 d of age. After weaning the levels of IGFBP-1 declined with development and no treatment effects were apparent.

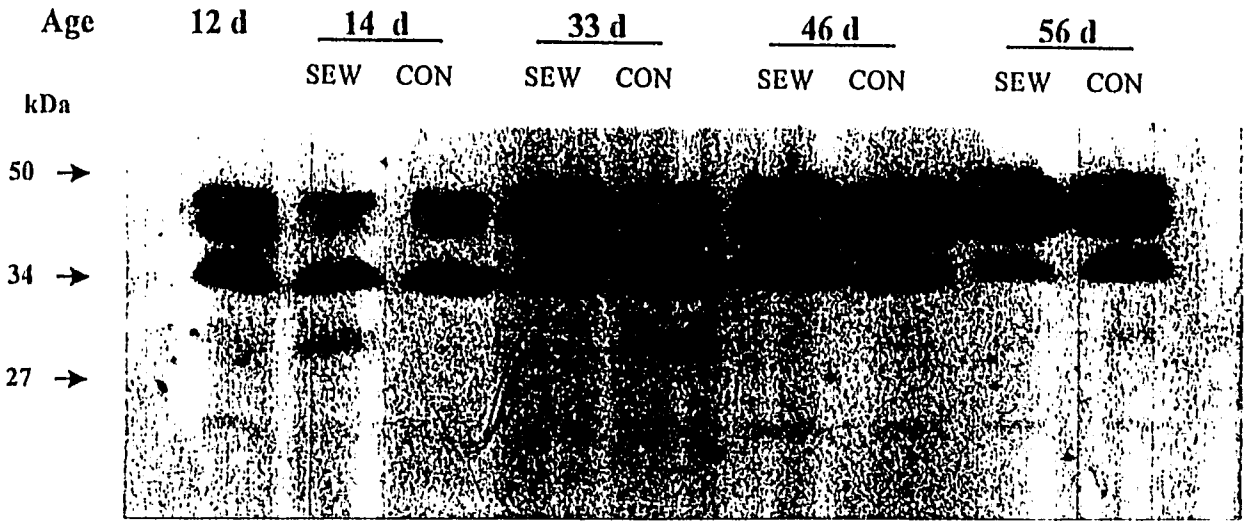

Figure 5.4 Autoradiograph of a ligand blot demonstrating the relative concentrations of plasma IGFBPs from SEW and CON pigs at 12, 14, 33, 46 and 56 d of age

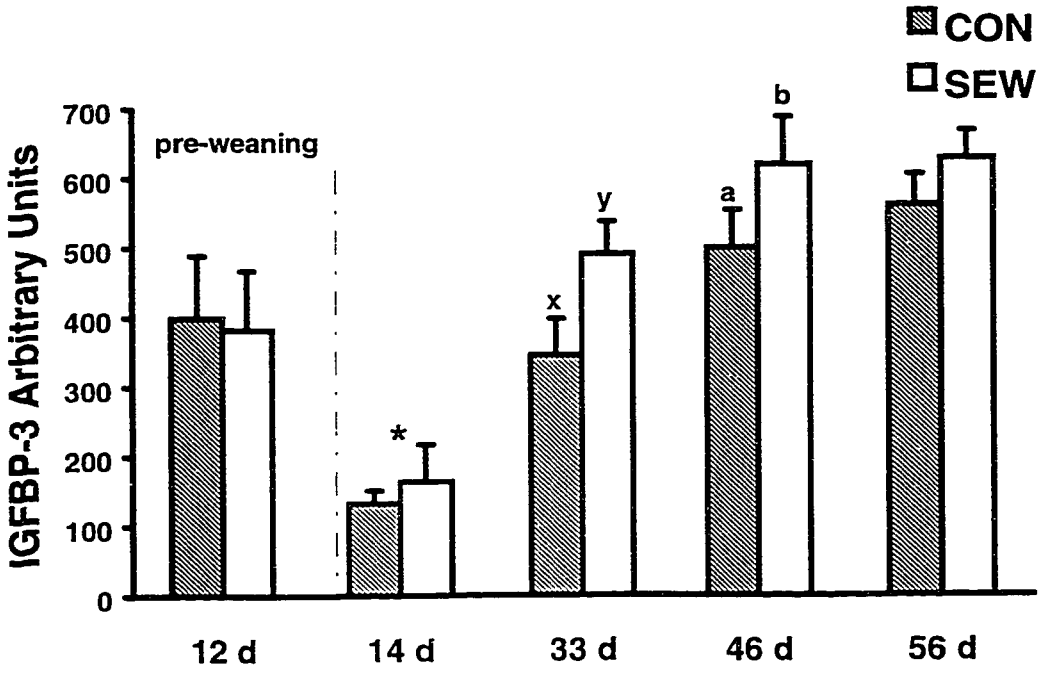

Figure 5.5A The concentrations of plasma IGFBP-3 in SEW and CON pigs. The densitometric measurements of the autoradiograms are expressed in arbitrary units. Values are means ± SEM (n=10). Bars within day with different letters, a or b and x or y, are different at $P < 0.1$ and $P < 0.05$. * between bars indicates ($P < 0.05$) effect of age versus day 12, 33, 46 and 56 d.

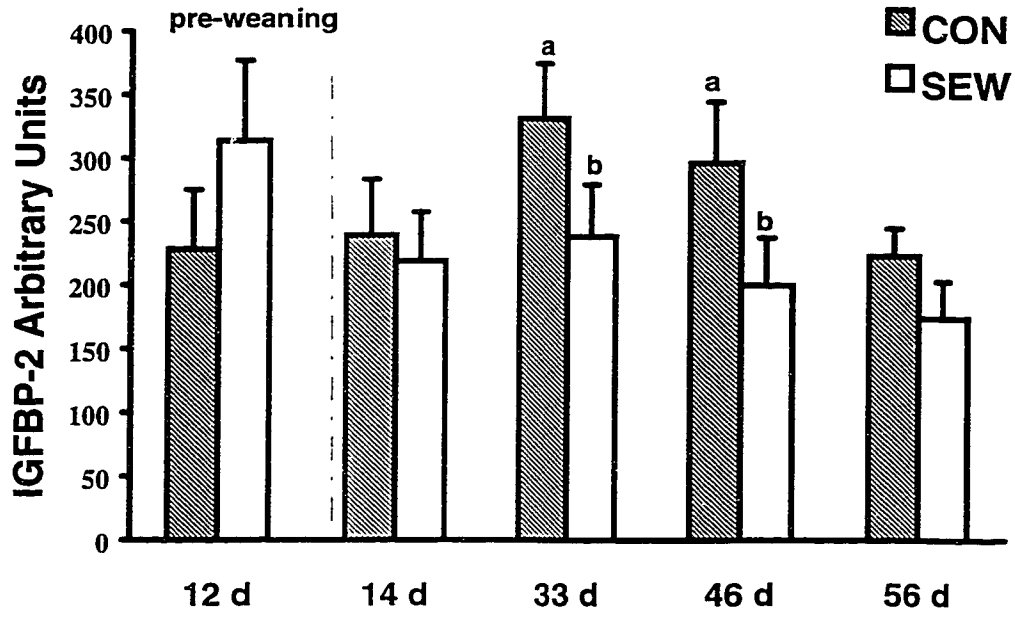

Figure 5.5B The concentrations of plasma IGFBP-2 in SEW and CON pigs. The densitometric measurements of the autoradiograms are expressed in arbitrary units. Values are means ± SEM (n=10). Bars within day with different letters, a or b, are different at $P < 0.1$.

