MATERNAL DIETARY GLUCOSE INTAKE AFFECTS NEONATAL GASTROINTESTINAL DEVELOPMENT IN RATS

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February 1999

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

ACKNOWLEDGEMENTS

I would like to thank Dr. Koski for her enthusiasm for this research project, and for her guidance and encouragement through the years. This has been a challenging, exciting and overall irreplaceable learning experience. Thank-you also to Dr. Wykes and Dr. Bissonnette for their support and their input into the process.

Thanks also go to Erika – thank-you for being there as a co-investigator and friend. The encouragement and support (BA!) you offered enriched the experience. To Dr. Cecile Logan, thanks for sharing your knowledge and experience of histology and opening my eyes to the fascinating world under the high-powered microscope. Tina Fisher and Elizabeth Murad, thank-you for your interest and time – this work is more complete because of you.

My deepest gratitude to all my friends, both on campus and elsewhere, for your constant support and validation. You made my last years at Mac great!

I would especially like to express my appreciation for the support of my family. You are always there for me.

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ABSTRACT

To test the hypothesis that maternal glucose restriction (GR) would compromise small intestine (SI) growth and development, we used a diet induced model of IUGR. Pregnant rats and offspring were fed isoenergetic diets {0% (deficient), 12, 24% (restricted), 60% (adequate) glucose} from gestation day (gd) 0 through adolescence. SI tissue was collected at gd20, birth, 12-24h, postnatal day (pd) 7, 15, 21, 28, 35, 49 and in controls. GR affected pup weight at early timepoints resulting in IUGR; beyond effects due to differences in body wt, GR compromised SI length at 12-24h and promoted SI growth during peak lactation (pd15; total and distal wts). Dietary glucose regulated *in utero* (gd20) expression of sodium-dependent glucose co-transporter (SGLT1) protein. Diet-induced precocious maturation of lactase and sucrase was observed in glucose deficient animals. In summary, there were periodic but no permanent effects of dietary glucose intake on gut growth and development.

RÉSUMÉ

Pour évaluer l'hypothèse qu'une restriction de glucose maternel influence la croissance et le développement du petit intestin (SI), nous avons utilisé un modèle de croissance intra-utérine retardée (IUGR) produit par le régime alimentaire. Des rates enceintes et leurs portées ont suivi un régime alimentaire isoénergétique {0% (déficient), 12, 24% (réduit), 60% (adéquat) de glucose} du jour de gestation (gd) 0 à l'adolescence. Des prélèvements du SI ont été effectués à gd20, à la naissance, entre 12 et 24 h, à 7, 15, 21, 28, 35 et 49 jours après naissance (pd) et chez le group temoin. La restriction de glucose a eu un effet sur le poids des rejetons durant la période périnatale résultant en une IUGR. Au-delà des effets dus aux différences de poids, la restriction du glucose a eu des effets négatifs sur la longueur du SI entre 12 et 24 h et a encouragé la croissance du SI pendant la période maximale d'allaitement (pd15, poids total et distal). It a été observé *in utero* que la protéine du co-transporteur du glucose dépendant du sodium (SGLT1) était réglé par le taux de glucose de la diète. Une maturation précoce de lactase et sucrase influencée par le régime alimentaire a été notée chez les animaux déficients en glucose. En résumé, la prise de glucose alimentaire a eu des effets périodiques mais non permanent sur la croissance et de développement du SI.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AUC	area under the curve
BBMV	brush border membrane vesicle
β-HBA	β-hydroxybutyrate
BLM	basolateral membrane
BLOTTO	bovine lacto transfer technique optimizer
EGF	epidermal growth factor
gd	gestation day
GGM	glucose-galactose malabsorption
Glut2	mRNA for the facilitative glucose transporter GLUT2
GLUT2	protein level of the faciltative glucose transporter GLUT2
IGF	insulin-like growth factor
IUGR	intra-uterine growth retardation
MVM	microvillous membrane
pd	postnatal day
SEM	standard error of the mean
SGLT1	sodium-dependent glucose co-transporter
TAFF	trans-amniotic fetal feeding
TTBS	Tris buffered saline

CHAPTER 1: OVERVIEW

"The influences of diet on growth and development are critical issues in nutrition research. They are especially evident for the intestine, as it responds to qualitative and quantitative changes in dietary inputs as animals develop from fetuses to adults....Because of difficulties inherent in studying human infants, it is essential that investigators not only use appropriate animal models but also recognize their limitations. Care and management of pre-term infants would benefit from a better understanding of developmental events during the final trimester...infants with digestive and nutritional problems will benefit from research directed at identifying specific dietary components that influence intestinal development, the mechanisms of actions, and the periods of responsiveness." (Buddington, 1994). This time period is difficult to study in human fetuses. The rat model provides a suitable alternative, as rat neonates are altricial, and therefore relatively immature at birth compared to full-term human infants. The period of rapid postnatal intestinal maturation which occurs in rats makes them a suitable model for the study of pre-term infant nutrition and intestinal development.

Ontogenic development of the small intestine largely follows an internal program. It is dependent upon (1) genetic endowment, which provides the ultimate control over differentiation, (2) endogenous regulatory mechanisms, including steroid and thyroid hormones which may act to control the expression of genetic endowment, and (3) an intrinsic biological clock which provides a controlled temporal sequence for developmental events including enzymatic development, cellular proliferation and differentiation. However, external environmental influences may also act to influence the dynamic relationship between regulatory mechanisms and the timing of developmental events (Lebenthal, 1989; Figure 1). Attainment of full genetic potential thus requires the presence of an optimal external environment, including diet.

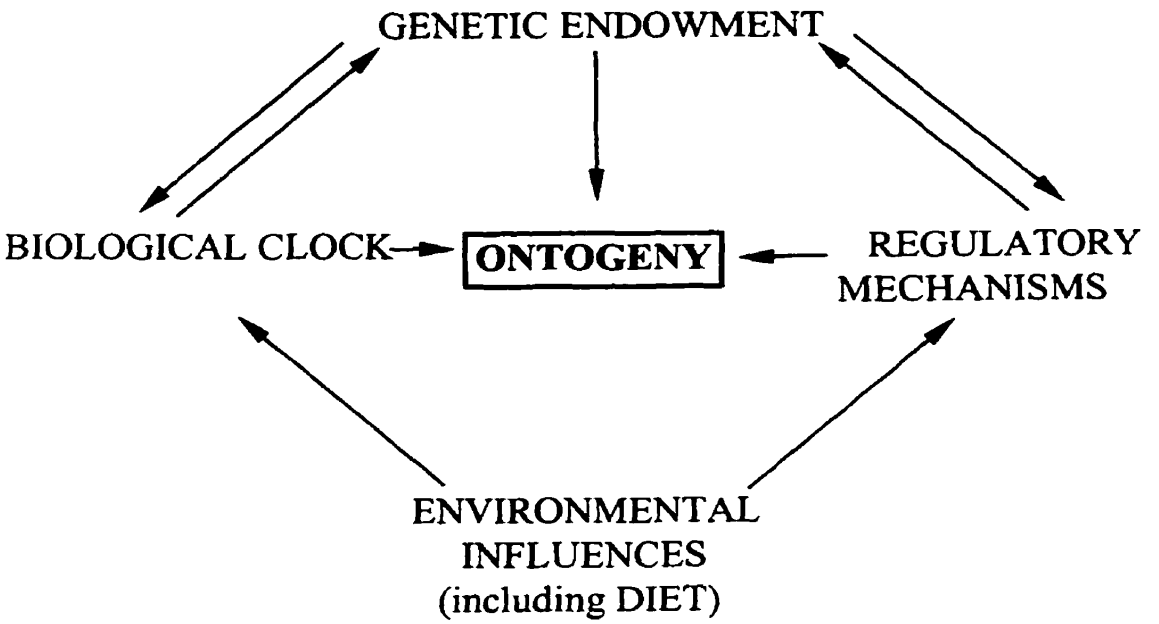
The adaptability of the intestine to dietary intake has been thoroughly studied for adult and young adult animals, however the impact of diet during early development remains largely unstudied. It has been shown that maternal dietary intake can impact upon gastrointestinal ontogeny as predicted by Lebenthal's model of ontogeny. Maternal dietary protein intake has been shown to influence in utero pancreatic structural and functional development in rats (Lebenthal, 1989). Postnatal malnutrition in suckling rats

reduces intestinal weight, retards the disaccharidase profile, and augments glucose uptake as well as altering brush border membrane biochemical properties (Patlak *et al.*, 1981). In fatal cases of human kwashiorkor, atrophy of the intestinal mucosa has been found while marasmus has been shown to result in structural as well as functional impairment of the intestine including blunting of villi, elongated crypts, branching of microvilli, thickened basolateral membrane, and lactose and sucrose intolerance (Lebenthal, 1989). Maternal and neonatal dietary intake may also affect critical period programming and influence intestinal structural development, as demonstrated in rats (Karasov *et al.*, 1985).

The ability of the adult small intestine to adapt to qualitative and quantitative changes in diet is well documented (Diamond and Karasov, 1984, 1987; Karasov *et al.*, 1985; Buddington *et al.*, 1991; Ferraris *et al.*, 1992a and 1992b), however the onset of the this adaptive capability for the sodium-dependent glucose co-transporter (SGLT1) protein and disaccharidase levels during the development of the small intestine has not been clearly established.

We know that glucose is an important metabolic fuel for the developing intestine (Windmueller and Spaeth, 1980; Karasov *et al.*, 1985; Kimura, 1987), however the specific impact of any restriction in maternal dietary carbohydrate intake on neonatal gastrointestinal structural and functional development has not been studied. The small intestine mucosa of suckling rats contains high levels of gluconeogenic enzymes (Hahn and Wei-Ning, 1986), suggesting that a glucose production is essential for the mucosa. No study to date has investigated whether glucose uptake or levels of glucose transporters can be regulated by levels of dietary glucose in the maternal diet in utero or in early postnatal life. A diet model developed in our lab provides the opportunity to investigate the effects of a diet restricted specifically in glucose. This study will use this diet model to investigate the following hypotheses; does the restriction of dietary glucose during pregnancy and through early adulthood of the offspring (1) result in alterations in small intestine structure as assessed by weight, length and histology and (2) affect small intestine functional development as assessed by glycogen reserves, disaccharidase activities and the level of sodium-dependent glucose co-transporter protein (SGLT1)?

Figure 1: A MODEL OF GASTROINTESTINAL ONTOGENY
(Based on Lebenthal, 1989)



CHAPTER 2: LITERATURE REVIEW

1. INTRODUCTION

The literature review to follow will begin with a discussion of the normal sequence of developmental events for the fetal and neonatal small intestine, focusing of structural and functional markers of development. Superimposed upon normal gut development will be the theme of the impact of diet, specifically dietary glucose, upon the described developmental characteristics. The importance of luminal nutrients in gut development will focus upon amniotic fluid for in utero development followed by the role of postnatal diet; the role of hormones and growth factors on pre- and postnatal gut development will also be discussed. The significant and important role of glucose as a metabolic fuel for the developing gut will then be followed by a discussion of specific functional outcomes of normal gut development, including the acquisition of a fully adult profile of disaccharidases and glucose transporters, including mature proximal-to-distal gradients for these enzymes and transporters.

2. DIET AND ONTOGENY OF THE NEONATAL SMALL INTESTINE

2.1 Structural Ontogeny of the Small Intestine

Structural and functional development of the small intestine occurs in five distinct phases, the first three occur prenatally in humans (Weaver and Walker, 1989) and in rats Klein, 1989). Firstly, the gut tube is formed with the coordination of regulatory peptides. In the second stage, villi are formed next and cell proliferation becomes limited to the primordial crypts. Cellular differentiation then occurs with the appearance of enterocytes, microvilli, the development of brush-border hydrolases and nutrient transporters. Developmental timing for the appearance of hydrolases and transporters differs between species, with the appearance of adult-like digestive capacity by 22 weeks gestation in human fetuses. The third prenatal stage involves continued maturation and functional development of the intestine with the appearance of motility, secretory and absorptive functions (Buddington 1994; Klein, 1989). In less precocious species, such as rats, enzyme and transporter development appear much later during gestation (about 80% of

gestation) (Buddington, 1992, 1994).

The final two phases of small intestine development occur postnatally. Neonatal intestinal development requires two sequential sets of adaptive changes. First, the transition from an intra- to extrauterine environment with the introduction of a milk based diet of relatively stable macronutrient composition, and secondly the maturation required for weaning to a solid diet (Lebenthal, 1989; Buddington, 1994). The first of these two postnatal developmental phases includes preparation of the epithelium, with fully functional intestinal hydrolases and nutrient transporters, capable of digesting and absorbing milk components and in order to meet the high nutritional requirements of the neonate (Buddington, 1994). Rapid intestinal growth immediately after birth is characteristic of many species, including precocial pigs whose mucosal mass doubles in the 24h period immediately after birth (Widdowson *et al.*, 1976 in Buddington, 1994), however this growth is less rapid in more altricial species such as rats (Tolaza and Diamond, 1992). For rodents this period of small intestinal development is characterized by the presence of vacuolated enterocytes along the villus profile from around birth until the end of the second postnatal week (Klein, 1989). These immature enterocytes are capable of absorbing proteins, both immunological and nutritional. The second postnatal developmental phase occurs around weaning, when the animal will undergo a second postnatal dietary transition from a milk based diet to a solid food diet. This involves maturation of the small intestine mucosa, with the acquisition of rapidly migrating non-vacuolated enterocytes, displaying fully functional adult digestive and absorptive capabilities (Buddington, 1994; Klein, 1989).

2.1.1 Diet and Structural Ontogeny of the Intestine

Both prenatal and postnatal diet have been shown to have an impact upon small intestine development. The role of amniotic fluid in fetal growth and development has clearly demonstrated that in the absence of ingested amniotic fluid, small intestine growth is retarded (Mulvihill *et al.*, 1985, 1986; Jacobs *et al.*, 1989; Trahair *et al.*, 1986, 1997; Avila and Harding, 1991). Conversely, the supplementation of amniotic fluid termed transamniotic fetal feeding (TAFF) with galactose resulted in a significant increase in

proximal mucosal weight (Buchmiller et al., 1992). Intrauterine growth retardation (IUGR) induced by reduction in blood flow across the placenta was shown to decrease small intestine and pancreas weight as well as to decrease the total level of disaccharidases in rats (Lebenthal *et al.*, 1980, 1981).

During postnatal development, undernourished sucklings have been found to have reduced small intestine and pancreas weights, and similar perturbances in disaccharidase levels to those suffering from IUGR (Guiraldes and Hamilton, 1981). In humans, small intestine and pancreatic atrophy was been found in cases of fatal kwashiorkor, while marasmus resulted in blunted villi with elongated crypts, branched microvilli, and accompanied by thickening of the basolateral membrane as well as lactose and sucrose intolerance (Lebenthal, 1989). In mice fed either a high-carbohydrate or a carbohydrate-free diet from the end of gestation (gd 15), body weight was permanently reduced in animals fed the carbohydrate-free diet; the small intestines were proportionally shorter and the proximal intestine was proportionally lighter in the carbohydrate-free group (Karasov *et al.*, 1985). The net result on gastrointestinal development of these dietary deficits was an apparent retardation of the biological clock with a structurally and functionally immature small intestine mucosa.

2.2 Functional Ontogeny of the Small Intestine

Carbohydrate assimilation by the small intestine requires the presence of a functionally developed mucosa, capable of carbohydrate digestion utilizing enterocyte associated disaccharidases, absorption via monosaccharide transporters and storage. The sodium-dependent glucose co-transporter (SGLT1) is solely responsible for glucose and galactose entry into the intestinal enterocyte. The timing of the appearance of SGLT1 varies by species. In humans, it appears early in gestation, at around week 9-10, or 25% of gestation, whereas in altricial species such as rats, it does not appear until gd 16 or at 80% of gestation (Buddington, 1994). "Dietary carbohydrate polymers enter the duodenum, are split by pancreatic amylase and by intestinal amylase and disaccharidases, and the resulting glucose is absorbed. Thus, glucose concentration should rise proximally along the intestine to a peak due to polymer hydrolysis, then decrease distally due to

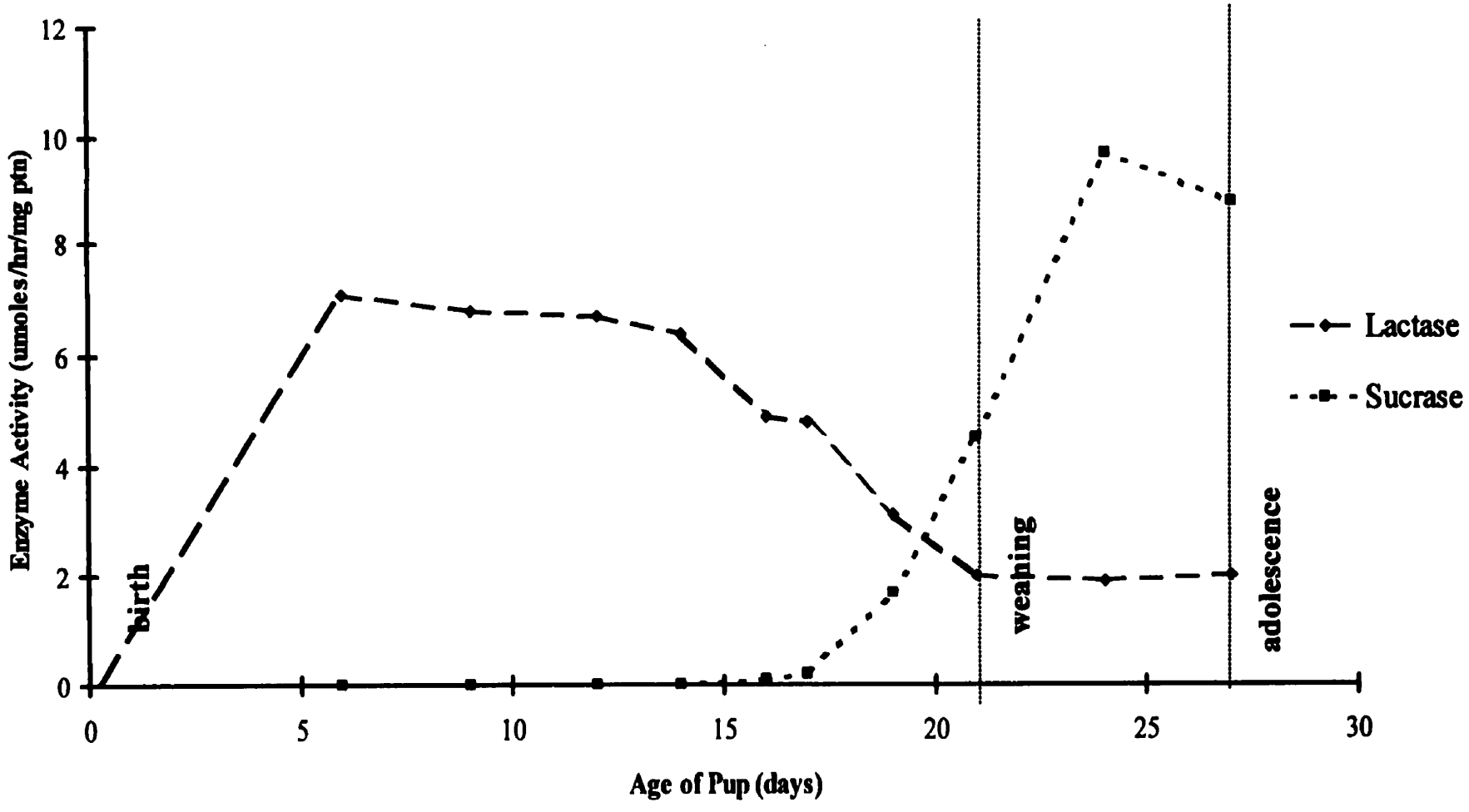
absorption." (Diamond & Karasov, 1984). Correspondingly, higher levels of SGLT1 protein are expected in proximal than in distal gut, matching the absorptive capability of the gut to the pattern of carbohydrate digestion along its length. The establishment of the proximal-distal gradient of transporters is established at approximately 55% of gestation in humans, but not until around postnatal day 15 in rats. Freeman and Quamme (1986) found that younger animals (<7-8 wks) had a greater overall capacity for glucose transport (per mg protein) than older animals (>12-13 wks).

