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INFLUENCE OF MATERNAL DIET ON THE DEVELOPMENTAL PROFILE OF POSTNATAL GLUCOSE TRANSPORTERS

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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Finally, I would like to dedicate this thesis to my mother and father for their never ending support, constant understanding and love.

ABSTRACT

To test the hypothesis that maternal dietary glucose restriction throughout pregnancy and lactation would perturb glucose transporter (GLUT) protein levels in offspring, isoenergetic diets containing graded levels of glucose (0, 12, 24 and 60%) were fed to pregnant rats and their offspring from gestation day (gd) 0 through postnatal day (pd) 49. Diets were defined as deficient (0%), restricted (12, 24%) or adequate (60%) in glucose. Plasma, small intestine, liver and kidney tissues were collected perinatally (gd20, birth, 12-24hrs postnatal), during lactation (pd7, 15, 21), post-weaning (pd28, 35, 49) and in adult controls. The proximal and distal regions of the small intestine responded differently to the dietary glucose restriction. Proximal small intestine GLUT2 protein levels did not change throughout postnatal development and remained unaltered with dietary glucose restriction, while distal small intestine GLUT2 protein expression changed throughout postnatal development and with dietary glucose restriction. These findings, together with information from the literature, indicate a dissociation between small intestine GLUT2 mRNA expression, GLUT2 protein levels and small intestine glycogen reserves.

RÉSUMÉ

Pour mettre à l'épreuve l'hypothèse que la restriction de glucose diététique maternel pendant la grossesse et la lactation perturberait le niveaux de protéines transporteurs de glucose (GLUT) chez la progéniture, les diètes isoénergétiques contenant des niveaux gradués de glucose (0, 12, 24 et 60%) ont été données à des rats femelles en gestation ainsi qu'à leurs bébés à partir du jour de gestation (gd) 0 jusqu'à jour postnatal (pd) 49. Les diètes ont été définies comme présentant une carence en glucose (0%), un taux de glucose restreint (12, 24%) ou adéquat (60%). Le plasma, l'intestin grêle, les tissus du foie et des reins ont été prélevés durant la période périnatale (gd20, naissance, 12 à 24 heures postnatal), durant la période de lactation (pd7, 15, 21), après sevrage (pd28, 35, 49) et chez les adultes. Les régions proximales et distales de l'intestin grêle ont répondu différemment à la restriction de glucose diététique. Les niveaux de protéines GLUT2 de l'intestin grêle proximal n'ont pas changé pendant le développement postnatal et sont restés intactes avec la restriction de glucose diététique, alors que les niveaux de protéines GLUT2 de l'intestin grêle distal ont été modifiés pendant toute la période de développement postnatal et avec la restriction de glucose diététique. Ces découvertes, en plus des renseignements trouvés dans la documentation, indiquent une dissociation entre l'expression ARNm de GLUT2 de l'intestin grêle, les niveaux de protéines GLUT2 et les réserves de glycogène de l'intestin grêle.

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LIST OF ABBREVIATIONS

ANOVA:	analysis of variance
ATP:	adenosine triphosphate
AUC:	area under curve
BLOTTO:	bovine lacto transfer technique optimizer
BSA:	bovine serum albumin
FFA:	free fatty acids
gd:	gestational day
GLU:	glucose
GLUT:	glucose transporter
G6P:	glucose-6-phosphate
hrs:	hours
IUGR:	intrauterine growth retardation
LSM:	least square mean
NAD:	nicotinamide adenine dinucleotide
pd:	postnatal day
RNA:	ribonucleic acid
SELSM:	standard error least square mean
SGLT:	sodium-dependent glucose transporter
TBS:	tris-buffered saline

CHAPTER 1 - OVERVIEW

Glucose is the main metabolic fuel of the fetus (Jones & Rolph, 1985) and newborn early after birth (Girard, 1989). Maternal dietary glucose restriction during gestation results in decreased pup survival, decreased pup weight, reduced plasma glucose and insulin concentrations and reduced liver, heart and brain glycogen concentrations (Taylor *et al.*, 1983; Koski & Hill, 1986; Fergusson & Koski, 1990; Koski & Hill, 1990; Koski *et al.*, 1990; Koski *et al.*, 1993; Cobrin & Koski, 1995).

Recently, knowledge of the role of sodium-dependent (SGLT) and facilitative glucose transporters (GLUT) in the cellular uptake and release of glucose has been described (Burant *et al.*, 1991; Devaskar & Mueckler, 1992; Bell *et al.*, 1993; Maher *et al.*, 1994; Mueckler, 1994; Thorens, 1996). These glucose transporters have tissue specific distributions and physiological functions. In the small intestine and kidney, SGLT1 is responsible for the uptake of dietary glucose and reabsorption of filtered glucose respectively while GLUT2 is responsible for the release of absorbed glucose in both the small intestine and kidney (Burant *et al.*, 1991; Thorens, 1996). In the liver, GLUT2 is responsible for both the uptake and release of glucose by the liver (Burant *et al.*, 1991).

Glucose transporters not only exhibit distinct tissue distributions (Burant *et al.*, 1991), they are also developmentally regulated (Burant *et al.*, 1991), resulting in distinct patterns of tissue expression. In the small intestine, conflicting results have been reported on the developmental expression of SGLT1 and GLUT2 mRNA (Miyamoto *et al.*, 1992; Reimer *et al.*, 1997) while no studies to date have analyzed small intestine SGLT1 and GLUT2 protein levels during development. In the kidney, developmental patterns of mRNA expression for the facilitative glucose transporters have been well described (Chin *et al.*, 1993) while little is known of the developmental patterns of mRNA expression for the sodium-dependent glucose transporters (You *et al.*, 1995). As it is in the small intestine, it remains unknown whether the developmental patterns of glucose transporter mRNA expression in the kidney are mimicked by their respective proteins.

Although information on glucose transporter protein levels is lacking in the small intestine and kidney, the developmental patterns of glucose transporters expressed in the liver have been established for both mRNA and protein (Postic *et al.*, 1994).

It is also known that glucose transporter expression can be modulated by various factors. The level of glucose transporters expressed in adult rat tissues can be changed with exercise (Rodnick *et al.*, 1990; Neufer *et al.*, 1992), fasting (Berger *et al.*, 1989; Charron & Kahn, 1990; Thorens *et al.*, 1990), refeeding (Berger *et al.*, 1989; Charron & Kahn, 1990; Thorens *et al.*, 1990) and with changes in the macronutrient composition of the diet, particularly by changes in the fat and carbohydrate content of the diet (Pederson *et al.*, 1991; Kahn & Pederson, 1993; Burant & Saxena, 1994). Much less is known about factors which modulate glucose transporter expression during development. A few studies have examined the effect of the macronutrient composition of the diet on glucose transporter expression during development and found that glucose transporter levels are also changed during development, as in the adult, by the fat and carbohydrate content of the diet (Leturque *et al.*, 1991; Wallace *et al.*, 1992; Postic *et al.*, 1994).

The purpose of this study is to determine the role, if any, that maternal dietary intake, in particular dietary glucose, may play in modulating glucose transporter protein levels in offspring during development. This study focuses on glucose transporter protein levels, rather than mRNA, in order to expand on the knowledge already available in the literature. Using diets containing graded levels of dietary glucose, we investigated whether maternal dietary glucose restriction alters glucose transporter protein levels in the small intestine, kidney and liver of offspring.

CHAPTER 2 - LITERATURE REVIEW

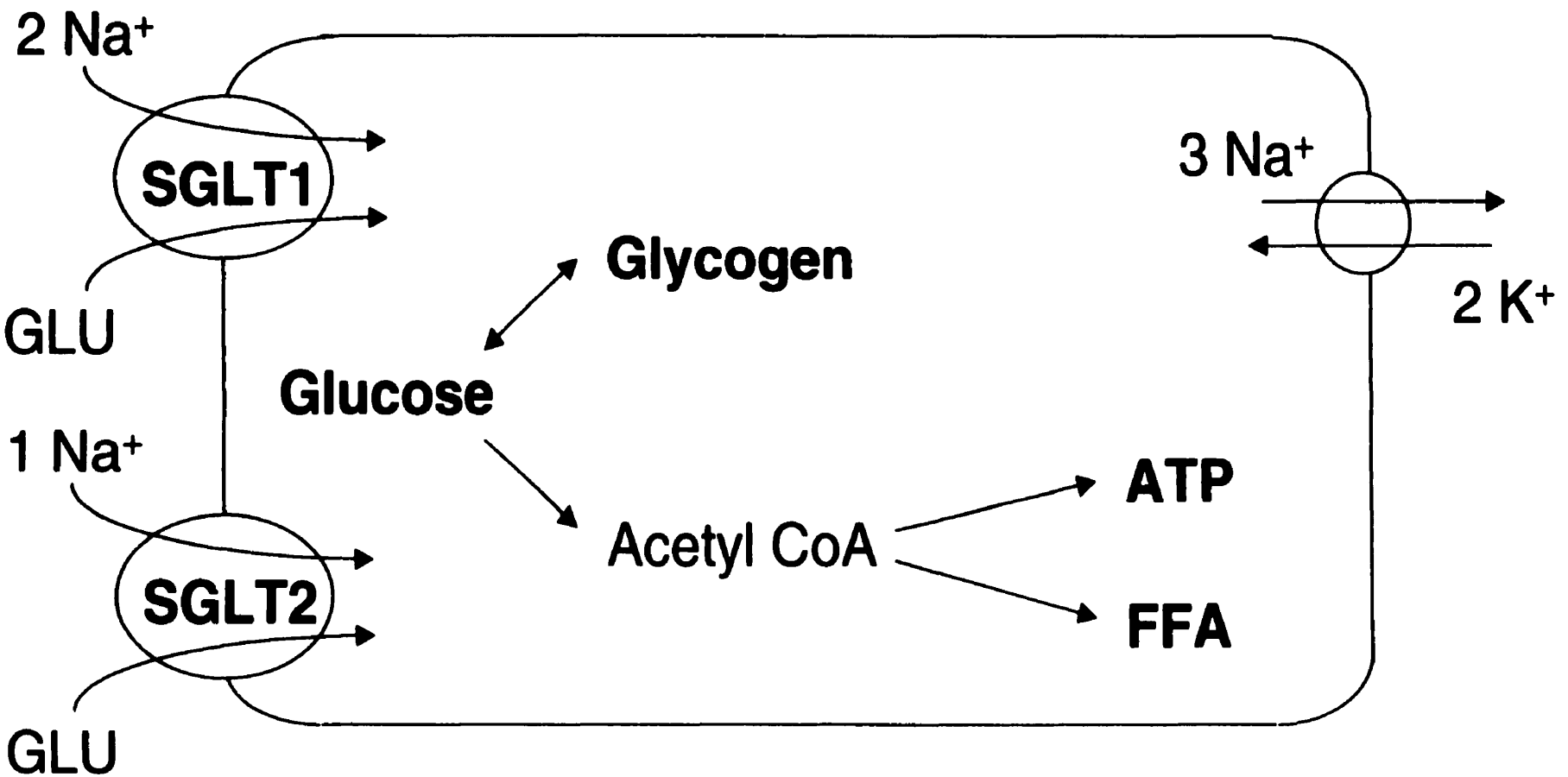
Glucose is a key metabolic substrate for mammalian cells (Devaskar & Mueckler, 1992). It supplies ATP under aerobic and anaerobic conditions (Bell *et al.*, 1993) and is a precursor for the biosynthesis of glycogen and fat as well as various sugar-containing macromolecules such as glycoproteins, glycolipids, and nucleic acids (Devaskar & Mueckler, 1992). It is also the main metabolic fuel of the fetus (Jones & Rolph, 1985) and the placenta (Hay & Sparks, 1985) which are supplied with glucose by the mother (Shelley, 1975) as well as the newborn early after birth (Girard, 1989) which is supplied with glucose through the mobilization of the liver glycogen stores accumulated in the liver during the latter part of gestation (Shelley, 1961).

Cellular uptake of glucose is mediated by membrane carrier proteins called glucose transporters (Bell *et al.*, 1993). Two distinct families of glucose transporters have been identified (Devaskar & Mueckler, 1992): first, the sodium-dependent glucose transporter family, in which two isoforms (SGLT1 and SGLT2) have been characterized (Thorens, 1996); and second, the facilitative glucose transporter family, in which six functional isoforms (GLUT1, GLUT2, GLUT3, GLUT4, GLUT5 and GLUT7) have been characterized (Waddell *et al.*, 1992; Thorens, 1996). These isoforms exhibit distinct tissue distributions and developmental patterns of expression (Burant *et al.*, 1991).

2.1 SODIUM-DEPENDENT GLUCOSE TRANSPORTERS

The sodium-dependent glucose transporters (SGLT1 and SGLT2) simultaneously transfer glucose against its concentration gradient and sodium down its concentration gradient by means of a secondary active transport system (**Figure II-1**) (Devaskar & Mueckler, 1992). No energy is used directly in the transport of glucose against its concentration gradient. Operation of the sodium-glucose cotransporter is driven by the sodium concentration gradient which is maintained by the energy using sodium-potassium ATPase pump (Thorens,

Figure II-1: Secondary Active Transport of Glucose by Sodium-Dependent Glucose Transporters



Based on Devaskar & Mueckler, 1992; Kanai *et al.*, 1994 and Thorens, 1996

1996). SGLT1 is a high affinity (K_m = 0.35 mM), low-capacity isoform which can transport glucose and galactose (Turner & Moran, 1982; Pajor *et al.*, 1992; Kanai *et al.*, 1994) while SGLT2 is a low affinity (K_m = 6 mM), high capacity isoform which transports only glucose (Turner & Moran, 1982; Pajor *et al.*, 1992; Kanai *et al.*, 1994). Two sodium molecules are required to transport one glucose molecule by the high energy cotransporter SGLT1 while only one sodium molecule is required for the transport of a glucose molecule by the SGLT2 cotransporter (Kanai *et al.*, 1994).

2.1.1 Tissue Specific Distribution and Physiological Function

SGLT1 is present at high levels in the intestine and at somewhat lower levels in the kidney (Thorens, 1996) while SGLT2 is almost exclusively present in the kidney (**Table II-1**) (Thorens, 1996). In the small intestine, SGLT1 is responsible for the uptake of dietary glucose (Burant *et al.*, 1991). In the kidney, SGLT2, found in the convoluted part of the proximal tubule, is responsible for the reabsorption of the bulk of the filtered glucose (Turner & Moran, 1982; Pajor *et al.*, 1992; Kanai *et al.*, 1994) while SGLT1, found in the straight part of the proximal tubule, is responsible for the reabsorption of the remaining glucose (Turner & Moran, 1982; Pajor *et al.*, 1992).

2.1.2 Developmental Changes in Expression

Sodium-dependent glucose transporter expression changes throughout development (You *et al.*, 1995; Reimer *et al.*, 1997). Developmental changes have been described for SGLT1 and SGLT2 mRNA in the kidney (You *et al.*, 1995) and SGLT1 mRNA in the small intestine (Reimer *et al.*, 1997). The precise timing of some developmental changes in expression as well as whether these developmental changes in mRNA expression are mimicked by their respective proteins remains unknown.

Kidney:

In the rat kidney, SGLT1 mRNA appears on gestational day 18 with levels gradually increasing until birth (You *et al.*, 1995). SGLT2 mRNA appears on gestational day 17 with levels gradually increasing until gestational day 19 and then decreasing until birth (You *et al.*, 1995). The appearance of SGLT1 and

Table II-1: Major Sites of Expression and Physiological Function of Sodium-Dependent and Facilitative Glucose Transporters

Glucose Transporter	Major Sites of Expression	Physiological Function
SGLT1	Small Intestine and Kidney	Uptake of dietary glucose from the lumen of the small intestine and reabsorption of filtered glucose in the straight part of the proximal tubule of the kidney
SGLT2	Kidney	Reabsorption of filtered glucose in the convoluted part of the proximal tubule of the kidney
GLUT1	Fetus, Placenta and Brain	Basal uptake of glucose by cells and transport of glucose across blood-tissue barriers
GLUT2	Liver, Small Intestine and Kidney	Uptake and release of glucose by hepatocytes and release of absorbed glucose across the basolateral surface of absorptive epithelial cells of the small intestine and kidney
GLUT3	Placenta and Brain	Basal uptake of glucose by the brain
GLUT4	Skeletal Muscle, Heart and Adipose Tissue	Insulin-stimulated glucose uptake; important in whole-body glucose disposal
GLUT5	Small Intestine	Intestinal absorption of fructose
GLUT7	Liver	Mediates glucose flux across endoplasmic reticulum membrane

Based on Turner & Moran, 1982; Burant *et al.*, 1991; Pajor *et al.*, 1992; Waddell *et al.*, 1992; Kanai *et al.*, 1994; Mueckler, 1994 and Thorens, 1996

SGLT2 mRNA on gestational days 18 and 17 respectively is consistent with the expression of brush border membrane proteins in the developing kidneys (You *et al.*, 1995). Both SGLT1 and SGLT2 mRNA appear at higher levels in the adult than in the fetus (You *et al.*, 1995). When during development the expression of SGLT1 and SGLT2 mRNA increase to attain adult levels as well as whether such changes are mimicked by SGLT1 and SGLT2 proteins has not yet been determined.

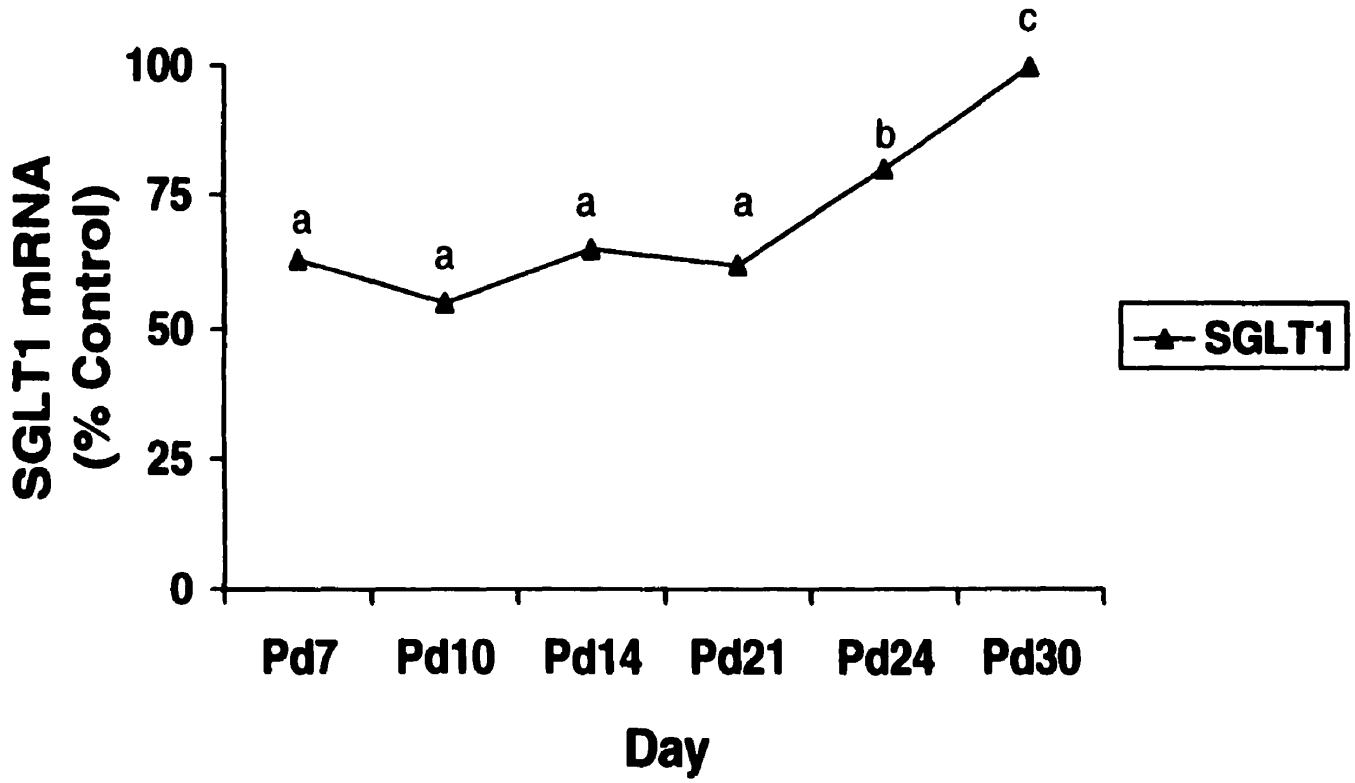
Small Intestine:

Although Miyamoto *et al.* in 1992 found that the level of SGLT1 mRNA in the proximal small intestine did not change significantly between postnatal day 5 and postnatal day 25, a more recent study by Reimer *et al.* in 1997 found significantly higher levels of SGLT1 mRNA in the proximal small intestine at postnatal days 24 and 30 than at postnatal days 7, 10, 14 and 21 (**Figure II-2**). Whether such developmental changes also occur in the distal small intestine and whether SGLT1 proteins change in parallel with SGLT1 mRNA remains unknown.

2.2 FACILITATIVE GLUCOSE TRANSPORTERS

The facilitative glucose transporters are stereospecific, saturable proteins that transport glucose down its concentration gradient using the energy-independent process of facilitative diffusion (Williams *et al.*, 1968). GLUT1 is a high affinity transporter capable of transporting glucose, galactose and mannose (Thorens, 1996). Like GLUT1, GLUT3 and GLUT4 are high affinity transporters but unlike GLUT1, GLUT3 is only capable of transporting glucose and galactose and GLUT4 is only capable of transporting glucose (Bell *et al.*, 1993; Thorens, 1996). The Michaelis constants (K_m) of the high affinity transporters are below normal blood glucose concentrations, therefore the high affinity transporters function at rates close to maximal velocity. Their level of cell surface expression therefore greatly influences the rate of glucose uptake into the cells in which they are found. GLUT2 is a low affinity, high capacity transporter capable of carrying

Figure II-2: Developmental Changes in SGLT1 mRNA Expression in Rat Jejunum as a Percentage of Control (Day 30 Rats)



Based on Reimer *et al.*, 1997

glucose, galactose, mannose and fructose (Bell *et al.*, 1993; Mueckler, 1994; Thorens, 1996). The rate of glucose uptake by GLUT2, because of its higher K_m, increases in parallel with the rise in blood glucose over the physiological concentration range. Therefore, in contrast to GLUT1, GLUT3 and GLUT4, in which the number of carriers present on the cell surface determines the rate of glucose uptake, the rate of glucose uptake by GLUT2 is determined by the blood glucose concentration. GLUT7, the most recently discovered glucose transporter isoform, transports glucose (Waddell *et al.*, 1992) while GLUT5 is primarily a fructose carrier (Burant *et al.*, 1992). GLUT6 is a pseudogene that is not expressed at the protein level (Kayano *et al.*, 1990).

2.2.1 Tissue Specific Distribution and Physiological Function

GLUT1 is the primary glucose transporter of fetal tissues and, in adult tissues, is expressed at highest levels in blood tissue barriers such as the blood/brain barrier (**Table II-1**) (Mueckler, 1994). GLUT1 is responsible for the basal uptake of glucose by cells (Burant *et al.*, 1991). GLUT2 is present in the liver, small intestine and kidney where it mediates the uptake and release of glucose by hepatocytes and results in the release of absorbed glucose across the basolateral surface of absorptive epithelial cells of the small intestine and kidney (Burant *et al.*, 1991). GLUT3 is primarily found in the placenta and brain where it is responsible for the basal uptake of glucose (Burant *et al.*, 1991). GLUT4 is expressed in skeletal muscle, heart and adipose tissue and is responsible for insulin-stimulated glucose uptake (Mueckler, 1994). It is thus important in whole-body glucose disposal (Mueckler, 1994). The fructose carrier, GLUT5, is expressed at highest levels in the small intestine, where it is responsible for the absorption of fructose, and at lower levels in the kidney, skeletal muscle, adipose tissue and brain (Mueckler, 1994). GLUT7 is the microsomal glucose transporter of the liver (Waddell *et al.*, 1992). It comprises part of the glucose-6-phosphatase complex and mediates glucose release from the endoplasmic reticulum (Waddell *et al.*, 1992).