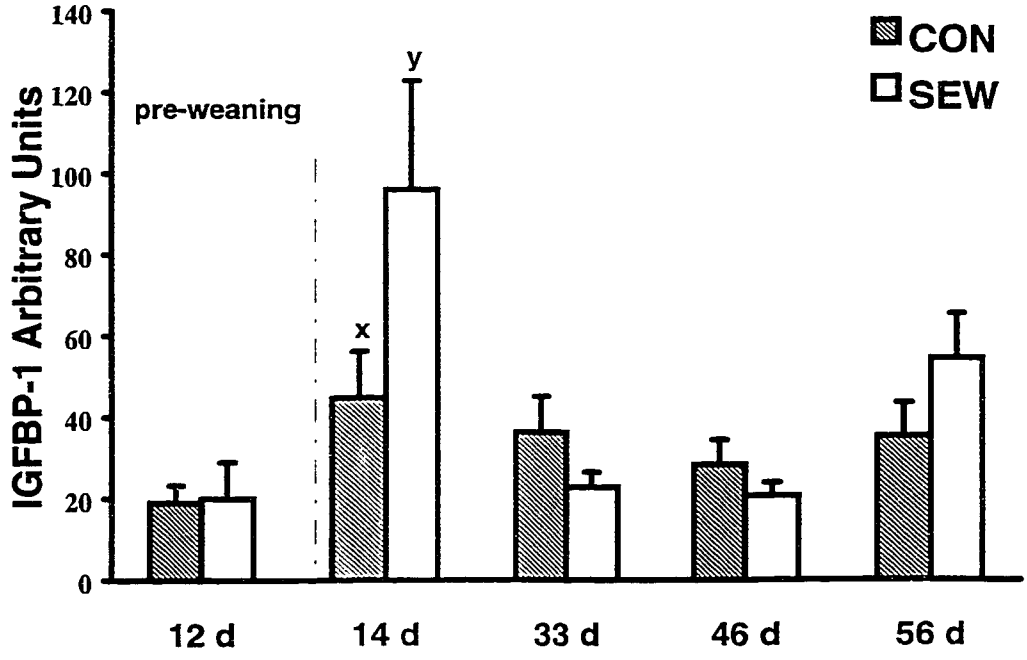

Figure 5.5C The concentrations of plasma IGFBP-1 in SEW and CON pigs. The densitometric measurements of the autoradiograms are expressed in arbitrary units. Values are means ± SEM (n=10). Bars within day with different letters, x or y, are different at $P < 0.05$.

IGFBP-4 represented a minor portion of the total IGF-binding activity in plasma and was not affected by age or treatment (Fig. 5.5.D).

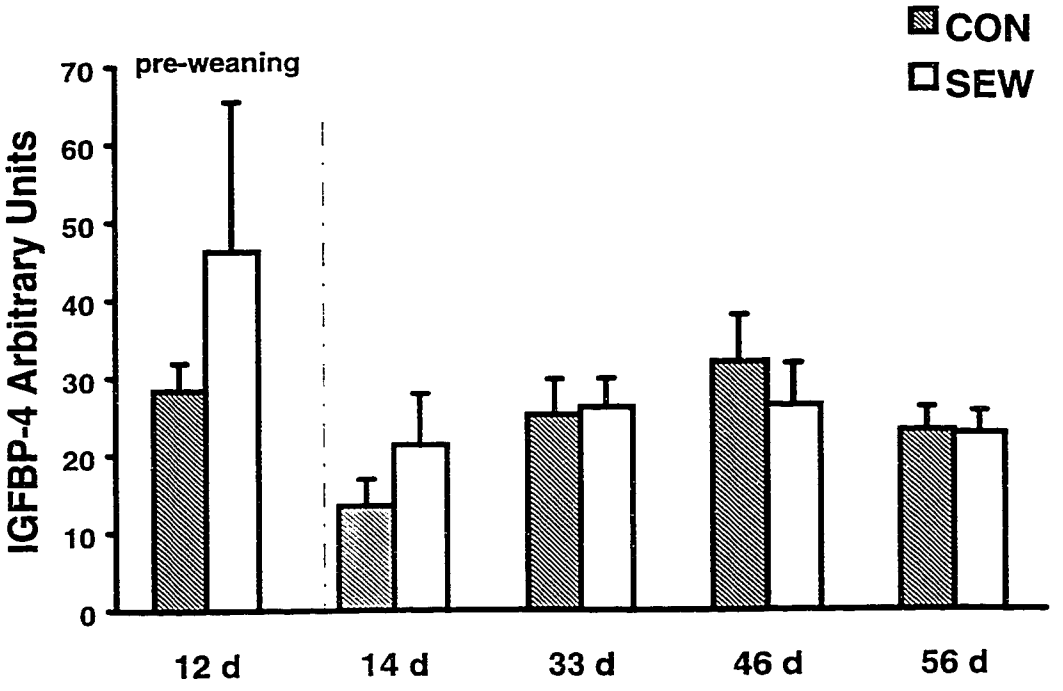

Figure 5.5D The concentrations of plasma IGFBP-4 in SEW and CON pigs. The densitometric measurements of the autoradiograms are expressed in arbitrary units. Values are means ± SEM (n=10).

Expression of IGFBPs mRNA in the Liver

Weaning was associated with dramatic decline ($P < 0.01$) in hepatic IGFBP-3 mRNA levels in both groups of animals (Fig. 5.6A). Abundance of hepatic IGFBP-3 mRNA transcript did not change from 15 to 34 d of age and no significant difference between SEW and CON pigs at both 15 and 34 d of age was apparent.

Hepatic IGFBP-2 mRNA abundance was not affected by weaning and was similar between SEW and CON pigs at 3 d post-weaning (Fig. 5.6B). In contrast, hepatic IGFBP-2 mRNA levels were significantly lower ($P < 0.01$) in SEW pigs than in CON pigs at 34 d of age.

Weaning reduced hepatic IGFBP-4 mRNA expression in SEW pigs (12%) and CON pigs (34%, $P < 0.05$) (Fig. 5.6C). SEW pigs showed a higher ($P < 0.05$) level of hepatic IGFBP-4 mRNA than CON pigs at 3 d post-weaning, but this difference was absent at 34 d of age. Hepatic IGFBP-6 mRNA was greatly reduced ($P < 0.05$) as a result of weaning and no effect of post-weaning environment was observed at 15 and 34 d of age (Fig. 5.6D). Expression of IGFBP-1 mRNA transcript in the liver was highly variable and hepatic IGFBP-5 mRNA was not detectable in all pigs examined (data not shown).