The mucosal disaccharidases play a key role in the liberation of monosaccharides for uptake into brush border enterocytes. The normal developmental profile for lactase shows peak lactase activity at birth in most placental animals, preparing the animal for the digestion of lactose, the major carbohydrate in milk (Henning, 1987). At human parturition, lactase reaches a level twice that of the adult (Buddington, 1994). Prior to weaning the intestine must be prepared to digest and absorb a mixed solid diet, requiring additional brush border hydrolases such as sucrase, maltase, and isomaltase. In rats these enzymes are virtually undetectable in the first two postnatal weeks, however they rise rapidly from approximately d16, peaking at adult levels by the beginning of week four (Henning, 1987; Castillo *et al.*, 1992) (**Figure 2**). A surge in the level of plasma corticosterone at d12 precedes the postnatal increase in sucrase suggesting a potential hormonal triggering of weaning. Although diet is not likely a trigger of weaning, the composition of the diet consumed during postnatal weeks three and four may determine plateau levels of certain gastrointestinal hormones (Henning, 1987).

2.2.1 Diet and Functional Ontogeny of the Small Intestine

Intestinal hydrolytic capabilities, nutrient transporter levels and uptake capacity can be affected by diet. Pre- and postnatal malnutrition affects intestinal hydrolase levels (Lebenthal, 1989; Guiraldes and Hamilton, 1981), resulting in a relative retardation of the developmental profile of the small intestine. Evidence for critical-period programming of intestinal nutrient transport by dietary substrates was shown by Thomson et al. (1989). In this elegantly designed study, the ratio of polyunsaturated to saturated fatty acids in the diet during early postweaning was shown to irreversibly affect jejunal and ileal glucose

Figure 2: Developmental Pattern of Lactase and Sucrase



(adapted from Henning, 1978)

and galactose uptake later in life. Maintenance of intestinal SGLT1 protein requires the presence of its luminal substrates glucose or galactose, suggesting that the appearance of SGLT1 is diet dependent (Shirazi-Beechey *et al.*, 1991). Glucose uptake capacity was reduced in proportion to reduced intestine length and weight in mice fed a carbohydrate-free diet from the end of gestation until postnatal week 9 (Karasov *et al.*, 1985). Although Buchmiller *et al.* (1992) showed that adaptive regulation of glucose and galactose uptake occurs prenatally, it is still commonly held that adaptive regulation of glucose transport is not achievable until postweaning (Buddington, 1994; Lynne Searles, M.Sc. thesis).

Small intestine functional development is further characterized by the development of proximal-distal or jejunoileal gradients of intestinal hydrolases, nutrient transporters and structural parameters. Consideration of region specific information from the literature is critical to the proper evaluation of gut growth and development. The timing of appearance of this gradient varies by species and by functional parameter. For example, postweaning, villus height decreases twofold from duodenum to ileum (Ferraris *et al.*, 1992b). The proximal-distal gradient for lactase and sucrase is established by the end of weaning (Henning, 1987). For lactase, the proximal-distal gradient is established by postnatal day 21, and is due to a maturational decline in ileal lactase levels, without any significant decrease in lactase levels proximally. The presence of intraluminal nutrients is essential for the normal maturational decline in ileal lactase levels, but not for jejunal lactase (Castillo *et al.*, 1992). The proposed mechanism for this decrease in lactase activity in the postweaned animal is the presence of cytogenetic changes, which result in a reduction of enterocyte life-span (Tsuboi *et al.* 1981). Sucrase is virtually undetectable until the third postnatal week (Henning, 1987). In contrast to the establishment of the proximal-distal gradient for lactase, jejunal rather than ileal sucrase levels do not achieve normal adult levels in the absence of luminal nutrients; dietary stimulation is partly responsible for the mature profile of sucrase in the small intestine (Castillo *et al.*, 1992).

Diamond and Karasov (1984) demonstrated that glucose uptake capacity in adult mice fed carbohydrate peaks in the jejunum and is lowest in the ileum. They note that

intestinal mass also peaks in this region, but determined that the gradient in uptake exists even when differences in mass are accounted for. Freeman and Quamme (1986) also found that the glucose transport capacity of the gut is greater in proximal than in distal portions for both young and old rats; in fact in rats older than 21 days, glucose uptake was 1.6 to 3 times higher per mg and per cm of gut respectively in the proximal and middle than in the distal small intestine (David *et al.*, 1995). In adult mice, feeding a high carbohydrate diet resulted in a 3.3-fold increase in sucrase in the duodenum, a 2.1-fold increase in upper jejunum, but no increase in the lower jejunum or ileum (Ferraris *et al.*, 1992b). The impact of diet on the establishment of the proximal-distal gradient of glucose uptake capacity and glucose transporter protein levels has not been studied.

3. FACTORS AFFECTING NEONATAL GASTROINTESTINAL DEVELOPMENT

Luminal nutrients as well as circulating hormones and growth factors play a major role in development of the gastrointestinal tract both in utero and postnatally. These contribute significantly not only to gut growth, but to overall somatic growth of the developing fetus (Mulvihill *et al.*, 1985). Natural surges in hormones and growth factors accompany developmental changes; exogenous administration of hormones and growth factors, notably corticosterone, thyroxine, and epidermal growth factor, can also induce precocious gut development, and functional changes in the gut, such as the appearance of sucrase and maltase and the decrease in lactase occurring postnatally (Lebenthal, 1989; Henning, 1987).

3.1 Luminal Nutrients

3.1.1 Amniotic Fluid Nutrients

The developing prenatal small intestine is bathed in amniotic fluid and is thus exposed to the nutrients contained in amniotic fluid. Amniotic fluid is considered to be a nutritionally dilute fluid, however the impact of swallowing a large volume of this fluid on small intestine growth and development may be more significant than that predicted solely on the basis of its nutrient content. Amniotic fluid glucose and protein

concentrations are low near term, however they are much higher earlier in gestation (Jacobs *et al.*, 1989). Their disappearance during gestation suggests they play a nutritional role. Furthermore, energy requirements of the gut are approximately 25% of the basal metabolic requirement, even though the gut comprises less than 25% of total body weight (Trahair, 1993). Therefore, the fetus may in fact be dependent upon swallowing of amniotic fluid to provide 10-14% of its energy requirements (Mulvihill *et al.*, 1985). High protein turnover (Nakshabendi *et al.*, 1995) and energy requirements (Trahair, 1993) of the gastrointestinal tract may make it very sensitive to the nutrients contained in amniotic fluid or to nutritional deprivation via maternal circulation.

The significant role of intraluminal nutrients in normal prenatal small intestine development has been demonstrated in studies which eliminated the swallowing of amniotic fluid. Nutrients and/or hormones contained within amniotic fluid are required for normal gastrointestinal development, specifically for mucosal growth (**Table 1**). It is known that the normal human fetus swallows up to 750 mL of amniotic fluid daily (Pritchard, 1966) and has attained the capacity to absorb carbohydrate and protein from amniotic fluid during the third trimester (Buchmiller, 1992). Intestinal nutrient transport capacity for monosaccharides and selected amino acids develops later in the gestation of altricial species: by 80% of gestation in rats (Buddington, 1994). [^{14}C] D-Glucose and [^{3}H]proline infused into the amniotic fluid were absorbed by the developing small intestine, and most highly concentrated into fetal lung and small intestine including the stomach (Phillips *et al.*, 1991). Normal intestinal development therefore requires the presence of nutrient transporters in utero to allow for absorption of ingested nutritive constituents from amniotic fluid.

Fetal as well as neonatal and adult nutrient transport is known to adapt to changes in dietary composition (Buddington, 1992). Adaptive regulation of glucose transporters has been studied extensively in the postnatal period (Ferraris and Diamond, 1989; Shirazi-Beechey *et al.*, 1991; Diamond and Karasov, 1984), however few studies have examined the capability of the small intestine to adapt to dietary changes prenatally. Phillips *et al.* (1990) describe transamniotic fetal feeding (TAFF) whereby amniotic fluid is supplemented to provide nutrients for possible treatment of intrauterine growth

retardation (IUGR). TAFF bypasses the dysfunctional placenta to provide nutrients to the developing fetus via the amniotic fluid, thus exploiting the potential for upregulation of the nutrient transporters present along the fetal intestine. Transamniotic administration of galactose to fetal rabbits resulted in the increased total uptake of glucose and galactose (Buchmiller *et al.*, 1992). Thus amniotic fluid nutrients have the potential to regulate nutrient uptake in utero, and ultimately could impact upon small intestine and whole body growth and development.

Table 1: Esophageal Ligation and Prenatal Small Intestine Growth

Authors	Species	Effects of Ligation
Mulvihill *et al.* (1985)	rabbits	•• reduced fetal somatic and SI growth • decreased amniotic fluid volume
Mulvihill *et al.* (1986)	rabbits	• reversal of growth deficit by infusion of bovine amniotic fluid, epidermal growth factor and pentagastrin
Jacobs *et al.* (1989)	rabbits	•• growth retarded regardless of timing or duration of ligation • no consistent histological changes found
Trahair (1986)	sheep	•• mucosal growth affected • diminished epithelium, reduced villus height, increased density of villi and crypts
Trahair *et al.* (1997)	sheep	• lighter SI with ligation over 1st half of gestation • enterocyte maturation was delayed • SI growth deficits may have long-term etiology
Avila & Harding (1991)	sheep	•• reduced SI growth; regional differences • ↓ villus height, ↑ crypt depth proximally • thicker submucosa distally • reduced enterocyte migration along villus

3.1.2 Postnatal Diet

During postnatal life, luminal contents, either milk or food, have also been shown to influence the morphologic and functional development of the postnatal small intestine. Differences in intestinal growth were observed in suckling pigs fed milk replacer diets with varying sources of carbohydrate (Vega *et al.*, 1992). Dietary carbohydrate source (lactose vs. 60:40 maltodextrin:sucrose) was found to exert a non-specific effect on amino acid uptake without affecting monosaccharide uptake, and pigs fed the

maltodextrin:sucrose diet were smaller and had proportionally smaller small intestines than those fed the lactose containing diet. Karasov *et al.* (1985) investigated postnatal critical period programming of intestinal transport in the young of pregnant mice fed a high-carbohydrate (54%) versus carbohydrate-free diet from the end of pregnancy through postnatal week nine. A permanent reduction in body weight, rate of growth, small intestine length as well as total glucose uptake capacity was seen with the feeding of the carbohydrate-free diet between the end of gestation and postnatal week 9. The critical effect was suggested to occur close to birth, however no measurements were made at that timepoint.

Diet composition may also play a critical role in glucose transporter function. Maintenance of intestinal sodium dependent glucose co-transport protein (SGLT1) in sheep requires the presence of its luminal substrates, glucose or galactose; this suggests that the appearance of SGLT1 is diet dependent. In ruminant sheep, the normal age-related decline in SGLT1 protein is associated with decreases in SGLT1 mRNA, and has been prevented by the intestinal infusion of glucose to adult sheep (Shirazi-Beechey *et al.*, 1991).

3.2 Hormones and Growth Factors

3.2.1 Amniotic Fluid and Prenatal Environment

Although esophageal ligation has clearly demonstrated the importance of fetal swallowing to normal growth and development, discrepancies in tissue protein and energy accrual between that predicted from current assumptions about amniotic fluid composition, energy content and consumption and fetal growth found in Jacobs *et al.* (1989) indicate that these assumptions are likely inaccurate, and that trophic factors in amniotic fluid likely also play an important role.

Amniotic fluid contains growth factors and hormones including gastrin, epidermal growth factor and insulin-like growth factor (Jacobs *et al.*, 1989; Trahair *et al.*, 1986; Trahair, 1993) all of which may enhance fetal small intestine growth. Other trophic factors present in amniotic fluid include an unidentified factor present at gd19 which specifically induces differentiation of villi and crypts, accelerating the intrinsically

programmed differentiation of the small intestine mucosa (Calvert *et al.*, 1991). This crypt differentiation factor may be derived from fetal membranes.

Avila *et al.*, 1989, investigated the role of gastrin in the prenatal development of the gastrointestinal tract. The presence of fluid in the stomach stimulates gastrin secretion, which has trophic effects on the stomach mucosa in adult animals. In this study, the gastric antrum, and thus the major source of gastrin, was surgically removed from gd 90 fetal sheep. Fetuses were killed at gd 135 and the entire gastrointestinal tract removed. No major changes in gastrointestinal growth or morphology were found, however crypt density and crypt-to-villus ratio were significantly reduced in the proximal gut, but only villi density was reduced in the distal SI when compared to sham operated controls. They concluded that gastrin has a trophic role on the gut mucosa, however this effect is only apparent under the experimental conditions used and not during normal fetal development. Unfortunately, the experiment was terminated at gd135 of a 145 day gestation, thus the authors failed to examine an important period of rapid small intestine growth and development immediately prior to birth.

Epidermal growth factor (EGF) is secreted by the submandibular glands, Brunner's glands of the small intestine, and is found in amniotic fluid, colostrum, milk, saliva and plasma (Lebenthal, 1989). The presence of EGF in these fluids suggests a trophic role of EGF in pre- and postnatal gastrointestinal development. Insulin-like growth factor-I (IGF-I), in culture, stimulates intestinal crypt cell proliferation (Winesett *et al.*, 1995). Glucocorticoids are well recognized as influencing small intestine development, both pre- and postnatally (Lebenthal, 1989).

3.2.2 Hormones and Growth Factors in Postnatal Intestinal Development

Evidence of the importance of hormones and growth factors in postnatal intestinal development comes from a variety of sources. Epidermal growth factor (EGF) receptors are required for this polypeptide hormone to exert its effects. Levels of EGF receptors are especially high at times of major morphologic change in the developing intestine (Bird *et al.*, 1994); in the neonate, EGF has been shown to stimulate cellular proliferation of the esophagus, stomach, small and large intestine (Lebenthal, 1989). Bird *et al.* (1994)

also noted that EGF administration enhanced glucose transport by 30% in the small intestine of adult mice without tissue hyperplasia or changes in passive glucose transport. Glucocorticoids are also closely related to postnatal small intestine maturation. Increases in serum corticosterone precede the brush border enzymatic changes associated with weaning by 48 hours (Lebenthal, 1989; Henning, 1987), and injections of cortisol to suckling animals induces premature intestinal maturation (Lebenthal, 1989).

In a review of growth factors in milk and their effects on infant development, Donovan and Odle (1994) described studies investigating the role of EGF, insulin, and ILF-I and IGF-II in colostrum and milk. Results have shown that these are all potent stimulators of small intestine growth and maturation, however these hormones may only modulate growth during key timepoints; insulin stimulated SI maturation during suckling, but had no effects during weaning. Additionally, the stimulatory effect of human milk on SI maturation far exceeds the effects of physiological doses of individually administered EGF or IGF. This review (Donovan and Odle, 1994) presented effects of growth factors in SI maturation, however this also highlights not only the complex interactions and possible synergistic effects of hormones and growth factors present in milk, but also the importance of macronutrients contained in the milk.

4. METABOLIC FUELS OF THE GASTROINTESTINAL TRACT

4.1 Prenatal Fuels of the Small Intestine

It has long been held that glucose is the principle metabolic fuel of the developing fetus. Human amniotic fluid glucose concentration is 53.7 mg% (2.98 mmol/L) at 10 weeks gestation, and decreases to 22.2 mg% (1.23 mmol/L) at term (Phillips *et al.*, 1990). Glucose is not necessarily the single source of fetal energy as previously accepted, however studies in lambs and monkeys indicate that fetal umbilical uptake of glucose accounts for 50% of oxidative metabolism, with amino acids and lactate providing the remaining substrate (Phillips *et al.*, 1990).

The importance of glucose as a metabolic fuel for the developing gastrointestinal tract has been shown by the appearance of glucose transporters in the developing human

gut at 25% of gestation, and at 80% of gestation in the less precocious rat. Human fetuses, in fact, demonstrate a proximal to distal gradient of these transporters by week 22 of gestation (Buddington, 1994). The fetal rat enterocyte, as well as the colonocyte, which is similar in configuration to the small intestine enterocyte, actively transports glucose during development (Thomson and Keelan, 1986), corresponding to the period of rapid intestinal glycogen accumulation prior to birth, suggesting the need for glucose transporter development prior to glycogen deposition. These large epithelial cell glycogen stores prior to hepatic glycogen appearance, may serve as the major glycogen repository during fetal development and differentiation (Lev and Weisberg, 1969). In the developing rabbit, uptake and incorporation of infused [^{14}C]-D-glucose between gd 23-26 in controls animals versus those with esophageal ligation was significantly higher in the small intestine (Phillips *et al.*, 1991). The incorporation and concentration of labeled carbon appeared to be due to nutrient utilization and not due to distribution through the bloodstream, indicating the importance of glucose as a fuel for the growing small intestine during gestation.