2.2.2 Developmental Changes in Expression

Facilitative glucose transporter expression is developmentally regulated (Burant *et al.*, 1991). These developmental changes result in organ specific patterns of glucose transporter expression. These patterns of expression have been described for the brain (Werner *et al.*, 1989; Maher *et al.*, 1994; Vannucci, 1994), brown adipose tissue (Postic *et al.*, 1994), skeletal muscle (Santalucia *et al.*, 1992; Wallace *et al.*, 1992; Postic *et al.*, 1994), heart (Werner *et al.*, 1989; Wang & Hu, 1991; Postic *et al.*, 1994), kidney (Chin *et al.*, 1993), liver (Postic *et al.*, 1994) and small intestine (Miyamoto *et al.*, 1992; Reimer *et al.*, 1997).

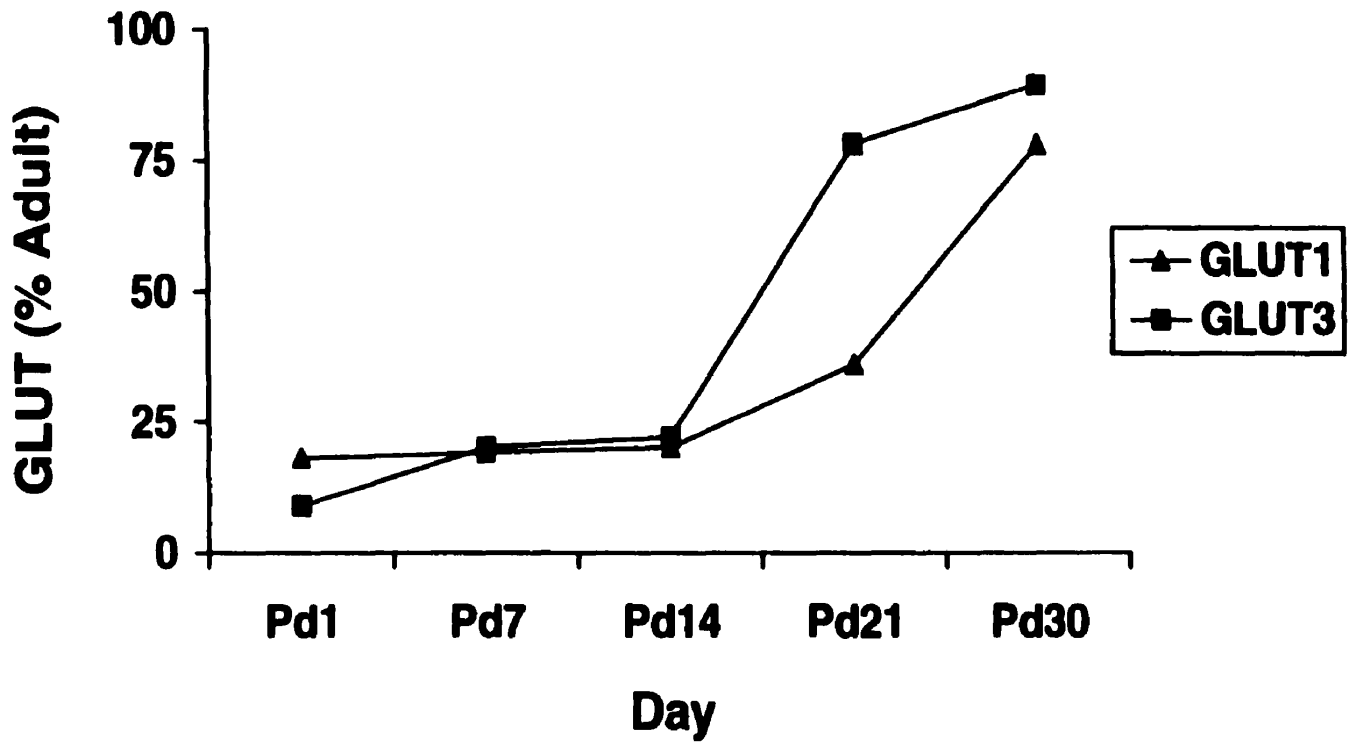
Brain:

In the rat brain, GLUT1 mRNA levels are low for the first 15 days of life (Werner *et al.*, 1989). They then increase by postnatal day 22 and reach maximal levels at 50 days of age (Werner *et al.*, 1989), a pattern paralleled by GLUT1 proteins in the rat brain (**Figure II-3**) (Maher *et al.*, 1994). The low levels of GLUT1 proteins during the first two weeks of postnatal life are in keeping with the low rates of cerebral glucose utilization while the increasing levels of GLUT1 during the next week of life probably reflects overall brain growth (Maher *et* al., 1994; Vannucci, 1994). GLUT3 protein levels in the brain are low during the first postnatal week of life (Maher *et al.*, 1994; Vannucci, 1994). GLUT3 levels then increase slightly between postnatal day 7 and 14 with a more rapid increase occurring between postnatal day 14 and 21, corresponding to the period of neuronal maturation and synaptogenesis (Maher *et al.*, 1994; Vannucci, 1994). The highest levels of brain GLUT3 are again seen in the adult rat (**Figure II-3**) (Maher *et al.*, 1994; Vannucci, 1994). Thus, the expression of both major glucose transporter isoforms present in the brain increases throughout postnatal development.

Brown Adipose Tissue:

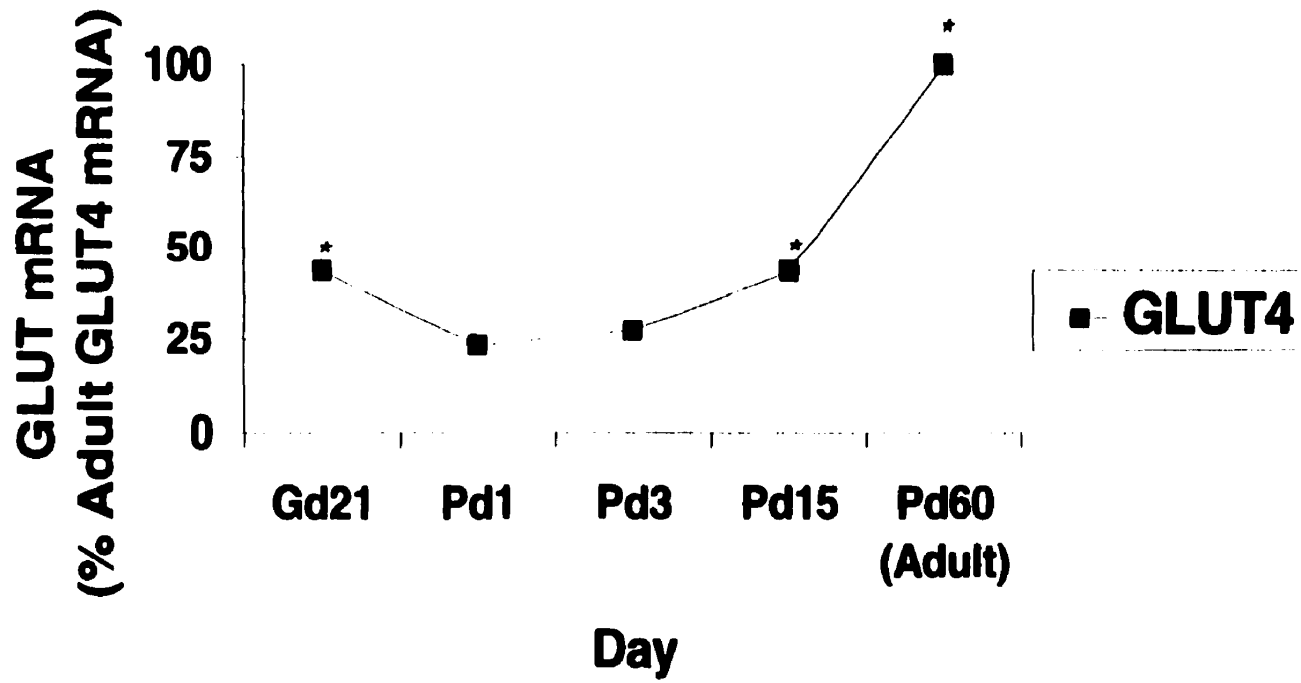
In the rat, GLUT4 mRNA is detectable in fetal brown adipose tissue (**Figure II-4**) (Postic *et al.*, 1994). This level of GLUT4 mRNA decreases by approximately 50% immediately after birth only to increase again, to its highest level, in adulthood (Postic *et al.*, 1994). Brown adipose tissue GLUT4 protein

Figure II-3: Developmental Changes in GLUT Expression in the Rat Brain



Based on Maher *et al.*, 1994

Figure II-4: Developmental Changes in GLUT mRNA Expression in Rat Brown Adipose Tissue



* Statistically significantly different from values obtained from 1 day old rats
Based on Postic *et al.*, 1994

levels follow the same pattern as brown adipose tissue GLUT4 mRNA (Postic *et al.*, 1994). In contrast to the changing GLUT4 mRNA and protein, GLUT1 mRNA and protein are detected in brown adipose tissue at low levels throughout development (Postic *et al.*, 1994).

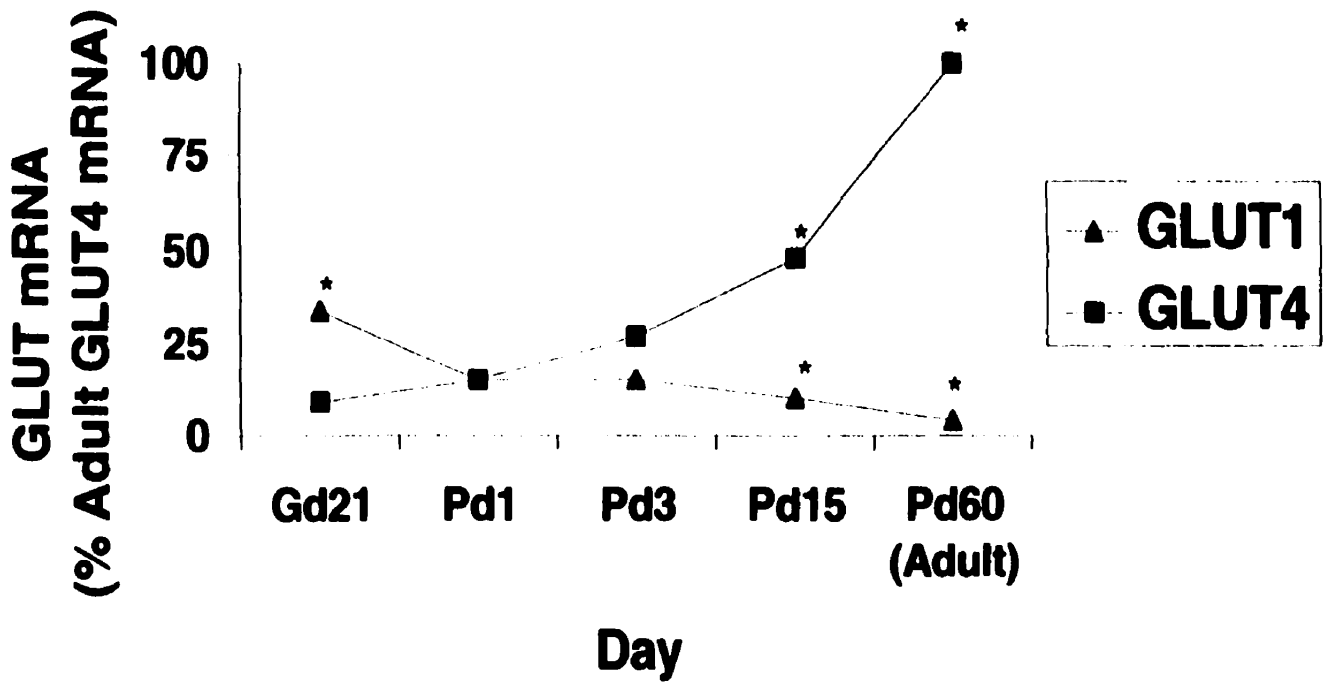
Skeletal Muscle:

In the rat, skeletal muscle GLUT1 mRNA levels are much higher in the fetus than in the adult (**Figure II-5**) (Postic *et al.*, 1994). These high GLUT1 mRNA levels decrease by more than 50% immediately after birth (Postic *et al.*, 1994). Throughout postnatal development, GLUT1 mRNA levels continue to decrease, although more progressively, with levels more than 50% lower at pd60 than at pd1 (Postic *et al.*, 1994). Skeletal muscle GLUT4 mRNA is found at 10% of the adult level in the fetus (Postic *et al.*, 1994). It increases approximately 2 fold immediately after birth and another 7.5 fold after weaning, a change which occurs concomitantly with the development of insulin sensitivity (Postic *et al.*, 1994). Developmental changes in GLUT1 and GLUT4 proteins parallel changes in their corresponding mRNAs (Postic *et al.*, 1994). Thus, the major type of glucose transporter in rat skeletal muscle switches from GLUT1 to GLUT4 during postnatal development. These developmental changes in skeletal muscle GLUT1 and GLUT4 mRNA and protein as described by Postic *et al.* in 1994 are similar to those described by Santalucia *et al.* and Wallace *et al.* in 1992.

Heart:

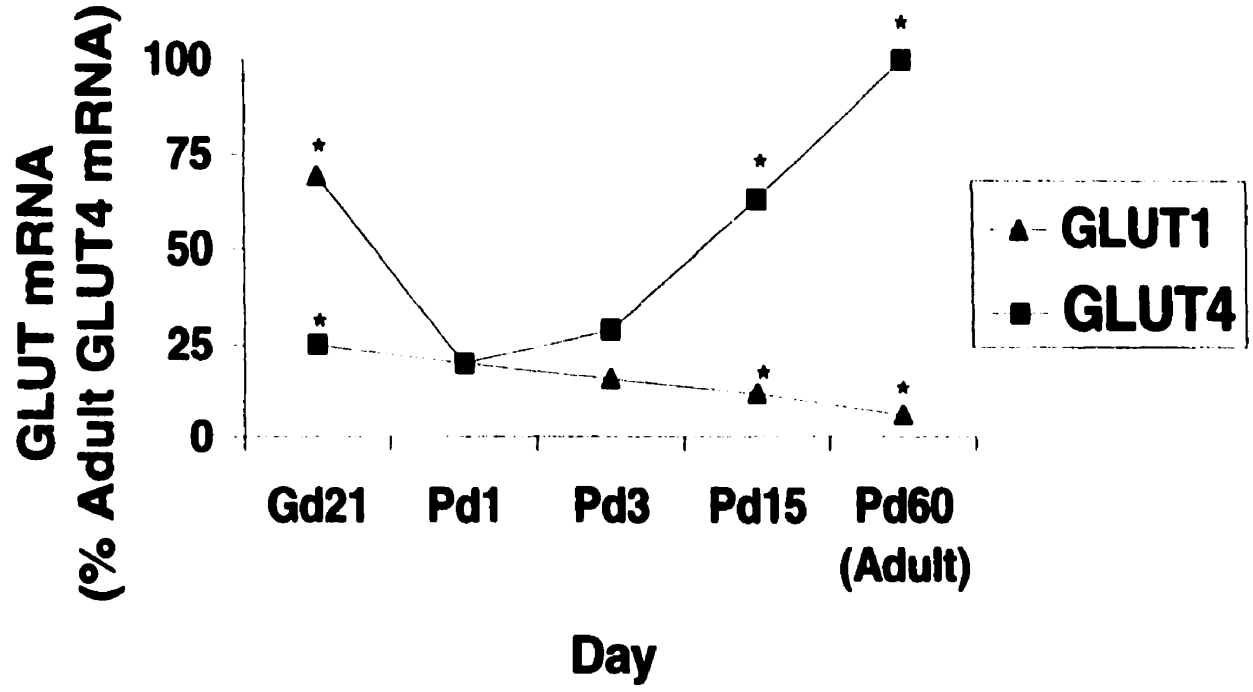
In the heart, GLUT1 mRNA levels are 20 fold higher in the fetus than in the adult (**Figure II-6**) (Werner *et al.*, 1989; Postic *et al.*, 1994). This high level of expression decreases by approximately 75% 3 days after birth with GLUT1 protein levels changing in parallel with its mRNA (Postic *et al.*, 1994). GLUT4 expression in the heart shows an opposite pattern to that of GLUT1. GLUT4 mRNA is found at 20% of the adult level in the fetus and neonate (Postic *et al.*, 1994). It increases approximately 3 fold by day 15, with the highest levels of GLUT4 mRNA observed in the adult (Postic *et al.*, 1994). Once again, GLUT4 protein levels in the heart follow the same pattern as GLUT4 mRNA (Postic *et al.*, 1994). As was seen in rat skeletal muscle, the major type of glucose

Figure II-5: Developmental Changes in GLUT mRNA Expression in Rat Skeletal Muscle



* Statistically significantly different from values obtained from 1 day old rats
Based on Postic *et al.*, 1994

Figure II-6: Developmental Changes in GLUT mRNA Expression in Rat Heart



* Statistically significantly different from values obtained from 1 day old rats
Based on Postic *et al.*, 1994

transporter in the rat heart switches from GLUT1 to GLUT4 during embryonic/postnatal development (Wang & Hu, 1991).

Kidney:

In the rat kidney, GLUT1 and GLUT5 mRNA can be detected by gestational day 20 with adult levels being reached by postnatal day 16 (Chin *et al.*, 1993). GLUT2 mRNA is abundant by gestational day 20 with levels declining through postnatal development until day 40 (Chin *et al.*, 1993) while GLUT4 mRNA is not detected in the perinatal kidney but is faintly discernible by postnatal day 16, more abundant by postnatal day 20 with the adult pattern expressed by day 40 (Chin *et al.*, 1993). Whether these developmental changes in mRNA expression are mimicked by their respective proteins remains unknown.

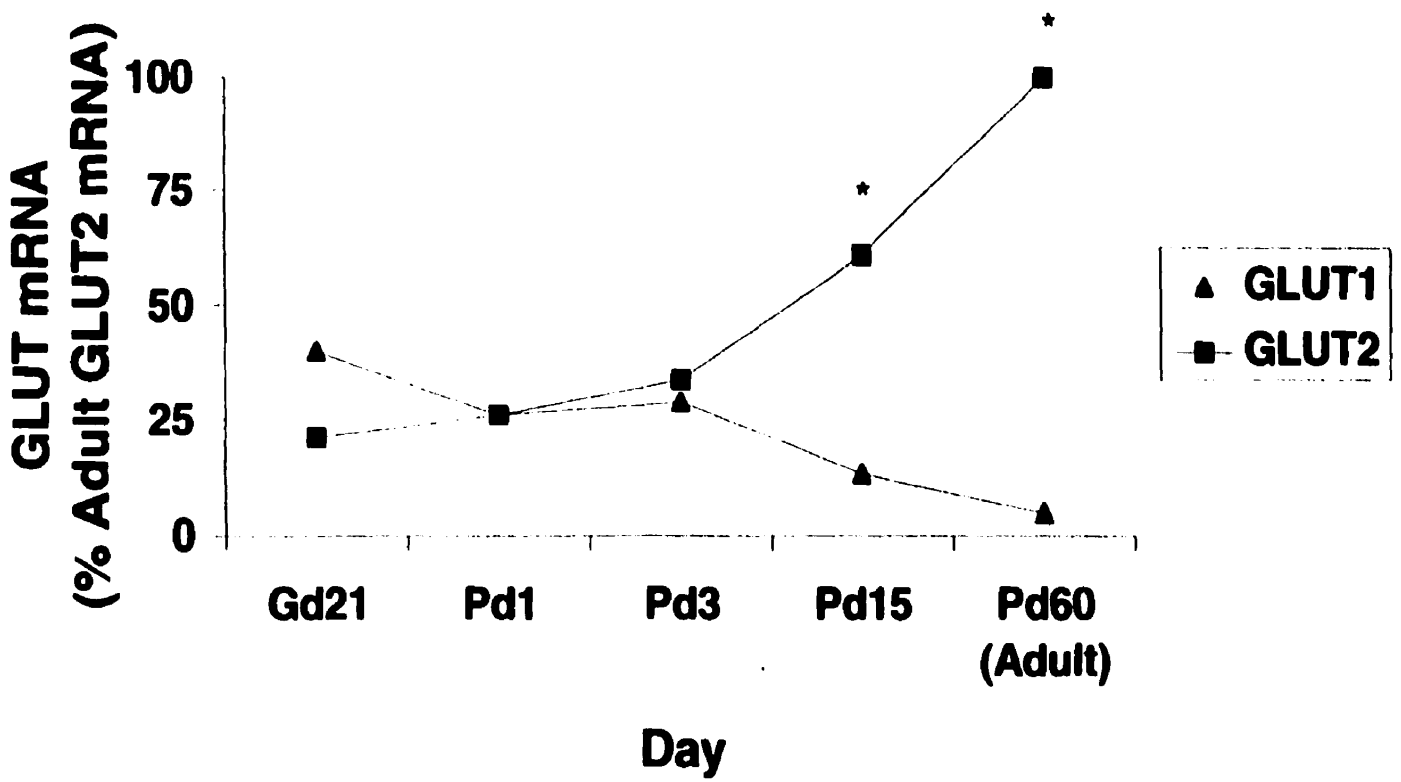
Liver:

In the rat liver, GLUT1 mRNA is expressed during fetal life and up to day 3 after birth at a higher level than in adult rats (**Figure II-7**) (Postic *et al.*, 1994) with its corresponding protein detectable in the liver of 1 day old newborn rats but not thereafter (Postic *et* al., 1994). GLUT2 mRNA is detectable in the fetal liver on gestational day 18 and increases during subsequent fetal life (Postic *et al.*, 1994). GLUT2 mRNA levels continue to increase in the first 3 days after birth with an additional increase occurring after weaning (Postic *et al.*, 1994). GLUT2 protein, following a similar pattern to that of GLUT2 mRNA, is present at low levels in fetal liver and shows a large increase in the first 3 days after birth (Postic *et al.*, 1994). Once again, the major type of glucose transporter in the rat liver switches during postnatal development, from GLUT1 to GLUT2.

Small Intestine:

Studies examining developmental changes in facilitative glucose transporter expression in the small intestine have been, to date, limited to the proximal small intestine. Miyamoto *et al.* in 1992 found that the GLUT2 mRNA level in proximal small intestine tissue was low in 5 and 10 day old rats but GLUT2 mRNA levels subsequently increased, reaching the adult level of expression by day 25 after birth (**Figure II-8**). Reimer *et al.* in 1997 found a

Figure II-7: Developmental Changes in GLUT mRNA Expression in the Rat Liver



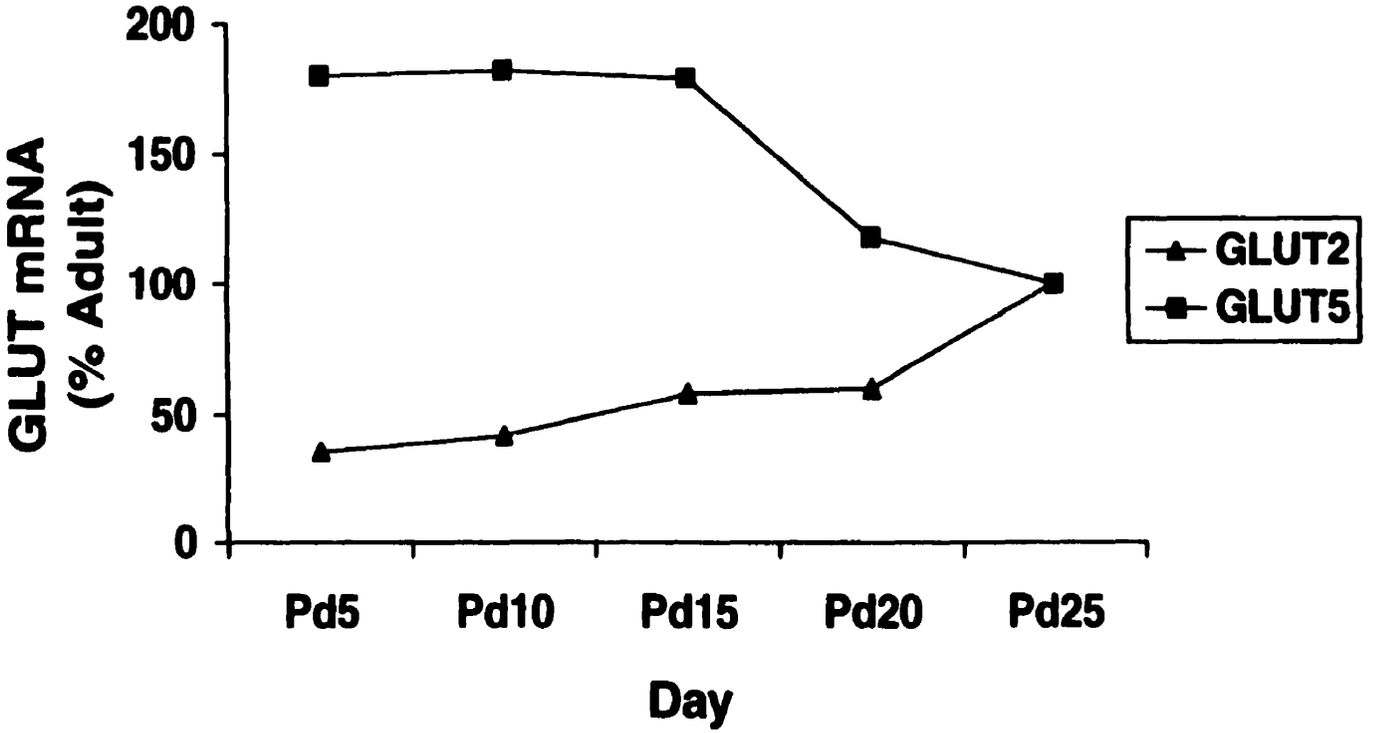
* Statistically significantly different from values obtained from 1 day old rats
Based on Postic *et al.*, 1994

different developmental pattern of GLUT2 mRNA expression from that described previously by Miyamoto *et al.* (**Figure II-9**). The pattern described by Reimer *et al.* is one in which GLUT2 mRNA levels increase between postnatal day 7 and postnatal day 10, decrease between postnatal day 10 and postnatal day 21, only to increase again between postnatal day 21 and postnatal day 30. Similarly, differences exist between the developmental patterns described by Miyamoto *et al.* in 1992 and Reimer *et al.* in 1997 for proximal small intestine GLUT5 mRNA. Miyamoto *et al.* found high GLUT5 mRNA levels in rats 5 and 10 days old. They found these high levels decrease with age to reach adult levels by day 25 after birth. Reimer *et al.* however found a GLUT5 mRNA pattern in proximal small intestine similar to the pattern they found for GLUT2 mRNA. They found that GLUT5 mRNA levels increase between postnatal day 7 and postnatal day 10, decrease between postnatal day 10 and postnatal day 21, only to increase again between postnatal day 21 and postnatal day 30. The conflicting findings of these two studies indicate the need for further research in this area in order to determine the true developmental GLUT2 and GLUT5 mRNA patterns. Further investigation is also required to determine whether the developmental patterns of jejunal GLUT2 and GLUT5 protein mimic their respective mRNA patterns as this has not yet been determined.