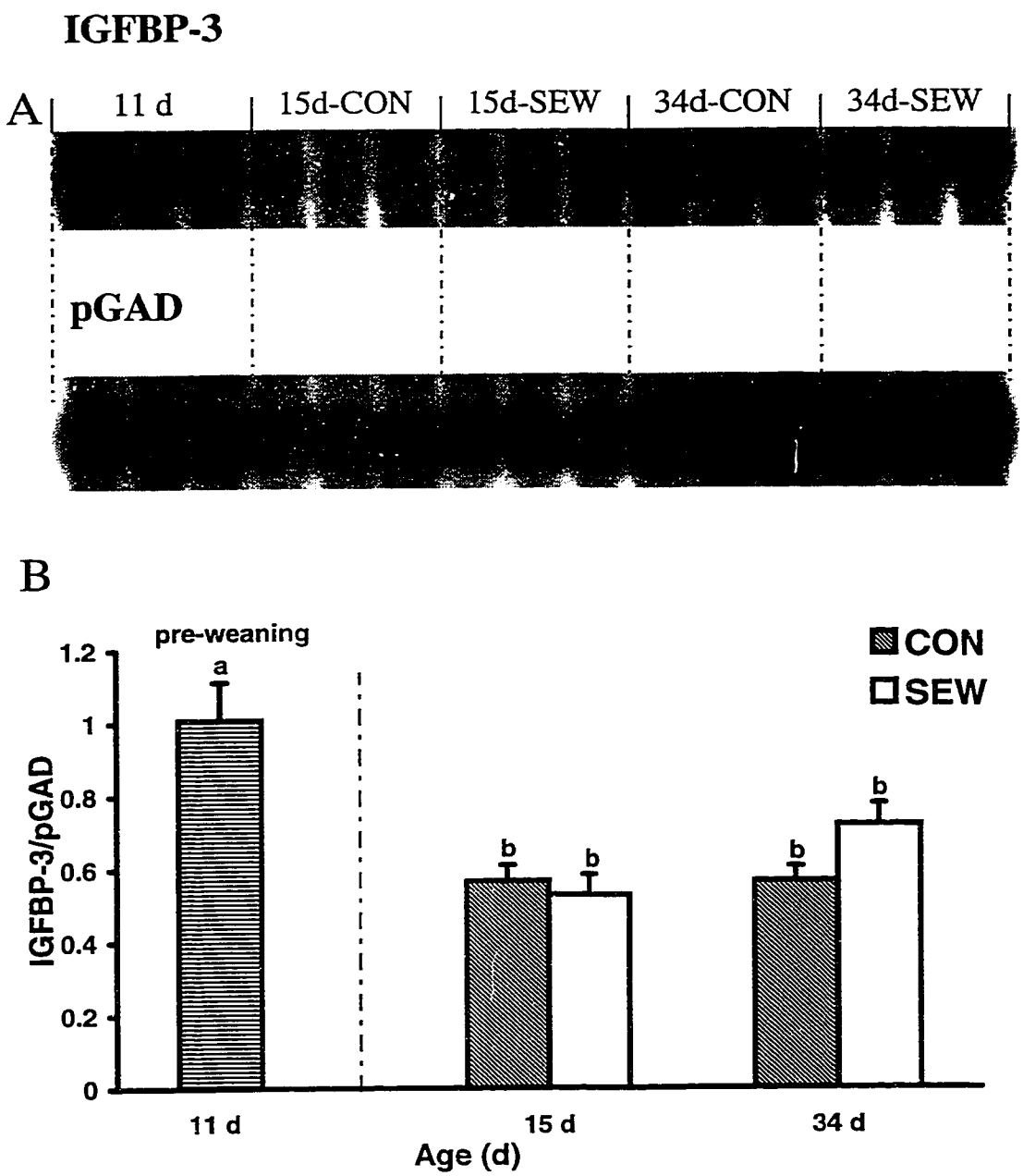

Figure 5.6A Northern blot analysis of IGFBP-3 mRNA abundance in the liver of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery (SEW). A 2.9 kb IGFBP-3 transcript (Upper panel A) was detected and subjected to densitometric measurement. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P<0.05$) different.

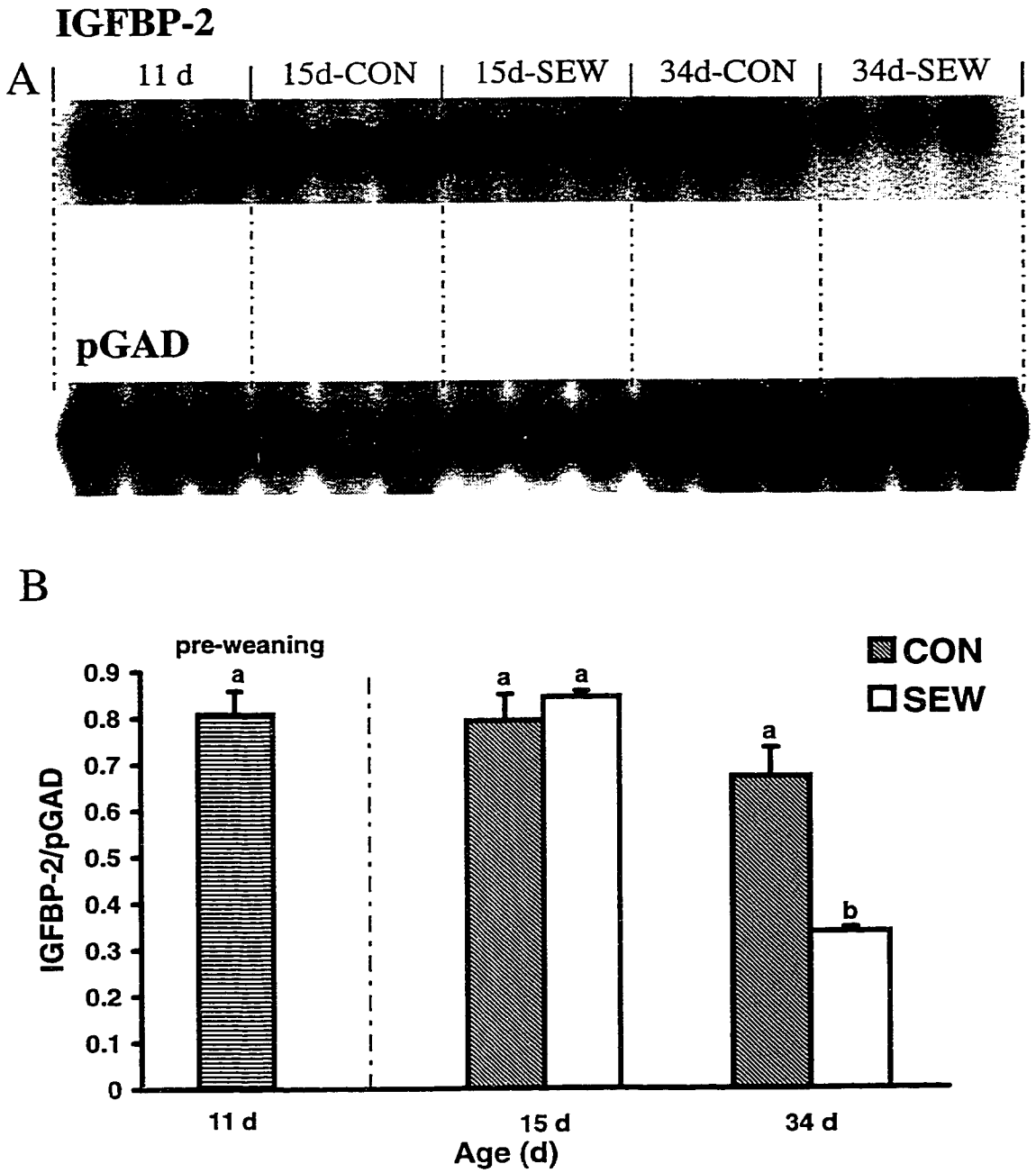

Figure 5.6B Northern blot analysis of IGFBP-2 mRNA abundance in the liver of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery (SEW). A 1.4 kb IGFBP-2 transcript (Up panel A) was detected and subject to densitometric scanning. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Low panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P<0.05$) different.