4.2 Postnatal Fuels of the Small Intestine

The importance of glucose as a metabolic fuel of the postnatal small intestine is demonstrated by the presence of glycogen reserves and high levels of gluconeogenic enzymes present in the small intestine mucosa (Hahn and Wei-Ning, 1986). Gluconeogenesis occurring in the guts of suckling rats provides glucose to the muscle layers of the SI. Glycogen reserves in the small intestine increase significantly between suckling and weaning (Hahn and Wei-Ning, 1986), and can be mobilized by glucagon administration (Várkonyi *et al*, 1979). This suggests that there is an increased importance of a readily available glucose source for intestinal energy requirements in weaned animals. In the post-absorptive adult rat it has been shown that the small intestine preferentially utilizes glucose and glutamine as metabolic substrates, with glucose contributing to 10% of the carbon dioxide produced by the jejunum (Kight and Flemming, 1993; Windmueller and Spaeth, 1978). In fasted rats glutamine and ketone bodies are the preferred fuels (Windmueller and Spaeth, 1978).

Several research groups have attempted to determine the preferred metabolic substrates of the small intestine, in ranked order of preference, however these studies have also served to illustrate that the preferred substrates of the isolated enterocytes and whole intestinal slices differs and that the pattern of fuel use changes with stage of development (Windmueller and Spaeth, 1978, 1980; Nagy and Kretchmer, 1988; Kimura, 1987, Kimura and Ilich, 1991; Al-Mahroos *et al.*, 1990). Glutamine oxidation by intestinal tissue slices of suckling rats reportedly increased significantly in the presence of glucose, however glucose oxidation to CO_2 decreased with the addition of glutamine in suckling and adult rats (Kimura, 1987). In contrast, in isolated enterocytes, glutamine oxidation did not change with the addition of glucose in suckling animals, but it did increase for weaned animals (Kimura and Ilich, 1991). Glucose metabolism to lactate was not affected by the addition of glutamine in either the suckling or adult animals in isolated enterocytes. These contrasting results for intestinal slices versus enterocytes suggest that while glutamine may be the preferred substrate for developing rat jejunal enterocytes in sucklings and weanlings, glucose may be the more important fuel for non-enterocyte metabolism. Using isolated enterocytes, the authors also found that although β-HBA oxidation decreases from suckling to weaning, glucose (and glutamine) oxidation increased from suckling to weaning. Glutamine transport into intestinal enterocytes across the basolateral membrane had a higher V_{max} in sucklings (2 weeks) than in weanling (3 weeks) or in adult rats, likely corresponding to a higher metabolic demand during this period of rapid cellular proliferation (Al-Mahroos *et al.*, 1990). These authors concluded that glutamine uptake changes during development, with higher uptakes per mg protein in sucklings than in adults.

Glutaminase activity in developing rat jejunum increases significantly from the first, to the second, to the third week of postnatal life in rats (Nagy and Kretchmer, 1988). At birth, glutamine oxidation may be even less significant than at d5; experiments used pooled d1-5 intestine, which may be inappropriate given the rapid increases in glutamine oxidation occurring perinatally. At d5, activity is only 50% of adult levels, but overshoots adult levels at d15-18. The ability of glucose to support DNA synthesis was significantly improved with the addition of glutamine for d1-5 and d18-21 rats,

demonstrating that glucose in synergy with glutamine works as an important metabolic fuel source in the suckling rat small intestine. Rates of oxidation of glutamine to CO_2 increased six-fold between the first and the third week of life.

In summary, glucose metabolism by the small intestine increases between suckling and weaning. This does not underscore the importance of glucose during suckling as suggested by high levels of gluconeogenic enzymes and glycogen reserves (Hahn and Wei-Ning, 1986) in the gut of suckling rats. Glutamine uptake and oxidation seems to peak during late suckling, and decreases with weaning and into adulthood. Studies using isolated enterocytes and intestinal slices have illustrated the apparent partitioning of fuels of the small intestine; while isolated enterocytes seem to prefer glutamine as a metabolic fuel, in whole tissue, glucose seems to play a bigger role. Thus glucose is an important fuel for the developing gastrointestinal tract, although its exact significance at critical developmental timepoints around birth and through postnatal development has not been fully examined.

5. INTESTINAL GLUCOSE ABSORPTION

5.1 Glucose Transport in the Small Intestine

Glucose absorption by the small intestine epithelium requires the presence of a specialized glucose transport protein. Glucose or galactose is transported into the intestinal enterocyte by the brush border sodium-dependent glucose co-transporter, SGLT1, which couples the transport of these monosaccharides to that of two ions of sodium. Once inside the enterocyte, glucose diffuses out of the cell and into the blood through the facilitative glucose transporter GLUT2 located in the basolateral membrane which transports glucose, galactose and fructose out of the enterocyte into the extracellular space. The Na^+-K^+-ATPase, also located in the basolateral membrane, maintains the inwardly directed sodium gradient required for SGLT1 transport of glucose by actively pumping sodium ions out of the cell (Hediger and Rhoads, 1994). A second, and equally essential role of the SGLT1 transporter, is the absorption of fluid from the intestinal lumen, driven by the sodium gradient across the enterocyte. The presence of glucose or galactose in the intestinal lumen thus stimulates sodium absorption, which

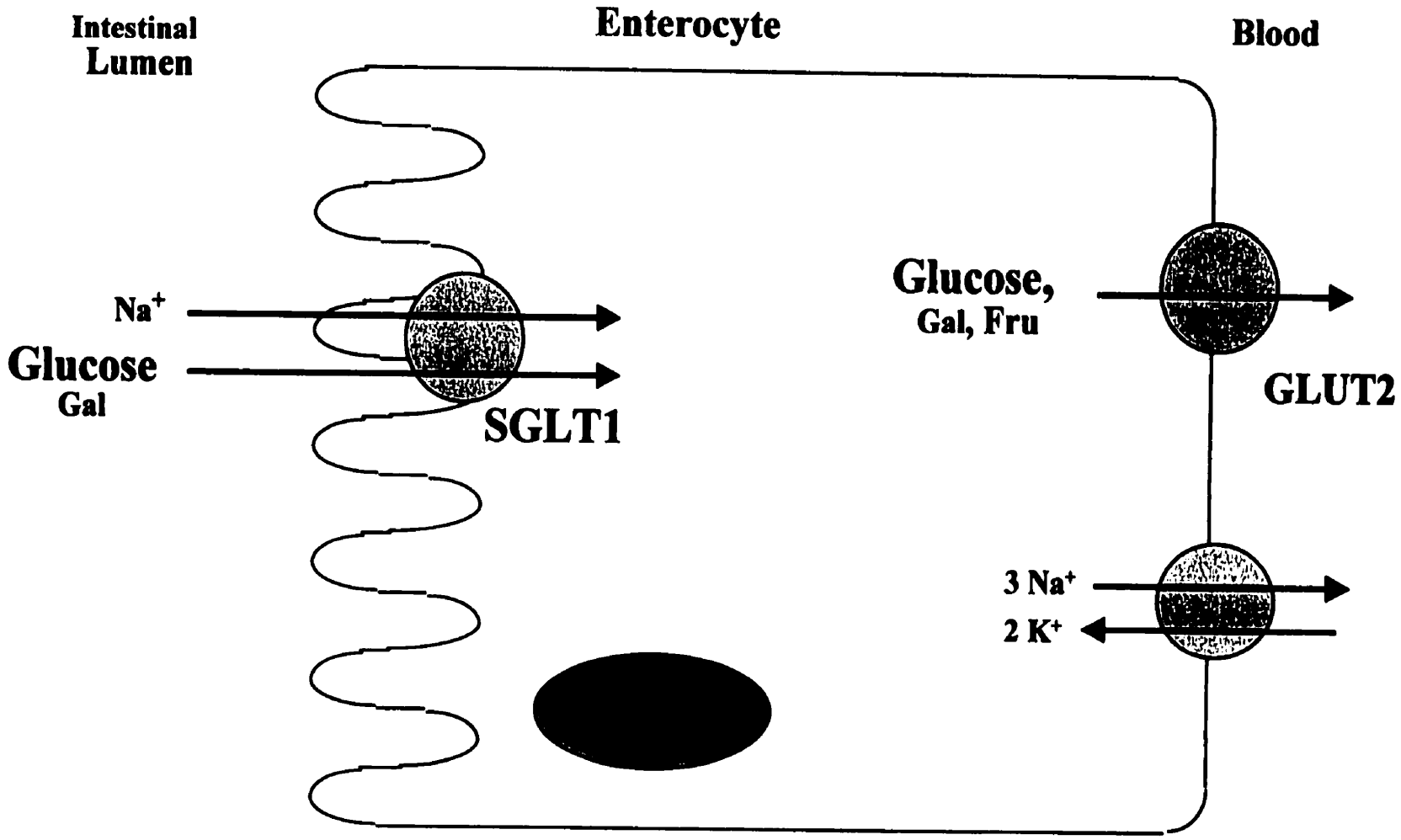
creates an osmotic gradient, stimulating water absorption (Hediger and Rhoads, 1994). The nutrient transporters required for the absorption of dietary glucose are illustrated in **Figure 3**.

5.2 SGLT1 - Discovery and Cloning

The sodium-dependent glucose co-transporter SGLT1 is located along the brush border membrane of the intestinal enterocyte, and is responsible for the transport of glucose and galactose into the enterocyte. Evidence of the essential role of SGLT1 in glucose and galactose transport is the rare and potentially fatal autosomal recessive disease glucose/galactose malabsorption (GGM), which causes severe watery diarrhea treatable only by complete removal of all glucose, galactose and lactose from the diet; absorption of fructose and xylose is normal (Turk *et al.*, 1991). In sisters affected by the disease, a single base change in the gene encoding SGLT1 changed a single amino acid ($Asp_{28} \rightarrow Asn_{28}$), resulting in GGM (Turk *et al.*, 1991). Examination of DNA in seven other GGM patients did not reveal this mutation, thus there is at least one other defect in the SGLT1 gene which can cause GGM (Wright, 1993).

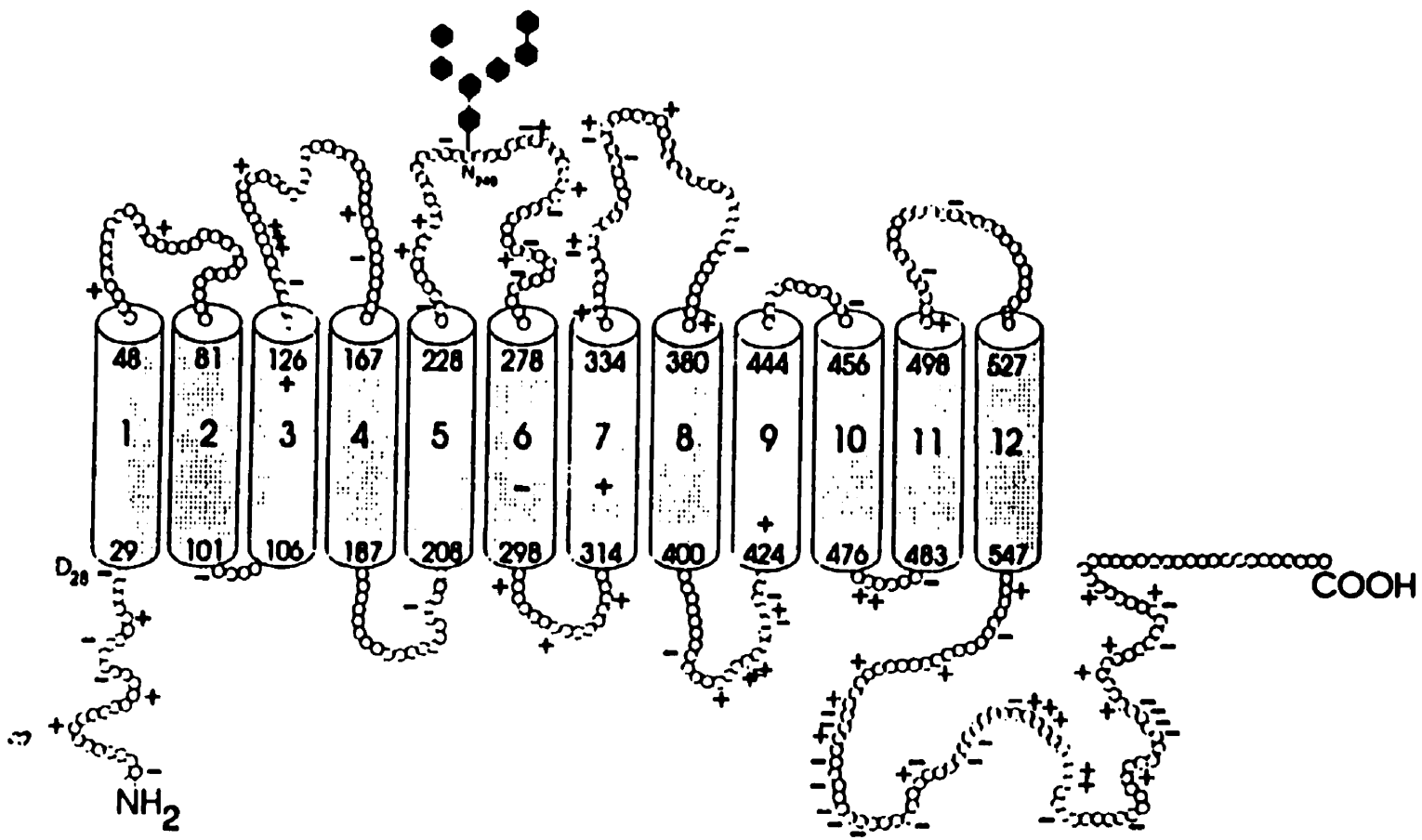
The cDNAs encoding SGLT1 in both rabbit and human intestine, as well as rat kidney, have been cloned, sequenced and expressed (Hediger *et al.*, 1987; 1989; 1991; Lee et al., 1994). The protein is composed of 664 amino acids, is predicted to have twelve membrane spanning regions (Turk *et al.*, 1991), and is glycosylated at Asn-248, although this glycosylation is not required for normal transporter activity (Hediger *et al.*, 1991; Hirayama and Wright, 1992) (**Figure 4**). The SGLT1 protein has an apparent molecular weight of 74kDa by SDS-PAGE (Hediger *et al.*, 1987), but may exist in the brush border membrane as a homotetramer (Stevens *et al.*, 1990). In adult rabbits, SGLT1 mRNA was localized by *in situ* hybridization to the cells on the villus, with a 6-fold increase from villus base to tip (Hwang *et al.*, 1991). SGLT1 protein was only found in the brush border towards the tip of the villus. No SGLT1 protein was found in the cytoplasm of the enterocytes, thus no intracellular store of glucose transporter protein is available to the enterocyte. This supports the principle of migration of mature, pre-programmed enterocytes from the crypts up along the villus. Lee *et al.* (1994) localized

Figure 3: Intestinal Glucose Transport



(adapted from Wright, 1993)

Figure 4: Structural Model of Human Sodium-dependent Glucose Co-transporter (SGLT1)



Taken from Hediger and Rhoads, 1994

SGLT1 mRNA to the lower two-thirds of the villi in rats, in this case decreasing towards the villus tip; these studies indicate the presence of species differences in SGLT1 mRNA localization.

The presence of more than one glucose transporter in the small intestine has long been debated in the literature. In 1986, Freeman and Quamme suggested that there are two sodium-dependent glucose carriers in the intestine of young rats (7-8 weeks); a high capacity, low affinity transporter present in the jejunum and a low capacity, high affinity transporter present throughout the jejunum and ileum. In older rats, both transport systems were found throughout the entire small intestine. Ferraris and Diamond (1992), and Ferraris *et al.* (1992b) further investigated the possibility of two Na^+-dependent glucose co-transporters on the intestinal enterocyte. A low-affinity binding site was found to be expressed in the crypts and a high-affinity site expressed along the villi. Two major reviews of the sodium-glucose co-transporter (Hediger and Rhoads, 1994; Wright, 1993) dispute kinetic evidence of multiple glucose transport systems, and suggest that the genetic mutation associated with GGM provides additional evidence that SGLT1 is, if not the only, certainly the major glucose transport system in the small intestine.

5.3 SGLT1 - Developmental Profile and Regulation

Glucose absorption in the small intestine is dependent upon the translation of sodium-dependent glucose co-transporter (SGLT1) mRNA into functional SGLT1 protein, which provides the means for glucose uptake into the enterocyte in the presence of glucose and sodium. In a developmental study in neonatal and adult rats, it was demonstrated that in postnatal life, SGLT1 mRNA levels remain relatively constant (Miyamoto et al., 1992), and that glucose uptake/mg intestine peaks around birth and then exhibits a gradual decline over the lifespan in rats and rabbits (Buddington, 1992). Glucose transport decreases from the proximal to the distal small intestine, with levels of uptake 1.6-3 times higher in proximal and middle than in distal small intestine (David *et al.*, 1995); this gradient of glucose uptake is not established until weaning (postnatal day 16-22) in the rat.

The presence of dietary glucose in the intestinal lumen is the external signal for

the maintenance of SGLT1 protein. In ruminant sheep, the normal age-related decline in SGLT1 protein is associated with decreases in SGLT1 mRNA. In adult sheep, levels of SGLT1 protein are undetectable. This age-related decrease in SGLT1 protein was prevented by the infusion of glucose to adult sheep intestine (Shirazi-Beechey *et al.*, 1991). In a more recent case study, analysis of human biopsy samples from normal intestinal tissue which had received luminal nutrients and a sample from a herniated segment of intestine which had not received luminal nutrients revealed that SGLT1 protein and glucose uptake decreased dramatically in the absence of luminal nutrient stimulation (Dyer *et al.*, 1997). The decreases in SGLT1 protein and glucose uptake were greater than was expected with the intestinal atrophy which accompanied the hernia.