2.3 FACTORS MODULATING GLUCOSE TRANSPORTER EXPRESSION

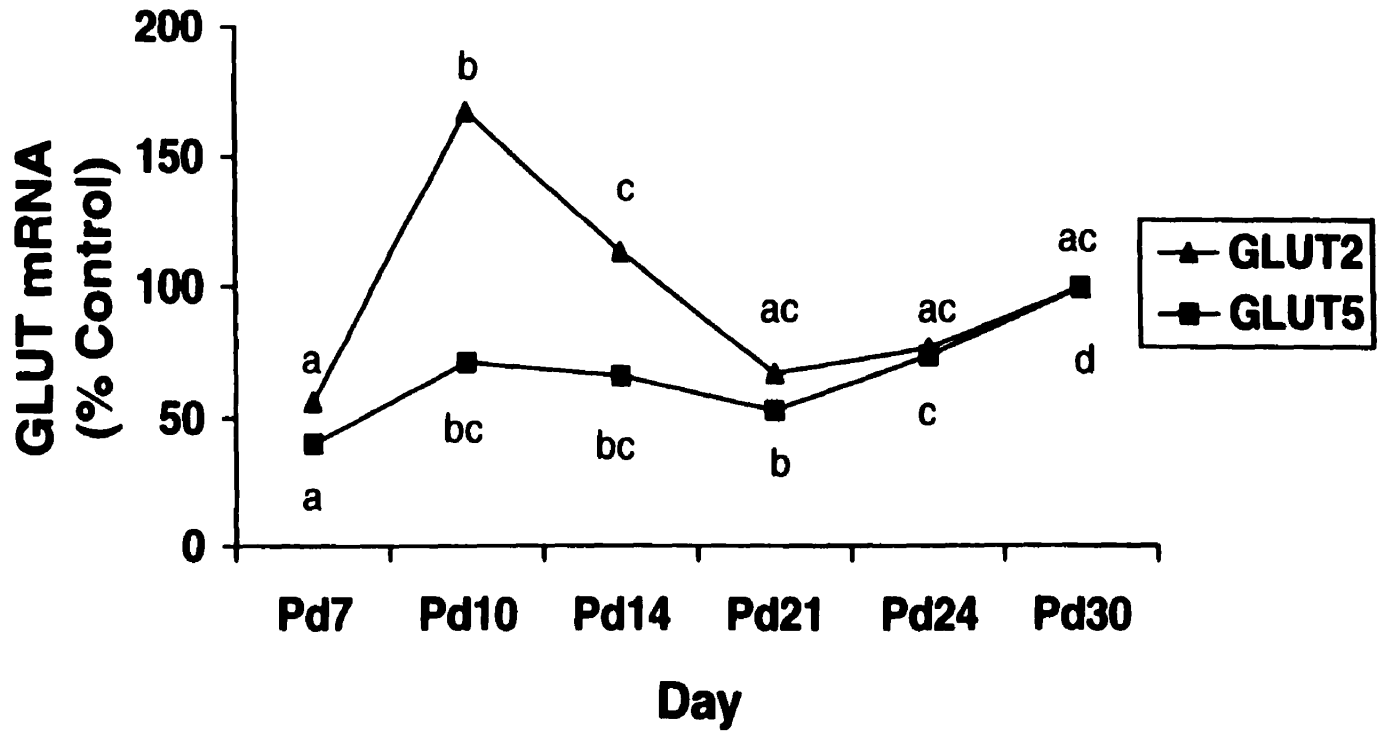
As described earlier, glucose plays a very important and multidimensional role in the mammalian system. It is a key metabolic substrate for mammalian cells (Devaskar & Mueckler, 1992), supplying ATP under aerobic and anaerobic conditions (Bell *et al.*, 1993), it is a precursor for the biosynthesis of glycogen and fat as well as sugar-containing macromolecules such as glycoproteins, glycolipids and nucleic acids (Devaskar & Mueckler, 1992) and it is a required energy source for some tissues (*e.g.* brain) (Devaskar & Mueckler, 1992). Knowing the important role glucose plays in the mammalian system and the fact that glucose transporters are the only means by which glucose can enter or

Figure II-8: Developmental Changes in GLUT mRNA Expression in the Rat Jejunum



Based on Miyamoto *et al.*, 1992

Figure II-9: Developmental Changes in GLUT mRNA Expression in the Rat Jejunum as a Percentage of Control (Day 30 Rats)



Based on Reimer *et al.*, 1997

leave a cell, the importance of understanding factors which change glucose transporter expression is evident. Several of these factors, which alter glucose transporter expression, have been studied in adults and during development. They are summarized in the following sections.

2.3.1 Factors in Adulthood

Exercise:

The effect of exercise training on glucose transporter expression has been studied using human as well as animal models. In order to examine if changes in skeletal muscle GLUT4 levels occur with exercise training and to determine the time course over which such changes might occur, Neufer *et al.* in 1992 analyzed GLUT4 levels in rat skeletal muscle after 1 day, 1 week and 6 weeks of treadmill exercise training. The authors of this study found no change in skeletal muscle GLUT4 levels after 1 day or 1 week of training but when the length of exercise training was extended to 6 weeks, significant increases were seen in the GLUT4 content of rat skeletal muscle. This finding supported previous work done by Rodnick *et al.* in 1990 which also showed an increase in skeletal muscle GLUT4 levels with 6 weeks of voluntary running in exercise-wheel cages. This increase in rat skeletal muscle GLUT4 seen with exercise training was however reversed with only 1 week of detraining (Neufer *et al.*, 1992). Similar results have been found in human studies looking into changes in skeletal muscle GLUT4 levels with exercise training. In an effort to determine if GLUT4 levels increase with training using a human model, a possible explanation for the improved insulin sensitivity seen with exercise, Houmard *et al.* in 1993 placed previously sedentary men on a 14 week exercise training schedule. The authors of this study showed that GLUT4 levels in skeletal muscle did indeed increase, as they did in rats, with 14 weeks of endurance-oriented exercise training while McCoy *et al.* in 1994 showed that skeletal muscle GLUT4 levels decreased significantly, as they did in rats, with detraining.

Fasting:

Hypothesizing that decreases in the level of glucose transporter expression in insulin-sensitive tissues may be in part responsible for the insulin

resistance seen with fasting, Berger *et al.* in 1989 and Charron & Kahn in 1990 investigated the effect of fasting on GLUT1 and GLUT4 expression in rat adipose tissue and skeletal muscle. These authors found that the changes seen in glucose transporter levels with fasting differed between tissues. In adipose tissue, GLUT1 levels did not change with 3 days of fasting (Berger *et al.*, 1989) while GLUT4 levels decreased to 40% of the control level with 3 days of fasting (Berger *et al.*, 1989; Charron & Kahn, 1990). On the other hand, in skeletal muscle, GLUT1 and GLUT4 levels both increased, 2 fold and 3 fold respectively, with 2-4 days of fasting (Charron & Kahn, 1990). Thorens *et al.* in 1990 took this investigation one step further by examining alterations in glucose transporter expression in the liver, a non-insulin sensitive tissue, with fasting. These authors found that liver GLUT1 levels increased 4 fold with 4 days of fasting while GLUT2 levels remain unchanged with 4 days of fasting (Thorens *et al.*, 1990). Taken together, these studies not only indicated that adult glucose transporter levels were altered with fasting, they point out the tissue specific nature of these changes. This tissue specific regulation of glucose transporter expression indicates an adaptive response to maintain adequate cellular nutrition in times of altered nutrient availability (Kahn, 1994).

Refeeding:

Berger *et al.* in 1989 and Charron & Kahn in 1990, in conjunction with their studies investigating how fasting modulates glucose transporter expression, also investigated how refeeding following a fast modulates glucose transporter levels in adipose tissue and skeletal muscle. Adult adipose tissue GLUT4 levels increased, following a 3 day fast, with only 1 day of refeeding (Berger *et al.*, 1989). These levels continued to increase, with daily refeeding, for 7 days resulting in GLUT4 levels 1.9 fold higher than control levels (Berger *et al.*, 1989). Adipose tissue GLUT1 levels were not examined. Skeletal muscle GLUT1 and GLUT4 levels, after increasing with fasting, returned to control values with refeeding (Charron & Kahn, 1990). Thorens *et al.* in 1990 did not examine how refeeding modulates adult liver GLUT1 levels but did examine how refeeding alters liver GLUT2 levels and found they increased with refeeding, after showing

no change with fasting. The results from these studies indicated once again a tissue specific effect. Refeeding following a fast resulted in increased adult glucose transporter levels in adipose tissue and liver while skeletal muscle glucose transporter levels return to normal with refeeding following a fast.

Diet Composition:

Glucose transporter levels can also be altered by diet composition in the adult rat (Pederson *et al.*, 1991; Kahn & Pederson, 1993; Burant & Saxena, 1994). Pederson *et al.* in 1991 fed a high fat (80% of energy) diet to adult male rats for 7 weeks at which time adipocyte GLUT1 and GLUT4 levels were measured. The results from this study showed a significant decrease in adipocyte GLUT1 and GLUT4 levels with 7 weeks of high fat feeding. Similar findings emerged from a 1993 study by Kahn & Pederson which also showed significant decreases in GLUT1 and GLUT4 levels, this time in skeletal muscle, with high fat feeding. Burant & Saxena, in 1994, examined GLUT2 and GLUT5 levels in the kidney, GLUT2 levels in the liver and SGLT1, GLUT2 and GLUT5 levels in the small intestine after feeding fructose and glucose enriched (~60% of energy) diets. The authors of this study found no change in kidney GLUT2 levels with the fructose enriched diet while a small increase in kidney GLUT2 levels was seen with the glucose enriched diet. Opposite results were found when kidney GLUT5 levels were examined. Kidney GLUT5 levels increased with the fructose enriched diet but did not change with the glucose enriched diet. Liver GLUT2 levels did not change with the feeding of either the fructose or glucose enriched diets. Small intestine SGLT1 levels did not increase with the fructose enriched diet while large increases in SGLT1 were seen with the glucose enriched diet. Similar results were found for small intestine GLUT2 levels. GLUT2 levels increased only slightly with the fructose enriched diet while a significant rise in GLUT2 was seen after feeding of the glucose enriched diet. Small intestine GLUT5 proteins behaved in opposing fashion to the SGLT1 and GLUT2 proteins. GLUT5 protein levels increased in the small intestine with fructose enriched feeding while glucose feeding had no effect on small intestine GLUT5. Returning rats to a control diet (~46% glucose as starch) for 7 days after

the feeding of either fructose or glucose enriched diets resulted in the return of both GLUT2 and GLUT5 proteins to control levels. It is apparent from these studies that changes in the macronutrient composition of the diet, particularly the carbohydrate or fat content in the diet, can alter glucose transporter levels.

2.3.2 Factors During Development

During development, adult levels of glucose transporters have not yet been achieved (Werner *et al.*, 1989; Wang & Hu, 1991; Santalucia *et al.*, 1992; Wallace *et al.*, 1992; Chin *et al.*, 1993; Maher *et al.*, 1994; Postic *et al.*, 1994; Vannucci, 1994; You *et al.*, 1995; Reimer *et al.*, 1997). This increases the importance of understanding factors which alter glucose transporter expression during development because alterations in expression during development may result in permanent changes in adult glucose transporter expression as was found by Ozanne *et al.* in 1996. Despite this, few studies to date have examined factors which modulate glucose transporter expression during development. The following summaries describe the knowledge gained to date on how diet composition and maternal dietary intake alter glucose transporter expression during development.

Diet Composition:

Only three studies to date have examined the effect of diet composition on level of glucose transporter expression during development (Leturque *et al.*, 1991; Wallace *et al.*, 1992; Postic *et al.*, 1994). Insulin sensitivity in rats emerges after weaning from the suckling of high fat milk to the nibbling of high carbohydrate chow (Issad *et al.*, 1988). In order to investigate the role of glucose transporters in the development of this insulin sensitivity in adipose tissue and skeletal muscles at weaning in rats, Leturque *et al.* in 1991 compared glucose transporter levels in 30 day old rats weaned at day 21 to a high fat (70% of energy) diet or to a high carbohydrate (60% of energy) diet. For comparison purposes, glucose transporter levels in 15 day old suckling rats were also measured. The authors found no difference in adipose tissue or skeletal muscle GLUT1 mRNA expression between the three groups while adipose tissue and skeletal muscle GLUT4 protein levels were significantly higher in the group

weaned to the high carbohydrate diet compared to either the suckling group or the group weaned to the high fat diet. Similar results were found in 1992 by Wallace *et al.* who also examined the effect weaning to either a high carbohydrate (69% carbohydrate) or high fat (59% fat) diet. These authors also found that skeletal muscle GLUT4 mRNA was significantly higher in the group weaned to the high carbohydrate diet compared to the level of expression found in the group weaned to the high fat diet. Postic *et al.* in 1994, using a study design similar to that of Leturque *et al.*, examined the effect of weaning to either a high fat (70% of energy) or high carbohydrate (60% of energy) diet on GLUT2 mRNA levels in the liver and GLUT4 mRNA levels in the heart and skeletal muscle. The authors of this study found that the consumption of a high carbohydrate diet by rats after weaning increased GLUT2 mRNA levels in the liver and GLUT4 mRNA levels in the heart and skeletal muscle whereas consumption of a high fat diet after weaning prevented these changes, producing a pattern of developmental expression fundamentally different from that seen in the high carbohydrate group. The results of these studies indicate that changes in the macronutrient composition of the diet consumed during development, specifically the carbohydrate and fat content of the diet, not only produces changes in glucose transporter levels, it also alters the pattern of glucose transporter expression normally seen with the feeding of high carbohydrate chow at weaning.

Maternal Dietary Intake:

Only one study to date has examined the effect of maternal dietary intake on glucose transporter levels in offspring (Ozanne *et al.*, 1996). In this study, pregnant rat dams were fed either a low protein (8% protein) or control (20% protein) diet. The experimental diets were identical in fat content but differed in carbohydrate content, containing 67% and 54% carbohydrate respectively (Snoeck *et al.*, 1990). These diets were fed to the dams throughout pregnancy and lactation. At weaning, all pups were fed the control (20% protein, 54% carbohydrate) diet for 9 weeks at which time differences in liver GLUT2 levels were examined. Results from this study showed that the GLUT2 protein

concentration in the livers of the adult offspring of the dams fed the low protein (8% protein, 67% carbohydrate) diet was significantly higher, more than 3 fold higher, than that seen in the adult offspring of the dams fed the control (20% protein, 54% carbohydrate) diet, despite the consumption of the control diet by all offspring for 9 weeks. Thus, in this study, differences in maternal dietary intake produced permanent alterations in offspring glucose transporter levels.

2.4 SUMMARY

Glucose transporters not only exhibit distinct tissue distributions (Burant *et al.*, 1991), they are also developmentally regulated (Burant *et al.*, 1991), resulting in distinct patterns of tissue expression at critical timepoints during development. The level of glucose transporters expressed in adult rat tissues can be changed with exercise (Rodnick *et al.*, 1990; Neufer *et al.*, 1992), fasting (Berger *et al.*, 1989; Charron & Kahn, 1990; Thorens *et al.*, 1990), refeeding (Berger *et al.*, 1989; Charron & Kahn, 1990; Thorens *et al.*, 1990) and with changes in the macronutrient composition of the diet, in particular by changes in the fat and carbohydrate content of the diet (Pederson *et al.*, 1991; Kahn & Pederson, 1993; Burant & Saxena, 1994). During development, glucose transporter levels are also changed by the fat and carbohydrate content of the diet (Leturque *et al.*, 1991; Wallace *et al.*, 1992; Postic *et al.*, 1994). The three developmental studies by Leturque *et al.*, Wallace *et al.*, and Postic *et al.* showed that changes in the fat and carbohydrate content of the diet during development not only altered glucose transporter levels, they also altered the pattern of developmental expression, illustrated by the finding that weaning to a high fat diet prevented the normal increase in glucose transporter expression seen with weaning to a high carbohydrate diet. However, because the experimental diets used in these studies varied greatly in their fat and carbohydrate content and because the diets were only fed to the pups at weaning, these studies do not allow any insight into the effect of more moderate alterations in diet composition or into the effect such

macronutrient alterations might have if fed to the mother, throughout gestation and lactation.

2.5 STATEMENT OF PURPOSE

The effect of restricted maternal glucose intake during gestation on pup survival, pup weight, pup plasma parameters and pup tissue glycogen content has been well established (Taylor *et al.*, 1983; Koski & Hill, 1986; Fergusson & Koski, 1990; Koski & Hill, 1990; Koski *et al.*, 1990; Koski *et al.*, 1993; Cobrin & Koski, 1995). Feeding a glucose-restricted diet during gestation has been shown to result in increased pup mortality (Koski & Hill, 1986; Koski *et al.*, 1990) and reduced birth weights (Koski & Hill, 1986; Koski & Hill, 1990). Plasma glucose and insulin measured in offspring at term were also compromised by maternal dietary glucose restriction (Cobrin & Koski, 1995) as were brain (Koski *et al.*, 1993), liver and heart glycogen concentrations (Fergusson & Koski, 1990; Cobrin & Koski, 1995).

Previous studies have also established that the fat and carbohydrate contents of the diet at weaning can alter not only glucose transporter levels (Leturque *et al.*, 1991; Wallace *et al.*, 1992; Postic *et al.*, 1994) but also the developmental pattern of glucose transporter expression (Leturque *et al.*, 1991; Wallace *et al.*, 1992; Postic *et al.*, 1994). The effect of restricted maternal intake during gestation on pup glucose transporter levels however has only been examined in one study (Ozanne *et al.*, 1996). This study examined the influence of maternal diet on GLUT2 protein levels in the livers of offspring at 12 weeks of age. Two experimental diets were used in this study, a control diet containing 20% protein and 54% carbohydrate and a low protein diet which contained 8% protein and 67% carbohydrate. This type of study design, using only two experimental diets, does not permit one to determine which macronutrient is responsible for any changes seen. Also, because results in this study were only obtained at one timepoint, it does not allow the reader to know if the developmental pattern of glucose transporter expression changed. It was

therefore the aim of this study to investigate if differences in the macronutrient content of the maternal diet, specifically carbohydrate differences, would perturb not only glucose transporter levels in offspring but also their developmental pattern of expression. The hypothesis was that low maternal dietary glucose intake throughout pregnancy and lactation would perturb both the quantity of glucose transporters expressed as well as the developmental pattern of glucose transporter expression in offspring. The specific objectives were to:

1) describe the developmental profile of glucose transporter proteins in the small intestine, liver and kidney;

2) determine if dietary glucose restriction alters the aforementioned developmental profiles; and

3) determine whether any changes in glucose transporter levels are associated with tissue specific glycogen levels.

CHAPTER 3 - MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

This study was designed to examine the effect of feeding graded levels of dietary carbohydrate to pregnant and lactating rat dams on the developmental expression of glucose transporters in the offspring. Pregnant rat dams, received on day 0 of gestation, were randomized into one of four dietary treatment protocols: 0, 12, 24 or 60% glucose diets. In order to assess the developmental profile of glucose transporters in the offspring of these dams, within each dietary group, offspring were killed at gestation day (gd) 20, birth, 12-24hrs postpartum, postnatal day (pd) 7, 15, 21, 28, 35 and 49.

3.2 EXPERIMENTAL PROTOCOL

Experimental diets containing graded levels of glucose (0, 12, 24 and 60% glucose by weight) were fed to pregnant rat dams from gd 0 through weaning. These diets were categorized as glucose-deficient (0% glucose), glucose-restricted (12% and 24% glucose) and adequate glucose (60% glucose). Plasma, small intestines, livers and kidneys from the offspring of these dams were collected at the following developmental time points: gd20, birth, 12-24hrs postpartum, pd7, pd15, pd21, pd28, pd35 and pd49. These time points were in turn categorized as perinatal (gd20, birth and 12-24hrs postpartum), suckling (pd7, pd15 and pd21) and post-weaning (pd28, pd35 and pd49). Plasma, kidneys, livers and small intestines were also collected from the dams at gd20 and, for the dams used in the postnatal portion of the experiment, at pd49. These pd49 dams are considered adult controls as they consumed the experimental diets from the time of arrival at gd0 through pd49. Whole litters were pooled for measurements at the gd20, birth and 12-24hrs postpartum timepoints while individual pups from the same litters were used during the postnatal portion of the experiment. The postnatal litters were culled to seven

pups at pd7 to allow for 2 pups from each litter to be sacrificed at pd15 and pd21 and for 1 pup from each litter to be sacrificed at pd28, pd35 and pd49. Only three of the experimental diets (12, 24 and 60% glucose) were used in the postnatal portion of the experiment because of the high levels of perinatal mortality seen with the feeding of 0% carbohydrate diets in this experiment and a previous experiment using these same diets (Koski & Hill, 1986). The total number of litters sacrificed for the perinatal timepoints are shown in **Table III-1**. The total number of litters and pups sacrificed for the suckling and post-weaning timepoints are shown in **Table III-2**.

Table III-1: Number of litters sacrificed for perinatal timepoints

	Perinatal		
Diet	Gd20[1]	Birth[1]	12-24Hrs Postpartum[1]
0%	9	5	4
12%	9	8	7
24%	9	8	8
60%	9	8	8

[1] Number of litters

Table III-2: Number of litters and pups sacrificed for the suckling and post-weaning timepoints

		Suckling			Post-Weaning		
Diet	n[1]	Pd7[2]	Pd15[2]	Pd21[2]	Pd28[2]	Pd35[2]	Pd49[2]
12%	8	variable	16	16	8	8	8
24%	9	variable	18	18	9	9	9
60%	9	variable	18	18	9	9	9

[1] Number of litters
[2] Number of pups

3.3 EXPERIMENTAL ANIMALS

Time-bred female Sprague-Dawley rats (180-200g) were obtained from Charles River (St. Constant, QC) at gd0 and randomly assigned to one of the four experimental diets. The rats were maintained in a temperature controlled animal room (22-25°C) with a 12 hour light cycle from 07h00 to 19h00. The dams were housed individually in maternity cages with the experimental diets and water available *ad libitum*. All procedures used were approved by the McGill

University Animal Resource Center and conformed with the Canadian Council on Animal Care (1984).