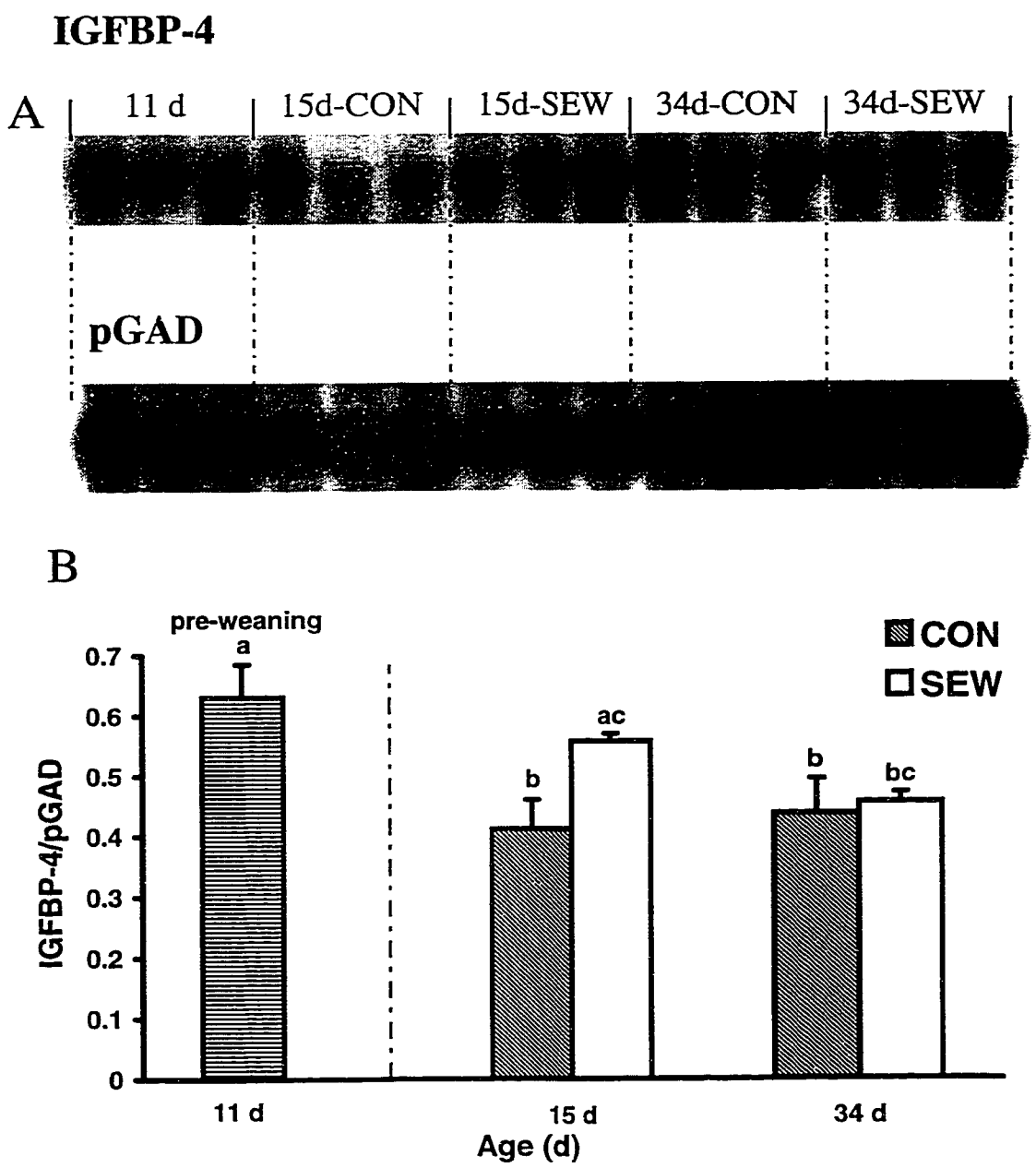

Figure 5.6C Northern blot analysis of IGFBP-4 mRNA abundance in the liver of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery (SEW). A 2.6 kb IGFBP-4 transcript (Upper panel A) was detected and subject to densitometric measurement. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P<0.05$) different.

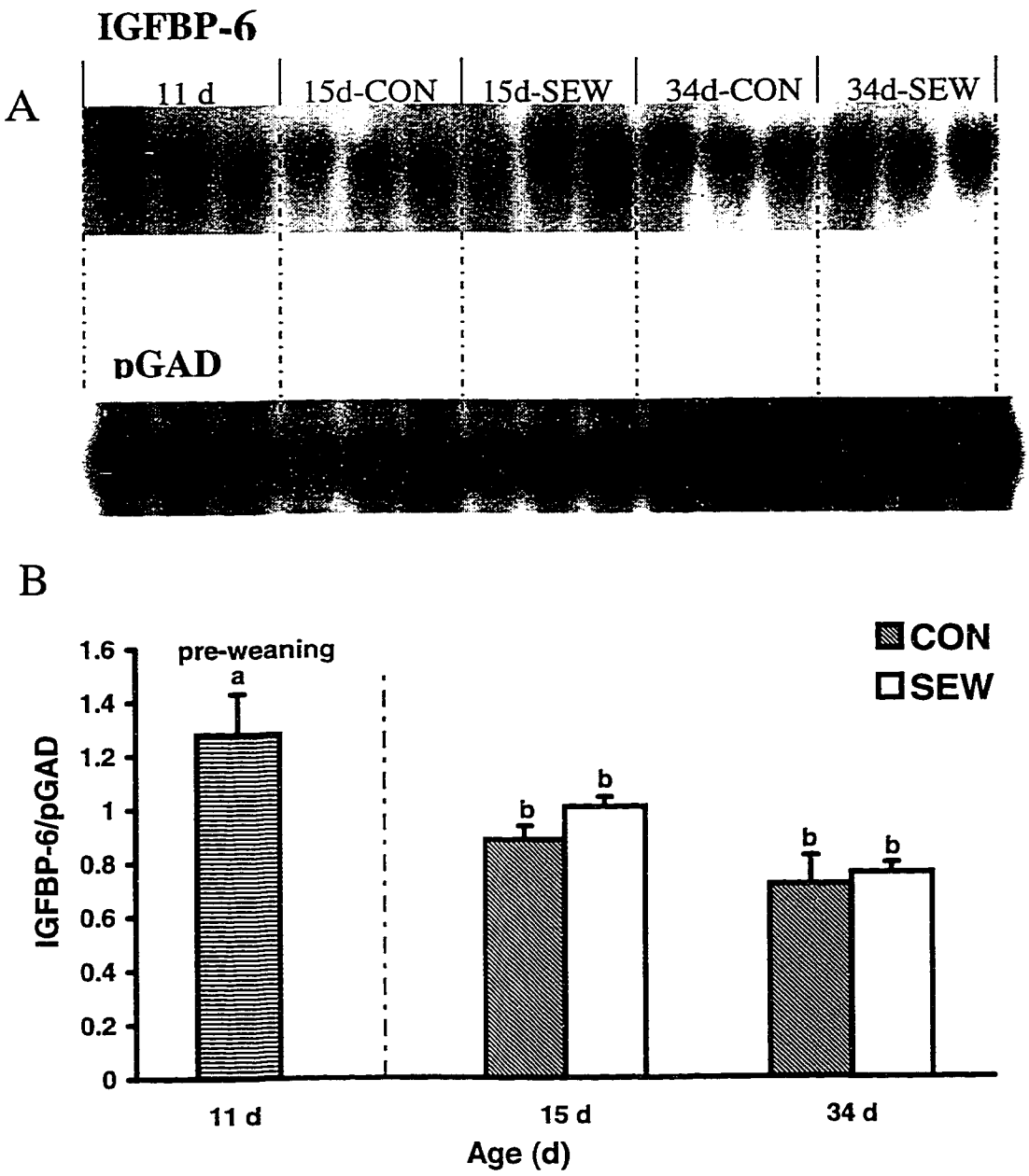

Figure 5.6D Northern blot analysis of IGFBP-6 mRNA abundance in the liver of pigs at 11, 15 and 34 days of age. Pigs were weaned at 12 days of age and housed in a conventional on-site nursery (CON) or in a segregated off-site nursery (SEW). A 1.3 kb IGFBP-6 transcript (Upper panel A) was detected and subject to densitometric measurement. Mean ± SEM (n=3) band intensity was expressed in arbitrary units relative to pGAD (Lower panel A) and presented in a bar graph (Panel B). Bars labeled with different letters are significantly ($P<0.05$) different.