Adaptive, reversible regulation of a nutrient transporter occurs when the level of dietary substrate regulates the level of its own transporters (Diamond and Karasov, 1984). In the example of glucose transport, high levels of dietary glucose lead to higher levels of glucose transport, termed upregulation. This regulation is thought to proceed when the energetic benefit of regulation exceeds the biosynthetic cost of synthesizing the additional transport protein (Diamond and Karasov, 1987). The following are studies which have attempted to determine whether adaptive regulation in glucose uptake (transport) occurs at the level of transcription of the glucose transporter gene into SGLT1 mRNA or at the level of translation of the mRNA into SGLT1 protein.

In pioneering research work, adaptive regulation of glucose uptake for adult mice was clearly demonstrated by Diamond and Karasov (1984) who showed that within one day of switching adult mice from a no-carbohydrate to a high-carbohydrate (55% sucrose) diet, the V_{max} for glucose uptake was increased by 81-116%. While upregulation of glucose uptake occurred within one day of a switch from the no-carbohydrate (NC) to the high-carbohydrate (HC) diet, downregulation of uptake when mice were changed from the HC to NC diet took 3 days to begin to decrease and 7 days to reach the level of previously NC fed mice. Galactose absorption occurs via the sodium-dependent glucose co-transporter; transamniotic administration of galactose to fetal rabbits resulted in increased galactose and glucose uptake by fetal rabbit small intestine (Buchmiller *et al.*, 1992).

To investigate the potential of postnatal diet to regulate intestinal glucose and fructose uptake, David et al. (1995) fed weaning and post-weaning pups either a high glucose, fructose or sucrose diet (65% carbohydrate) or a no-carbohydrate (NC) diet for 6-7 days. Glucose uptake per mg tissue by all regions of the small intestine (proximal, middle and distal) was found to be independent of diet. The authors concluded that glucose absorption could not be induced by diet during early weaning, whereas fructose absorption could be induced. Fructose transport does not appear until weaning, thus enhancement of fructose transport by weaning diet is an interesting finding. The study attempted to establish whether glucose transport could be induced by weaning diet, however glucose transport is established prenatally, and thus a potentially crucial developmental timeframe for induction was likely missed. Other limitations of this study were that the carbohydrate containing diets were not isoenergetic to the no-carbohydrate diet, and that the NC diet contained 70% casein.

Phlorizin is a competitive inhibitor of the brush-border glucose transporter, and can thus be used to determine glucose transporter content of isolated enterocytes. Using phlorizin binding, delays in SGLT1 protein adaptation to changes in dietary glucose content have been shown to be due to the time required for enterocyte migration (Ferraris and Diamond, 1992). Reversible regulation as determined by glucose uptake methodology versus phlorizin binding has been variably reported to take 24-36 hours for upregulation (Diamond and Karasov, 1984; Ferraris and Diamond 1992) and 36 hours (Ferraris and Diamond, 1992) to 3-7 days (Diamond and Karasov, 1984) for downregulation. No change in mucosal mass or passive permeability to glucose occurs in response to dietary substrate levels (Diamond and Karasov, 1984), rather the upregulation of the transporter occurs via increased transporter site density along the crypt-villus axis, with full expression from midvillus to villus tip (Ferraris *et al.*, 1992b). The signal for regulation is perceived in the crypts, where pre-programmed cells then migrate up the crypt-villus axis (Ferraris and Diamond, 1992). These mature enterocytes cannot be reprogrammed. Both up- and downregulation in response to changes in dietary carbohydrate levels occur sequentially from crypt to villus tip (Ferraris and Diamond, 1992), however dietary induced upregulation of transporters occurs along the entire

terminal portion of the villus (Ferraris *et al.*, 1992b). It was calculated that adult mice enterocytes contain approximately 2.8 x 10^6 glucose transporters per enterocyte on a high-carbohydrate diet and 1.4 x 10^6 following feeding of a low-carbohydrate diet (Ferraris *et al.*, 1992b), a difference in magnitude of two fold.

Miyamoto *et al.* (1993) endeavored to determine whether SGLT1 mRNA levels were regulated by level and type of dietary substrate. Adult rats fed test diets containing 55% carbohydrate (source either D-glucose, D-galactose, 3-*O*-methylglucose, D-fructose, D-mannose or D-xylose) for five days following a 0% carbohydrate diet showed increases of 2.4 and 2.1-fold in SGLT1 and GLUT2 (the basolateral membrane transporter) mRNA with the high glucose diet. This increase was noted three days after the diet change, reaching a maximum of a 3-fold increase by the fifth day post-diet change. Thus reversible regulation of glucose transport is at least partially controlled by level of mRNA; either at the level of transcription, or possibly by increased stability of pre-existing mRNA (Miyamoto *et al.*, 1993). Further attempting to characterize the level of control for the reversible regulation of glucose transport, measurements of both mRNA and SGLT1 protein were used by Lescale-Matys *et al.* (1993). During postnatal development in ruminant sheep, there is a large (200-fold) decrease in SGLT1 activity (uptake?) and SGLT1 protein content, however this natural developmental decrease is associated with only a 4-fold decrease in SGLT1 mRNA. This dissociation of SGLT1 mRNA abundance from protein content has shown that regulation of SGLT1 is translational or post-translational (Lescale-Matys, *et al.*, 1993). Freeman *et al.* (1993) in their investigations of SGLT1 expression in postnatal lamb small intestine, also found that decreased levels of SGLT1 mRNA did not result in any decrease in quantity or activity of SGLT1 protein, further supporting post-transcriptional regulation of SGLT1 during postnatal development. Delézay *et al.* (1995) provided additional evidence that SGLT1 activity is regulated at the post-translational level. In HT-29-D4 epithelial clone cells, full differentiation into mature intestinal absorptive cells with SGLT1 protein at the apical membrane is regulated by protein kinase C. SGLT1 protein is present in undifferentiated cells, but the cells are unable to absorb glucose. Post-translational targeting of SGLT1 protein into the plasma membrane was required for glucose uptake,

and this was shown to require intracellular signaling via pathways regulated by the phosphorylation/ dephosphorylation of protein kinase C.

Although Buchmiller *et al.* (1992) showed that adaptive regulation of glucose and galactose uptake occurs prenatally, it is still commonly held that adaptive regulation of glucose transport is not achievable until postweaning (Buddington, 1994; Lynne Searles M.Sc. thesis). To date assessment of adaptive regulation of the process of glucose transport have employed a variety of methodologies at various developmental timepoints, but no one has fully characterized the developmental profile of small intestine SGLT1 protein or the possibility for adaptive regulation across all important developmental timepoints.

CHAPTER 3: STATEMENT OF PURPOSE

Ontogenic development of the small intestine is known to be affected by environmental influences, including diet (Lebenthal, 1989). The acquisition of an adult, mature small intestine with fully developed proximal-distal gradients of nutrient digestion and absorption requires the presence of an optimal nutritional environment. Restriction of luminal nutrients to the fetal small intestine *in utero* has clearly shown that amniotic fluid nutrients are required for the normal structural and functional development of the small intestine (Mulvihill *et al.*, 1985, 1986; Jacobs *et al.*, 1989; Trahair *et al.*, 1986 and 1997; Avila and Harding, 1991; Lebenthal *et al.* 1980, 1981). Furthermore, supplementation of the amniotic fluid with carbohydrate increased small intestine growth in rabbits (Buchmiller *et al.*, 1992). Postnatal malnutrition similarly reduces small intestine growth and development, resulting in a structurally and functionally immature mucosa (Guiraldes and Hamilton, 1981; Lebenthal, 1989; Karasov *et al.*, 1985), representing a delay in the normal sequence of developmental events for the small intestine. The mechanisms of this retardation in the biological clock for small intestine development have not been thoroughly investigated. Although nutrient imbalances both *in utero* and postnatally clearly have a profound impact upon gastrointestinal ontogeny, the specific mechanisms and importance of the macronutrient composition of the diet have not been investigated.

It has long been held that glucose is the principle metabolic fuel of the developing fetus (Battaglia and Meschia, 1978). However the role of glucose as a fuel for the developing small intestine is complex. Prenatal fuel preference has yet to be established. During suckling and postweaning, glutamine is the preferred substrate to glucose in the enterocyte, however there is evidence of an increased preference for glucose for whole intestine slices (Kimura and Ilich, 1991). During late gestation and early postnatal life, the accumulation of glycogen reserves (Lev and Weisberg, 1969; Nathan, 1967) and high levels of gluconeogenic enzymes (Hahn and Wei-Ning, 1986) suggest that readily available glucose production is important for the rapidly growing small intestine. To address the importance of glucose, an important metabolic fuel of the developing small

intestine, in fetal and neonatal small intestine growth and functional development a dietary model was used which restricts dietary glucose intake throughout pregnancy. Diets to be fed were adequate in glucose (60%), glucose restricted (12 or 24%) or glucose deficient (0%). This model allowed the examination of the impact of feeding such diets during pregnancy on gut structural development, the appearance of prenatal glycogen reserves, the establishment of the normal developmental pattern of lactase and sucrase enzymes and the potentiality of prenatal dietary substrate levels to regulate the expression of the sodium-dependent glucose co-transporter protein (SGLT1) in offspring. Given that this diet model results in significantly lower levels of glucose in amniotic fluid it was hypothesized that the reduced dietary signal/substrate required for transporter ontogeny would negatively impact upon SGLT appearance and could negatively affect fetal intestinal glycogen reserves and thus reduce an important energy store for the rapidly developing postnatal small intestine. As these diets were fed through lactation and to the weaned offspring, this allowed further investigation of the importance of dietary glucose on levels of postnatal gut growth, lactase and sucrase levels, SGLT1 protein levels, and the establishment of the normal proximal-distal gradients of these enzymes and transporter levels.

CHAPTER 4: EXPERIMENTAL DESIGN

1. RESEARCH QUESTIONS

This study was designed to examine the following research questions about neonatal small intestine growth and functional development.

1. What is the developmental profile of the sodium-dependent glucose co-transporter (SGLT1) in the rat proximal and distal small intestine? Can prenatal diet modify this profile in early neonatal life? What are the periods of responsiveness of SGLT1 to level of dietary glucose?
2. Are any differences in intestinal growth with glucose restriction accompanied by differences in nutrient transporter levels? Could these differences partially explain IUGR and perinatal mortality seen with the diet model?
3. Does maternal diet during pregnancy affect small intestine glycogen reserves in offspring? Is there an altered pattern of mobilization of these glycogen reserves in glucose restricted pups?
4. Are levels of glucose transporters and glycogen reserves correlated?
5. Could the level of dietary glucose (1) delay the normal developmental profile of the intestinal disaccharidases lactase and sucrase, or (2) alter plateau levels of these enzymes or (3) affect the development of the proximal-distal gradient normally established during the third postnatal week?

2. STUDY HYPOTHESES

In order to test the preceding research questions, the following study hypotheses were developed. The primary hypothesis is that a restriction in maternal dietary glucose will result in irreversible structural and biochemical changes in the proximal and/or distal segments of neonatal rat small intestine. We hypothesize the following for the neonatal small intestine:

1. Intestinal architecture will be perturbed by restrictions in maternal dietary glucose, as measured by intestinal length, weight and histologic changes.
2. The relative quantity of intestinal sodium-dependent glucose co-transporter protein (SGLT1) will be regulated in response to the level of glucose in the

diet.

3. Glycogen reserves will be diminished with lower levels of dietary glucose.
4. The developmental profile/pattern of intestinal disaccharidases (lactase and sucrase) will be influenced by maternal dietary glucose.

3. EXPERIMENTAL DESIGN

This study examined the effects of graded levels of maternal dietary carbohydrate, specifically glucose, fed throughout pregnancy and through weaning on gastrointestinal development in rats. The four levels of dietary glucose which were tested included 0, 12, 24, and 60% glucose by weight; the 0% group was designated glucose-deficient, 12 and 24% groups as glucose-restricted and the 60% group as control or adequate in glucose. Tissue collection occurred at the following timepoints; gd 20, birth, 12-24 hours after birth, and postnatal days (pd) 7, 15, 21, 28, 35, and 49, which in turn were categorized as **perinatal** (gd 20, birth and 12-24h), **suckling** (pd 7, 15 and 21), and **post-weaning** (pd 28, 35, and 49). Dams were also killed at two timepoints; necropsy at gd 20 with collection of blood and amniotic fluid, and at pd49 when all tissues and blood were collected. All dams were fed experimental diets from the time of receipt at gd0, 1) through pregnancy (gd20 necropsy), or 2) through pregnancy, lactation and post-weaning of their pups (pd49 necropsy). The pd49 dams were the dams of the post-weaning experimental litters, killed on postnatal day 49 and considered adult controls. The total number of pregnant dams included in the perinatal and postnatal timepoints is shown in **Table 2**.

Table 2: Number of Pregnant and Nursing Dams Included in Perinatal and Postnatal Sampling Times[1]

	PERINATAL[1]			POSTNATAL[1,2]
Diet Group	**gd 20**	**birth**	**12-24h**	**(suckling & post-weaning)**
0%	9	5	4	
12%	9	8	7	8
24%	9	8	8	9
60%	9	8	8	9

[1]Numbers represent number of dams received, i.e. total number of litters for each category. [2]Postnatal includes number of dams at beginning of postnatal sampling.

Table 3: Sampling Protocol for Postnatal Timepoints

Diet Group	n[1]	SUCKLING[2]			POST-WEANING[2]		
		pd7	pd15	pd21	pd28	pd35	pd49
12%	8	variable	16(2)	16(2)	8(1)	8(1)	8(1)
24%	9	variable	18(2)	18(2)	9(1)	9(1)	9(1)
60%	9	variable	18(2)	18(2)	9(1)	9(1)	9(1)

[1]Number of litters, as per Table 1. [2]Total number of pups killed at each timepoint; number of pups per litter in parentheses.

At each of the perinatal timepoints, the small intestine of all pups in a litter was collected. Length and weight measurements were made on individual intestines, however to obtain adequate tissue for laboratory analyses, the intestines within a litter were pooled. The sampling protocol for postnatal timepoints differed from that for perinatal. At pd7, a variable number of pups were killed, culling the litters so that 7 pups remained per litter. Of the remaining seven pups in each litter, two from each litter were killed on pd 15 and 21, and one from each litter on days 28, 35 and 49 (**Table 3**). Again, length and weight measurements were made on individual intestines, however tissues within litters were pooled following weighing to obtain adequate tissue for laboratory analyses. Previous use of this diet model has shown almost 100% perinatal mortality by 12-24h in the 0% glucose-deficient groups (Koski and Hill, 1986, 1990), and we wished to ascertain if poor in utero gastrointestinal development was contributing to this high perinatal mortality.

4. ANIMALS AND HOUSING

One-hundred and forty-one time-bred female Sprague Dawley rats (180-200 g) were obtained from Charles River (St. Constant, QC). Pregnant dams were individually housed in plastic maternity cages with wire lids. The experimental diets and water were available *ad libitum*. Cages and bedding were cleaned biweekly. Rats were maintained in a temperature controlled animal room (22-25°C) with a 12 hour light cycle from 07h00 to 19h00. Rats were excluded from the study for the following reasons; rat not pregnant, pups present in only one uterine horn, overgrown teeth preventing normal eating, and infection. This left a final sample of 129 dams for whom food intake and maternal body weight data was used in analysis. Offspring data was only available for the offspring of

118 dams for the following reasons; death during labour, abnormal cannibalism of pups and pup mortality. Therefore, this resulted in a total of 36 litters killed at gd20, 29 litters killed at birth, 27 litters killed at 12-24h and 26 litters killed for the postnatal part of the experiment.

5. DIET COMPOSITION

All purified diets were prepared in the laboratory and were formulated to meet the NRC nutrient requirements for pregnant rats (1995) except for level of carbohydrate. Diets were formulated to contain 0, 12, 24 or 60% glucose (anhydrous dextrose) by dry weight. The basal carbohydrate-free diet (0% glucose) for pregnant rat dams is based on work done by Koski et al., 1986. This diet is more correctly termed "carbohydrate-restricted" as it was triglyceride based and thus provided some glucose equivalents in the form of glycerol; for the purpose of this thesis this diet was called glucose-deficient to avoid confusion with the 12 and 24% glucose diet groups. The 12 and 24% glucose groups are referred to as glucose-restricted, and the 60% group as glucose adequate or control. Diets were formulated to contain 4.15 kcal (17.4 kJ) metabolizable energy per gram dry matter and were made isocaloric by the equienergetic substitution of carbohydrate with soy oil and replacement of the difference in weight with cellulose (Alphacel). Protein (casein), methionine, sodium bicarbonate, vitamin and mineral content remained constant across all four formulations. Protein was provided at a level which supplies adequate amounts of protein and essential amino acids, however it was not provided in excess as this would have provided additional glucogenic precursors. The level was set at 11 g/100g dry weight, and thus provided 26.5 mg protein/kcal of diet. This level is within the range of 25-31 mg lactalbumin protein/kcal of diet which has been reported to be adequate for maximum weight gain in young rats and is thus in accordance with the NRC guidelines (1995). Methionine was added to all diets at a level of 0.34 g/100 g dry weight; casein supplemented with methionine is considered to provide a nutritionally adequate protein source equivalent in quality to lactalbumin (NRC Press, 1995). Vitamins were added at levels exceeding the NRC recommendations by a factor of approximately 2 for the water soluble vitamins and 4-5 for the fat soluble

vitamins. Minerals were added at a level approximately twice that of the current NRC guidelines for gestation and even though there is no specified requirement given in the 1995 edition of Nutrient Requirements for Laboratory Animals, chromium was added at a level of 0.66 mg/kg diet as this level had been previously used in this laboratory under similar dietary conditions. These levels were set to ensure adequate micronutrient intake while allowing for the possibility of reduced total dietary intake. Sodium bicarbonate was added at the level of 1 g/100 g diet to correct for the metabolic acidosis expected with the feeding of carbohydrate restricted diets.

Table 4 shows the composition of 100g of each isoenergetically formulated experimental diet; calculations were based upon metabolizable energy of dietary constituents as outlined in **Table 5**. In **Table 6**, 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. Fat and water soluble vitamin mixtures were prepared separately, and were formulated to contain the weight of vitamin in the 800g mixture that when added to the diet at a level of 4g/kg diet would achieve the required mg/kg diet (**Table 7**). For example, for 800g of water soluble vitamin mixture containing 15.0g of niacin, addition of 4g of this mixture to 1kg of diet would yield 75mg/kg niacin in the diet (15g/800g x 4000mg/kg = 75mg/kg). Mineral mix was added at a level of 6.5% to the experimental diets, as determined from the combined weights of all mineral compounds (e.g. $CaHPO_4$) as required to achieve the desired level of elemental mineral (e.g. Ca) (**Tables 8** and **9**). The mineral mix (**Table 10**) was then formulated to contain the weight of mineral compound in 1kg mix that when added to the diet at a level of 6.5g/kg diet would achieve the required mg/100g diet (from **Tables 8** and **9**).