One hundred and forty one animals were received in total. Rats were excluded from all treatments in the study for the following reasons: not pregnant (6), pups present in only one uterine horn (2), dam unable to eat properly because of malaligned teeth (3) and infection (1). This resulted in a total of one hundred and twenty nine dams for whom data was collected for use in the food intake and maternal body weight analyses. Offspring data was only available for the offspring of one hundred and eighteen dams for the following reasons: maternal death during labor (1), abnormal cannibalistic behavior (2) and high within litter pup mortality (8). This total therefore allowed for 36 litters to be sacrificed at gd20, 29 litters to be sacrificed at birth, 27 litters to be sacrificed at 12-24hrs postpartum and 26 litters to be used for the postnatal portion of the experiment **(Tables III-1 and III-2)**.

3.4 EXPERIMENTAL DIETS

3.4.1 Diet Formulation

The experimental diets used in this study contained 0, 12, 24 and 60% glucose. The diets were made isocaloric by the equienergetic substitution of soy oil for carbohydrate and replacement of the difference in weight with cellulose. The 0% glucose diet was selected because it contains close to 4% glycerol which was previously determined as the amount of glucose equivalents required to support implantation and maintain pregnancy to term (Koski *et al.*, 1986). The 12% glucose diet was chosen because this level of glucose is known to be suboptimal, resulting in reduced growth (Lanoue & Koski, 1994) and reduced liver and heart glycogen concentrations (Cobrin & Koski, 1995). The 24% glucose diet was selected because it does not result in poor growth (Lanoue & Koski, 1994) but does compromise other parameters such as milk composition (Lanoue & Koski, 1994). The 60% glucose diet was used because it is the standard control diet used in reproductive studies. By selecting experimental

diets with graded levels of carbohydrate, it allowed determination of whether the carbohydrate or lipid content of the diets was responsible for any diet effects seen. For example, if any differences were seen between the 0% CHO and 12% CHO groups, this difference could be said to be due to a carbohydrate effect because these two diets have comparable lipid levels (39.47g and 34.74g respectively). However, since the lipid content of the 60% CHO diet is much lower (15.83g) than that of the 0% CHO diet, any difference seen between the 60% CHO group and the 0% CHO group could be due to a carbohydrate or lipid effect.

3.4.2 Diet Composition

The experimental diets used in this study were formulated to meet all NRC rat nutrient requirements for reproduction (Nutrient Requirements of Laboratory Animals, 1995) except for the level of carbohydrate. Each diet contained 4.15kcal (17.4kJ) metabolizable energy per gram dry matter. The protein, methionine, sodium bicarbonate, vitamin and mineral content of the diets was constant across formulations. The level of protein in the diets was set at 11g/100g dry weight, thus providing 26.5mg protein/kcal diet. Methionine was added to all diets at a level of 0.34g/100g dry weight. Casein supplemented with methionine is considered to provide a nutritionally adequate protein source equivalent in quality to lactalbumin (Nutrient Requirements of Laboratory Animals, 1995). Therefore, all diets provided 26.5mg protein/kcal diet with a quality equivalent to that of lactalbumin and thus fall within the range of 25-31mg lactalbumin protein/kcal diet outlined in the NRC guidelines (1995). Excess protein was not added to the diets as this would have provided additional gluconeogenic precursors. Vitamins were added at levels exceeding the NRC recommendations by a factor of approximately 2 for the fat soluble vitamins and 4-5 for the water soluble vitamins while the minerals were added at levels exceeding the recommendations by a factor of approximately 2.5. These levels were set to insure, should food intake be low, adequate vitamin and mineral intake. Sodium bicarbonate was added at a level of 1g/100g diet in order to

correct for the metabolic acidosis expected with the feeding of carbohydrate restricted diets.

Table III-3 shows the composition of 100g of each experimental diet. The metabolizable energy of the dietary constituents used in the experimental diets are outlined in **Table III-4**. In **Table III-5**, the NRC requirements for vitamins are presented, along with the previously described ratio for adequacy. The resulting mg/kg diet was used to determine the formulation of the vitamin mixtures. The fat and water soluble vitamin mixtures were prepared separately and were formulated to contain enough vitamin in the 800g mixture that when added to the diet at a level of 4g/kg diet would achieve the required mg/kg level (*e.g.* the 800g water soluble vitamin mixture contained 15g of niacin; when 4g of this water soluble vitamin mixture, which would contain 0.075g of niacin, was added to 1 kg of diet, the resulting niacin concentration was the required 75mg/kg diet) **(Table III-6)**. Mineral mix was added to the experimental diets at a level of 6.5%, a level determined from the combined weights of all mineral compounds (*e.g.* $CaHPO_4$) required to achieve the desired level of elemental mineral (*e.g.* Ca) **(Tables III-7 and III-8)**. The mineral mix **(Table III-9)** was formulated to contain enough mineral in the 1kg mix that when added to the diet at a level of 6.5g/kg diet would achieve the required mg/100g diet.

3.4.3 Diet Preparation

In the making of the experimental diets, vitamin and mineral mixes were prepared first. The experimental diets were then prepared using a two step procedure. All dry ingredients were mixed first, in ascending order of weight. The soy oil was then added and all ingredients were thoroughly mixed. The diets were then stored at 4°C in sealed containers until used in order to prevent spoilage or contamination.

Table III-3: Diet Compositions[1]

Ingredients	0% g	12% g	24% g	60% g
Carbohydrate[2]	0	12.0	24.0	60.0
Soy Oil[3]	39.47	34.74	30.01	15.83
Cellulose[4]	40.49	33.22	25.95	4.13
Casein[5]	11.0	11.0	11.0	11.0
Vitamin Mix[6]	1.2	1.2	1.2	1.2
Mineral Mix[7]	6.5	6.5	6.5	6.5
Methionine	0.34	0.34	0.34	0.34
Sodium Bicarbonate	1.0	1.0	1.0	1.0
Weight (g)	100.0	100.0	100.0	100.0
Metabolizable Energy (kcal/g)[8]	4.15	4.15	4.15	4.15
(MJ/g)	17.36	17.36	17.36	17.36

[1]Dry weight basis
[2]Dextrose (anhydrous): ICN Biomedicals Canada Ltd., Montreal, QC
[3]Degummed soybean oil: Canada Packers Inc., Montreal, QC
[4]Alphacel: ICN Biomedicals Canada Ltd., Montreal, QC
[5]High-nitrogen casein: ICN Biomedicals Canada Ltd., Montreal, QC
[6]See Table III-5
[7]See Table III-9
[8]See Table III-4

Table III-4: Metabolizable Energy of Dietary Ingredients

Ingredients	Metabolizable Energy (kcal/g dry matter)	Reference/Assumption
Glucose	3.64	Anderson *et al.*, 1958
Casein	4.45	Kriss & Miller, 1934
Amino Acid	4.00	Assuming complete absorption
Soybean Oil	9.24	Renner & Hill, 1961; Young, 1961

Table III-5: Composition of Vitamin Mixture

Ingredients	NRC Requirements[1] mg/kg diet	Experimental Diets mg/kg diet	Ratio[2]
Niacin	15.0	75.0	5.0
Calcium Pantothenate	10.0	40.0	4.0
Riboflavin	4.0	16.0	4.0
Pyridoxine Hydrochloride	6.0	24.0	4.0
Thiamin Hydrochloride	4.0	16.0	4.0
Folacin	1.0	4.0	4.0
Biotin	0.2	0.8	4.0
Cyanocobalamine	0.05	0.2	4.0
Alpha-tocopherol Acetate[3]	18.0	36.0	2.0
Menaquinone	-	-	-
Choline Chloride	-	4000	-
Cholecalciferol[4]	1000 IU	1500 IU	1.5
Vitamin A Palmitate[5]	2300 IU	4600 IU	2.0

[1]National Research Council (1995) Nutrient Requirements of Laboratory Animals, National Academy of Sciences, Washington, DC
[2]Ratio of Experimental Diets: NRC Requirements
[3]Supplied as 1 000 IU/g DL-alpha-tocopheryl acetate: ICN Biomedicals Canada Ltd., Montreal, QC
[4]Supplied as 400 000 IU Vitamin D_3/g: ICN Biomedicals Canada Ltd., Montreal, QC
[5]Supplied as 250 000 IU/g: ICN Biomedicals Canada Ltd., Montreal, QC

Table III-6: Formulation of Vitamin Mixture

Ingredients		grams	g/kg diet
FAT SOLUBLE			
Vitamin A Palmitate		3.68	
Cholecalciferol		0.75	
Alpha-tocopherol Acetate		10.8	
Phyllaquinone		0.8	
Cellulose[1]		783.97	
	TOTAL	800.0	4
WATER SOLUBLE			
Niacin		15.0	
Calcium Pantothenate		8.0	
Riboflavin		3.2	
Pyridoxine Hydrochloride		4.8	
Thiamine Hydrochloride		3.2	
Folacin		0.8	
Biotin		0.16	
Cyanocobalamine		0.04	
Butylated Hydroxytoluene		20.0	
Cellulose[1]		744.8	
	TOTAL	800.0	4
CHOLINE CHLORIDE			4
TOTAL VITAMIN MIXTURE			12 g/kg diet

[1]Alphacel: ICN Biomedicals Canada Ltd., Montreal, QC

Table III-7: Composition of Macroelements

Ingredients (MW)	mg/100g diet	Ca	K	Mg	Na	P	Cl	S
					mg/100g diet			
$CaHPO_4$ (136.06)	3422	1008				779		
$KHCO_3$ (100.11)	2028		792					
$MgSO_4$ (120.38)	654			132				174
NaCl (58.44)	282				111		171	
Totals[1]		1008	792	132	111	779	171	174
NRC Requirements[2]		630	360	60	50	370	50	-
Ratio[3]		1.6	2.1	2.2	2.2	2.1	3.4	-

[1]Does not include additional sodium supplied by $NaHCO_3$
[2]National Research Council (1995) Nutrient Requirements of Laboratory Animals, National Academy of Sciences, Washington, DC
[3]Ratio of Totals:NRC Requirements

Table III-8: Composition of Microelements

Ingredients (MW)	mg/kg diet	Fe	Zn	Mn	Cu	F	Cr	Se	Mo	I
		mg/kg diet								
$FeSO_4 \bullet 7H_2O$ (278.02)	746.7	150								
$ZnCO_3$ (125.38)	105.49		55.00							
$MnCO_3$ (114.94)	41.84			20.00						
$CuCO_3 \bullet Cu(OH)_2 \bullet H_2O$ (239.19)	33.10				17.6					
$KF \bullet 2H_2O$ (94.13)	10.90					2.2				
$CrK(SO_4)_2 \bullet 12H_2O$ (499.40)	6.34						0.66			
Na_2SeO_3 (172.92)	1.927							0.88		
$Na_2MoO_4 \bullet 2H_2O$ (241.95)	0.832								0.33	
KIO_3 (214.02)	0.556									0.33
Totals		150	55.00	20.00	17.6	2.2	0.66	0.88	0.33	0.33
NRC Requirements[1]		75.0	25.0	10.00	8.0	1.00	-	0.400	0.150	0.150
Ratio[2]		2.0	2.2	2.0	2.2	2.2	-	2.2	2.2	2.2

[1]National Research Council (1995) Nutrient Requirements of Laboratory Animals, National Academy of Sciences, Washington, DC
[2]Ratio of Totals:NRC Requirements

Table III-9: Composition of Mineral Mixture

Ingredients	g/kg salt	g/kg diet @ 6.5%
$CaHPO_4$	527.60	34.22
$KHCO_3$	312.67	20.28
$MgSO_4$	100.83	6.54
NaCl	43.48	2.82
$FeSO_4 \cdot 7H_2O$	11.512	0.7467
$ZnCO_3$	1.6266	0.1055
$MnCO_3$	0.6455	0.0418
$CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$	0.5103	0.0331
$KF \cdot 2H_2O$	0.1681	0.0109
$CrK(SO_4)_2 \cdot 12H_2O$	0.0971	0.0063
Na_2SeO_3	0.0293	0.0019
$Na_2MoO_4 \cdot 2H_2O$	0.0123	0.0008
KIO_3	0.0093	0.0006
Subtotal	999.19	64.81
Citric Acid	0.81	0.05
TOTAL	1000.00	64.86

3.5 FOOD INTAKE AND BODY WEIGHT

Maternal food intake was measured and recorded every second day throughout gestation. Daily food intakes were calculated by dividing this two day total in half. For those litters used in the postnatal portion of the experiment, food intake was determined for the cage as a unit, which included the dam and a specific number of pups which varied with the time course of the study but was comparable across all diet groups at any given time during the study.

Maternal body weights were also measured and recorded every second day throughout gestation. Daily body weights were determined by averaging two successive measurements. Maternal and pup body weights were measured every second day for those litters used in the postnatal portion of the experiment. Once again the maternal body weights were averaged in order to determine daily body weights however the same could not be done for the pups because individual pups were not tracked from day to day.

3.6 AMNIOTIC FLUID, PLASMA AND TISSUE COLLECTION

The pregnant dams sacrificed at gd20 were killed by ketamine-HCl injection at 30mg/kg body weight into the jugular region accompanied by exsanguination by cardiac puncture. The gd20 fetuses were then removed by cesarean section, with the collection and pooling of amniotic fluid for each litter. The pups sacrificed at birth, at 12-24hrs postpartum and those sacrificed as part of the postnatal experiment were also killed by ketamine-HCl injection and exsanguination. The blood collected during the maternal and pup exsanguination procedures was centrifuged and the plasma stored at -80°C until analyzed. Following the exsanguination, the abdomen of the pups were opened and the kidneys, livers and small intestines removed. The tissues were then frozen in liquid nitrogen and stored at -80°C until analyzed.

3.7 ANALYTICAL PROCEDURES

3.7.1 Plasma Albumin

Plasma albumin was determined colorimetrically (Sigma, St. Louis) using an Abbott VP Super System (Irving, Texas). The colorimetric determination of plasma albumin is based on the binding reaction of albumin to bromcresol green. This reaction produces a blue green color with an absorbance maximum at 628 nm. The intensity of the color produced is directly proportional to the albumin content in the sample. The procedure used is a modification of the method of Doumas *et al.* (1971).

3.7.2 Plasma and Amniotic Fluid Glucose

Plasma and amniotic fluid glucose was determined enzymatically (Sigma, St. Louis) using an Abbott VP Super System (Irving, Texas). The enzymatic determination of plasma and amniotic fluid glucose is based on the following reactions. Glucose is phosphorylated to glucose-6-phosphate (G6P) by hexokinase. G6P is then oxidized to phosphogluconate by G6P dehydrogenase with a concomitant reduction of NAD to NADH. NADH has an absorption maximum at 340 nm. The increase in absorbance at 340 nm is then proportional to the glucose concentration. The procedure used is similar to that described by Bondar and Mead (1974).

3.7.3 Plasma and Amniotic Fluid β-Hydroxybutyrate

Plasma and amniotic fluid β-hydroxybutyrate was determined enzymatically (Sigma, St. Louis) using an Abbott VP Super System (Irving, Texas). The enzymatic determination of plasma and amniotic fluid β-hydroxybutyrate is based on the oxidation of β-hydroxybutyrate to acetoacetate by β-hydroxybutyrate dehydrogenase. During this oxidation, NAD is reduced to NADH. NADH has an absorption maximum at 340 nm. The increase in absorbance at 340 nm is then proportional to the β-hydroxybutyrate concentration. The procedure used was initially described by Williamson *et al.* (1962).

3.7.4 Plasma and Amniotic Fluid Urea

Plasma and amniotic fluid urea was determined enzymatically (Sigma, St. Louis) using an Abbott VP Super System (Irving, Texas). The enzymatic determination of plasma and amniotic fluid urea is based on the following reactions. Urea is hydrolyzed to ammonia and carbon dioxide by urease. The ammonia produced then serves to aminate α-ketoglutarate to glutamate with the concurrent oxidation of NADH to NAD, a reaction catalyzed by glutamate dehydrogenase. The decrease in absorbance at 340 nm, resulting from the oxidation of NADH to NAD, is directly proportional to the urea content in the sample. The procedure used is based on Talke and Schubert (1965).

3.7.5 Liver and Kidney Glycogen

Liver and kidney samples from each experimental diet group at each developmental timepoint were analyzed for glycogen content using the spectrophotometric method of Lo *et al.*, 1970. All samples were analyzed in duplicate. In the method of Lo *et al.*, the tissue sample is digested with a strong alkali (KOH) after which the tissue glycogen is isolated by precipitation with ethanol. The glycogen is then hydrolyzed in the presence of sulfuric acid to glucose which combines with phenol to produce a colorimetric reaction. The glycogen content of the samples is then determined by reading the absorbance of the end product at 490 nm on a spectrophotometer and comparing the results obtained with the results from standards of known glycogen content.

3.7.6 Small Intestine GLUT2

Proximal and distal small intestine tissue samples were analyzed for GLUT2 content using a modified version of the methodology described by Jorgenson in 1988. All samples were analyzed in duplicate. Small intestine samples were prepared by homogenization of the tissue in 8mL of homogenization buffer, containing aprotinin, benzamidine, iodoacetamide, leupeptin and phenylmethylsulfonyl fluoride, using a Polytron (Brinkman) homogenizer. Homogenates were centrifuged for 15 minutes at 7100 RPM in the SS-34 rotor of an RC5B centrifuge (Sorvall). The resulting pellet was re-extracted in 2mL of homogenization buffer and centrifuged again for 15 minutes at 7100

RPM while the supernatant was kept on ice. The supernatant from the second centrifugation was then combined with that from the first and the resultant supernatant centrifuged for 55 minutes at 19 300 RPM in a Beckman 50.2 Ti rotor. The resulting pellet was resuspended in homogenization buffer and frozen at -80°C until subsequent protein determination. The protein content of the samples was determined using a dye-binding BioRad Protein Assay with BSA as a standard (BioRad, Mississauga, ON).

After the protein content of the samples was determined, the samples were combined with Laemmli sample buffer dye containing 4% SDS and resolved on 10% SDS-polyacrylamide gels (Laemmli, 1970). Electrotransfer to nitrocellulose membranes was performed at 4°C (Towbin et al., 1979). After the transfer, the membranes were washed in TBS (150mM NaCl, 30mM Tris) containing 0.5% Tween-20 for 10 minutes and then incubated in BLOTTO (5% nonfat dry milk, 0.5% Tween-20, 150mM NaCl, 30mM Tris) for 1 hour. After incubation in the BLOTTO, the membranes were incubated with primary antibodies diluted 1:500 in TBS containing 0.5% Tween-20 and dry milk. After primary antibody incubation, the membranes were washed in TBS containing 0.5% Tween-20 at room temperature for 30 minutes. The membranes were then incubated with secondary antibodies, again diluted 1:500 in TBS containing 0.5% Tween-20 and dry milk, for 45 minutes. The membranes were finally washed for 30 minutes in TBS containing 0.5% Tween-20 before exposure to Kodak BioMax Light films (Mandel Scientific, Hornby, ON).

3.7.7 Liver and Kidney GLUT2

Liver and kidney samples were analyzed for GLUT2 content using a modified version of the methodology described by Thorens *et al.* in 1988. All samples were analyzed in duplicate. Liver and kidney samples were prepared by homogenization of the tissue in 4mL of homogenization buffer, containing aprotinin, phenylmethylsulfonyl fluoride, and tosyl-lysine chloromethyl ketone, using a Polytron (Brinkman) homogenizer. Homogenates were centrifuged for 10 minutes at 8000 rpm in the SS-34 rotor of an RC5B centrifuge (Sorvall) and the supernatant centrifuged for 20 minutes at the same speed. The cytosol was then

centrifuged for 40 minutes at 43 000 rpm in a Beckman Ti 50.2 rotor and the membrane pellet resuspended in 0.25 M sucrose, 50 mM Tris (pH 7.4), 100 mM KCl and 5 mM $MgCl_2$. The protein content of the samples was determined using a dye-binding BioRad Protein Assay with BSA as a standard (BioRad, Mississauga, ON).

After the protein content of the samples was determined, the samples were combined with Laemmli sample buffer dye containing 4% SDS and resolved on 10% SDS-polyacrylamide gels (Laemmli, 1970). Electrotransfer to nitrocellulose membranes was performed at 4°C (Towbin *et al.*, 1979). After the transfer, the membranes were washed at room temperature for 10 minutes in distilled water, for 20 minutes in TBS (150 mM NaCl, 20 mM Tris) containing 0.1% Tween-20 and then for 30 minutes in BLOTTO (5% nonfat dry milk, 0.2% NP-40, 150mM NaCl, 20 mM Tris) at 37°C. After these washings, the membranes were incubated with primary antibodies, diluted 1:500 in BLOTTO, for 2 hours at room temperature. After the primary antibody incubation, the membranes were washed at room temperature for 20 minutes in TBS containing 0.2% NP-40, for 20 minutes in TBS containing 0.1% Tween-20 and then reblocked in BLOTTO. The blocked membranes were then incubated with secondary antibodies, again diluted 1:500 in BLOTTO, for 30 minutes at 37°C. The membranes were finally washed for 20 minutes in TBS containing 0.2% NP-40 and for 20 minutes in TBS containing 0.1% Tween-20 before exposure to Kodak BioMax Light films (Mandel Scientific, Hornby, ON).

3.8 STATISTICAL ANALYSIS

Homogeneity of variances were examined for all biochemical parameters using Bartlett's test (Steel & Torrie, 1980). Data sets with non-homogeneous variances were log transformed or, when this transformation did not result in homogeneous variances, observations were weighted by the reciprocal of the group variance. Cumulative food intake, maternal body weight, litter size and weight, pup body weight, amniotic fluid glucose, β-hydroxybutyrate and urea,

plasma albumin, glucose, β-hydroxybutyrate and urea as well as kidney and liver glycogen were analyzed for diet as a main effect using one-way ANOVAs. Small intestine GLUT2 content was analyzed for diet, region and time effects using a three-way ANOVA. When main effects were found, post hoc testing was done using Scheffe's method of multiple comparisons. For measurements made on individual pups, a nested design was used. A probability of $P < 0.05$ was accepted as the minimal level of significance. All results are presented as least square means (LSM) ± standard errors of least square means (SELSM). All statistical analyses were performed using SAS (Version 6.12, SAS Institute, Cary, OH). All statistical model statements used are described in Appendix 1.

CHAPTER 4 - RESULTS

Data tables and figures of results referred to in this section are presented at the end of this chapter. The figures presented are included to provide a visual representation of the data.

4.1 CUMULATIVE FOOD INTAKE AND MATERNAL BODY WEIGHT

4.1.1 Prenatal Cumulative Food Intake and Maternal Body Weight

Tables IV-1 and **IV-2** describe the effect of graded levels of dietary glucose on cumulative food intake and maternal body weight. Cumulative food intake during gestation (gestation day (gd) 0-19) was calculated and analyzed for diet effects. The only significant diet difference in cumulative food intake for this time period was between the 0% glucose (GLU) and 60% GLU groups with the 0% GLU group consuming significantly less food. No other group differences in cumulative food intake existed during pregnancy, thus the diets restricted in glucose (12% and 24% GLU) did not result in reduced food intake.

By the end of gestation (gd19) maternal body weights increased with the increasing glucose content of the diets while, at the time of arrival (gd0), maternal body weights did not differ. At the end of gestation (gd19), the dams fed the 0% GLU diet weighed significantly less than the dams fed the 12% or 24% GLU diets who in turn weighed less than the dams fed the 60% GLU diet. Thus, prepartum maternal body weights did not follow the same pattern as prenatal cumulative food intakes. Despite similar dietary intakes (12% and 24% GLU dams ate the same as the 60% GLU dams), inadequate glucose in the diet during gestation resulted in lower maternal body weights at the end of gestation (gd19).

4.1.2 Suckling Cumulative Food Intake and Maternal Body Weight

Cumulative food intake during suckling was calculated for each cage by week (postnatal day (pd) 0-7, pd8-15 and pd16-21), coinciding with the developmental timepoints at which plasma and tissues were collected. These

cumulative food intakes were then analyzed for diet effects. The litter size in each cage was constant across diets after culling of the litters at pd7. Cumulative food intake was significantly different between the 12% GLU group and the 60% GLU group for the suckling weeks (pd0-7, pd8-15 and pd16-21) with the 12% GLU group eating significantly more than the 60% GLU group. Neither the 12% GLU group nor the 60% GLU group differed significantly in cumulative food intake from the 24% GLU group during suckling.