5.5 Discussion

The relationship between hepatic mRNA for IGFBPs and IGF-I and the levels of the respective proteins in plasma in relation to weaning and differing post-weaning growth performance was investigated in SEW and CON pigs. Parallel changes in the concentrations of plasma IGF-I and IGFBP-3 in both groups were evident throughout the study period, reflecting a coordinate regulation of IGF-I and IGFBP-3 *in vivo* to maintain an ample reservoir of IGF-I in the vasculature (Clemmons 1997).

The observations reported here clearly demonstrate that high levels of IGF-I and IGFBP-3 and low levels of IGFBP-2 in plasma are associated with enhanced growth in SEW pigs compared with CON pigs. The distinct feature in this study is the observed discordance of hepatic IGF-I and IGFBP-3 mRNA abundance with the levels of these two proteins present in plasma. Plasma IGF-I and IGFBP-3 drastically decreased 2 d post-weaning and recovered during the post-weaning period with significantly higher levels of both in SEW pigs than in CON pigs. In contrast, hepatic IGF-I and IGFBP-3 mRNA transcript abundance was reduced in both groups at weaning, but the levels of these mRNA transcripts did not recover by 34 days of age nor were differences observed between the two treatments. This agrees with Lee *et al.* (1993) and Peng et al. (1996) who demonstrated discordance of hepatic IGF-I and IGFBP-3 mRNA abundance with the levels of the two proteins in the circulation during postnatal development in pigs. Our findings provide further evidence linking this discordance with growth performance. These findings also indicate that in the pig the liver is unlikely to be a primary source of circulating IGF-I and IGFBP-3 during post-weaning development.

Nutrition markedly affects synthesis of IGF-I and IGFBPs in multiple tissues as well as the ratio of circulating IGF-I and IGFBPs (Clemmons and Underwood 1991, McCusker et al. 1989). Weaning usually results in a marked decline in dietary intake associated with adaptation to a solid diet. This could explain the lower hepatic IGF-I and IGFBP-3 gene expression and the lower levels of these two proteins in plasma in pigs for both treatments immediately after weaning. Increased feed intake and feed efficiency (Patience et al. 1997) in SEW pigs could explain the subsequently higher levels of circulating IGF-I and IGFBP-3 in SEW pigs. Tang et al. (1999; section 3) observed that the SEW treatment, using the current experimental protocol, enhanced gut maturation as shown by consistently higher brush border enzyme activities, higher ratios of mucosal protein to DNA and villus height to crypt depth at 34 d of age. This would likely improve nutrient absorption and induce synthesis of IGF-I and IGFBP-3 in multiple tissues, contributing to higher levels of IGF-I and IGFBP-3 in the circulation as observed in SEW pigs.

Parallel changes in hepatic IGFBP-2 mRNA levels and IGFBP-2 levels in plasma were observed in this study. The pattern of hepatic IGFBP-2 gene expression during postnatal development is in a good agreement with other studies in pigs (Lee et al. 1993) and rats (Brown et al. 1989). A novel finding in this study is that attenuated growth performance induced by a different post-weaning environment is associated with increases in hepatic IGFBP-2 mRNA expression and IGFBP-2 levels in plasma. Higher levels of low molecular weight IGFBPs, such as IGFBP-2, could facilitate the transport of IGF-I to the target tissues, but the excess of unsaturated high affinity IGFBPs in the

plasma could also inhibit IGF-I action in target tissues by competing with the IGF-I receptor (Haffner et al. 1997). Increased low molecular IGFBPs in the circulation, especially that of IGFBP-2, may sequester IGFs that are released from the 150 kDa ternary complex (Powell 1997). The increased hepatic IGFBP-2 gene expression and higher IGFBP-2 plasma level observed in CON pigs could reduce somatic growth by changing the ratio of high and low molecular IGFBPs in the circulation or through competition with the IGF-I receptor for IGF-I anabolic actions (Powell 1997, Tonshoff et al. 1995). Elevated IGFBP-2 in the circulation may also result in increased IGF-I clearance (Monaco and Donovan 1997, Prosser and Schwander 1996) through formation of IGFBP-2:IGF-I complexes (Clemmons 1997). The half-life of the IGFBP-2: IGF-I complex is about 15-20 times shorter than that of the ternary complex in the circulation (Davis et al. 1989). Thissen et al. (1992) and Monaco and Donovan (1997) showed that protein restriction in rats increased serum IGFBP-2 and reduced serum IGF-I by increasing IGF-I clearance from the circulation. Furthermore, increased IGFBP-2 in serum of rats with experimental uremia suggests that IGFBP-2 may play a pathogenic role in catabolism and growth failure (Tonshoff et al. 1997). However, evidence for a role for plasma IGFBP-2 in the regulation of somatic growth in healthy rats or in apparently normal animals is not available. This study does not provide a conclusive explanation why hepatic IGFBP-2 mRNA expression in CON pigs was stimulated since nutritional status is a weaker regulator of this protein (Clemmons 1997). The speculation in current study is that IGFBP-2 gene expression in the liver may be stimulated as a result of the observed increased pathogenic challenge of CON pigs under the same

experimental protocol (Van Kessel et al. 1997). This hypothesis would, however, contrast findings in a study by Elsasser et al. (1995) in which endotoxin administration in steers reduced plasma IGFBP-2 concentration independent of nutrient status. Our study provides the first evidence that increased levels of IGFBP-2 in plasma are associated with reduced somatic growth in apparently healthy animals, and suggests that the concentrations of IGFBP-2 in plasma play a role in regulation of somatic growth.

This study showed an acute rise in circulating IGFBP-1 level at 2 d post-weaning, which would be a consistent response to weaning stress-induced glucocorticoids (Luo et al. 1990) and reduced feed intake (Straus 1994). IGFBP-4, which is a minor IGFBP in plasma, showed minor changes in response to treatment and development and may have a negligible effect on the action of circulating IGF-I (Lee et al. 1991) and growth regulation. We demonstrated for the first time the pattern and abundance of hepatic IGFBP-6 mRNA in pigs during post-weaning development. However, the role of IGFBP-6 *in vivo* remains unclear.

In summary, data of this study demonstrate an important role of circulating IGFBPs in the regulation of somatic growth, especially for IGFBP-3 and IGFBP-2. It is likely that the circulating IGFBP-3 is a positive regulator of somatic growth. Increased hepatic IGFBP-2 mRNA expression accompanied by elevated plasma IGFBP-2 exerts a negative effect on somatic growth. Findings in this study also show that gene expression of hepatic IGFBP-2 was stimulated in pigs in a conventional weaning environment, indicating that varying degrees of sub-clinical infection in apparently normal animals

could impact IGFBP-2 synthesis and secretion and directly influence IGF-I biological activity and growth itself.

6 GENERAL DISCUSSION

The effects of early weaning and post weaning environment on post-weaning gut development and intestinal IGFBPs and IGF-I expression were elucidated in pigs. Furthermore, the differences in growth performance of pigs between the two post weaning environment systems allowed us to investigate the importance of endogenous IGFBPs and IGF-I in the regulation of somatic and intestinal growth. A general summary of the effects of early weaning and weaning environment on the intestinal and hepatic IGF-I and IGFBPs mRNA expression and these proteins in plasma, as well as post-weaning gut maturation, is presented in Table 6.1.

The observations reported here demonstrate that the concentrations of circulating IGF-I increase with age. This is in good agreement with studies in pigs (Scanes et al. 1987; Lee et al. 1991) and rats (Daughaday et al. 1982; Donovan et al. 1991; Kikuchi et al. 1992) and reflects developmental regulation of IGF-I production (section 2.2.1). The findings also suggest that elevated circulating IGF-I is associated with enhanced somatic growth, which is consistent with reports of IGF-I stimulating somatic growth in pigs (Owens et al. 1990) and in rats (Schenle et al. 1982 & 1985).