Table 4: Composition of Diets[1]

Ingredients	0% g	12% g	24% g	60% g
Carbohydrate[2]	0	12.0	24.0	60
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 (g). [2]Dextrose (anhydrous), ICN Biomedicals Canada Ltd., Montreal, QC. [3]Degummed soybean oil. [4]Alphacel, ICN Biomedicals Canada Ltd., Montreal, QC. [5]High-nitrogen Casein, ICN Biomedicals Canada Ltd., Montreal, QC. [6]See Tables 3. [7]See Tables 5. [8]See Table 2 for ME of Ingredients.

Table 5: Metabolizable Energy of Dietary Ingredients

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

Table 6: Composition of Vitamin Mixture

Ingredient	NRC Requirements mg/kg diet[1]	Ratio	mg/kg diet
Niacin	15.0	5.0	75
Calcium pantothenate	10.0	4.0	40.0
Riboflavin	4.0	4.0	16.0
Pyridoxine hydrochloride	6.0	4.0	24.0
Thiamin hydrochloride	4.0	4.0	16.0
Folacin	1.0	4.0	4.0
Biotin	0.2	4.0	0.8
Cyanocobalamine	0.05	4.0	0.2
Alpha-tocopherol acetate[2]	18.0	2.0	36.0
Menaquinone	-	-	0.6
Choline Chloride	-	-	4000
Cholecalciferol[3]	1000 IU	1.5	1500 IU
Vitamin A palmitate[4]	2300 IU	2.0	4600 IU

[1]Nutritional Requirements of Laboratory Animals. Fourth Revised Edition NRC. National Academy of Sciences, Washington, DC, 1995. [2]Supplied as 1,000 IU/g DL-alpha-tocopheryl acetate, ICN Biomedicals Canada Ltd., Montreal, QC. [3]Supplied as 400,000 IU of Vitamin D_3/g, ICN Biomedicals Canada Ltd., Montreal, QC. [4]Supplied as 250,000 IU/g, ICN Biomedicals Canada Ltd., Montreal, QC.

Table 7: Formulation of Vitamin Mixture

Ingredient	grams	g/kg diet
FAT SOLUBLE (A)		
Vitamin A palmitate	3.68	
Cholecalciferol	0.75	
Alpha-tocopherol acetate	10.8	
Phyllaquinone	0.8	
Cellulose[1]	783.97	
TOTAL (A)	**800.0**	**4**
WATER SOLUBLE (B)		
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	0.040	
Butylated hydroxytoluene	20.0	
Cellulose[1]	744.8	
TOTAL (B)	**800.0**	**4**
CHOLINE CHLORIDE		**4**
TOTAL		**12 g/kg diet**

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

Table 8: Elemental Composition of Macroelements[1,2]

Ingredient (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	
Microelements	100							
TOTAL (A)[1]	6486 6.486g/100 g	1008	792	132	111	779	171	174
NRC Requirement for Reproduction 1995 (B)[2]		**630**	**360**	**60**	**50**	**370**	**50**	-
Ratio A:B		1.6	2.1	2.2	2.2	2.1	3.4	-

[1]Not including additional sodium as $NaHCO_3$ at a rate of 1g/100g diet in order to correct the metabolic acidosis resulting from feeding low carbohydrate diets. [2]Nutrient Requirements of Laboratory Animals. Fourth Revised Edition. National Academy of Sciences, Washington, DC, 1995.

Table 9: Elemental Composition of Microelements

Ingredient (MW)	mg/kg diet	Fe	Zn	Mn	Cu	F	Cr	Se	Mo	I
		mg element / kg diet								
$FeSO_4 \cdot 7H_2O$ (278.02)	746.7	150								
$ZnCO_3$ (125.38)	105.49		55							
$MnCO_3$ (114.94)	41.84			20						
$CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$ (239.19)	33.1				17.6					
$KF \cdot 2H_2O$ (94.13)	10.90					2.2				
$CrK(SO_4)_2 \cdot 12H_2O$ (499.40)	6.34						0.66			
Na_2SeO_3 (172.92)	1.927							0.88		
$Na_2MoO4 \cdot 2H_2O$ (241.95)	0.832								0.33	
KIO_3 (214.02)	0.556									0.33
Citric acid	52.31									
TOTAL (A)	1000.00	150	55	20	17.6	2.2	0.66	0.88	0.33	0.33
NRC Requirement for Reproduction 1995 (B)[1]		**75**	**25**	**10**	**8**	**1.0**	**-**	**0.40**	**0.15**	**0.15**
Ratio A:B		2.0	2.2	2.0	2.2	2.2	-	2.2	2.2	2.2

[1]Nutrient Requirements of Laboratory Animals. Fourth Revised Edition. National Academy of Sciences, Washington, DC, 1995.

Table 10: Composition of Mineral Mix[1,2]

Ingredient	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**
		6.5 g/kg diet

[1]See **Table 9** for Elemental Composition of Macroelements. [2]See **Table 10** for Elemental Composition of Salt Mixture.

5.1 Diet Preparation

Based on Lanoue and Koski (1994), sufficient experimental diet was prepared to allow for expected intake of 18-20 g/day for pregnant dams and approximately 40-60 g/day for lactating dams. In total, 200 kg of the experimental diets was prepared, which included a factor of 30% to accommodate loss due to spillage or mishandling. Diets were prepared in a two step procedure. Component vitamin and mineral mixes were prepared in advance in quantities adequate for the entire experiment. All experimental diets were then prepared from the resulting vitamin and mineral mixes and from quantities of the remaining ingredients according to Table 3. All ingredients came from the same lot from the supplier to ensure uniformity within the study. Diets were stored at 4°C in sealed containers to prevent contamination or spoilage.

6. EXPERIMENTAL PROTOCOL

On gd 0, 141 time-bred female Sprague-Dawley rats were received, weighed, and randomly assigned to one of four experimental diets (0, 12, 24, or 60% dietary glucose). The animals were received in a series of groups of approximately 12-15. The receipt of small groups of animals was necessary because of space and time limitations. Maternal weight and food intake were measured and recorded every econd day and on tissue collection days to monitor weight gain and food acceptance.

6.1 Nutritional Outcomes

Food intake was determined every second day throughout the experimental protocol by subtracting the weight of the food remaining in the feeder from the weight of food given two days previously. Daily food intakes were calculated by dividing this two day intake in two. For the litters included in the postnatal sampling, food intake was determined for the cage as a unit. This measurement unit included the dam and a number of pups that varied with the time course of the study, but was consistent across litters at any given time during the study, thus allowing intakes to be compared. Maternal and pup body weights were measured every second day. To allow for analysis of patterns of maternal weight gain, daily maternal body weight was determined by taking the weight at the midpoint of any two successive measurements of weight. Body weight for individual pups was taken every second day, however individual patterns of weight gain or loss could not be determined as individual pups were not tracked for weight. Analysis of pup weight was thus done using mean pup weight in a litter with litter size remaining constant between litters. Adequacy of food intake and weight gain were ensured throughout the experiment. Initial body weight was also controlled for through this method.

6.2 Collection of Tissues and Experimental Techniques

One of two experimental protocols was used for sacrifice and collection of tissues. Pregnant dams were killed in a post-absorptive state by Ketamine-HCl injection (Ketalean, MTC Pharmaceuticals; Cambridge, ON) at 30 mg/kg body weight into the jugular region followed by exsanguination through cardiac puncture. Maternal blood was

collected into heparinized tubes and centrifuged at 3,200 rpm x 5 minutes (Sorvall RT6000B Refrigerated Centrifuge, DuPont) and the plasma layer collected. The fetuses were then removed by caesarian section. Amniotic fluid was collected from each amniotic sac and pooled for the litter. Fetuses were killed by exsanguination into heparinized microhematocrit capillary tubes, the blood pooled and centrifuged for collection of plasma. For all the postnatal tissue collections, pups were killed by intraperitoneal injection of Ketamine-HCl followed by exsanguination. All amniotic fluid and plasma samples was stored at -80°C until the time of analysis.

The small intestine of each animal was excised from the ligament of Treitz to the ileocecal valve and laid flat for the measurement of length to the nearest millimeter. At birth, 12-24h after birth and pd 7, 15, 21, 28, 35, 49 and in the adult controls the intestine was divided by length into two equal segments which were designated as proximal and distal. Tissues from animals younger than 21 days were weighed and stored without removal of gut contents. These segments were weighed individually on an analytical balance (Mettler AE200). From pd21 forward, intestines were opened longitudinally and gut contents gently removed and rinsed with ddH_2O. At all timepoints, intestinal segments within litters were pooled to maximize tissue weight available for analysis. The only exception was that for the second block of animals received, one intestine was randomly selected from each litter and frozen separately for glycogen analysis. Tissues were collected onto dry ice, frozen in liquid nitrogen and stored at -80°C until the time of analysis.

6.2.1 Amniotic Fluid and Plasma Parameters

Amniotic fluid and plasma glucose, urea nitrogen, and β-hydroxybutyrate were measured using the Abbott VP SuperSystem (Irving, TX); all were analyzed using kits from Sigma Chemicals (Ohio). β-hydroxybutyrate was measured using an enzymatic assay (Sigma β-HBA Kit, procedure 310-A). Glucose was also measured using a enzymatic method (Sigma Glucose HK Kit, procedure 16-20). Albumin was analyzed using Sigma Albumin BCG (procedure 631-2), and urea using the Sigma BUN 20 Endpoint Kit (Procedure 66-20).

6.2.2 Histology

One sample was randomly selected from each diet group and sampling time for histological examination. After measuring the small intestine length and dividing the intestine into two segments of equal length, a short piece (~1-3cm) of intestine was snipped from the distal most end of the proximal intestine segment, thus the samples represent mid-small intestine, or mid-jejunum. Tissues were stored in 10% buffered formalin (W/V) and prepared for microscopic examination by embedding in paraffin, staining with hematoxylin and eosin (H/E) by the Department of Pathology, St. Mary's Hospital, McGill University (as per Avila and Harding, 1991). A qualitative assessment of intestinal development including villus to crypt ratio, villus height, branching of villi, and mucosal thickness was done under the direction of Dr. Cecile Logan, Pathologist, St. Mary's Hospital.

6.2.3 Tissue Glycogen Determination

One entire small intestine for perinatal timepoints was randomly selected for each litter from the second block of animals for analysis of glycogen concentration using the spectrophotometric method of Lo et al., 1970. From pd7 forward, proximal and distal intestine tissue was analyzed separately. For these timepoints, a small amount (200-500mg) tissue was cleaved from the pooled frozen sample. All samples were analyzed in duplicate. According to the method of Lo *et al.*, tissue samples are digested in a strong alkali (KOH) and then the glycogen is precipitated with ethanol (95%). Sulfuric acid hydrolyzes this glycogen, releasing glucose which then combines with phenol to produce a colorimetric endproduct. The glycogen content of the samples can then be determined by reading the resulting absorbance at 490nm on a spectrophotometer (Beckmann Instruments, California) and comparing the mean absorbance with standards of known initial glycogen content undergoing the same sulfuric acid hydrolysis and phenol endpoint analysis.

6.2.4 Preparation of Brush Border Membrane Vesicles

Brush border membrane vesicles (BBMV) were prepared using a modified magnesium chloride precipitation methodology, based upon Kessler *et al.* (1978). These

vesicles were used for determination of intestinal disaccharidase levels and for the quantitation of SGLT1 protein content. All steps in the preparation of BBMV were carried out on ice; centrifugation was done at a temperature <10°C. Homogenization of 0.8-1.0g frozen intestinal tissue in a mannitol-HEPES buffer containing the protease inhibitors aprotinin, leupeptin and PMSF was followed by centrifugation at 3000xG (15mins) in a Sorvall Refrigerated Centrifuge; the resulting supernatant was then spun at 3000xG (15mins) in the same centrifuge. The pellet was then resuspended and spun again at 20,000xG (30mins). The final pellet was resuspended in a volume of 10mM Tris buffer in order to achieve a protein concentration of >6.7μg/μL. This minimum protein concentration was required so that 80μg of total protein could be loaded into a volume of 12μL for Western Blots (80μg/12μL = 6.7μg/μL). Total protein content of the resultant BBMV fractions were analyzed using a dye-binding BioRad Protein Assay with bovine serum albumin as a standard (BioRad, Mississauga, ON). All samples were assayed in duplicate. Concentrations were generally between 8-20μg/μL. Where necessary, this protocol was adapted to accommodate the much smaller tissue weights available; tissues were homogenized and resuspended in a proportionately smaller volume of 10mM Tris resuspension buffer, in order to achieve the aforementioned protein concentration.

6.2.5 Intestinal Disaccharidase Activity

Intestinal disaccharidase activity (lactase, sucrase) in proximal and distal segments was assayed in brush border membrane fractions prepared for Western Blotting. Using the classic method of Dahlqvist (1964) modified into a one-step procedure in 1966 by Messer and Dahlqvist, 20μL of diluted BBMV fractions containing disaccharidases, were incubated in the presence of the appropriate substrate (lactose or sucrose) to release glucose. The glucose released was then oxidized using a glucose oxidase containing reagent, and the colorimetric product read at 530nm. Enzyme activity was calculated as per Messer and Dahlqvist (1966) and reported in μmoles substrate catalyzed per minute (UE); enzyme specific activity was then calculated as UE/mg protein using available protein concentrations of BBMV fractions as described in section 6.2.4.

6.2.6 Western Blotting

Detection, quantification and identification of a protein in a complex protein solution can be accomplished through the combined use of sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting ("immunoblotting").

Pre-cast 10-well 7.5% Tris-HCl gels were purchased from BioRad (Mississauga, ON). The use of a two-gel system allows better resolution of protein bands than traditional one-phase slab gel electrophoresis. In a two-gel system, a stacking gel is layered over the resolving gel. The pH of the stacking gel is around 2 units lower than that of the resolving gel, and the acrylamide content is lower. When current is applied, the lower pH causes the buffer ions in the stacking gel to become uncharged. This results in an increased electrical field, which increases migration rates of the proteins. When the migrating proteins reach the lower region of the stacking gel, they slow down, and thereby approach the resolving gel as stacks of very narrow bands (~0.01mm thick) which are ordered by mobility. Separation of proteins then proceeds normally in the resolving gel with improved resolution (Voet and Voet, 1990).

Brush border membrane vesicle (BBMV) fractions were prepared as described in section **6.2.4**. A control sample was prepared from the distal small intestine of adult animals fed the 60% glucose diet and was run on all Western Blots. All samples were assayed in duplicate; 80μg protein was loaded into each lane for SDS-PAGE electrophoresis in the Mini-Protean II Electrophoresis System (BioRad, Mississauga, ON). Molecular weight markers were loaded into the outer lanes of the polyacrylamide gel for determination of molecular weight of the final protein band on the blot.

Following electrophoresis, protein bands are classically transferred to a nitrocellulose or nylon membrane by electrotransfer. For this experiment protein bands separated by SDS-PAGE were transferred to PVDF membrane (BioRad, Mississauga, ON) using the Mini-Trans Blot Electrophoretic Transfer Cell (BioRad, Mississauga, ON). Unoccupied sites on the membrane were then blocked with a dilute protein solution (BLOTTO: 5% bovine serum albumin or reconstituted partly-skimmed powdered milk) to prevent non-specific binding of antibodies to the membrane during incubation. The

blot was then incubated in the primary antibody (the probe), a sequence specific antibody prepared against a synthetic peptide corresponding to the known amino acid sequence of SGLT1 (Hirayama and Wright, 1992). Rabbit anti-rat SGLT1 purified antibody was purchased from Cedarlane Laboratories (Hornby, ON) a supplier for Biogenesis (Sandown, NH), and blots were incubated in a 1:500 dilution of antibody for 2 hours. The blot was incubated in the secondary antibody, goat anti-rabbit IgG, which has been conjugated to alkaline phosphatase (Cedarlane Laboratories, Hornby, ON) in a 1:500 dilution for 1 hour.

Exposure of the blot was accomplished by incubation in an alkaline phosphatase based luminescent substrate (CSPD, Boehringer-Mannheim, Laval, QC), followed by exposure to Kodak BioMax Light film (Mandel Scientific, Hornby, ON), chosen for its performance with chemiluminescent detection systems. Quantitative densitometry was accomplished by scanning the film, and using free imaging software (Scion Image, Scion Corporation, www.scioncorp.com) to determine peak areas for each band. Within each blot, the area under the curve for each sample were averaged and were compared to a control sample also run on the blot, and relative band density (area under the curve, AUC) was calculated. These relative band densities were used for statistical analysis. A relative value greater or less than one is considered to represent SGLT1 abundance which is greater or lower than that of adult controls.

7. STATISTICS

Analysis was performed using the SAS statistical analysis package (SAS, Version 6.12; SAS Institute, Cary, NC). A one-way analysis of variance (ANOVA) was done using diet as the main effect for outcome measurements including food intake by cage, maternal and pup body weights, litter size and litter weight, plasma and amniotic fluid parameters, and small intestine growth parameter. These small intestine outcome parameters included length, and weight (total, proximal and distal). A two-way ANOVA was used to assess the regional as well as diet effects for postnatal glycogen content, SGLT1 protein content, lactase and sucrase specific activity. Since proximal and distal small intestine segments were expected to behave differently, a one-way ANOVA with diet as the main effect was used on region specific data (proximal and distal separately) to more specifically determine which region was affected by diet. Covariates were included in the ANOVA where appropriate. Bartlett's test for the homogeneity of variances was used for parameters normally subject to homeostatic control, including plasma and amniotic fluid measurements, and glycogen content. Outcomes such as food intakes, body weights and gut growth parameters (especially for growing pups) were not expected to be homogeneous, as growth patterns and peaks may be quite different at different timepoints and be independent of dietary treatment group. A nested design was used only for measurements made on individual pups including pup weight and gut growth parameters. Post hoc comparisons were done using Scheffé's test to determine differences between dietary treatments when a main effect of diet was found. Data has been presented as mean ± standard error of the mean (SEM). A p-value less than 0.05 was considered significant for all statistical analyses.