While differences in cumulative food intake were found during the suckling timepoints (12% GLU group ate significantly more than the 60% GLU group), no differences in maternal body weights, which were analyzed at pd7, pd15 and pd21, were found during suckling. Therefore, although the 12% GLU group was eating significantly more than the 60% GLU group during suckling, no difference in maternal body weights existed between these groups during suckling. Thus, cumulative food intake in the 12% GLU group had to be higher in order to maintain a maternal body weight similar to that seen in the control glucose group (60% GLU group).

4.1.3 Post-Weaning Cumulative Food Intake and Maternal Body Weight

Post-weaning cumulative food intake was calculated for each cage by week (pd22-28, pd29-35, pd36-42 and pd43-48), coinciding with the developmental timepoints measured post-weaning, with litter size in each cage being constant across diets. Cumulative food intake was significantly different between the 12% GLU group and the 60% GLU group for the week pd29-35, with the 12% GLU group eating significantly more than the 60% GLU group. No other significant group differences in cumulative food intake were seen for the post-weaning timepoints.

Post-weaning maternal body weights were analyzed for diet effects at pd28, pd35, pd42 and pd49. At pd28 and pd35, the maternal body weights of the 12% GLU and 24% GLU groups differed significantly from the 60% GLU group, but did not differ significantly from one another. The dams fed the 60% GLU diet were heavier than those fed either the 12% GLU or 24% GLU diets. Therefore, although the 12% GLU group ate significantly more than the 60% GLU group for

the week pd29-35, the 12% GLU group weighed significantly less than the 60% GLU group, indicating that for the 12% GLU group, an increased food intake for the week pd29-35 was not enough to maintain a weight similar to that seen in the 60% GLU group. At pd42, the only significant group difference in maternal body weight was between the 12% GLU group and the 60% GLU group, with the 60% GLU group again being heavier. At pd49, an overall effect of diet was found but further analysis revealed no significant differences between diet groups when simple pairwise comparisons were used. Therefore, further analysis was done using other possible contrasts and it was found that the overall effect of diet at pd49 was due to a significant difference between the average of the 12% and 24% GLU diets and the 60% GLU diet. Graphic presentation of these results is presented in **Figures IV-1** and **IV-2**.

4.2 LITTER SIZE AND WEIGHT

Litter size and weight data are described in **Table IV-3** while a graphic representation of these results is presented in **Figure IV-3**. Litter size was analyzed for a main effect of diet using the litter data available from all perinatal timepoints (gd20, birth and 12-24hrs postpartum). Litter size was significantly different between the 0% GLU group and all other diet groups with the size of the litters being smaller for the 0% GLU group. No other diet group differences were found.

Litter weight was also analyzed for a main effect of diet using the litter weight data available from all perinatal timepoints (gd20, birth and 12-24hrs postpartum). Once again, the 0% GLU group differed significantly from all other diet groups with the weight of the litters being lighter for the 0% GLU group. No other diet group differences were found.

4.3 PUP BODY WEIGHT

Pup body weight data is described in **Table IV-4** while a graphic representation of these results is presented in **Figure IV-4**. In order to analyze the effect of diet on pup body weight a nested design was used. Results from this analysis showed that for those pups sacrificed at gd20 as well as for those sacrificed at 12-24hrs, significant differences existed in body weight between the 0% GLU group and all other diet groups with the 0% GLU group having significantly lower body weights than those from the other diet groups. For those pups sacrificed at birth, significant differences in body weight existed between the 0% GLU group and the 24% and 60% GLU groups but not between the 0% GLU group and the 12% GLU group. Once again, the 0% GLU group had significantly lower body weights than those seen in the 24% and 60% GLU groups.

No significant difference in pup body weights existed between diet groups at pd7 but at pd15, pd21 and pd28 significant differences in pup body weights were found between the 12% GLU and 60% GLU groups with the 12% GLU pups weighing significantly more than the pups from the 60% GLU group. While differences at these timepoints existed between the 12% and 60% GLU groups, the 24% GLU group did not differ significantly from either the 12% or 60% GLU groups. The higher pup body weights seen in the 12% GLU group at these timepoints coincides with the higher cumulative food intake seen in the 12% GLU group indicating a compensatory response by the 12% GLU group. At pd35 and pd49 no significant differences in pup weight were seen between any of the diet groups.

4.4 AMNIOTIC FLUID AND PLASMA PARAMETERS

4.4.1 Plasma Albumin

Plasma albumin data is described in **Table IV-5** while a graphic representation of these results is presented in **Figure IV-5**. No significant difference in plasma albumin was found at any timepoint between diet groups, indicating adequate protein intakes to prevent protein deficiency for all experimental diet groups.

4.4.2 Amniotic Fluid and Plasma Glucose

Table IV-6 describes the effect of graded levels of dietary glucose on amniotic fluid and plasma glucose. Results from the amniotic fluid analysis showed a significant difference in amniotic fluid glucose between the 0% GLU group and all other diet groups with the 0% GLU group having a significantly lower amniotic fluid glucose concentration than the concentrations found for the other diet groups. Dam plasma glucose analyses showed, for the gd20 dams, a significant difference in plasma glucose concentrations between the 0% GLU group and the 12% GLU group but not between any other diet groups. The plasma glucose concentration was significantly lower in the 0% GLU group compared to the 12% GLU group. For the control dams, no significant difference in plasma glucose was found between diet groups.

A significant difference in plasma glucose concentrations was found for gd20 pups. The 0% GLU group, although not differing significantly from the 24% GLU group, had a significantly lower plasma glucose concentration than that found in the 12% and 60% GLU groups. No differences in plasma glucose concentrations were found for any other timepoints except at pd15 where a significant difference was found between the 12% GLU group and the average of the 24 and 60% GLU groups with the 12% GLU group having a significantly higher plasma glucose level. Graphic presentation of these results is presented in **Figure IV-6**.

4.4.3 Amniotic Fluid and Plasma β-Hydroxybutyrate

Amniotic fluid and plasma β-hydroxybutyrate data is described in **Table IV-7** while a graphic representation of these results is presented in **Figure IV-7**. Results from the amniotic fluid analysis showed a significantly higher level of amniotic fluid β-hydroxybutyrate in the 0% GLU group than in the 12% GLU group which in turn had a significantly higher level of amniotic fluid β-hydroxybutyrate than either the 24% or 60% GLU groups. Results from the gd20 dam plasma β-hydroxybutyrate analysis showed a significant difference in plasma β-hydroxybutyrate concentrations between the 0% GLU group and all other diet groups with the 0% GLU group having a significantly higher plasma β-hydroxybutyrate concentration. Results from the analysis of β-hydroxybutyrate in the plasma of the control dams also showed significant differences in β-hydroxybutyrate levels, with higher levels found in the plasma of the 12% GLU group compared to the plasma of the 24% or 60% GLU groups.

A significant difference in pup plasma β-hydroxybutyrate concentration was found for all perinatal timepoints with the 0% GLU group always having a significantly higher β-hydroxybutyrate concentration. At pd7 and pd15, no significant group differences in β-hydroxybutyrate were found. However, for all further timepoints analyzed, higher levels of β-hydroxybutyrate were found in the 12% GLU group than in the 60% GLU group with the 24% GLU group differing significantly from the 12% GLU group at pd21, from the 60% GLU group at pd28 and from neither group at pd35 and pd49. These results indicate that carbohydrate restriction results in physiological stress, as indicated by elevated β-hydroxybutyrate levels, in both dam and pup systems, except in the pup during lactation.

4.4.4 Amniotic Fluid and Plasma Urea

Table IV-8 describes the effect of graded levels of dietary glucose on amniotic fluid and plasma urea. Results from the amniotic fluid analysis showed no significant differences in amniotic fluid urea levels between diet groups.

Similarly, no significant differences in plasma urea levels were found between diet groups for either the gd20 or control dams.

Results from pup plasma urea analysis however, showed a significant difference in plasma urea levels for all three perinatal timepoints with the 0% GLU group always having higher levels and differing significantly from the 60% GLU group at gd20, from all other diet groups at birth and from the 24% and 60% GLU groups at 12-24hrs postpartum. No significant differences were found between diet groups for any suckling or post-weaning timepoints. Graphic presentation of these results is presented in **Figure IV-8**.

4.5 KIDNEY AND LIVER GLYCOGEN

4.5.1 Kidney Glycogen

Kidney glycogen data is described in **Table IV-9** while a graphic representation of these results is presented in **Figure IV-9**. No significant difference in kidney glycogen content was found between diet groups at any timepoint except at pd7. At pd7, the 12% GLU group had significantly more kidney glycogen than the average of the 24% and 60% GLU groups. This elevated kidney glycogen level in the 12% GLU group at pd7 may result from the higher cumulative food intake at pd7 in the 12% GLU group, although this is unlikely since elevated kidney glycogen levels in the 12% GLU group were not found at pd15 and pd21 when cumulative food intakes were also elevated, or it may indicate a decreased capacity for glycogen mobilization in this group at this time point.

4.5.2 Liver Glycogen

Liver glycogen data is described in **Table IV-10** while a graphic representation of these results is presented in **Figure IV-10**. Results from this analysis showed a significant difference in fetal liver glycogen content at gd20 between the 0% GLU group and the 60% GLU group with the 0% GLU group having significantly lower liver glycogen levels. This difference in liver glycogen content seen at gd20 between the 0% GLU group and the 60% GLU group was

enhanced by birth where the 0% GLU group had significantly lower liver glycogen levels than the 12% and 24% GLU groups as well as the 60% GLU group. No significant differences existed between the 12%, 24% and 60% GLU groups at gd20 or birth. By 12-24 hours postpartum, offspring liver glycogen levels were low across all diet groups with no significant differences found between any of the diet groups. At pd7 as well as at pd15, no difference in liver glycogen levels existed between diet groups. Differences in liver glycogen levels between diet groups returned at pd21 where the 12% GLU group had significantly lower liver glycogen levels than the 24% GLU group which in turn had significantly lower levels than the 60% GLU group. Differences in liver glycogen content were also found for all post-weaning timepoints with the 12% and 24% GLU groups having significantly lower liver glycogen levels than the 60% GLU group. No diet difference in liver glycogen content was found in the adult control group.

4.6 LIVER AND KIDNEY GLUT2

The GLUT2 protein analysis was first attempted on liver tissue because Postic *et al.* (1994) had previously determined the presence of GLUT2 protein in neonatal liver tissue. The first liver tissue samples analyzed produced a blot which had an overall high background and it appeared as if the bulk of each sample had not migrated down the gel perhaps due to insufficient denaturation of the samples prior to loading into the gel. To alleviate these problems, the blocking time was increased to reduce the overall high background and the samples were heated for a longer period of time in order to insure adequate denaturation of the proteins. In the next attempt, the high background was reduced with the increased blocking time and the samples did migrate down the gels however the blots obtained in this next attempt had vertical streaking and multiple bands. Vertical streaking is most often due to sample overloading or sample precipitation while the appearance of multiple bands may be the result of protein breakdown during tissue preparation. To alleviate these problems many

different strategies were used: the samples were diluted in order to prevent any vertical streaking due to sample overloading; different percentages of SDS were used (4%, 5%, 8% and 10%) in an attempt to attain an SDS to protein ratio that allowed for enough SDS to coat each protein molecule, a strategy used to prevent sample precipitation and therefore in turn prevent any vertical streaking: during tissue preparation, all steps were done on ice to prevent protein breakdown, a possible cause of the appearance of multiple bands; and freshly prepared protease inhibitors were used in the preparation of the samples, once again to prevent any protein breakdown and the production of multiple bands. Even after applying all of these problem solving strategies to the analysis, the results obtained were still poor, with the ever persistent problem of multiple bands. After the multitude of unsuccessful attempts at isolating liver GLUT2 proteins, attempts were made at isolating kidney GLUT2 proteins with the same, unsuccessful results.

4.7 SMALL INTESTINE GLUT2

Although the problems encountered in the liver and kidney GLUT2 analysis remain unresolved, the small intestine GLUT2 analysis was successful and produced the following results. All relative area under the curve (AUC) values used in the small intestine GLUT2 protein analysis were obtained by comparing sample band intensities with the intensity of an adult control band. The tissue used to obtain the adult control band was acquired from dams exposed to the 60% GLU diet for 10 weeks. This methodology allowed a comparison to be made between postnatal tissue levels and adult tissue levels. Any relative AUC value below one indicated a level of GLUT2 protein lower than that found in an adult while anything higher that one indicated a level of GLUT2 protein higher than that found in an adult. Relative AUC values of one indicated postnatal levels of GLUT2 protein similar to adult levels of GLUT2 protein.

Small intestine GLUT2 data is described in **Table IV-11** while a graphic representation of these results is presented in **Figures IV-11** and **IV-12**. Results

from the small intestine GLUT2 analysis showed a significant three-way interaction between diet, region and time. This indicated that the proximal and distal regions of the small intestine differed and should be analyzed separately.

4.7.1 Proximal Small Intestine GLUT2

When diet and time were analyzed for significance in the proximal region of the small intestine, neither the interaction between diet and time nor the main effects of diet or time were significant (**Figure IV-11**).

4.7.2 Distal Small Intestine GLUT2

Results for the distal region of the small intestine differed significantly from those found for the proximal region of the small intestine. In the distal region of the small intestine, both the interaction between diet and time as well as the main effects of diet and time were significant. Also, the level of GLUT2 protein expression in the distal small intestine reached a mature, adult level of expression by pd28.

Diet Effects:

In the distal small intestine, GLUT2 protein levels at pd7, pd15 and pd21 were altered by dietary intake (**Figure IV-12**), suggesting that the distal small intestine is more susceptible to dietary influences during suckling. At pd7, both the 12% and 24% GLU groups had significantly lower relative GLUT2 signals than the 60% GLU group. At pd15, only the 12% GLU group differed significantly from the 60% GLU group, with the 12% GLU group again having a lower relative GLUT2 signal than the 60% GLU group. At pd21, only the 24% GLU group differed significantly from the 60% GLU group however, at this timepoint, the relative GLUT2 signal in the 24% GLU group was higher than that of the 60% GLU group. At pd28 and pd49, no significant differences existed in relative GLUT2 signals between experimental diet groups and the relative GLUT2 signals obtained were comparable to adult control values.

Time Effects:

GLUT2 protein levels in the 60% GLU group were highest at pd7, with levels more than 3.5 fold higher than those found in adult tissue, were lower at pd15, with levels approximately 2.5 fold higher than those found in adult tissue,

lower again at pd21 and lower once again, although not that different from the level seen at pd21, at pd28. At pd49, GLUT2 protein levels in the 60% GLU group appeared higher than those seen at pd28 although no significant difference was found between pd28 and pd49. Significant differences in relative GLUT2 signals for the 60% GLU group were found between pd7 and pd21, pd28 and pd49. Changes in GLUT2 protein levels over time in the 12% and 24% GLU restricted groups differed fundamentally from those changes found in the 60% GLU adequate group. GLUT2 protein levels in the distal small intestine did not change significantly over time for the 12% GLU group. GLUT2 protein levels in the 12% GLU group decreased slightly between pd7 and pd15, increased by pd21, decreased again by pd28 and remained relatively steady up to pd49 (**Figure IV-12**). A similar pattern of expression was demonstrated by the 24% GLU group, however the increased level of GLUT2 protein expressed at pd21 in the 24% GLU group differed significantly from that found at pd28 and pd49, with levels almost 3 fold higher at pd21 than at pd28 and pd49.

*Diet*Time Interaction Effects:*

Dietary GLU restriction not only produced altered levels of GLUT2 protein expression at specific timepoints in the distal small intestine, dietary GLU restriction resulted in developmental patterns of GLUT2 protein expression entirely different from that observed in the control, 60% GLU adequate group. The developmental patterns of GLUT2 protein expression in the 12% and 24% GLU restricted groups, although very similar to each other, differed fundamentally from the developmental pattern found in the control, 60% GLU adequate group (**Figure IV-12**). GLUT2 protein expression in the 12% and 24% GLU restricted groups increased between pd7 and pd21 and decreased between pd21 and pd49, rather than decreasing constantly from pd7 to pd28 with a slight but insignificant rise by pd49 as occurred in the 60% control group. Although the developmental pattern between pd7 and pd49 was altered by dietary GLU restriction, the long term effects of such a change may be minimal since the groups do not differ significantly from one another at pd49.

Table IV-1: Effect of graded levels of dietary glucose on cumulative food intake (g).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[7]		
Time	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd0-gd19	127[4]	330 ± 8^{a}	352 ± 7^{ab}	345 ± 6^{ab}	359 ± 6^{b}	Diet	3.04	0.0316
pd0-pd7	25[5]	-	214 ± 9^{a}	193 ± 9^{ab}	177 ± 8^{b}	Diet	4.42	0.0244
pd8-pd15	25[5]	-	283 ± 14^{a}	248 ± 14^{ab}	217 ± 13^{b}	Diet	5.82	0.0094
pd16-pd21	25[6]	-	236 ± 10^{a}	217 ± 10^{ab}	200 ± 9^{b}	Diet	3.53	0.0469
pd22-pd28	25[6]	-	269 ± 9	262 ± 9	259 ± 9	Diet	0.27	0.7677
pd29-pd35	24[6]	-	279 ± 13^{a}	256 ± 14^{ab}	228 ± 12^{b}	Diet	3.88	0.0368
pd36-pd42	24[6]	-	197 ± 13	206 ± 12	177 ± 12	Diet	1.57	0.2320
pd43-pd48	23[6]	-	196 ± 12	183 ± 12	160 ± 10	Diet	2.90	0.0783

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of cages
[4]One dam per cage
[5]One dam plus suckling pups per cage
[6]One dam plus nibbling pups per cage
[7]Results from analysis of variance (ANOVA)

Figure IV-1: Effect of Graded Levels of Dietary Glucose on Cumulative Food Intake

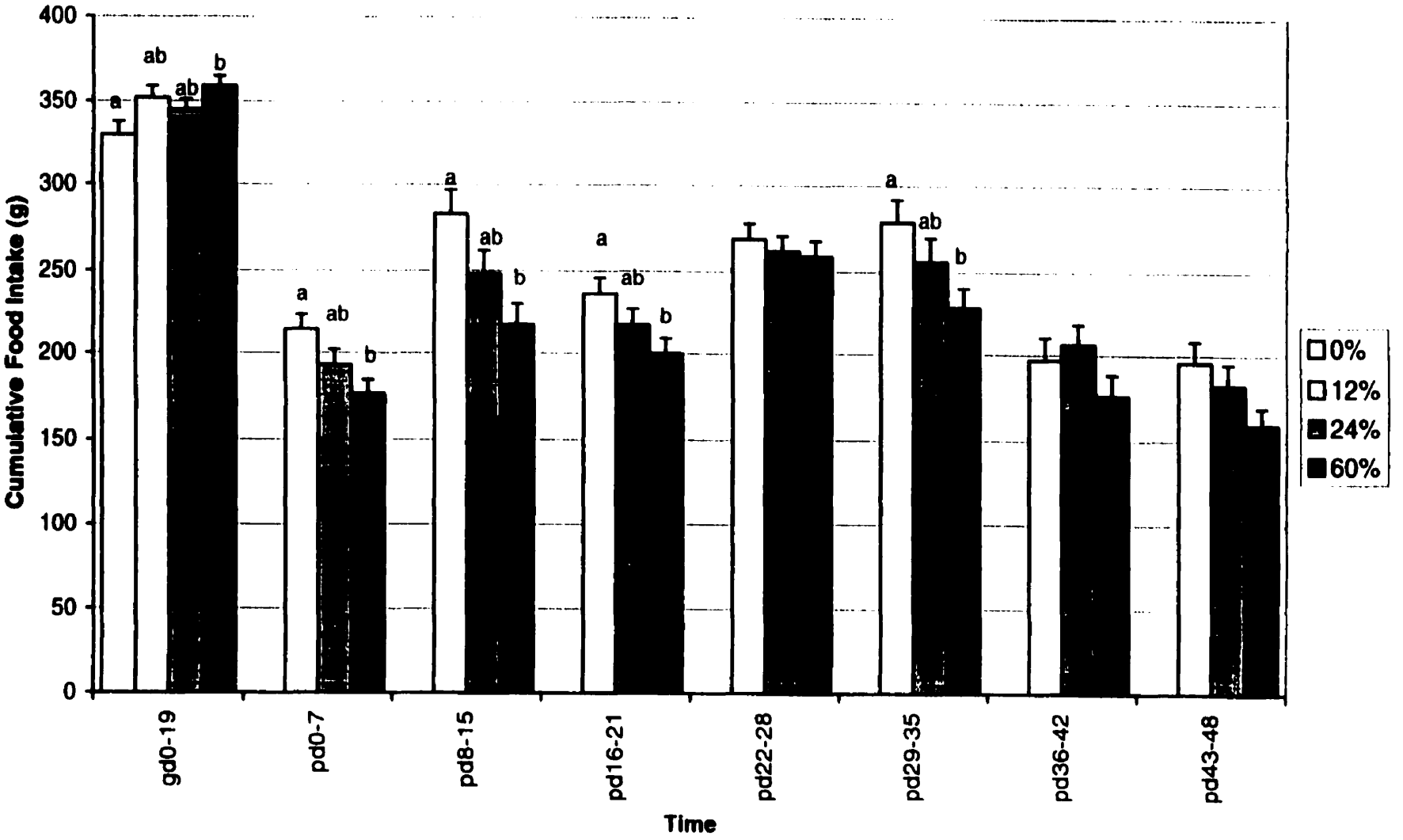
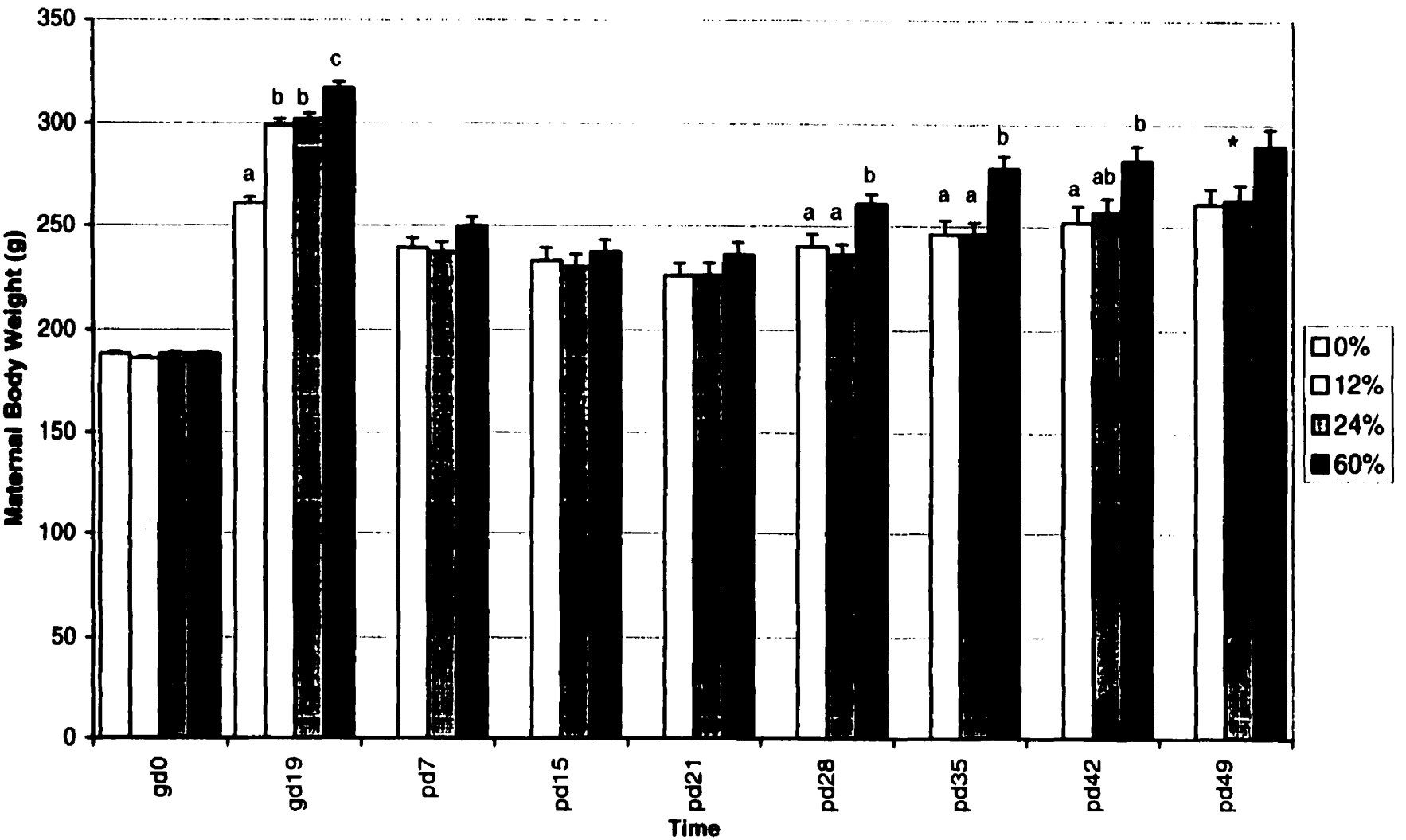