Nutrition markedly influences synthesis of IGF-I and IGFBPs in multiple tissues as well as the ratio of circulating IGF-I and IGFBPs (Clemmons and Underwood 1991, McCusker et al. 1989). Weaning reduced hepatic IGF-I and IGFBP-3 gene expression

Table 6.1 Summary of the effects of weaning and weaning environment on intestinal and hepatic IGF-I and IGFBPs mRNA, these proteins in plasma, and gut maturation.

	Pre-weaning	3 d Post-weaning		22 d Post-weaning	
Items[1]	**11 d**	**15d-CON**	**15d-SEW**	**34d-CON**	**34d-SEW**
Intestinal IGF-I mRNA	0.47 ± 0.04^{a}	0.53 ± 0.03^{a}	0.77 ± 0.08^{b}	0.65 ± 0.04^{ab}	0.55 ± 0.02^{a}
Intestinal IGFBP-3 mRNA	0.45 ± 0.09	0.54 ± 0.01	0.53 ± 0.03	0.50 ± 0.07	0.42 ± 0.04
Intestinal IGFBP-5 mRNA	0.28 ± 0.05^{a}	0.29 ± 0.06^{a}	0.37 ± 0.05^{ab}	0.43 ± 0.02^{b}	0.26 ± 0.01^{a}
Hepatic IGF-I mRNA	0.62 ± 0.09^{a}	0.36 ± 0.08^{b}	0.46 ± 0.07^{ab}	0.27 ± 0.05^{b}	0.37 ± 0.06^{bc}
Plasma IGF-I	122 ± 23^{a}	44.7 ± 3.1^{b}	47.3 ± 2.2^{b}	152.9 ± 36.3^{c}	65.3 ± 6.3^{d}
Hepatic IGFBP-3 mRNA	1.00 ± 0.10^{a}	0.57 ± 0.04^{b}	0.53 ± 0.05^{b}	0.57 ± 0.04^{b}	0.72 ± 0.06^{b}
Plasma IGFBP-3	390 ± 87^{a}	132 ± 19^{b}	163 ± 53^{b}	345 ± 52^{c}	490 ± 46^{d}
Hepatic IGFBP-2 mRNA	0.81 ± 0.09^{a}	0.79 ± 0.06^{a}	0.84 ± 0.01^{a}	0.67 ± 0.06^{a}	0.34 ± 0.01^{b}
Plasma IGFBP-2	272 ± 55	240 ± 39	219 ± 44	332 ± 41	238 ± 43*
[2]Mucosal protein to DNA ratio	0.58 ± 0.03^{a}	1.19 ± 0.07^{b}	0.89 ± 0.08^{c}	0.71 ± 0.13	0.93 ± 0.04*
[2]Villus height to crypt depth ratio	6.0 ± 0.3^{a}	1.9 ± 0.8^{b}	1.8 ± 0.6^{b}	1.5 ± 0.8^{b}	2.6 ± 0.1^{c}
Mucus layer in the ileum	N/A	N/A	N/A	thicker	thinner

[1] The units of the values are the same as in each section of the thesis. [2] Jejunum.

[a, b, c] All the values are contrasted to pre-weaning (11 d). Values with different letters ($P < 0.05$) are different between SEW and CON pigs of the same age or different from pre-weaning pigs. * Indicates difference ($P < 0.1$) between SEW and CON pigs of the same age.

and the levels of these two proteins in plasma in both SEW and CON pigs. This could be explained, at least partially, by the reduction in dietary intake associated with adaptation to a solid diet. Higher levels of circulating IGF-I and IGFBP-3 in SEW pigs at 34 d could be attributed to advanced maturation of their small intestine and enhanced digestive and absorptive capacity (Tang et al. 1999; section 3.4) as well as higher feed intake (Patience et al. 1997). SEW resulted in consistently higher brush border enzyme activities, higher ratios of mucosal protein to DNA and villus height to crypt depth at 34 d of age. This would likely improve nutrient absorption and induce synthesis of IGF-I and IGFBP-3 in multiple tissues, contributing to higher levels of IGF-I and IGFBP-3 in the circulation as observed in SEW pigs.

The distinct feature in this study is the discordance between hepatic IGF-I and IGFBP-3 mRNA expression and the levels of these two proteins in plasma (section 5.4). This agrees with Lee et al. (1993) and Peng et al. (1996) who demonstrated discordance between hepatic IGF-I and IGFBP-3 mRNA abundance and the levels of the two proteins in the circulation during postnatal development in pigs. The findings in the current study provide further evidence linking this discordance with different growth performance. These findings also indicate that in the pig the liver is unlikely to be a primary source of circulating IGF-I and IGFBP-3 during post-weaning development. Furthermore, somatic growth is associated with the increased concentrations of plasma IGF-I and IGFBP-3 and not with hepatic IGF-I and IGFBP-3 mRNA levels. In addition, parallel changes in the concentrations of plasma IGF-I and IGFBP-3 reflect a coordinate regulation of IGF-I and

IGFBP-3 *in vivo* to maintain an ample reservoir of IGF-I in the vasculature (Clemmons 1997).

A novel finding in this study is that attenuated growth performance induced by a different post-weaning environment is associated with an increase in hepatic IGFBP-2 mRNA expression and IGFBP-2 levels in plasma. Increased levels of IGFBP-2 in plasma of CON pigs may sequester IGFs that are released from the 150 kDa ternary complex (Powell 1997). This could increase IGF-I clearance from the circulation (Monaco and Donovan 1997) or inhibit IGF-I action in target tissues by competing with the IGF-I receptor (Haffner et al. 1997). Together these actions could reduce somatic growth by inhibiting the anabolic and growth promoting effects of IGF-I. This study does not proves a conclusive explanation why hepatic IGFBP-2 mRNA expression in CON pigs was stimulated since nutrition is a weaker regulator of this protein (Clemmons 1997). The speculation is that IGFBP-2 gene expression in the liver may be stimulated as a result of the observed increased pathogenic challenge of CON pigs under the same experimental protocol (Van Kessel et al. 1997). This study provides the first evidence that increased levels of IGFBP-2 in plasma are associated with reduced somatic growth in apparently healthy animals, and suggests that the concentrations of IGFBP-2 in plasma play a role in regulation of somatic growth.

The current study showed an acute rise in circulating IGFBP-1 level at 2 d post-weaning in both groups of pigs, which would be consistent with effects of weaning stress-induced glucocorticoids (Luo et al. 1990) and reduced feed intake (Straus 1994). Hepatic IGFBP-1 mRNA and IGFBP-1 plasma levels in both groups of pigs were highly

variable throughout the study. IGFBP-4, which is a minor IGFBP in plasma, showed minor changes in response to treatment and development and may have a negligible effect on the action of circulating IGF-I (Lee et al. 1991) and growth regulation. This study demonstrated for the first time the pattern and abundance of hepatic IGFBP-6 mRNA in pigs during post-weaning development. However, the role of IGFBP-6 *in vivo* remains unclear.

The results of this study demonstrate that differences in circulating IGFBPs, especially IGFBP-3 and IGFBP-2, are associated with different post-weaning somatic growth. This suggests that IGFBP-3 and -2 affect the anabolic and growth promoting effect of IGF-I and thus growth itself. It is likely that circulating IGFBP-3 stimulates and IGFBP-2 inhibits the biological action of IGF-I and growth performance. The increased hepatic IGFBP-2 expression accompanied by an elevated plasma IGFBP-2 in CON pigs and the higher plasma IGFBP-3 in SEW pigs indicate that weaning environment affects IGFBPs synthesis and secretion and directly influences IGF-I biological activity and growth itself.