CHAPTER 5: RESULTS

1. FOOD INTAKE

Food intake during pregnancy was analyzed over the period gd 0 through 19 (gd0-19). Postnatal food intake for the period between postnatal day 0 through 48 (pd0-48) was analyzed for periods between sequential kill days including the kill day (approximately weekly), as the number of animals in the cage (dam plus pups) was stable during these intervals and thus the measurement unit (food intake per cage) was comparable. Results are presented in **Table 11** and **Figure 5**.

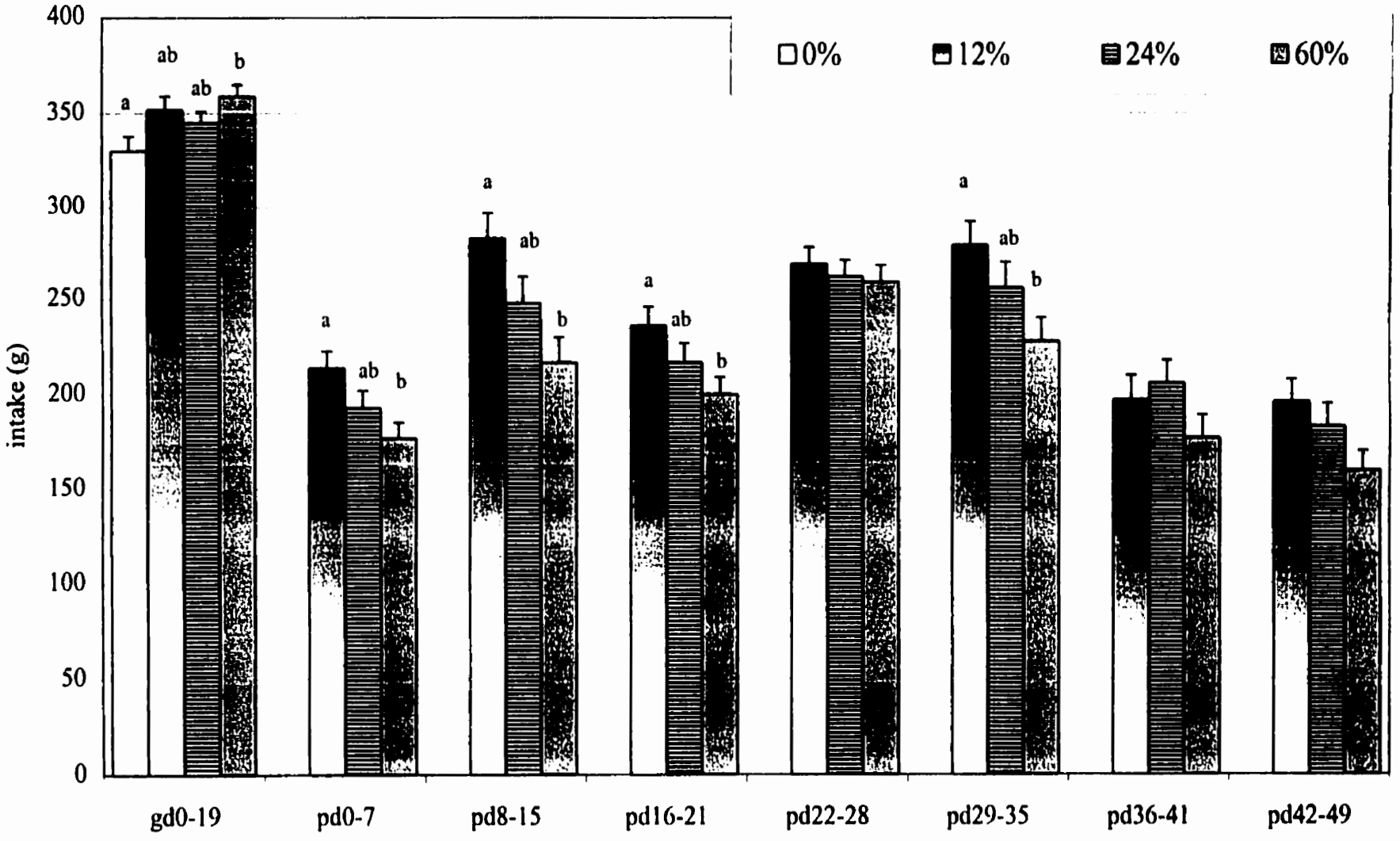
Table 11: Effect of Graded Levels of Dietary Glucose on Cumulative Food Intake (g)[1,2]

		Diet Group				p value[7]
Time	n[3]	0%	12%	24%	60%	(diet)
gd0-19[A]	127[4]	330 ± 8^{a}	352 ± 7^{ab}	345 ± 6^{ab}	359 ± 6^{b}	0.0316
pd0-7[A]	25[5]	-	214 ± 9^{a}	193 ± 9^{ab}	177 ± 8^{b}	0.0244
pd8-15[A]	25[5]	-	283 ± 14^{a}	248 ± 14^{ab}	217 ± 13^{b}	0.0094
pd16-21[A]	25[6]	-	236 ± 10^{a}	217 ± 10^{ab}	200 ± 9^{b}	0.0469
pd22-28	25[6]	-	269 ± 9	262 ± 9	259 ± 9	ns
pd29-35[A]	24[6]	-	279 ± 13^{a}	256 ± 14^{ab}	228 ± 12^{b}	0.0368
pd36-42	24[6]	-	197 ± 13	206 ± 12	177 ± 12	ns
pd43-48	23[6]	-	196 ± 12	183 ± 12	160 ± 10	ns

[1]Values are least square means (LSM) ± SELSM. [2]Multiple comparisons between diet groups by timepoint were done using Scheffé's test, indicated across rows with different lowercase letters. [3]Number of measurement units (cages). [4]One dam per cage. [5]One dam plus suckling pups. [6]One dam plus nibbling pups. [7]P-value from ANOVA. [A]Significant main effect of diet from ANOVA.

Food intake was significantly affected by diet during gestation (gd 0-19), between pd 0-7, pd 8-15, pd 16-21 and pd 29-35. During gestation, the only significant difference in food intake was between the 0% vs. 60% glucose groups, with dams fed the 0% glucose deficient diet eating significantly less food ($p<0.03$). No other diet differences existed during pregnancy, thus a diet restricted in glucose (12 or 24%) did not result in reduced food intake. All postnatal periods significantly affected by diet (pd0-7, pd8-15, pd16-21, pd29-35) showed a similar pattern. For each of these periods the 12% carbohydrate restricted animals ate significantly more than the control group; this difference ranged between 18% (pd16-21) and 30% (pd8-15) more food for the 12%

Figure 5: Effect of Graded Levels of Dietary Glucose on Cumulative Food Intake



group.

2. MATERNAL BODY WEIGHTS

Body weight was measured at the time of arrival and every second day thereafter. Dams were also weighed on kill days. Data are presented in **Table 12** and **Figure 6**. Significant differences in maternal body weight were seen at gd19, pd28, 35, 42, and 49. While maternal body weights were not different at the time of arrival in the animal care area (gd0), by the end of gestation (gd19) there was a stepwise reduction in body weight with decreasing glucose content of the diet. Scheffé's test revealed that 0% dams weighed significantly less than 12, 24 or 60% dams, while 12 and 24% glucose restricted dams also weighed less than the 60% glucose control dams. Maternal body weights (**Table 11**) did not follow the same pattern as food intakes. This confirms that without adequate glucose in the diet during pregnancy, even with adequate total energy intake (12 and 24% ate same as 60%), maternal gain found is less than that found in controls. This represents a specific effect of glucose restriction alone on weight gain during pregnancy.

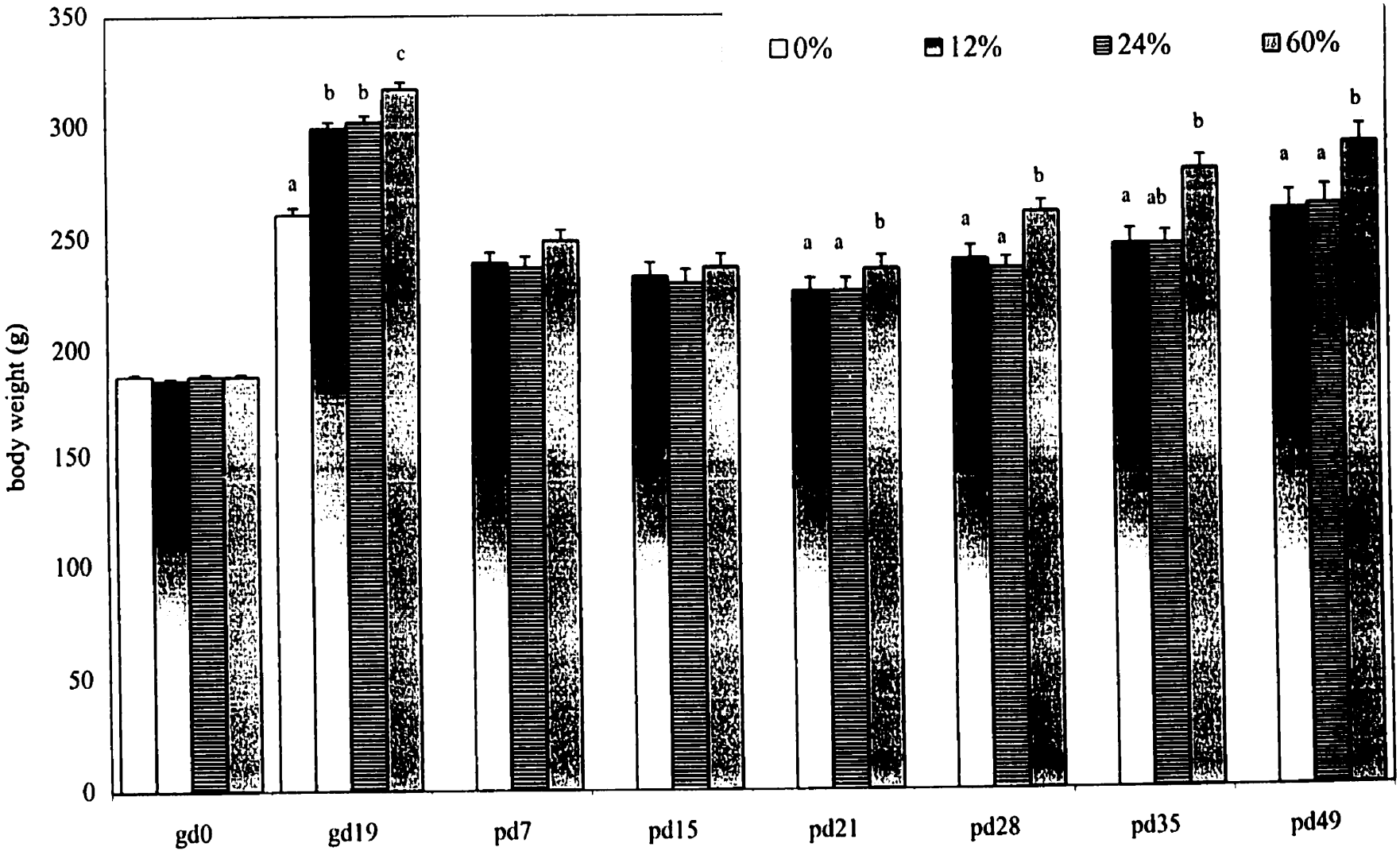
Table 12: Effect of Dietary Glucose Intake on Maternal Body Weight (g) During Gestation, Through Lactation and Post-Weaning[1,2]

		Diet Group				p value[7]
Time	n[3]	0%	12%	24%	60%	(diet)
gd0	129[4]	188±1	186±1	188±1	188±1	ns
gd19[A]	129[4]	261±3[a]	299±3[b]	302±3[b]	317±3[c]	0.0001
pd7	26[5]	-	239±5	237±5	249±5	ns
pd15	26[5]	-	233±6	230±6	237±6	ns
pd21	26[6]	-	226±6	226±6	236±6	ns
pd28[A]	26[6]	-	240±6[a]	236±5[a]	261±5[b]	0.0048
pd35[A]	26[6]	-	246±7[a]	246±6[a]	279±6[b]	0.0013
pd42[A]	26[6]	-	252±8[a]	257±7[ab]	283±7[b]	0.0139
pd49[A]	26[6]	-	261±8[a]	263±8[a]	290±8[b]	0.0268[8]

[1]Values are least square means (LSM) ± SELSM. [2] Multiple comparisons between diet groups by timepoint were done using Scheffé's test, indicated across rows with different lowercase letters. [3]Number of measurement units (cages). [4]One dam per cage. [5]One dam plus suckling pups. [6]One dam plus nibbling pups. [7]P-value from ANOVA. [8]Post hoc contrast between average of 12 and 24% vs. 60% group. [A]Significant main effect of diet.

During lactation, (pd 7, 15 and 21), maternal body weight of all animals was statistically the same, thus glucose restricted dams were not weight compromised. For all

Figure 6: Effect of Dietary Glucose Intake on Maternal Body Weight During Gestation, Through Lactation and Post-Weaning



post-weaning timepoints (pd28, 35, 42, and 49) glucose intake affected maternal body weight; at pd28 and 35 60%-adequate dams weighed significantly more than the 12 and 24% restricted dams, and significantly more than 12%-restricted dams only on pd42. At pd49 a significant effect of diet remained, however individual post hoc comparisons with the 12 and 24% groups failed to reveal where the effect lay. Further analysis, using contrasts, showed a significant difference between the 60% dams and the average of the 12 and 24% dams. The 60% glucose dams weighed more than the average of the 12 and 24% glucose dams.

3. LITTER SIZE AND WEIGHT

Litter size and weight data is presented in **Table 13**. Litter size for the combined perinatal timepoints was significantly smaller for 0% dams, 9±1 pups, than for all other dietary groups (11±1, 12±1 and 12±1 pups for 12, 24, and 60% dams respectively; p=0.0004). Combined litter weight for all living pups at gd20, birth and 12-24h were all similarly affected (p<0.0001) by dietary treatment. At all three timepoints, combined litter weight was significantly lower in 0% litters compared either 12, 24 or 60% litters. Diet did not compromise litter size of those litters designated for the postnatal part of this experiment. It is also clear from the results that the litter weights of all diet groups increased between gd20 and birth, however after birth, litter weight decreased and had not returned to birthweight by 12-24h for 0% litters.

Table 13: Effect of Dietary Glucose Intake on Litter Size and Weight[1, 2]

		Diet Group				p value[4]
Litter Size (#)	**n[3]**	**0%**	**12%**	**24%**	**60%**	**(diet)**
Perinatal	92	9 ± 1^{a}	11 ± 1^{b}	12 ± 1^{b}	12 ± 1^{b}	0.0004
Litter Weight (g)						
gd20	36	23 ± 2^{a}	39 ± 2^{b}	41 ± 2^{b}	44 ± 2^{b}	0.0001
birth	28	28 ± 4^{a}	60 ± 3^{b}	59 ± 3^{b}	63 ± 3^{b}	0.0001
12-24h	27	23 ± 6^{a}	50 ± 4^{b}	59 ± 4^{b}	62 ± 4^{b}	0.0001

[1]Values are least square means (LSM) ± SELSM. [2] Multiple comparisons between diet groups by timepoint were done using Scheffé's test, indicated across rows with different lowercase letters. [3]Number of litters. [4]P-value from one-way analysis of variance (ANOVA).

Litter size for the combined perinatal timepoints was significantly affected by diet; there were significantly fewer pups in 0% litters than for all other diet groups ($p=0.0004$). Litter weight at all three timepoints was significantly lower in 0%-glucose deficient litters as compared to all other diet groups. It is also clear from the results that the litters of all diet groups were increasing in weight between gd20 and birth, however after birth, litter weight decreased or did not increase further by 12-24h. This may in part be due to pup mortality decreasing litter size and thus reducing litter weight.

4. PUP BODY WEIGHTS

Pup body weights were measured every second day and immediately preceding necropsy. The results of the statistical analysis using a nested one-way ANOVA are presented in **Table 14**. Significant main effects of diet on pup weight were found at gd20, birth, 12-24h, pd15, 21 and 28.