Table IV-2: Effect of graded levels of dietary glucose on maternal body weight (g).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[4]		
Time	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd0	129	188 ± 1	186 ± 1	188 ± 1	188 ± 1	Diet	0.65	0.5831
gd19	129	261 ± 3[a]	299 ± 3[bc]	302 ± 3[bc]	317 ± 3[d]	Diet	60.00	0.0001
pd7	26	-	239 ± 5	237 ± 5	249 ± 5	Diet	1.70	0.2056
pd15	26	-	233 ± 6	230 ± 6	237 ± 6	Diet	0.34	0.7141
pd21	26	-	226 ± 6	226 ± 6	236 ± 6	Diet	0.95	0.4004
pd28	26	-	240 ± 6[a]	236 ± 5[a]	261 ± 5[b]	Diet	6.79	0.0048
pd35	26	-	246 ± 7[a]	246 ± 6[a]	279 ± 6[b]	Diet	8.95	0.0013
pd42	26	-	252 ± 8[a]	257 ± 7[ab]	283 ± 7[b]	Diet	5.18	0.0139
pd49	26	-	261 ± 8	263 ± 8	290 ± 8	Diet	4.25	0.0268*

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of dams
[4]Results from analysis of variance (ANOVA)
*Diet effect due to a significant difference between the average of the 12 and 24% GLU diets and the 60% GLU diet

Figure IV-2: Effect of Graded Levels of Dietary Glucose on Maternal Body Weight



* Diet effect due to a significant difference between the average of the 12 and 24% GLU diets and the 60% GLU diet

Table IV-3: Effect of graded levels of dietary glucose on litter size and weight (g).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[4]		
Litter Size	n[3]	0	12	24	60	Main Effect	F Value	p Value
Perinatal	92	9 ± 1^a	11 ± 1^b	12 ± 1^b	12 ± 1^b	Diet	6.74	0.0004
Litter Weight								
Perinatal	91	25 ± 3^a	49 ± 2^b	53 ± 2^b	55 ± 2^b	Diet	29.24	0.0001

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of litters
[4]Results from analysis of variance (ANOVA)

Figure IV-3: Effect of Graded Levels of Dietary Glucose on Litter Size and Weight

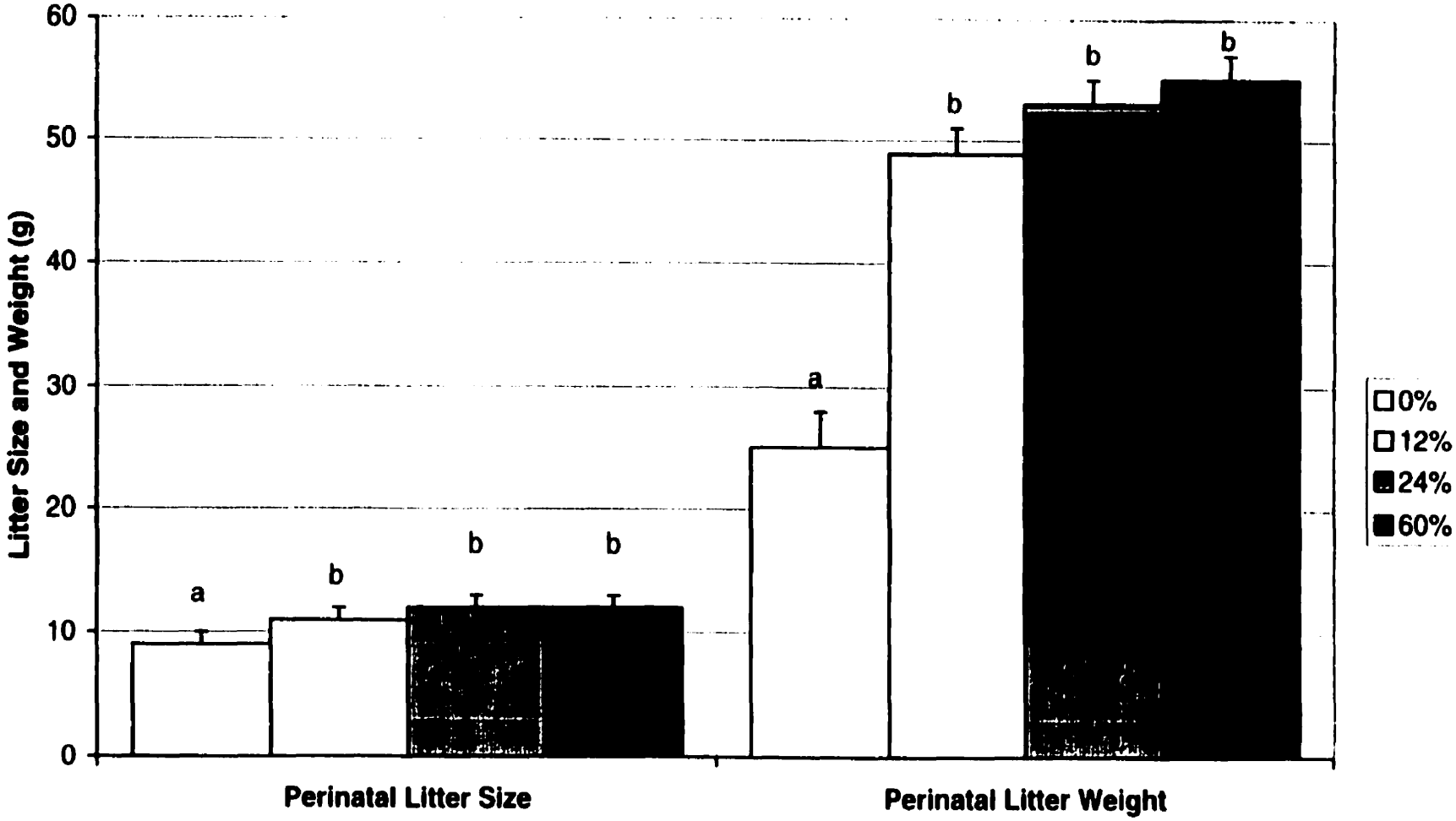


Table IV-4: Effect of graded levels of dietary glucose on pup body weight (g).[1,2,3]

		Dietary Glucose Level (%)				Statistical Analysis[5]		
Time	n[4]	0	12	24	60	Main Effect	F Value	p Value
gd20	36(421)	2.3 ± 0.1^{a}	3.3 ± 0.1^{b}	3.3 ± 0.1^{b}	3.6 ± 0.1^{b}	Diet	34.60	0.0001
birth	29(316)	4.0 ± 0.3^{a}	5.0 ± 0.2^{ab}	5.2 ± 0.2^{b}	5.6 ± 0.2^{b}	Diet	7.54	0.0010
12-24hrs	27(280)	3.2 ± 0.3^{a}	5.0 ± 0.3^{b}	5.4 ± 0.2^{b}	5.6 ± 0.2^{b}	Diet	12.16	0.0001
pd7	26(306)	-	11.1 ± 0.4	10.9 ± 0.4	10.8 ± 0.4	Diet	0.14	0.8688
pd15	26(182)	-	27.7 ± 0.8^{a}	26.1 ± 0.7^{ab}	24.2 ± 0.7^{b}	Diet	5.54	0.0109
pd21	26(130)	-	44.7 ± 1.4^{a}	42.6 ± 1.3^{ab}	38.6 ± 1.3^{b}	Diet	5.06	0.0151
pd28	26(78)	-	69.5 ± 2.2^{a}	65.2 ± 2.1^{ab}	61.4 ± 2.1^{b}	Diet	3.49	0.0475
pd35	26(52)	-	88.2 ± 2.6	89.0 ± 2.5	86.3 ± 2.5	Diet	0.32	0.7301
pd49	26(26)	-	133.9 ± 6.1	148.0 ± 5.8	143.6 ± 5.8	Diet	1.44	0.2566

[1]Nested design was used in the analysis
[2]Values are least square means (LSM) ± SELSM
[3]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[4]Number of dams with number of pups in parentheses
[5]Results from analysis of variance (ANOVA)

Figure IV-4: Effect of Graded Levels of Dietary Glucose on Pup Body Weight

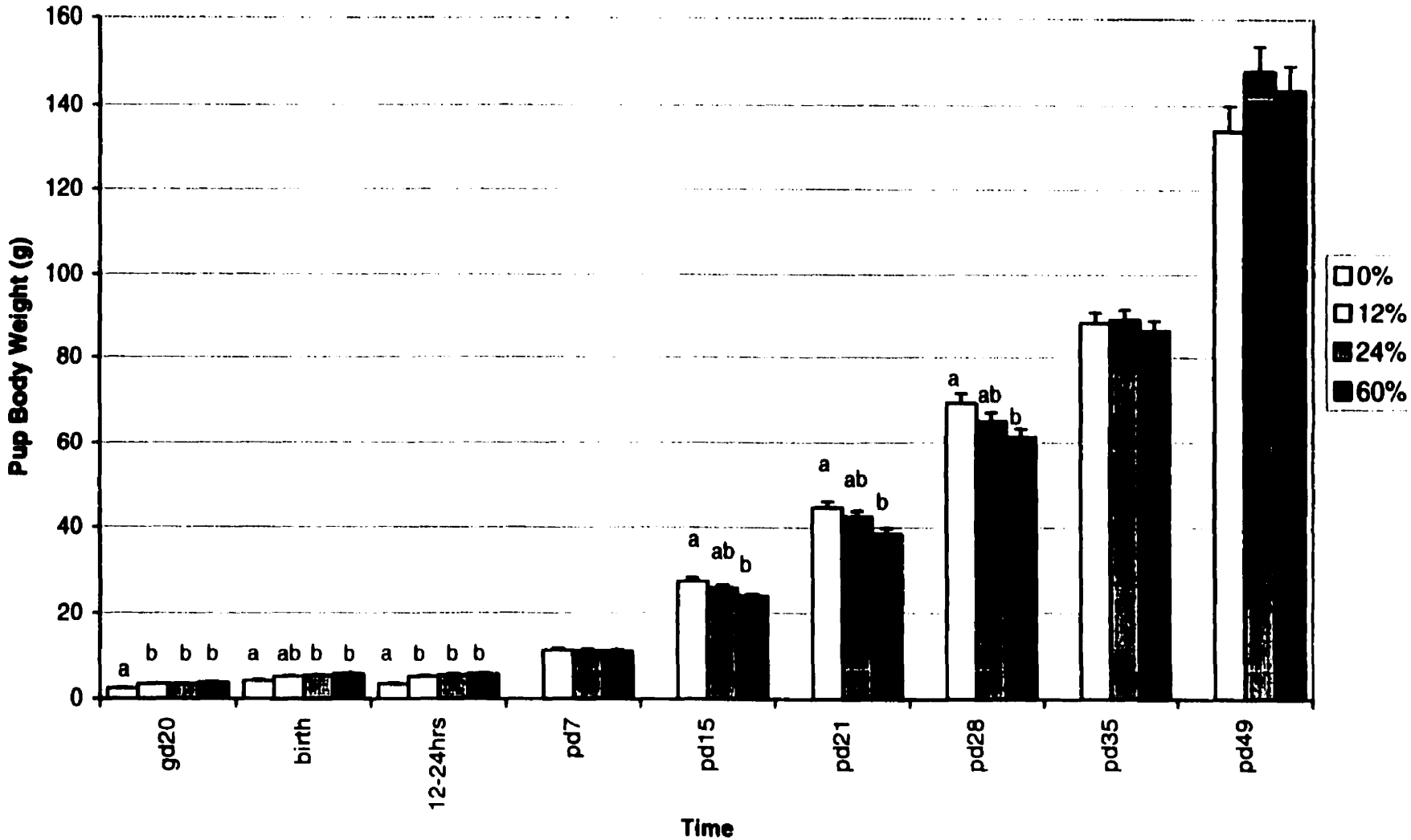


Table IV-5: Effect of graded levels of dietary glucose on plasma albumin (g/L).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[9]		
Time	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd20 dams	35[4]	39.81 ± 2.05	39.01 ± 2.17	40.36 ± 2.05	45.69 ± 2.05	Diet	2.15	0.1139
gd20 pups	34[5]	11.76 ± 1.26	13.07 ± 1.26	14.62 ± 1.19	16.09 ± 1.19	Diet	2.36	0.0917
birth	26[5]	15.69 ± 2.19	14.86 ± 1.34	14.41 ± 1.43	16.30 ± 1.34	Diet	0.36	0.7817
12-24hrs	25[5]	16.20 ± 2.88	16.58 ± 2.04	13.30 ± 1.77	15.69 ± 1.77	Diet	0.61	0.6186
pd7	25[6]	-	23.53 ± 2.02	22.60 ± 1.78	23.10 ± 1.78	Diet	0.06	0.9414
pd15	25[7]	-	26.58 ± 1.39	27.87 ± 1.39	27.11 ± 1.31	Diet	0.22	0.8063
pd21	26[7]	-	29.24 ± 2.30	30.53 ± 2.17	30.45 ± 2.17	Diet	0.10	0.9027
pd28	24[8]	-	40.58 ± 1.09	40.00 ± 1.16	37.14 ± 1.02	Diet	3.05	0.0686
pd35	26[8]	-	39.41 ± 2.79	38.33 ± 2.63	38.97 ± 2.63	Diet	0.04	0.9605
pd49	25[8]	-	49.02 ± 2.44	44.44 ± 2.30	46.18 ± 2.44	Diet	0.94	0.4060
adult dams	19[4]	-	64.27 ± 3.22	57.11 ± 3.22	59.37 ± 2.98	Diet	1.30	0.3010

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of plasma samples analyzed
[4]Individual dam plasma samples
[5]Plasma samples pooled by litter
[6]Plasma samples pooled from variable number of pups sacrificed
[7]Plasma samples pooled from two pups sacrificed
[8]Individual pup plasma samples
[9]Results from analysis of variance (ANOVA)

Figure IV-5: Effect of Graded Levels of Dietary Glucose on Plasma Albumin

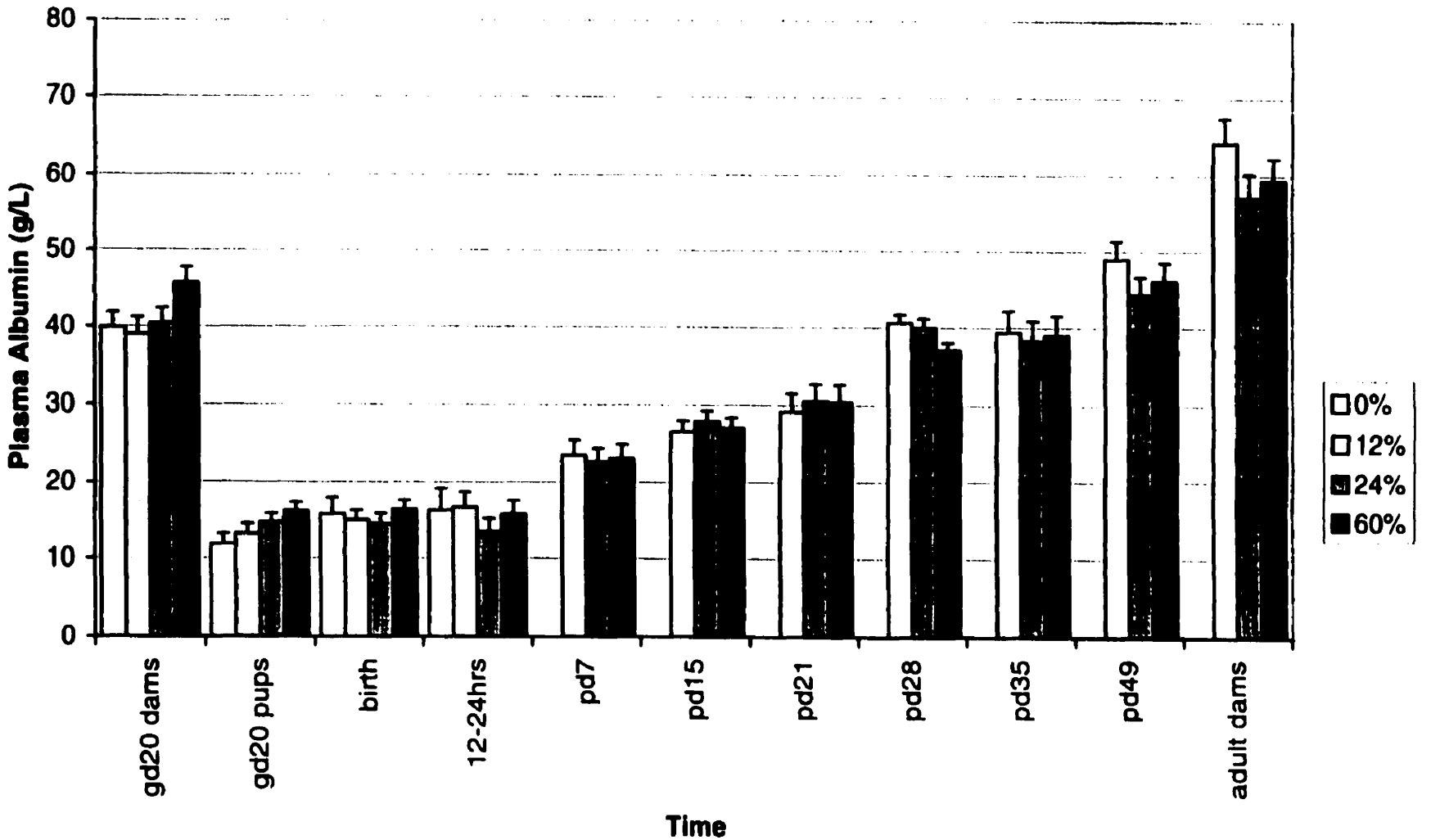
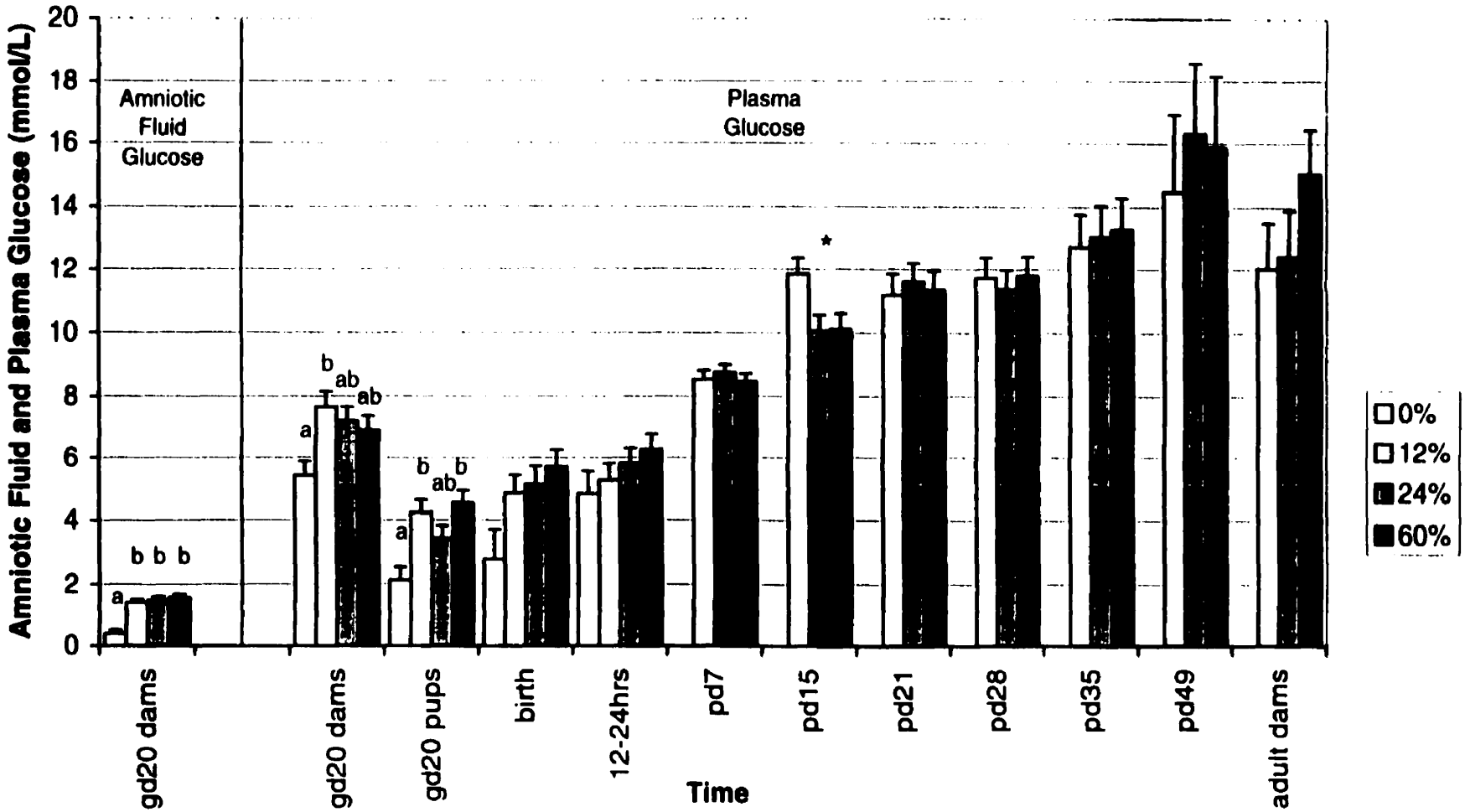


Table IV-6: Effect of graded levels of dietary glucose on amniotic fluid and plasma glucose (mmol/L).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[10]		
Amniotic Fluid	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd20 dams	36[4]	0.38 ± 0.11[a]	1.40 ± 0.11[b]	1.49 ± 0.11[b]	1.57 ± 0.11[b]	Diet	23.76	0.0001
Plasma								
gd20 dams	35[5]	5.40 ± 0.46[a]	7.67 ± 0.49[b]	7.21 ± 0.46[ab]	6.92 ± 0.46[ab]	Diet	4.47	0.0101
gd20 pups	35[6]	2.12 ± 0.42[a]	4.26 ± 0.40[b]	3.44 ± 0.40[ab]	4.55 ± 0.40[b]	Diet	6.92	0.0011
birth	26[6]	2.79 ± 0.93	4.85 ± 0.57	5.11 ± 0.61	5.69 ± 0.57	Diet	2.40	0.0953
12-24hrs	27[6]	4.82 ± 0.73	5.25 ± 0.55	5.80 ± 0.52	6.27 ± 0.52	Diet	1.10	0.3694
pd7	25[7]	-	8.55 ± 0.28	8.77 ± 0.25	8.49 ± 0.25	Diet	0.35	0.7099
pd15	26[8]	-	11.85 ± 0.53	10.06 ± 0.50	10.11 ± 0.50	Diet	3.82	0.0369*
pd21	25[8]	-	11.18 ± 0.67	11.61 ± 0.59	11.34 ± 0.59	Diet	0.12	0.8857
pd28	26[9]	-	11.73 ± 0.65	11.36 ± 0.62	11.80 ± 0.62	Diet	0.14	0.8673
pd35	26[9]	-	12.73 ± 1.05	13.06 ± 0.99	13.30 ± 0.99	Diet	0.02	0.9788**
pd49	26[9]	-	14.48 ± 2.44	16.31 ± 2.30	15.88 ± 2.30	Diet	0.16	0.8523
adult dams	19[5]	-	12.02 ± 1.49	12.42 ± 1.49	15.06 ± 1.38	Diet	1.35	0.2863

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of amniotic fluid/plasma samples analyzed
[4]Amniotic fluid samples pooled by litter
[5]Individual dam plasma samples
[6]Plasma samples pooled by litter
[7]Plasma samples pooled from variable number of pups sacrificed
[8]Plasma samples pooled from two pups sacrificed
[9]Individual pup plasma samples
[10]Results from analysis of variance (ANOVA)
*Diet effect due to a significant difference between the 12% GLU diet and the average of the 24 and 60% GLU diets
**Statistical analysis was performed on transformed data; LSM ± SELSM presented are non-transformed values