Early weaning resulted in a marked increase in expression of intestinal IGF-I mRNA, which is similar to responses observed in inflammatory bowel disease (Lund and Zimmermann 1996) and bowel resection (Ziegler et al. 1998). Up-regulated IGF-I mRNA expression in the small intestine post-weaning in this study therefore may reflect a trophic effect of gut IGF-I in repairing or maintaining gut structure and function (Clark 1997). Gene expression of IGFBP-3 in the small intestine was unaffected by weaning, indicating that IGFBP-3 is unlikely to play a role in the adaptive response to weaning.

This finding agrees with Ziegler et al. (1998) who observed that IGFBP-3 mRNA abundance was not changed in the jejunum following bowel resection.

The increased IGF-I and marginally increased IGFBP-3 mRNA expression in the gut following weaning contrast the dramatic reductions in serum IGF-I (Carroll et al. 1998), hepatic IGF-I and IGFBP-3 mRNA abundance and plasma IGF-I and IGFBP-3 (Tang et al. 1998a & b; section 5.4). These findings demonstrate for the first time tissue specificity in the regulation of IGF-I gene expression post weaning (section 2.2.1.2). The changes observed may also reflect a complex response to weaning, which includes significant aspects of intestinal damage and repair related to the change in diet composition and possible inflammatory responses to dietary antigens or invasive pathogens (Stokes et al. 1994). The fasting model alone may therefore not adequately reflect the adaptive changes observed in the gut at weaning, although reduction of jejunal IGF-I and IGFBP-3 mRNA expression in fasted rats was observed (Winesett et al. 1995).

An inverse relationship was observed between intestinal IGF-I mRNA and IGF-IR numbers and mRNA abundance after weaning. This probably reflects receptor desensitization in response to increased local expression of IGF-I. The intestinal IGFBP-2 mRNA declined rapidly after weaning, which agrees with Orlowski et al. (1990). SEW pigs showed higher intestinal IGF-I and IGFBP-5 mRNA and lower abundance of IGF-I receptor, IGFBP-4 and IGFBP-6 mRNA. These changes may accelerate the transition from the 'neonatal-type' to the 'adult-type' intestine when compared with CON pigs at 3 d post-weaning.

CON pigs showed lower levels of intestinal IGF-I and IGFBP-5 mRNA transcript at 3 d post-weaning, but higher levels of these two protein mRNA transcripts in the small intestine at 34 d of age, when compared with SEW pigs. This indicates that rearing environment influences the local IGF-I and IGFBPs expression in the gut. Similar changes in IGF-I mRNA have also been observed in rats with experimental enterocolitis (Zimmermann et al. 1993) and in inflamed rat colon where IGFBP-5 mRNA expression was induced by up-regulated IGF-I mRNA (Zimmermann et al. 1997). Increased intestinal IGF-I and IGFBP-5 mRNA expression may reflect increased enteric infection or sub-clinical inflammation in the gut of 34 d old CON pigs. These observations suggest that a conventional rearing environment compromises post-weaning gut maturation due to a potentially increased pathogen load present in the gut lumen (Gaskins 1997; Van Kessel et al. 1997). In contrast, SEW pigs reared in a clean environment with reduced infection pressure may benefit from advanced gut maturation.

The data from this study confirm that elevated IGF-I expression in the bowel may play an important role in adaptive hyperplasia of the small intestinal mucosa in response to weaning and protect against mucosal damage during inflammation or sub-clinical inflammation. Increased or decreased intestinal IGFBPs mRNA expression may mediate bioactivity of IGF-I expressed in the gut.

The results of weaning and weaning environment on post-weaning gut maturation demonstrate that rearing environment can exert a significant impact on post-weaning biochemical and morphological gut development. The specific activities of brush border enzymes, lactase, sucrase and alkaline phosphatase, were consistently higher in SEW

pigs than in CON pigs at 34 d of age. This suggests that enterocytes in villi were more mature with greater synthesis of brush border enzymes (Hampson and Kidder 1986, Henning 1985). Abrams (1977) demonstrated that shorter villi and deeper crypts were caused by resident bacterial flora when compared with germ-free animals. The morphological results of this study showed longer villi, shorter crypts and significantly higher V/C ratios in the three regions of the small intestine in SEW pigs compared with CON pigs at 34 d of age. This indicates that CON pigs may have a larger proportion of functionally immature epithelial cells due to the more rapid migration of epithelial cells from crypts, accompanying a reduction in digestive enzymes (Thake et al. 1973).

Scanning electron microscopy and AB/PSA staining revealed a much thicker mucus covering on the villus surface in the ileum of all CON pigs at 34 d of age, but not in SEW pigs. This finding points to increased presence of potential pathogens in the gut lumen of CON pigs compared with SEW pigs (Neutra and Forstner 1987), and provides further evidence that higher levels of the intestinal IGF-I and IGFBP-5 mRNA in CON pigs at 34 d may represent an adaptive response to infection. This is consistent with the suggestion that SEW pigs experience reduced pathogen challenge (Boeckman 1996, Van Kessel et al. 1997). Such thicker mucus on the surface of epithelial cells in CON pigs may reduce digestion and absorption of nutrients by attenuating free diffusion of various nutrients, even water, to the apical surface of epithelial cells, which contains disaccharidases, peptidases, receptors and transport proteins (Forstner 1978). Therefore, in addition to the observed increase in specific activity of brush border enzymes in SEW pigs, the reduction in mucus thickness may also be a contributing factor in

improving feed efficiency as observed in SEW pigs (Patience et al. 1997). The data (section 3.4) overall support the proposed hypothesis that superior production performance in SEW pigs may be a result of advanced post-weaning gut maturation associated with reduced potential pathogen presence in the gut as compared with CON pigs. This would be expected to improve capacity of digestion and absorption of nutrients from the small intestine and thus increase growth performance and feed efficiency.

In summary, the findings of this study provide insight into the endocrine and paracrine/autocrine roles of IGFBPs and IGF-I in the regulation of intestinal and somatic growth in porcine. The data show that early weaning and weaning environment could impact synthesis and secretion of IGFBPs and IGF-I as well as influence post-weaning gut development.

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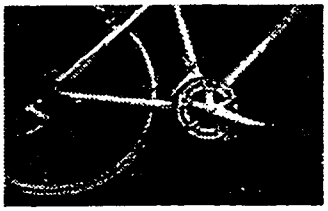
IGF-I AND IGFBPS IN GROWTH REGULATION 1999 M. TANG

Riding uphill

Climbing: Some hate it, some love it. Either way, you're going to have to do it. You might as well learn to do it right.

First, you need to know what happens to your gears when you shift: The left thumb controls the front chain ring and the big shifts in gears; the right thumb controls the rear chain ring, which lets you work between higher and lower gears within each of the front rings.

Climbing hills requires a little preparation. You need to look ahead, take a good look at the hill, and try and judge a few key things:



Learn how to use your gears efficiently.

1. What big chain should you be in given your strength and the steepness of the hill? If it's really steep and long, shift down into the smallest chain ring. If it's a medium, maybe the middle ring. Your left thumb controls this. It's important shift into in the big ring you want to climb in BEFORE you start uphill, otherwise you can run into problems.

Keep pedaling hard as you approach the hill.

2. Keep pedaling. As you approach the hill, pedal extra hard, then keep cranking. If you need to, work your way down in the smaller gears with your right thumb as you climb. As you shift, make sure you're pedaling smoothly. Do not stop pedaling, shift, then start cranking again. You want to try to shift without putting climbing pressure on

without putting climbing pressure on the chain.

Try to stay seated, weight over the rear tire.

Weight the front tire with your arms if the tire wobbles.

Pedal smoothly and "think you can."

3. Try not to change the big chain ring with your left thumb as you climb. If you do need to go down to a lower gear, make sure it's a smooth transition, without pressure on the chain.