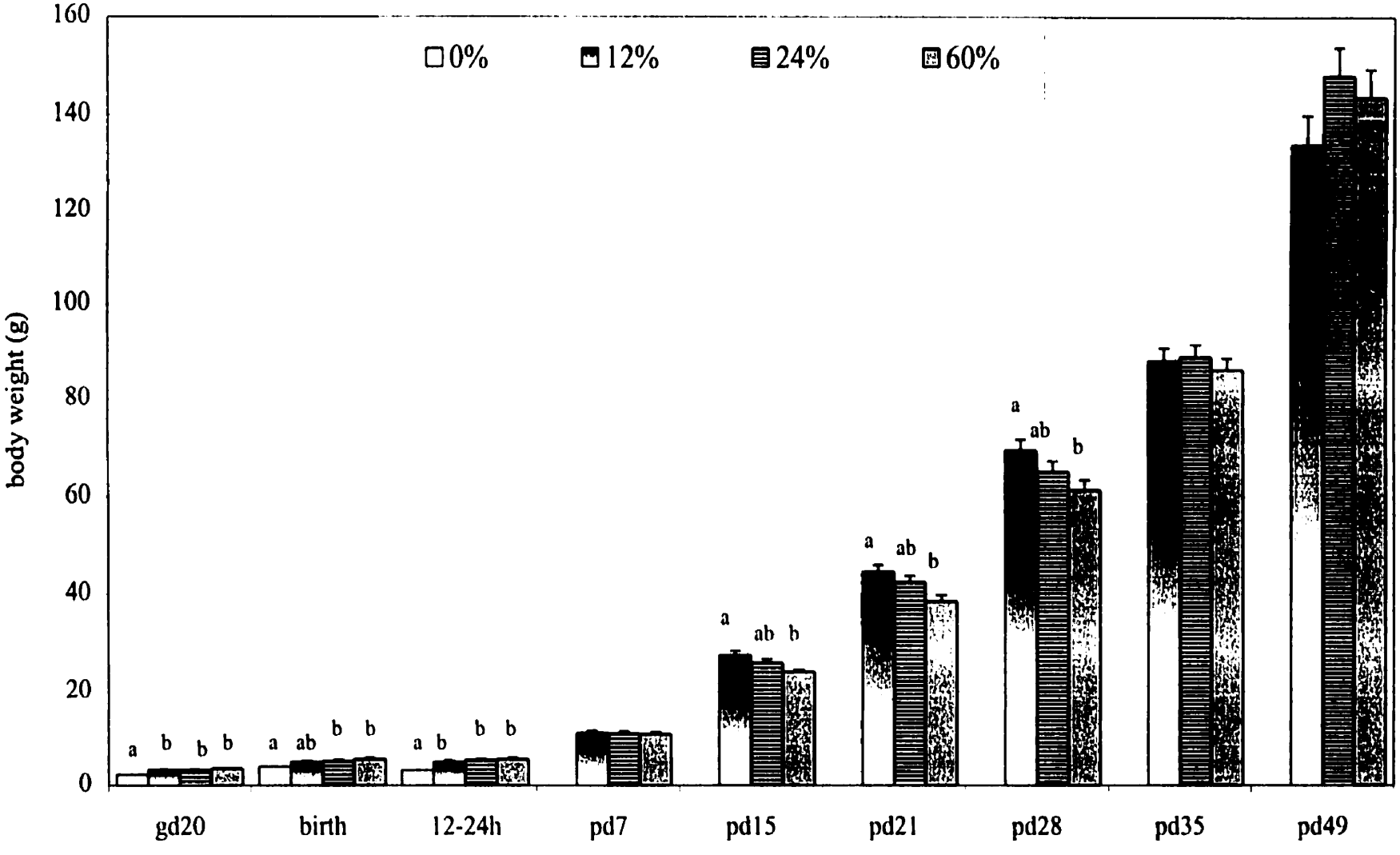
Table 14: Effect of Dietary Glucose Intake on Pup Body Weights (g)[1, 2, 3]

		Diet Group				p value[5]
Time	n[4]	0%	12%	24%	60%	(diet)
gd20[A]	36(421)	2.3 ± 0.1^{a}	3.3 ± 0.1^{b}	3.3 ± 0.1^{b}	3.6 ± 0.1^{b}	0.0001
birth[A]	29(316)	4.0 ± 0.3^{a}	5.0 ± 0.2^{ab}	5.2 ± 0.2^{b}	5.6 ± 0.2^{b}	0.0010
12-24h[A]	27(280)	3.2 ± 0.3^{a}	5.0 ± 0.3^{b}	5.4 ± 0.2^{b}	5.6 ± 0.2^{b}	0.0001
pd7	26(306)	-	11.1±0.4	10.9±0.4	10.8±0.4	ns
pd15[A]	26(182)	-	27.7 ± 0.8^{a}	26.1 ± 0.7^{ab}	24.2 ± 0.4^{b}	0.0109
pd21[A]	26(130)	-	44.7 ± 1.4^{a}	42.6 ± 1.3^{ab}	38.6 ± 1.3^{b}	0.0151
pd28[A]	26(78)	-	69.5 ± 2.2^{a}	65.2 ± 2.1^{ab}	61.4 ± 2.1^{b}	0.0475
pd35	26(52)	-	88.2±2.6	89.0±2.5	86.3±2.5	ns
pd49	26(26)	-	133.9±6.1	148.0±5.8	143.6±5.8	ns

[1]Values are least square means (LSM) ± SELSM. [2]Multiple comparisons between diet groups by timepoint were done using Scheffé's test; significant differences are indicated with different lowercase letters. [3]A nested design was used in the analysis. [4]Number of litters; number of pups in parentheses. [5]P-value in analysis of variance (ANOVA). [A]Significant main effect of diet.

Individual pup body weights were significantly affected by diet at gd20, birth and at 12-24h (**Table 14 and Figure 7**). At gd20 and 12-24h, 0% pups weighed an average of 2.3±0.1 and 3.2±0.3g respectively, significantly less than the 12, 24 or 60% pups ($p<0.0001$ for both timepoints). Mean birth weight of 0% pups was 4.0±0.3g, differing significantly only from the 24% and 60% pups ($p<0.001$). No significant difference in birth weight was found between the 0% and 12% pups or between the 12% and 24 or

Figure 7: Effect of Dietary Glucose Intake on Pup Body Weight



60% pups. Pup weights appeared to increase for all diet groups between gd20 and birth, however between birth and 12-24 hours, weights of the 0% pups appeared to decrease, while for the other diet groups weights appeared stable.

During lactation and early postweaning (pd15, 21 and 28), 12%-glucose restricted pups weighed significantly more than their glucose adequate counterparts (60%). There were no significant differences in weight between the 12 and 24% restricted pups or between the 24% and 60% pups at these timepoints. By postnatal day 35 and 49, there were no further detectable differences in pup weights across diet groups.

5. AMNIOTIC FLUID PARAMETERS

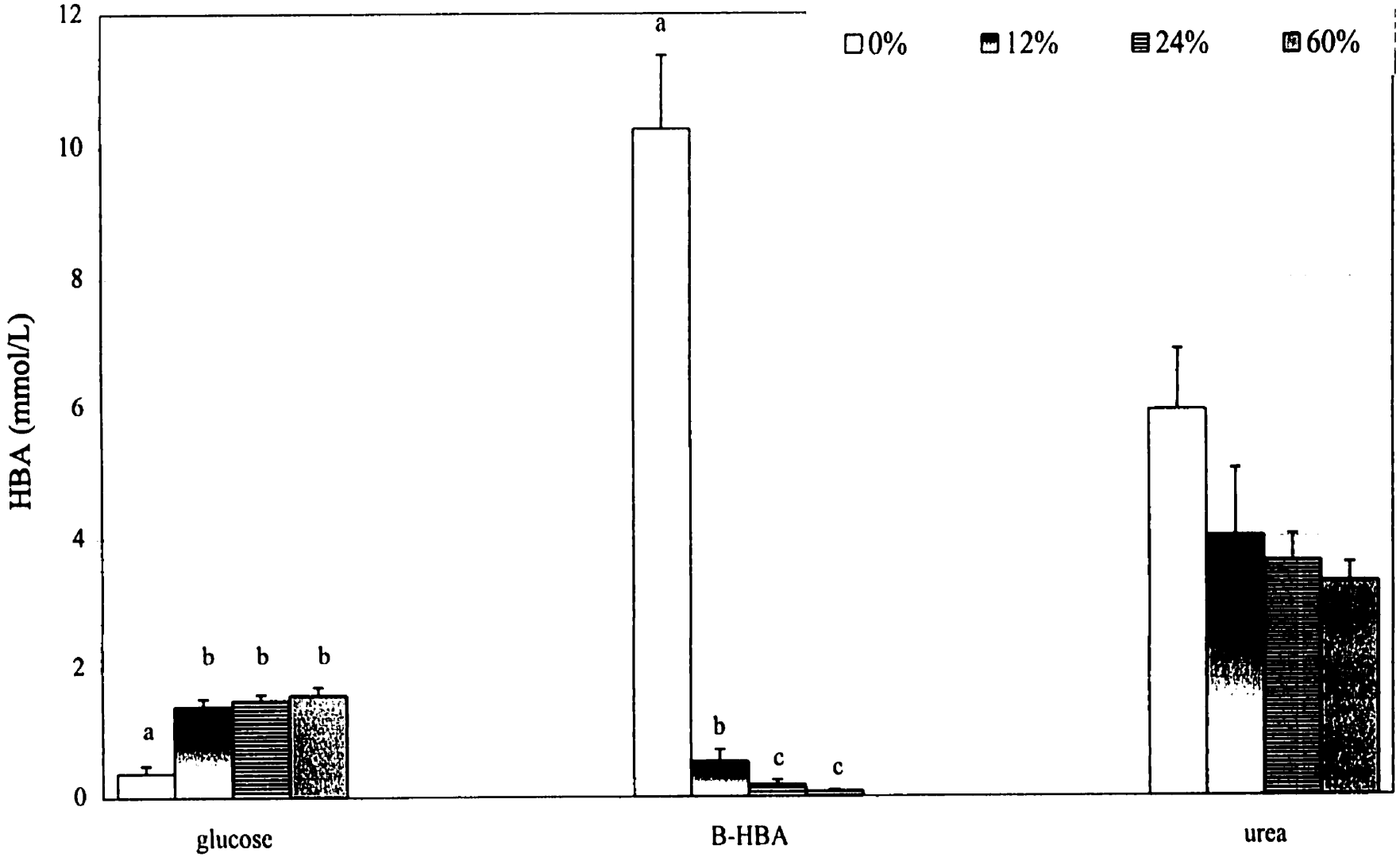
Amniotic fluid glucose and β-hydroxybutyrate levels were significantly affected by diet at gd20, while urea levels were unaffected (**Table 15** and **Figure 8**). Amniotic fluid glucose was significantly lower for 0% dams than for all other diet groups. Amniotic fluid β-hydroxybutyrate (β-HBA) levels increased with decreasing dietary glucose level. In 0% dams β-HBA was significantly higher than for all other diet groups (>19-fold higher); the 12% dams also had significantly higher β-HBA levels than the 24 and 60% dams (3-6-fold higher), which did not differ from each other.

Table 15: Effects of Dietary Glucose on Amniotic Fluid Glucose, Urea and β-Hydroxybutyrate Level at gd20[1, 2]

	Diet Group				p value[3]
	0%	12%	24%	60%	(diet)
Glucose (mmol/L)	0.37±0.12[a] (9)	1.40±0.12[b] (9)	1.49±0.09[b] (9)	1.57±0.13[b] (9)	0.0001
β-HBA (mmol/L)	10.27±1.12[a] (9)	0.54±0.18[b] (9)	0.18±0.07[c] (9)	0.08±0.02[c] (9)	0.0001[4]
Urea (mmol/L)	5.95±0.95 (9)	4.02±1.04 (9)	3.64±0.40 (9)	3.32±0.29 (9)	ns

[1]Values presented are mean±SEM. [2]Multiple comparisons between diet groups by timepoint were done using Scheffé's test; significant differences are indicated with different lowercase letters. [3]P-value in analysis of variance (ANOVA). [4]ANOVA run on log transformed data. Number of pooled litter samples presented in parentheses.

Figure 8: Effect of Dietary Glucose on Amniotic Fluid Glucose, B-Hydroxybutyrate and Urea Levels at gd20



6. PLASMA PARAMETERS

6.1 Plasma Albumin

There was no significant effect of dietary glucose level on plasma albumin levels at any timepoint. Results are presented in **Table 16** and **Figure 9**.

Table 16: Effect of Dietary Glucose Intake on Plasma Albumin (g/L)[1, 2]

	Diet Group			
Time	**0%**	**12%**	**24%**	**60%**
Dams				
gd20	39.8±1.9 (9)	39.0±2.9 (8)	40.4±2.2 (9)	45.7±1.1 (9)
pd49	-	64.3±1.6 (6)	57.1±2.8 (6)	59.4±4.0 (7)
Pups				
gd20	11.8±0.9 (8)	13.1±1.2 (8)	14.6±1.2 (9)	16.1±1.4 (9)
birth	15.7±4.8 (3)	14.9±1.1 (8)	14.4±0.5 (7)	16.3±1.3 (8)
12-24h	16.2±0.9 (3)	16.6±2.1 (6)	13.3±2.3 (8)	15.7±1.2 (8)
pd7	-	23.5±1.9 (7)	22.6±2.0 (9)	23.1±1.6 (9)
pd15	-	26.6±1.0 (8)	27.8±1.8 (8)	27.1±1.2 (9)
pd21	-	29.2±2.5 (8)	30.5±1.9 (9)	30.4±2.2 (9)
pd28	-	40.6±1.1 (8)	40.0±1.4 (7)	37.1±0.9 (9)
pd35	-	39.4±2.0 (8)	38.3±3.2 (9)	39.0±2.5 (9)
pd49	-	49.0±2.9 (8)	44.4±1.9 (9)	46.2±2.4 (8)

[1]Values are means±SEM. Number of dams or pooled litter samples presented in parentheses.

6.2 Plasma Urea

Results of the plasma urea analysis for both dams and pups are presented in **Table 17** and **Figure 10**. Maternal plasma urea concentration was not significantly affected by diet. In offspring, only at perinatal timepoints (gd20, birth, 12-24h) did diet have a significant impact on plasma urea levels. At gd20, pups from the glucose restricted litters had significantly higher plasma urea than the 60% control pups. There were no other differences by diet group. By birth, urea levels were higher in 0% restricted pups than in any of the other groups, which did not differ from one another. By 12-24 hours after birth, a more graded distribution of urea levels was seen, with 0% pups having significantly higher plasma urea levels than 24% and 60% pups, but not different than 12% pups; urea level did not differ between the 12, 24, and 60% groups. From pd7-49 there were no diet induced differences in plasma urea in dams or offspring.

Figure 12: Effect of Dietary Glucose on Plasma B-Hydroxybutyrate

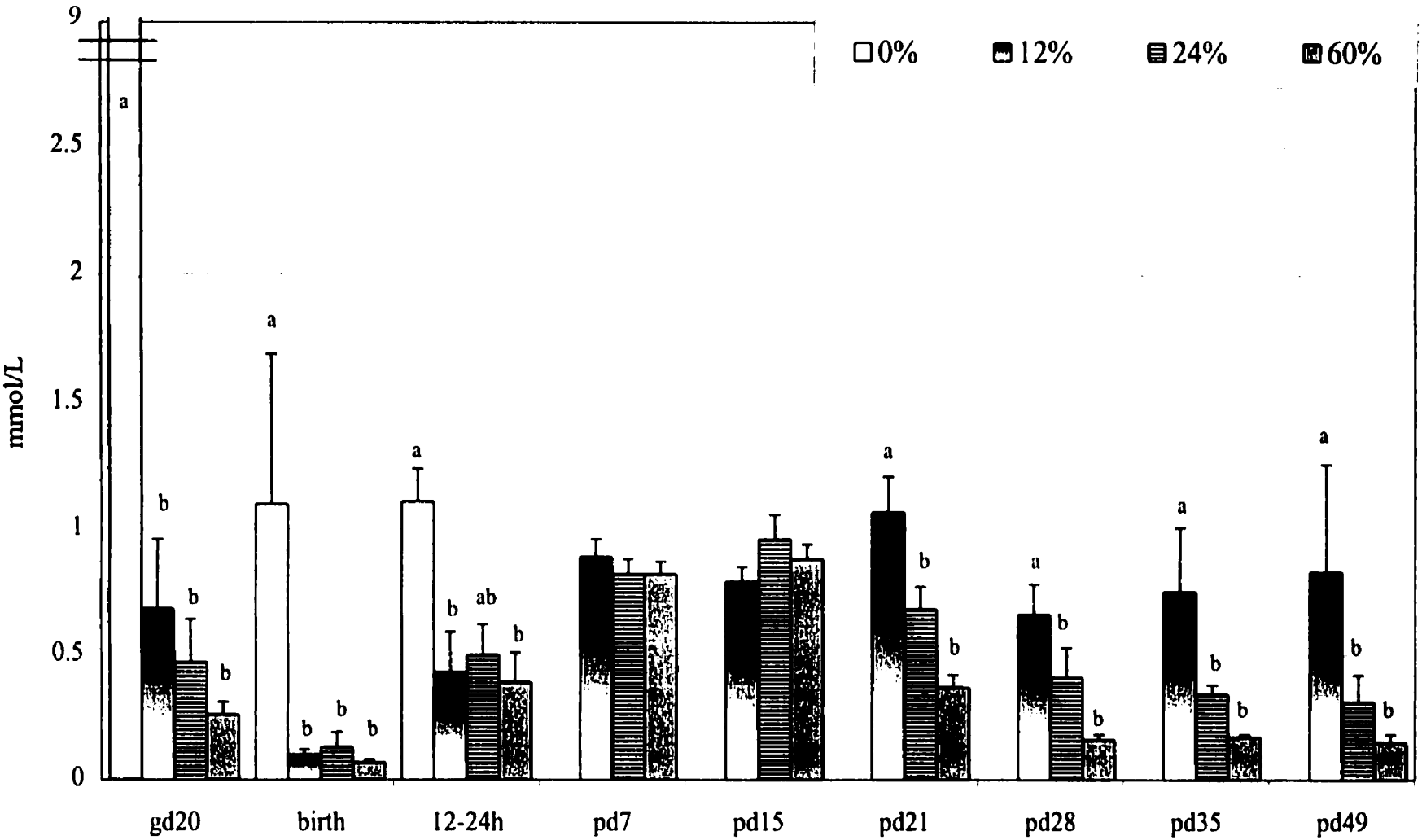


Table 17: Effect of Dietary Glucose Intake on Plasma Urea (mmol/L)[1, 2]

Time	Diet Group 0%	12%	24%	60%	p-value[3] (diet)
Dams					
gd20	5.01±0.96 (7)	3.82±0.50 (8)	3.70±0.36 (9)	3.35±0.15 (9)	ns[4]
pd49	-	6.01±0.31 (6)	4.91±0.32 (6)	5.32±0.32 (7)	ns
Pups					
gd20	6.87±0.94^{a} (8)	4.95±0.62^{ab} (8)	4.50±0.59^{ab} (9)	3.98±0.28^{b} (9)	0.0186
birth	21.47±0.22^{a} (2)	4.60±0.39^{b} (8)	4.76±0.46^{b} (7)	3.50±0.40^{b} (8)	0.0001
12-24h	16.74±0.60^{a} (3)	10.92±1.48^{ab} (6)	9.85±1.12^{b} (8)	7.08±0.64^{b} (8)	0.0005
pd7	-	8.53±0.71 (7)	7.93±0.85 (9)	7.79±0.87 (9)	ns
pd15	-	3.74±0.36 (8)	4.06±0.79 (9)	3.75±0.83 (9)	ns
pd21	-	3.73±0.50 (8)	3.27±0.25 (9)	2.62±0.43 (9)	ns
pd28	-	4.32±0.51 (8)	3.94±0.38 (9)	3.54±0.37 (9)	ns
pd35	-	2.49±0.19 (8)	3.40±0.46 (9)	3.36±0.40 (9)	ns
pd49	-	6.75±1.40 (8)	4.74±0.85 (9)	4.74±0.66 (9)	ns

[1]Values are mean±SEM. [2]Multiple comparisons between diet groups by timepoint were done using Scheffé's test; significant differences are indicated with different lowercase letters. [3]P-value from ANOVA. [4]ANOVA run on log transformed data. Number of dams or pooled litter samples presented in parentheses.