Figure IV-6: Effect of Graded Levels of Dietary Glucose on Amniotic Fluid and Plasma Glucose



* Diet effect due to a significant difference between the 12% GLU diet and the average of the 24 and 60% GLU diets

Table IV-7: Effect of graded levels of dietary glucose on amniotic fluid and plasma β-hydroxybutyrate (mmol/L).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[10]		
Amniotic Fluid	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd20 dams	36[4]	10.27 ± 0.57[a]	0.54 ± 0.57[b]	0.18 ± 0.57[c]	0.08 ± 0.57[c]	Diet	61.85	0.0001*
Plasma								
gd20 dams	35[5]	7.20 ± 0.42[a]	0.50 ± 0.45[b]	0.49 ± 0.42[b]	0.24 ± 0.42[b]	Diet	41.80	0.0001*
gd20 pups	34[6]	8.19 ± 0.63[a]	0.68 ± 0.63[b]	0.47 ± 0.60[b]	0.26 ± 0.60[b]	Diet	34.94	0.0001*
birth	26[6]	1.10 ± 0.22[a]	0.10 ± 0.16[b]	0.13 ± 0.18[b]	0.07 ± 0.16[b]	Diet	6.51	0.0026*
12-24hrs	26[6]	1.11 ± 0.21	0.43 ± 0.13	0.50 ± 0.12	0.39 ± 0.13	Diet	3.22	0.0423**
pd7	25[7]	-	0.89 ± 0.06	0.82 ± 0.05	0.82 ± 0.05	Diet	0.43	0.6588
pd15	26[8]	-	0.79 ± 0.07	0.96 ± 0.07	0.88 ± 0.07	Diet	1.35	0.2784
pd21	26[8]	-	1.07 ± 0.10[a]	0.68 ± 0.09[b]	0.37 ± 0.09[b]	Diet	13.21	0.0002
pd28	25[9]	-	0.66 ± 0.09[a]	0.41 ± 0.09[a]	0.16 ± 0.09[b]	Diet	12.48	0.0002*
pd35	23[9]	-	0.75 ± 0.15[a]	0.34 ± 0.13[ab]	0.17 ± 0.15[b]	Diet	8.33	0.0023*
pd49	26[9]	-	0.83 ± 0.25[a]	0.31 ± 0.23[ab]	0.15 ± 0.23[b]	Diet	5.91	0.0085*
adult dams	19[5]	-	0.32 ± 0.03[a]	0.15 ± 0.03[b]	0.19 ± 0.02[b]	Diet	11.24	0.0009

[1]Values are least square means (LSM) ± SELSM

[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons

[3]Number of amniotic fluid/plasma samples analyzed

[4]Amniotic fluid samples pooled by litter

[5]Individual dam plasma samples

[6]Plasma samples pooled by litter

[7]Plasma samples pooled from variable number of pups sacrificed

[8]Plasma samples pooled from two pups sacrificed

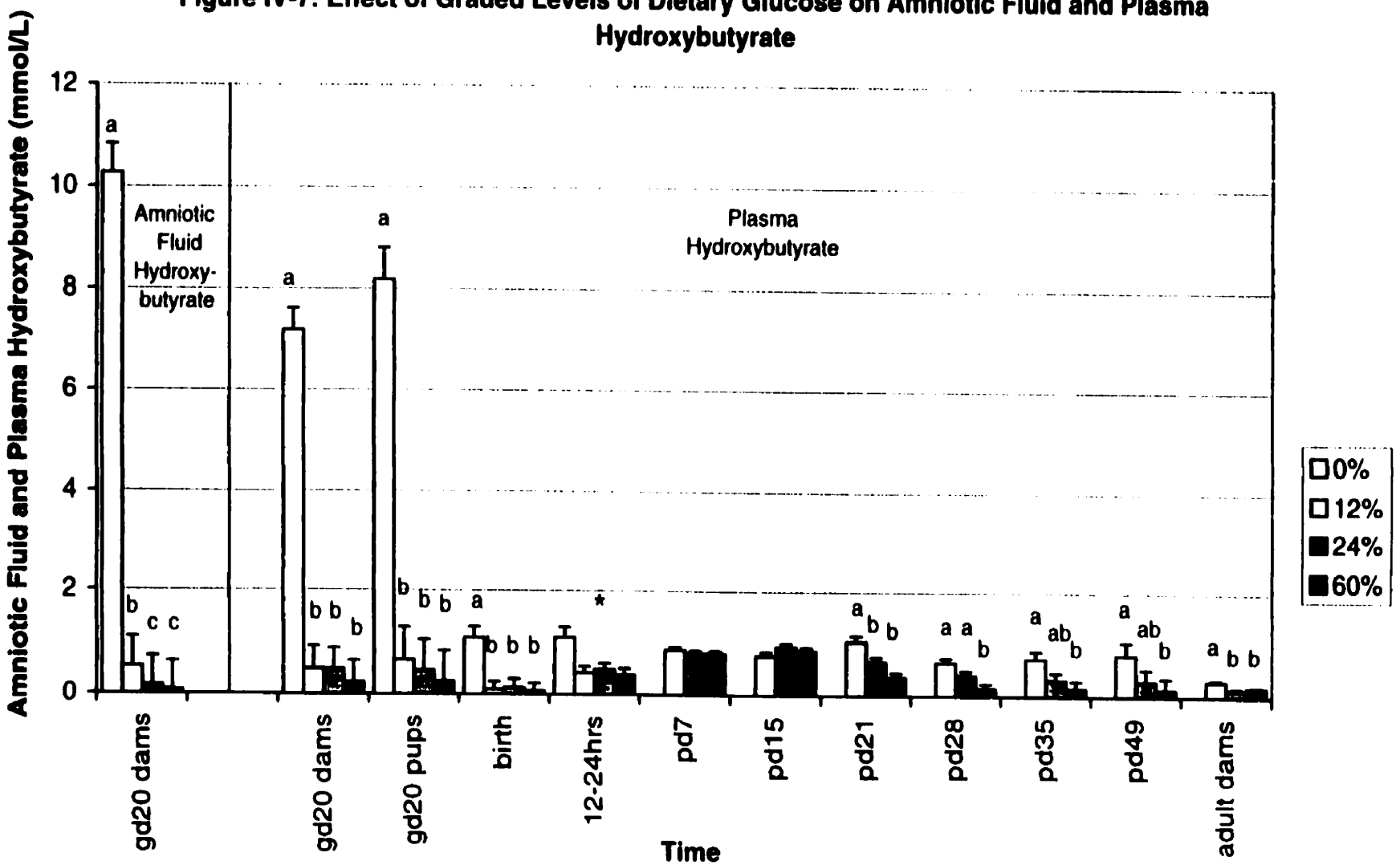
[9]Individual pup plasma samples

[10]Results from analysis of variance (ANOVA)

*Statistical analysis was performed on transformed data; LSM ± SELSM presented are non-transformed values

**Diet effect due to a significant difference between the 0% GLU diet and the average of the 12 and 60% GLU diets

Figure IV-7: Effect of Graded Levels of Dietary Glucose on Amniotic Fluid and Plasma Hydroxybutyrate



* Diet effect due to a significant difference between the 0% GLU diet and the average of the 12 and 60% GLU diets

Table IV-8: Effect of graded levels of dietary glucose on amniotic fluid and plasma urea (mmol/L).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[10]		
Amniotic Fluid	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd20 dams	36[4]	5.95 ± 0.56	4.02 ± 0.56	3.64 ± 0.56	3.32 ± 0.56	Diet	2.63	0.0667*
Plasma								
gd20 dams	33[5]	5.01 ± 0.56	3.82 ± 0.52	3.70 ± 0.49	3.35 ± 0.49	Diet	1.36	0.2735**
gd20 pups	34[6]	6.87 ± 0.65[a]	4.95 ± 0.65[ab]	4.50 ± 0.61[ab]	3.98 ± 0.61[b]	Diet	3.88	0.0186
birth	25[6]	21.47 ± 0.80[a]	4.60 ± 0.40[b]	4.76 ± 0.42[b]	3.50 ± 0.40[b]	Diet	145.91	0.0001
12-24hrs	25[6]	16.74 ± 1.60[a]	10.92 ± 1.13[ab]	9.85 ± 0.98[b]	7.08 ± 0.98[b]	Diet	9.10	0.0005
pd7	25[7]	-	8.54 ± 0.91	7.93 ± 0.80	7.79 ± 0.80	Diet	0.21	0.8135
pd15	26[8]	-	3.74 ± 0.75	4.06 ± 0.70	3.75 ± 0.70	Diet	0.07	0.9367
pd21	26[8]	-	3.73 ± 0.42	3.27 ± 0.39	2.62 ± 0.39	Diet	1.93	0.1673
pd28	26[9]	-	4.32 ± 0.43	3.94 ± 0.41	3.54 ± 0.41	Diet	0.86	0.4369
pd35	26[9]	-	2.49 ± 0.40	3.40 ± 0.37	3.36 ± 0.37	Diet	1.74	0.1971
pd49	26[9]	-	6.75 ± 1.02	4.74 ± 0.96	4.74 ± 0.96	Diet	1.34	0.2811
adult dams	19[5]	-	6.01 ± 0.33	4.91 ± 0.33	5.32 ± 0.30	Diet	2.89	0.0846

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of amniotic fluid/plasma samples analyzed
[4]Amniotic fluid samples pooled by litter
[5]Individual dam plasma samples
[6]Plasma samples pooled by litter
[7]Plasma samples pooled from variable number of pups sacrificed
[8]Plasma samples pooled from two pups sacrificed
[9]Individual pup plasma samples
[10]Results from analysis of variance (ANOVA)
*Statistical analysis was performed on transformed data; LSM ± SELSM presented are non-transformed values
**Statistical analysis was performed on weighted data; LSM ± SELSM presented are non-weighted values

Figure IV-8: Effect of Graded Levels of Dietary Glucose on Amniotic Fluid and Plasma Urea

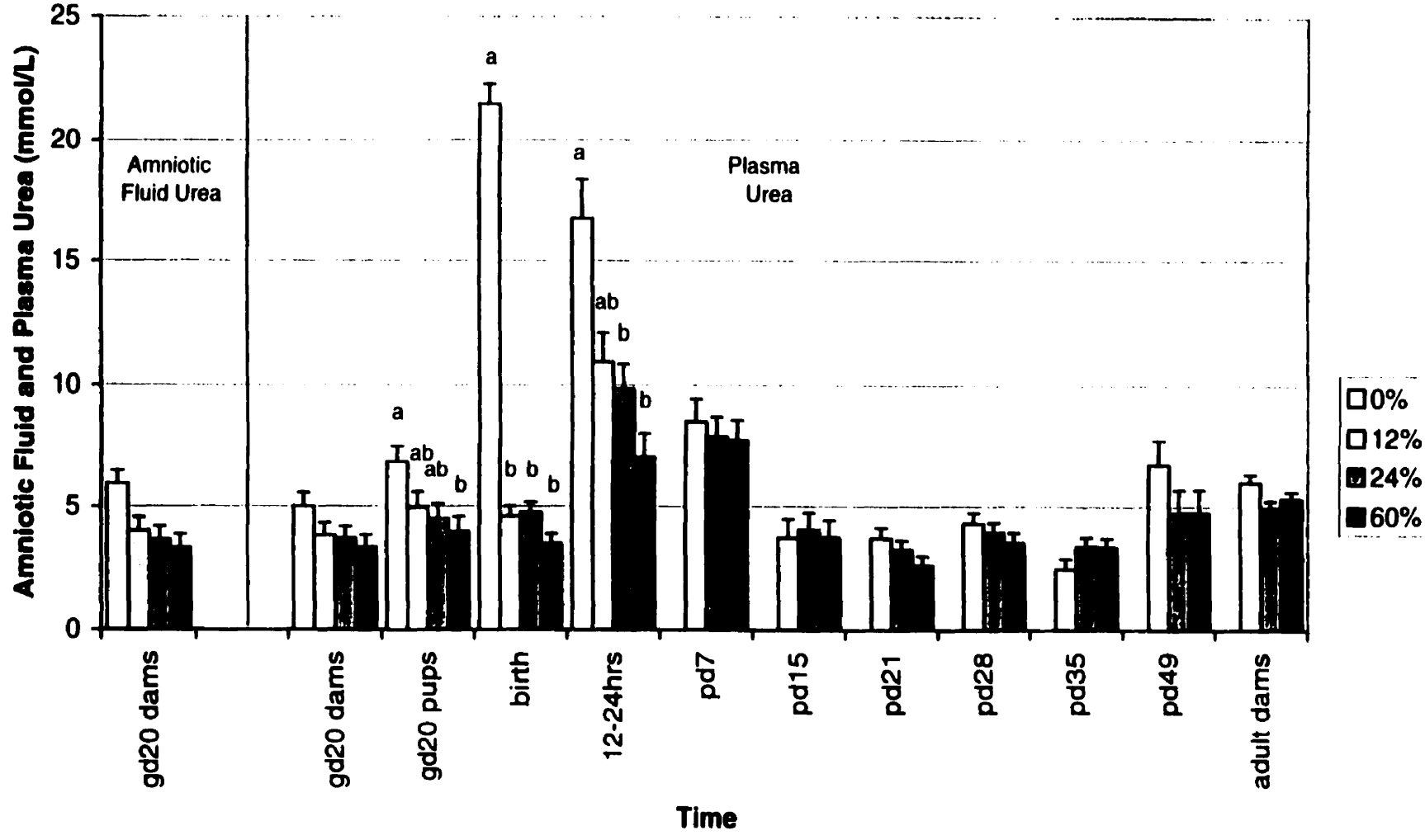
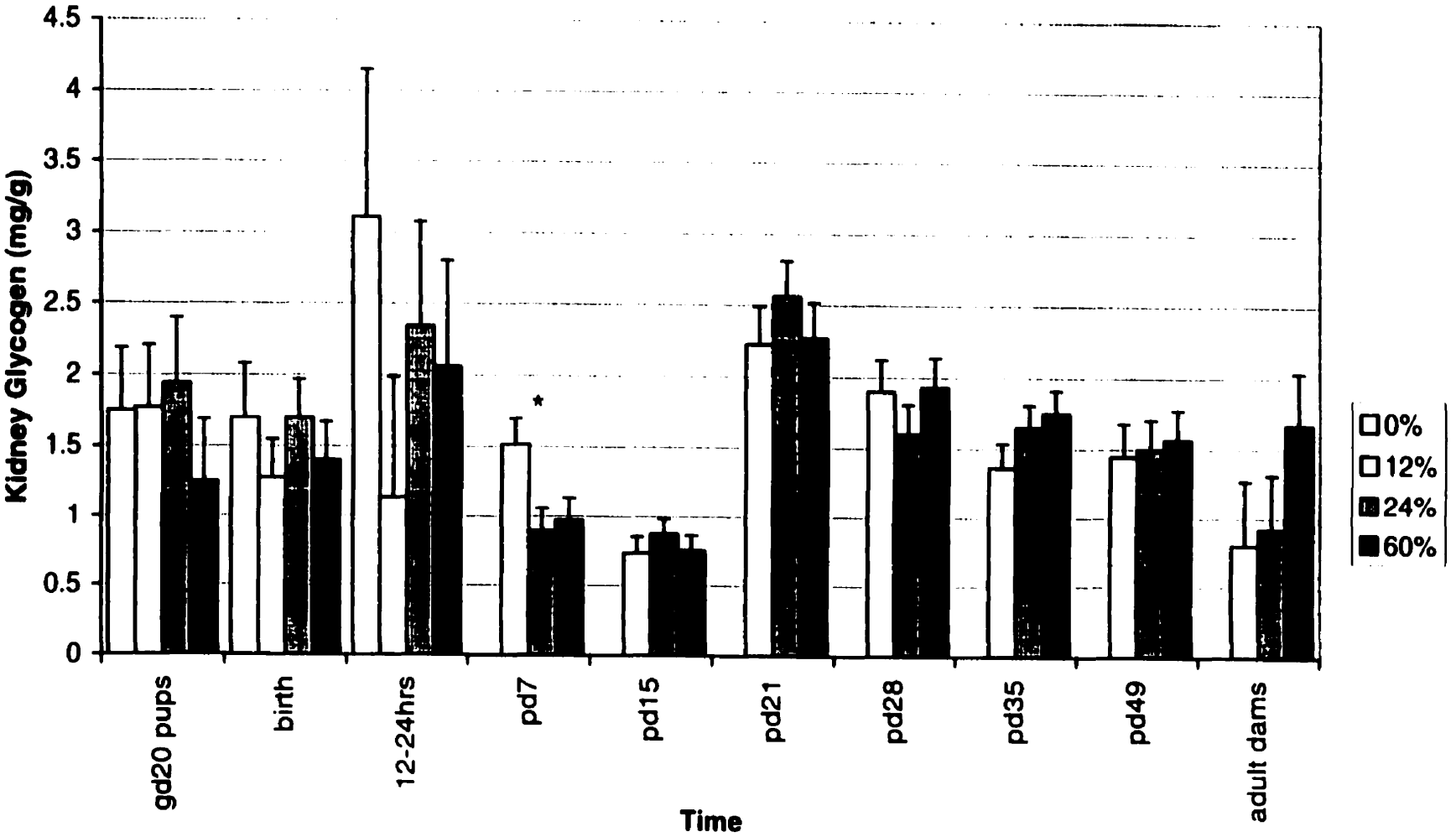


Table IV-9: Effect of graded levels of dietary glucose on kidney glycogen levels (mg/g).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[9]		
Time	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd20 pups	35[4]	1.75 ± 0.44	1.77 ± 0.44	1.94 ± 0.46	1.25 ± 0.44	Diet	0.45	0.7202
birth	28[4]	1.70 ± 0.38	1.28 ± 0.27	1.70 ± 0.27	1.40 ± 0.27	Diet	0.54	0.6578
12-24hrs	26[4]	3.11 ± 1.04	1.14 ± 0.85	2.34 ± 0.74	2.06 ± 0.74	Diet	0.78	0.5193
pd7	26[5]	-	1.52 ± 0.18	0.89 ± 0.17	0.97 ± 0.17	Diet	3.81	0.0372*
pd15	26[6]	-	0.73 ± 0.12	0.87 ± 0.11	0.75 ± 0.11	Diet	0.42	0.6601
pd21	26[6]	-	2.23 ± 0.26	2.56 ± 0.25	2.27 ± 0.25	Diet	0.51	0.6057
pd28	26[7]	-	1.90 ± 0.22	1.60 ± 0.21	1.93 ± 0.21	Diet	0.76	0.4776
pd35	26[7]	-	1.37 ± 0.17	1.65 ± 0.16	1.75 ± 0.16	Diet	1.46	0.2528
pd49	26[7]	-	1.45 ± 0.23	1.50 ± 0.21	1.57 ± 0.21	Diet	0.07	0.9296
adult dams	17[8]	-	0.80 ± 0.48	0.92 ± 0.40	1.67 ± 0.37	Diet	1.42	0.2749

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of kidney samples analyzed
[4]Kidney samples pooled by litter
[5]Kidney samples pooled from variable number of pups sacrificed
[6]Kidney samples pooled from two pups sacrificed
[7]Individual pup kidney samples
[8]Individual dam kidney samples
[9]Results from analysis of variance (ANOVA)
*Diet effect due to a significant difference between the 12% GLU diet and the average of the 24 and 60% GLU diets

Figure IV-9: Effect of Graded Levels of Dietary Glucose on Kidney Glycogen Levels



* Diet effect due to a significant difference between the 12% GLU diet and the average of the 24 and 60% GLU diets

Table IV-10: Effect of graded levels of dietary glucose on liver glycogen levels (mg/g).[1,2]

		Dietary Glucose Level (%)				Statistical Analysis[9]		
Time	n[3]	0	12	24	60	Main Effect	F Value	p Value
gd20 pups	36[4]	20.71 ± 3.75[a]	35.81 ± 3.75[ab]	36.07 ± 3.75[ab]	47.76 ± 3.75[b]	Diet	8.76	0.0002
birth	29[4]	11.42 ± 6.44[a]	38.56 ± 5.09[b]	43.29 ± 5.09[b]	48.12 ± 5.09[b]	Diet	7.36	0.0011
12-24hrs	25[4]	1.49 ± 1.76	1.41 ± 1.33	3.20 ± 1.33	4.65 ± 1.33	Diet	2.38	0.0982*
pd7	26[5]	-	14.49 ± 2.50	17.18 ± 2.36	15.21 ± 2.36	Diet	0.33	0.7192
pd15	26[6]	-	13.38 ± 1.67	17.13 ± 1.57	17.52 ± 1.57	Diet	1.95	0.1653
pd21	26[6]	-	18.24 ± 3.28[a]	27.76 ± 3.09[b]	57.36 ± 3.09[c]	Diet	39.06	0.0001*
pd28	26[7]	-	25.86 ± 4.11[a]	34.51 ± 3.87[a]	49.83 ± 3.87[b]	Diet	9.38	0.0011
pd35	26[7]	-	26.90 ± 2.99[a]	31.52 ± 2.82[a]	47.87 ± 2.82[b]	Diet	14.78	0.0001
pd49	26[7]	-	21.70 ± 3.60[a]	26.90 ± 3.39[a]	44.11 ± 3.39[b]	Diet	11.55	0.0003
adult dams	19[8]	-	17.99 ± 4.22	23.90 ± 4.22	31.54 ± 3.91	Diet	2.81	0.0900

[1]Values are least square means (LSM) ± SELSM
[2]LSM within a row were tested for main effect of diet; different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons
[3]Number of liver samples analyzed
[4]Liver samples pooled by litter
[5]Liver samples pooled from variable number of pups sacrificed
[6]Liver samples pooled from two pups sacrificed
[7]Individual pup liver samples
[8]Individual dam liver samples
[9]Results from analysis of variance (ANOVA)
*Statistical analysis was performed on transformed data; LSM ± SELSM presented are non-transformed values

Figure IV-10: Effect of Graded Levels of Dietary Glucose on Liver Glycogen Levels

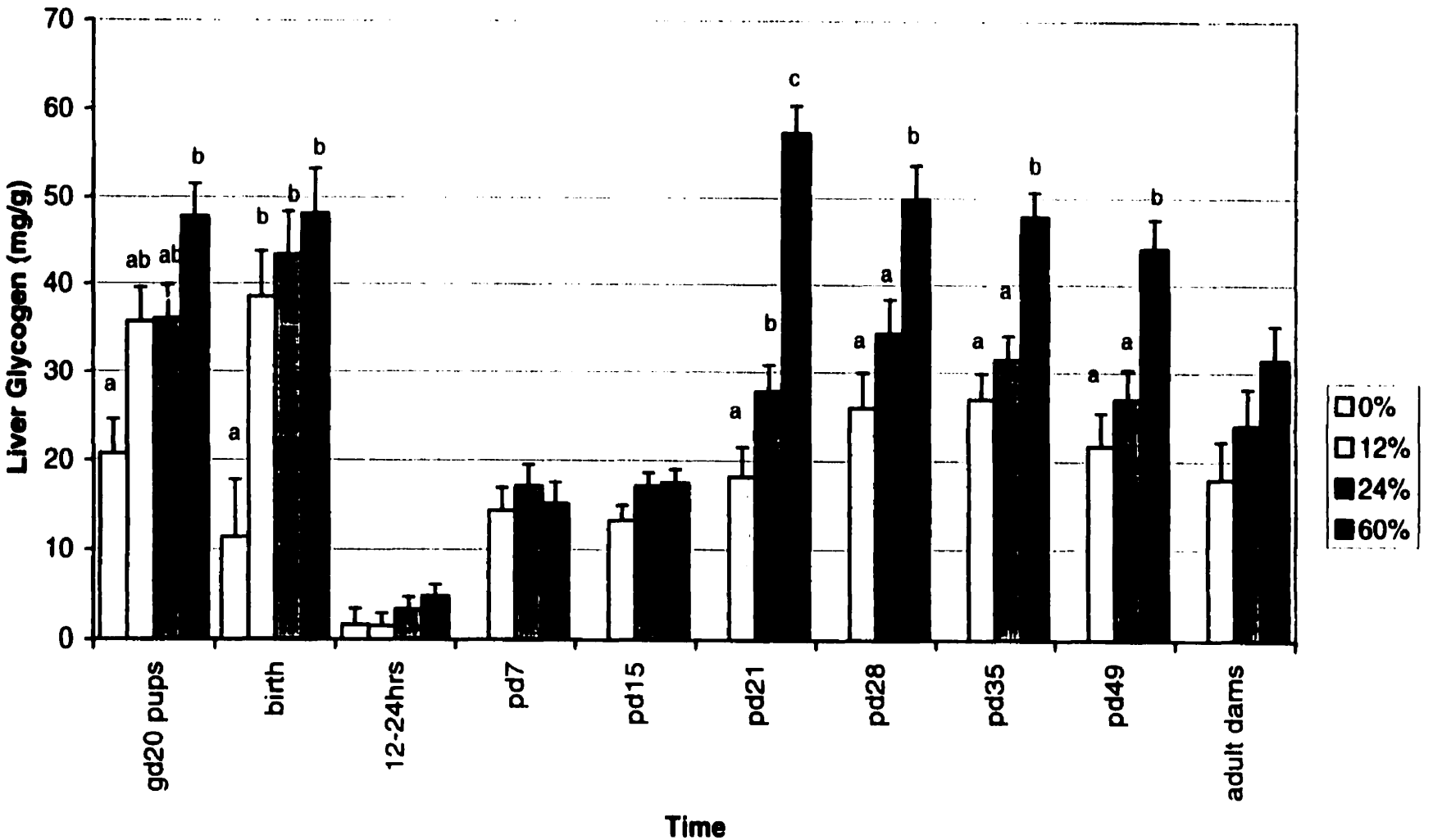


Table IV-11: Effect of graded levels of dietary glucose and time on small intestine GLUT2 (relative AUC).[1,2]