4. Overall, you want to be pedaling smoothly and efficiently. It should be work, but not impossible.

5. Stay seated if you can. Keep your weight over your rear tire. This gives you better traction.

6. If you find your front wheel wobbling or coming up, move forward on the seat a bit, but keep your weight on the rear tire.

Return to Gordo's Mountain Biking Tips

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ginger-cookies recipe

Date: Thu, 14 Aug 1997 21:43:24 -0400 (EDT)
From: JBennicoff@aol.com

From a Jean Carper article.

Fatfree Ginger Cookies

1 cup packed brown sugar (I use succanat)
one 2-1/2 oz jar prune baby food
1/4 cup molasses
1/4 cup water + 2 teaspoons powdered egg whites or 1/4 cup egg substitute
2 teaspoons ground ginger
2-1/4 cup combined unbleached and whole wheat flour
1/4 teaspoon ground cloves
2 tablespoons grated, peeled fresh ginger (optional)
1/4 cup granulated sugar or fructose or turbinado sugar

Combine brown sugar, prtune baby food, water and egg whites. Combine other ingredients except for the last sugar listed. Cover and refrigerate fpr 2 hours or overnight. Preheat oven to 350o. Spray cookie sheet with canola oil or use parchment paper. form dough into small, walnut size balls, roll in sugar and place 2 inches apart on cookie sheet. Bake 10-12 minutes. Cool on wire rack. Makes 4 dozen.
Per cookie: 43 calories, .8g protein, 9.7 carbohydrtes, .2g fiber (more with WW flour), .1g fat, 31 mg sodium.

kwovo ovo

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ginger-oat-cookies recipe

Date: Thu, 14 Aug 1997 06:50:50 +0100
From: Ellen Sentovich

I find this cookie delicious, and I think it would be a very nice "clif bar" type base. I've been meaning to try adding dried apricots, dried plums, or dried nectarines to make a heftier snack.

I love these cookies!

Ginger Oat Cookies

1.5 jars baby food (w/bananas)
1/2 c. molasses
2 tsp vanilla
1 c. wheat flour
2 c. oats
2 tsp baking soda
1 tsp ginger
3 tsp cinnamon

Mix and bake at 350F 10-15 minutes. The above makes 42 tiny cookies at 25 cal, .8g fat, .3g fiber each.

For the 1.5 jars baby food, sometimes I just use pure banana, though I think I like it better with baby food. Use your favorite combination of banana/apple sauce/prune puree. If I use only banana, I make sure it's ripe, obviously, and nuke it first to make it more liquidy and easier to mix.

kwvegan vegan

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oatmeal-cookies recipe

Date: Thu, 23 Sep 93 08:52:01 EDT
From: Christina Hulbe

Oatmeal Cookies (modified, a bit, from _Eating_Well_ mag)

1	Cup	packed brown sugar
1/4	Cup	unsweetened applesauce
1/4	Cup	egg equivalent (ie comercial replacer, two whites, 1/4 cup "egg beaters")
1	Tbsp	vanilla extract
1	Cup	raisins
2	Cups	rolled oats, toasted
1	Cup	cake flour (all-purpose is okay but not as tender)
1/2	Cup	whole wheat flour
1	tsp	baking soda
2	tsp	ground cinnamon
1/2	tsp	salt

oven: 350 F
line 2 baking sheets with parchment or coat with non-stick spray

Toasting oats: Heat a large skillet over medium-high flame and add half the oats. Toast, stiring frequently until the oats begin to color and become fragrant (15 minutes or so). Repeat with remaining oats.

In a large bowl, whisk together sugar, applesauce, egg replacer, and vanilla. Stir in raisins. In a medium bowl, stir together remaining (dry) ingredients. Add the dry ingredients to the wet and mix just until blended.

Drop the dough by tablespoonfuls onto prepared baking sheets, spacing cookies about 4 cm apart. Bake for 15 minutes, or until lightly browned. Transfer cookies to a wire rack to cool. Makes 3 dozen cookies.

Storage: no-fat-added cookies do not store well. They become stale quickly and stick together if stacked. As soon as they are cool, wrap the cookies, layers separated by waxed paper, and freeze. To serve, simply let them thaw to room temperature.

Notes: Because there is no fat to help them spread, the cookies will be kind of lumpy. Do toast the oats, it ads depth to the flavor.
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This information is an extract from
Gluten-free Cookery. The Complete Guide for Gluten-free or Wheat-free Diets

By Peter Thomson.

Published by Headway, Hodder Headline. 1995

This information is intended for educational purposes only. It is not medical advice.If you think you need medical advice consult a doctor. Many of the contributors are not health care professionals.

A gluten free or wheat free diet is essential for health for many people. It is not a diet that can be given up or forgotten about for a short time.

What you can eat on a gluten-free or wheat-free diet

- all cooking oils and fats
- all dried fruit
- all dried peas and beans
- all fresh fruit and vegetables
- all fresh meat and fish
- all herbs
- all pure spices
- agar
- ground almonds
- arrow root
- bicarbonate of soda
- buckwheat flour
- butter
- carob flour
- tinned unsweetened chestnut pureÉ
- cheese
- corn meal (maize)
- cornflour (maize)
- cream of tartar.
- dried banana chips
- eggs
- flaked millet
- flaked rice
- ground and chopped nuts
- lentils
- maize and maize flour

- milk
- millet
- pea flour
- potato
- polenta
- pure rice noodles
- quinnoa grain
- rice
- rice flour
- ground rice
- rice puffed cereal
- sago flour
- sesame seed
- sorghum flour
- sweet chestnut flour
- tapioca flour
- teff flour
- tofu- soya curd
- wild rice
- yogurt (except with cereal flavour)
- yam flour
    - But note; soya products and buckwheat are often indigestible for people on wheat-free diets.

Black List:

Food ingredients to avoid. What you must not eat.

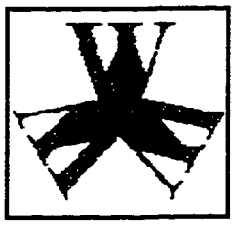
Wheat:
grain containing high levels of gluten.
Bulgar:
soaked and dried wheat.
Durum:
a type of wheat.
Strong flour, bread flour, brown flour, wholemeal flour, granary flour:
all made from wheat.
Oats:
contains some protein similar to wheat gluten but may not cause problems for all celiacs. Best avoided.
Barley:
contains some protein similar to wheat gluten.

Rye:
contains some protein similar to wheat gluten.
Triticale:
contains some protein similar to wheat gluten.
Spelt:
contains some protein similar to wheat gluten.
Semolina:
made from wheat.
Couscous:
made from wheat.
Pasta, macaroni, spaghetti:
made from wheat.
Baking powder:
may contain wheat flour.
Stock cubes and gravy cubes:
may contain wheat flour.
Mustard powder:
may contain wheat flour.
Soy sauce:
this is normally soya beans fermented with wheat flour.
Suet in packets:
may contain wheat flour to stop the suet sticking together.
Starch, Vegetable starch:
may be wheat starch.
Beer, stout, lager, wheat germ, vitamin E pills:
all made from wheat.
BEWARE,
Some brands of 'rice paper' are made using wheat flour.
BEWARE.
Sauce mixes, curry mixes, ice cream, packet and tinned soups, dried meals, gravy mixes, all may contain wheat flour, and do not always declare it on the contents list!
BEWARE.
Some cloudy lemonade and ginger beer now contain wheat flour
BEWARE.
Some sweets are dusted with wheat flour to prevent them sticking.

Links to all Celiac and Gluten-free infromation Food lists for Gluten-free and Wheat-free Diets

The Celiac condition

Recipes for Gluten-free and Wheat-free Diets



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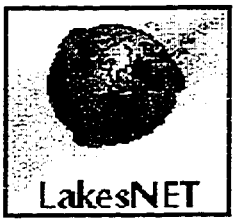

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