		Time					Statistical Analysis[4]		
PROXIMAL									
Diet	n[3]	pd7	pd15	pd21	pd28	pd49	Main Effect	F Value	p Value
12%	13	1.90 ± 0.55	0.65 ± 0.55	2.76 ± 0.45	1.54 ± 0.45	1.84 ± 0.45	Diet	0.38	0.6874
24%	14	3.43 ± 0.45	1.38 ± 0.55	1.63 ± 0.45	1.68 ± 0.45	1.24 ± 0.45	Time	2.24	0.0943
60%	12	1.77 ± 0.78	0.96 ± 0.55	1.10 ± 0.45	1.66 ± 0.45	2.46 ± 0.45	Diet*Time	2.18	0.0664
DISTAL									
Diet	n[3]	pd7	pd15	pd21	pd28	pd49	Main Effect	F Value	p Value
12%	14	1.35 ± 0.38^{a}	0.76 ± 0.47^{a}	1.92 ± 0.38^{ab}	0.86 ± 0.38	1.00 ± 0.38	Diet	4.69	0.0191
24%	13	1.48 ± 0.38^{a,AB}	1.33 ± 0.67^{ab,AB}	2.95 ± 0.38^{b,A}	0.76 ± 0.38^{B}	0.92 ± 0.38^{B}	Time	5.67	0.0023
60%	12	3.80 ± 0.67^{b,A}	2.47 ± 0.47^{b,AB}	1.30 ± 0.38^{a,B}	1.01 ± 0.38^{B}	1.54 ± 0.38^{B}	Diet*Time	3.08	0.0154

[1]Values are least square means (LSM) ± SELSM

[2]LSM within a column were tested for main effect diet, different lowercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons; LSM within a row were tested for main effect of time, different uppercase letters represent significant differences as indicated by post hoc testing using Scheffe's method of multiple comparisons

[3]Number of small intestine samples analyzed

[4]Results from analysis of variance (ANOVA)

Figure IV-11: Effect of Graded Levels of Dietary Glucose and Time on Proximal Small Intestine GLUT2

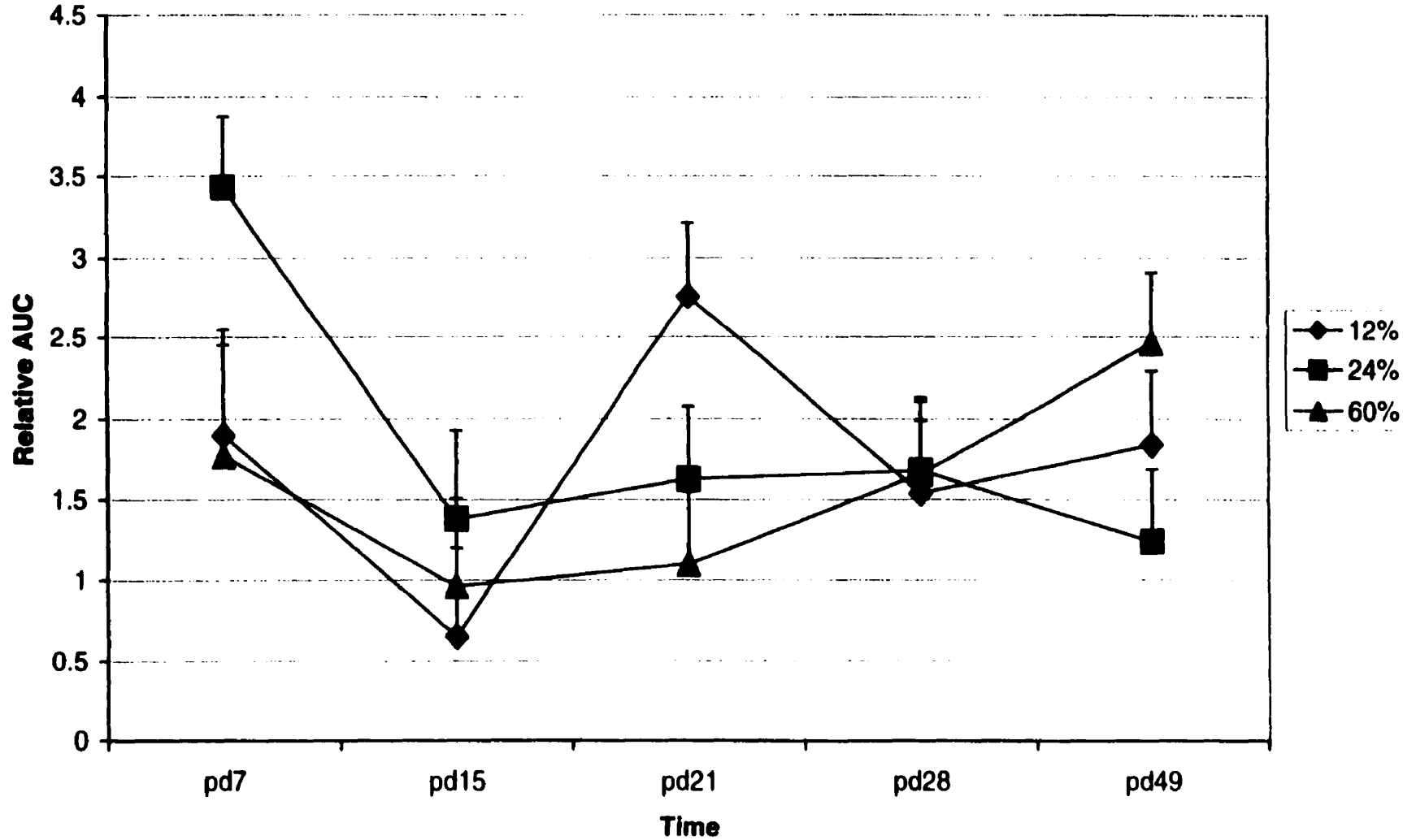
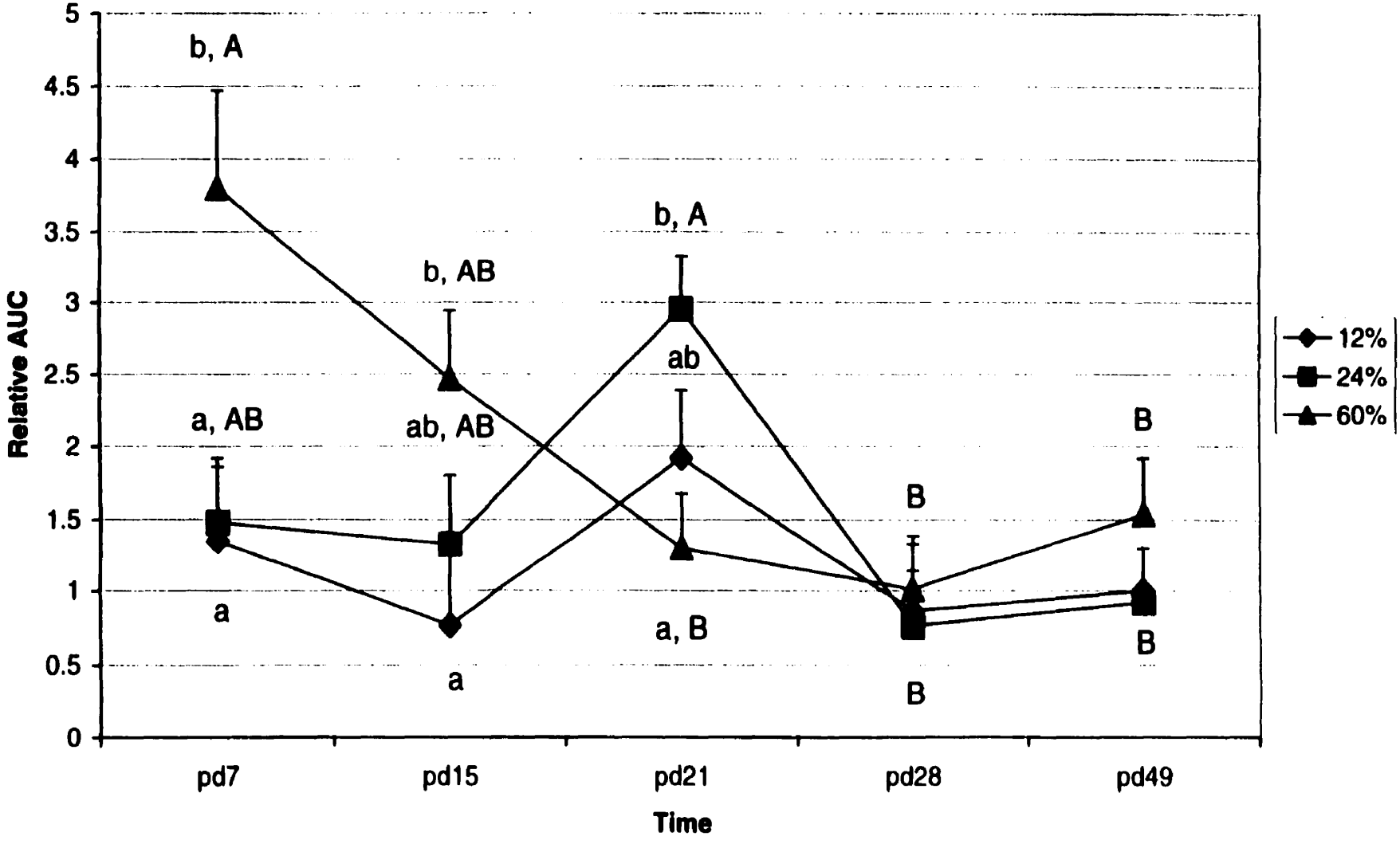


Figure IV-12: Effect of Graded Levels of Dietary Glucose and Time on Distal Small Intestine GLUT2



CHAPTER 5 - DISCUSSION

Nutritional Status:

The dietary model used in this study was designed to create animals who were carbohydrate stressed yet not in turn protein compromised. The level of protein in the experimental diets used, set at the most minimally adequate level so as not to provide extra gluconeogenic precursors, has been shown to be adequate to support normal pup growth (Koski *et al.*, 1986) and is considered adequate by the NRC (1995). Many biochemical markers can be used as indicators of protein status: retinol binding protein; prealbumin; and transferrin which have shorter half-lives; and albumin which has a longer half-life (Shils *et al.*, 1994). The animals used in this study were exposed to the experimental diets for at least 3 and up to 7 weeks, a period of time expanding well beyond the half-life of albumin in the rat which is 1.9-2.4 days (Reed *et al.*, 1988). Therefore, although it has a longer half-life, plasma albumin was the biochemical marker chosen for this study. If the diets had been inadequate in protein, we would have expected to see differences in plasma albumin during the timecourse of this study as well as compromised growth. No differences in plasma albumin were found in either the dams or the pups, at any timepoint with glucose restriction, and pup growth was not compromised in the glucose restricted groups, indicating uncompromised protein status. Brooks and Lampi (1996) had similar findings in their study which involved the feeding of diets containing varying levels of carbohydrate (32, 55 and 67%) to young and adult rats; diet had no effect on plasma albumin. The plasma urea findings in the perinatal period of our study (12% and 24% glucose restricted groups had comparable plasma urea levels to the 60% glucose group) also support the idea that the animals were carbohydrate stressed and not protein malnourished. Elevated plasma urea levels would have been expected in the glucose restricted groups, if the diets were protein deficient. The elevated levels of β-hydroxybutyrate in the glucose deficient pups during the perinatal period indicates the presence of carbohydrate stress. Again, Brooks and Lampi (1996) found similar results with β-

hydroxybutyrate levels increasing significantly with decreasing dietary carbohydrate in young animals.

Dietary carbohydrate restriction produced chronically carbohydrate stressed animals, as indicated by the elevated β-hydroxybutyrate levels, with one exception. The physiological stress experienced by these animals was absent during suckling. During suckling, no differences in plasma β-hydroxybutyrate levels existed between diet groups. This exception is supported by Lanoue & Koski who reported in 1994 that milk concentrations of lactose, glucose and protein were not affected by feeding graded levels of dietary glucose to rat dams. Therefore, pups suckling dams fed glucose restricted diets would be getting adequate glucose in their diets, resulting in lowered plasma β-hydroxybutyrate levels. The absence of any physiological stress during suckling is also supported by the results from the liver glycogen determination. In the present study, liver glycogen concentrations of pups suckling dams fed glucose restricted diets during lactation did not differ from those of pups suckling dams fed a glucose adequate diet. Despite the absence of any physiological stress during suckling, when a solid diet was introduced (at ~ pd21), elevated β-hydroxybutyrate and depressed liver glycogen levels re-emerged, indicative of metabolic stress.

During suckling and in post-weaning, glucose restriction did not have the same consequences on pup body weights as during the perinatal period where pup body weights were comparable. At pd7, no significant differences in pup body weights were produced by the feeding of the glucose restricted diets while at pd15, pd21 and pd28, pups suckling dams fed the 12% glucose restricted diet had significantly higher body weights than the pups of dams fed the 60% glucose adequate diet. This increased body weight among the pups of dams fed the 12% glucose restricted diet was due to higher cumulative food intakes in this group. This finding contrasts with Lanoue & Koski (1994) who found that pups suckling dams fed a 12% glucose restricted diet had significantly lower body weights than pups of dams fed a 60% glucose adequate diet. The pups in the Lanoue & Koski study however did not increase their food intake, preventing increases in body

weights. The results from our study indicate that consumption of an adequate quantity of a glucose restricted diet, while maintaining metabolic stress, as indicated by elevated β-hydroxybutyrate and depressed liver glycogen levels, does not compromise growth.

Small Intestine Glucose Transporters:

Investigation of the hypothesis of this study established the following novel findings: 1) GLUT2 protein levels changed with time during postnatal development in the distal but not proximal small intestine; and 2) dietary glucose intake influenced developmental GLUT2 protein levels in the distal small intestine.

To our knowledge a developmental profile for proximal small intestine GLUT2 protein has not been described to date. All previous studies examining developmental small intestine GLUT2 expression (Miyamoto *et al.*, 1992; Reimer *et al.*, 1997) have analyzed mRNA levels in the proximal small intestine. The developmental profile obtained for GLUT2 protein in the proximal small intestine in this study differs from both mRNA patterns obtained by Miyamoto *et al.* in 1992 and Reimer *et al.* in 1997. These groups both found changes in GLUT2 mRNA expression between the first and fourth weeks of postnatal life. In contrast, the results from our study indicate that GLUT2 protein levels did not change significantly between the first and fourth postnatal weeks of life. The results from our study, when considered against the findings of Miyamoto *et al.* (1992) and Reimer *et al.* (1997), suggest a dissociation between the level of GLUT2 mRNA present and the level of GLUT2 protein expressed.

The only study to date examining the effect of diet on small intestine GLUT2 protein expression (Burant & Saxena, 1994) analyzed proximal small intestine tissue in adult rats fed glucose enriched diets. Although a direct comparison cannot be done between this study and that of Burant & Saxena (1994) because the experimental diets differ and adult, not developmental, small intestinal tissue was examined by Burant & Saxena, an interesting point arises. Burant & Saxena found that feeding a glucose enriched (~60% of energy) diet to

adult rats resulted in increased proximal GLUT2 protein levels compared to the levels found in adult rats fed a control (46% glucose as starch) diet. In our study, which examined the effect of glucose restriction on developmental GLUT2 protein levels, no significant differences in proximal GLUT2 protein expression were found in suckling and post-weaning pups fed either a 12% or 24% glucose restricted diet compared to the levels found in suckling and post-weaning pups fed a 60% glucose adequate diet. The findings of our study suggest that, during postnatal development, the level of dietary substrate is not a regulatory factor in proximal GLUT2 protein levels while the findings of Burant & Saxena (1994) indicate that, in the adult rat, GLUT2 protein levels are upregulated by the level of dietary substrate.

In contrast to the findings for the proximal small intestine, our findings for the distal small intestine demonstrate that: 1) developmental GLUT2 protein levels decrease significantly during postnatal life with adult GLUT2 protein levels attained by postnatal day 28; and 2) GLUT2 protein levels are altered by dietary glucose restriction. The findings of our study suggest that in the absence of any physiological stress (*i.e.* during suckling), dietary substrate availability regulates distal GLUT2 protein levels. However, during suckling, pups from all experimental diet groups are consuming milk which, despite the feeding of glucose restricted diets, contains equivalent levels of lactose, glucose and protein (Lanoue & Koski, 1994). This suggests that substrate availability may not be regulating GLUT2 protein levels. Perhaps GLUT2 protein levels are lower during suckling in the glucose restricted groups due to exposure of the fetus to reduced amniotic fluid glucose (Koski & Fergusson, 1992) *in utero*, preventing the full expression of GLUT2 protein. In the presence of any physiological stress however, this residual *in utero* regulation of GLUT2 protein levels appears to be overridden since the glucose restricted animals upregulated their GLUT2 protein expression at postnatal day 21, when β-hydroxybutyrate and liver glycogen levels indicated the presence of metabolic stress. The results from postnatal day 28 and 49 (*i.e.* all experimental diet groups are at the same, mature GLUT2 protein

level) suggest that post-weaning GLUT2 protein levels are controlled by an intrinsic biological clock, an intrinsic regulatory mechanism which overrides the presence of any other extrinsic regulatory factors such as diet, *in utero* exposure or stress.

Liver and Kidney Glucose Transporters:

We failed to test fully the specific objectives outlined in this study as we did not isolate GLUT2 proteins in liver and kidney tissue. Although the reason for the persistent problems of vertical streaking and multiple bands encountered in the liver and kidney GLUT2 analyses eludes us, we can speculate on possible reasons for the problems encountered. While GLUT2 mRNA is abundant in the perinatal rat kidney (Chin *et al.*, 1993), the translation of this mRNA into protein may be low during neonatal development. However, because GLUT2 protein has been analyzed successfully in the adult kidney (Burant & Saxena, 1994) when the level of GLUT2 mRNA is low (Chin *et al.*, 1993), the level of GLUT2 protein expressed in the kidney during postnatal development may not contribute to our problem. In the case of the liver, the problem of multiple bands in the GLUT2 protein analysis may be due to the presence of different molecular weight forms of the GLUT2 protein as has been found previously for GLUT1 proteins in the brain (Maher *et al.*, 1994). This is unlikely however since Postic *et al.* (1994) did not report any such findings in their analysis of GLUT2 protein in neonatal liver tissue. A more probable explanation to the problems encountered in the kidney and liver GLUT2 protein analysis lies in the methodology used, most probably in the preparation of the kidney and liver tissue samples. We are continuing to modify the procedure used in an attempt to identify solutions to the methodological problem(s) that have troubled us thus far.

Glycogen:

Earlier work from our lab has established that fetal brain, heart and liver glycogen levels are significantly reduced with the feeding of glucose deficient diets (Fergusson & Koski, 1990; Koski *et al.*, 1993; Cobrin & Koski, 1995). We had therefore hypothesized that kidney glycogen reserves would be likewise

compromised with glucose deficiency. The results from our study support the liver glycogen findings of these previous studies however, in contrast to these previous results, we found that kidney glycogen reserves, during the perinatal period, were not vulnerable to this dietary deprivation of glucose during pregnancy. Similar to the kidney glycogen findings from this study, it has recently been established in our lab that small intestine glycogen reserves in the perinatal period were not vulnerable to glucose deficiency (Anderson, S.A., M.Sc. Thesis). Therefore, perinatal glycogen levels in organs which require glucose as an energy source (*e.g.* brain) or are major storage sites for glycogen (*e.g.* heart and liver) are perturbed by maternal dietary glucose deprivation while perinatal glycogen levels in organs which are not major storage sites for glycogen nor require glucose as an energy source (*e.g.* kidney and small intestine) are not perturbed by maternal dietary glucose deprivation. Postnatally, kidney glycogen levels were unaffected by dietary glucose intake except at pd7, where glycogen levels were higher in the 12% glucose restricted group. Interestingly, increased mortality occurs with glucose restriction at pd7 (Koski *et al.*, 1990), suggesting that a lack of glycogen mobilization in the kidney, an important source of glucose under stressed conditions (Kida *et al.*, 1978; Schoolwerth *et al.*, 1988), may play a role in this increased mortality. The post-weaning results from this study which indicate that kidney glycogen stores are not altered by dietary carbohydrate restriction support the findings of Brooks and Lampi (1996) who also found kidney glycogen concentrations to be unaffected by changes in dietary carbohydrate. The finding in this study that distal GLUT2 protein levels are altered postnatally with dietary glucose restriction indicate a dissociation between distal small intestine GLUT2 protein content and distal small intestine glycogen reserves as Anderson, S.A. (M.Sc. Thesis) found no changes in distal small intestine glycogen content with the feeding of glucose restricted diets during early postnatal development.

Summary:

Investigation into the hypothesis of this study established that GLUT2 protein levels are perturbed in the distal but not proximal small intestine with

maternal dietary glucose restriction. Our results show the distinct responses of the proximal and distal small intestine to the same developmental and dietary challenges. No significant changes in GLUT2 protein levels were found in the proximal small intestine between the first and seventh weeks of postnatal life, suggesting a dissociation between GLUT2 mRNA expression, which has been found by others to change throughout postnatal development, and GLUT2 protein levels. GLUT2 protein levels in the proximal small intestine were also unaltered by dietary glucose restriction. In contrast to the findings in the proximal small intestine, significant differences in GLUT2 protein levels were found in the distal small intestine between the first and seventh weeks of postnatal life and with dietary glucose restriction. It appears that distal GLUT2 protein levels may be suppressed by inadequate nutrition *in utero*, enhanced by stress and regulated by an intrinsic biological clock in post-weaning.

APPENDIX 1: STATISTICAL ANALYSIS

1) Cumulative Food Intake; Maternal Body Weight; Litter Size; Litter Weight; Amniotic Fluid Glucose, β-Hydroxybutyrate and Urea; Plasma Albumin, Glucose, β-Hydroxybutyrate and Urea; Kidney and Liver Glycogen

```
PROC GLM;
CLASSES DIET;
MODEL CFI MBW LTSZ LTWT AMFG AMFH AMFU PALB PGLU PHBA PU
KGLY LGLY = DIET;
```

where CFI = cumulative food intake, MBW = maternal body weight, LTSZ = litter size, LTWT = litter weight, AMFG = amniotic fluid glucose, AMFH = amniotic fluid β-hydroxybutyrate, AMFU = amniotic fluid urea, PALB = plasma albumin, PGLU = plasma glucose, PHBA = plasma β-hydroxybutyrate, PU = plasma urea, KGLY = kidney glycogen and LGLY = liver glycogen.

2) Pup Body Weight

```
PROC MIXED;
CLASSES DIET DAM;
MODEL PBW = DIET;
RANDOM DAM(DIET);
```

where PBW = pup body weight.

3) Small Intestine GLUT2

```
PROC GLM;
CLASSES DIET REGION TIME;
MODEL GL2 = DIET REGION TIME DIET*REGION DIET*TIME REGION*TIME
DIET*REGION*TIME;
```

where GL2 = small intestine GLUT2.

CHAPTER 6 - REFERENCES

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