6.3 Plasma Glucose

Plasma glucose levels are presented in **Table 18** and **Figure 11**. Maternal plasma glucose was affected by diet at gd20, where glucose levels were significantly lower in animals fed the 0% than the 12% diet. No other group differences existed at this time.

Table 18: Effect of Dietary Glucose Intake on Plasma Glucose (mmol/L)[1, 2]

Time	Diet Group 0%	12%	24%	60%	p-value (diet)
Dams					
gd20	5.40±0.37^{a} (9)	7.67±0.40^{b} (8)	7.21±0.37^{ab} (9)	6.92±0.65^{ab} (9)	0.0101
pd49	-	12.02±0.83 (6)	12.42±0.92 (6)	15.06±2.00 (7)	ns
Pups					
gd20	2.12±0.49^{a} (8)	4.26±0.39^{b} (9)	3.44±0.28^{ab} (9)	4.55±0.44^{b} (9)	0.0011
birth	2.79±1.31 (3)	4.85±0.35 (8)	5.11±0.71 (7)	5.69±0.59 (8)	ns
12-24h	4.82±1.40 (4)	5.27±0.47 (7)	5.80±0.29 (8)	6.27±0.48 (8)	ns
pd7	-	8.55±0.25 (7)	8.77±0.28 (9)	8.49±0.24 (9)	ns
pd15	-	11.85±0.71^{a} (8)	10.06±0.40^{b} (9)	10.11±0.41^{b} (9)	0.0369[3]
pd21	-	11.18±0.81 (7)	11.61±0.57 (9)	11.34±0.51 (9)	ns[4]
pd28	-	11.73±0.57 (8)	11.36±0.54 (9)	11.80±0.74 (9)	ns
pd35	-	12.73±0.43 (8)	13.06±0.69 (9)	13.30±1.49 (9)	ns[4]
pd49	-	14.48±1.21 (8)	16.31±2.72 (9)	15.88±2.58 (9)	ns

[1]Values are least square mean±SEM. [2]Multiple comparisons between diet groups by timepoint were done using Scheffé's test; significant differences are indicated with different lowercase letters. [3]Contrast

Figure 10: Effect of Dietary Glucose Intake on Plasma Urea

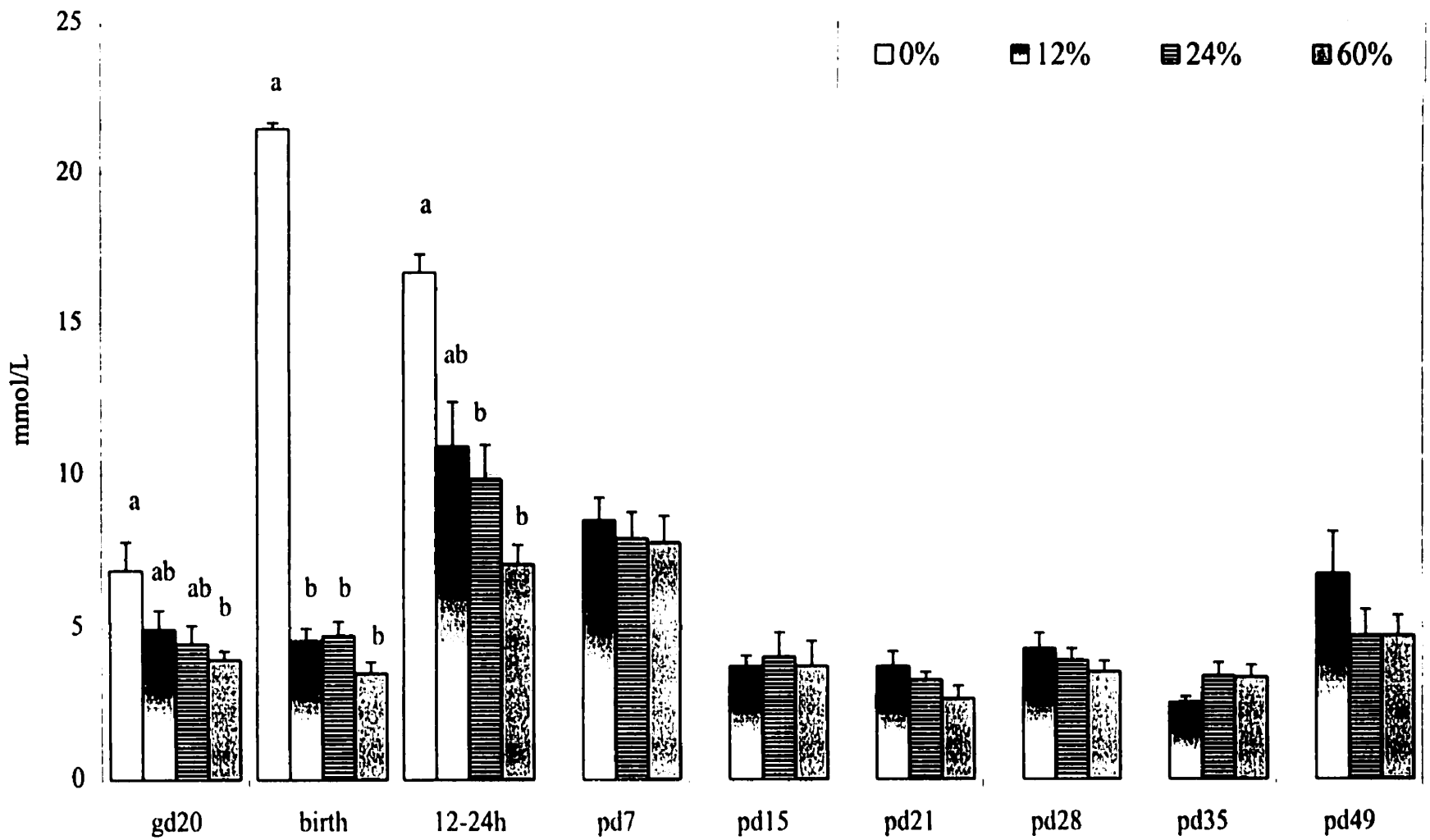
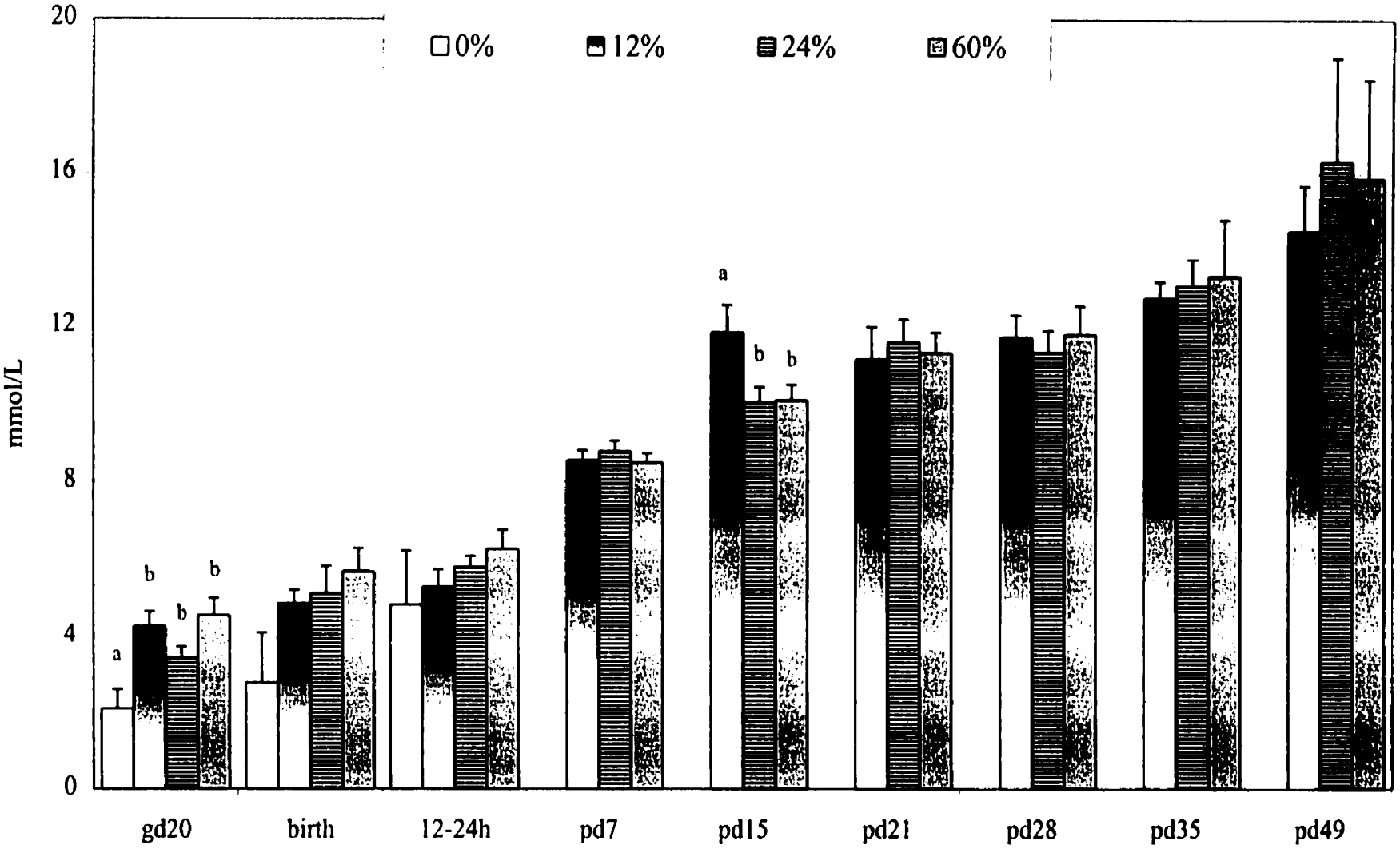


Figure 11: Effect of Dietary Glucose on Plasma Glucose



between 12% vs. average of 24 and 60% significantly different. [4]ANOVA run on log transformed data. Number of dams or pooled litter samples presented in parentheses.

Pup plasma glucose was also significantly affected by dietary glucose at gd20, with plasma glucose levels significantly lower in the 0% pups than in either the 12 or 60% pups; the 24% pups did not differ from any other diet group. The only other timepoint at which glucose levels were affected by diet was at pd15, where 12% pups had significantly higher plasma glucose than the average of the 24% and 60% pups (a contrast was required).

6.4 Plasma β-Hydroxybutyrate

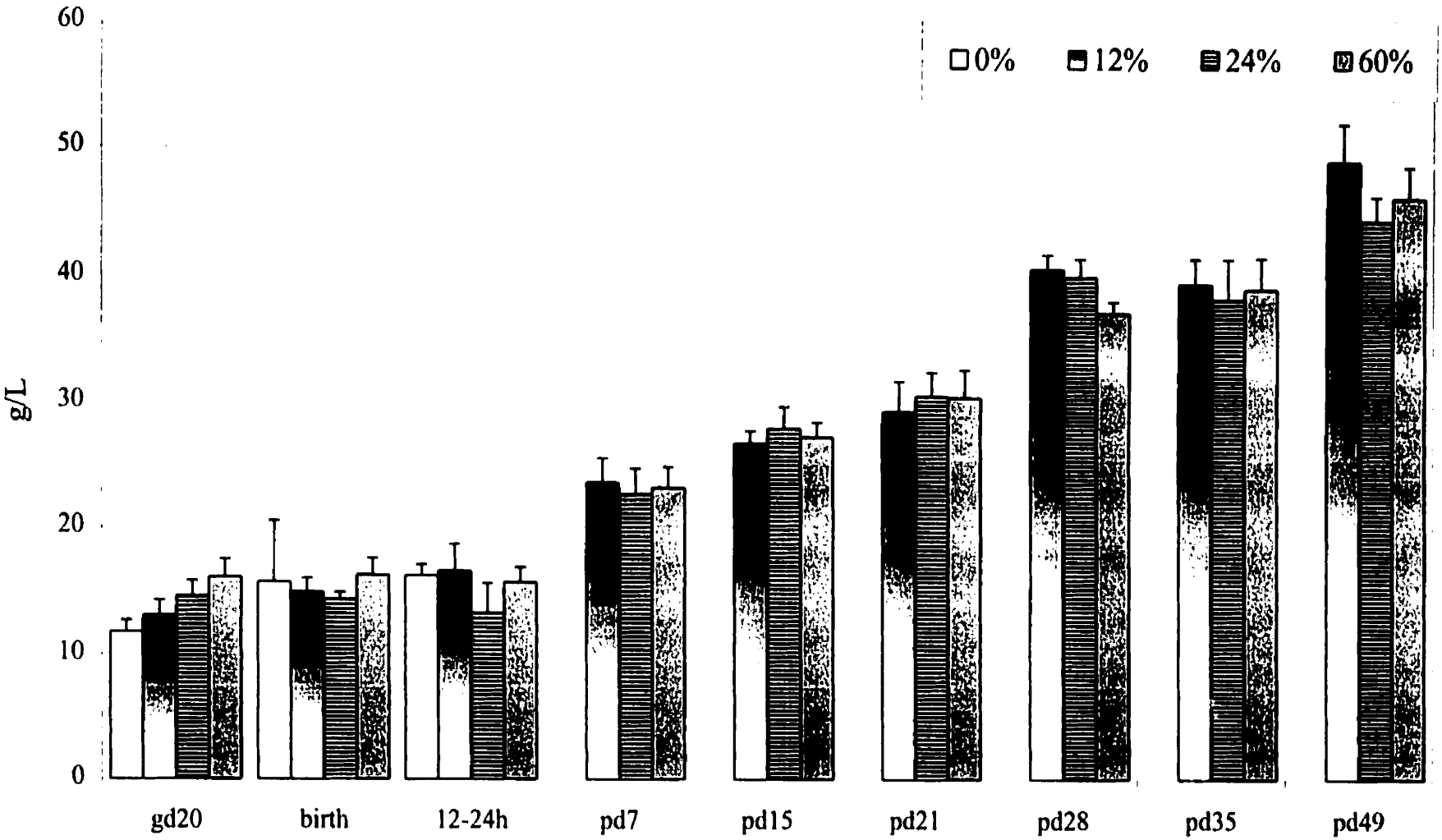
Plasma β-hydroxybutyrate (β-HBA) levels were affected by diet at all timepoints except pd7 and 15 (**Table 19** and **Figure 12**). Dams were affected at both gd20 and pd49, with the group fed the most restricted diet at each time having significantly higher levels than their counterparts; at gd 20, plasma β-HBA levels were approximately >25-fold higher in restricted dams.

Table 19: Effect of Dietary Glucose Intake on Plasma β-Hydroxybutyrate (mmol/L)[1, 2]

	Diet Group				p-value[3]
Time	0%	12%	24%	60%	(diet)
Dams					
gd20	7.20±0.81[a] (9)	0.50±0.11[b] (8)	0.49±0.18[b] (9)	0.24±0.06[b] (9)	0.0001[4]
pd49	-	0.32±0.04[a] (6)	0.15±0.03[b] (6)	0.19±0.01[b] (7)	0.0009
Pups					
gd20	8.19±1.26[a] (8)	0.68±0.28[b] (8)	0.47±0.17[b] (9)	0.26±0.05[b] (9)	0.0001[4]
birth	1.10±0.59[a] (4)	0.10±0.02[b] (8)	0.13±0.06[b] (6)	0.07±0.01[b] (8)	0.0026[4]
12-24h	1.11±0.13[a] (3)	0.43±0.16[b] (7)	0.50±0.12[ab] (9)	0.39±0.12[b] (7)	0.0423[5]
pd7	-	0.89±0.07 (7)	0.82±0.06 (9)	0.82±0.05 (9)	ns
pd15	-	0.79±0.06 (8)	0.96±0.10 (9)	0.88±0.06 (9)	ns
pd21	-	1.07±0.14[a] (8)	0.68±0.09[b] (9)	0.37±0.05[b] (9)	0.0002
pd28	-	0.66±0.12[a] (8)	0.41±0.12[b] (8)	0.16±0.02[b] (9)	0.0002[4]
pd35	-	0.75±0.26[a] (7)	0.34±0.04[ab] (9)	0.17±0.01[b] (7)	0.0023[4]
pd49	-	0.83±0.43[a] (8)	0.31±0.11[ab] (9)	0.15±0.03[b] (9)	0.0085[4]

[1]Values are mean±SEM. [2]Multiple comparisons between diet groups by timepoint were done using Scheffé's test; significant differences are indicated with different lowercase letters. [3]P-value in analysis of variance (ANOVA). [4]ANOVA run on log transformed data. [5]Contrast between 0% and average of 12 and 60% significantly different. Number of dams or pooled litter samples presented in parentheses.

Figure 9: Effect of Dietary Glucose Intake on Plasma Albumin



Pup plasma β-hydroxybutyrate levels were also perturbed at gd20 with 0% deficient pups having higher levels than either 24% or 60% pups only. Levels of β-HBA in maternal and pup plasma as well as amniotic fluid at gd20 closely paralleled each other by diet group; greatly elevated in the 0% group compared to all other groups. At birth, β-HBA levels were significantly elevated in 0% pups versus all other diet groups. At 12-24h the significant difference was found in the contrast between the 0% deficient dams and the average of the 12 and 60% dams. At pd21 and 28, 12% pups had significantly higher β-HBA levels than 24 and 60% pups. By pd35 and 49, β-HBA levels were higher in 12% vs. 60% animals only.

7. SMALL INTESTINE STRUCTURAL DEVELOPMENT

Diet was tested as a main effect for all gut growth outcome parameters. Diet was again tested for its effects when pup weight was included as a covariate in the model. This second analysis was done to determine whether pup weights were driving the significant differences seen in gut outcome parameters. The measurement of gut length was not feasible at gd20 due to the extreme friability and fragility of the guts, so total SI was collected and weighed. There is therefore no data available for gd20 gut length, and proximal or distal SI weight.

7.1 Gut Length

Small intestine length was measured from the ligament of Treitz to the ileocecal valve (**Table 20** and **Figure 13**). Length was significantly different by diet at birth, postnatal days 15 and 21. When pup weight was included in the model as a covariate, significant effects of diet were seen at 12-24h and in adults, but ceased to exist for the timepoints found significant when pup weight was not accounted for in the model. At 12-24h, guts of the glucose deficient pups were significantly shorter than for all other diet groups. In adult animals fed experimental diets postnatally only, guts were significantly shorter for the 24%-restricted group than the control animals.

7.2 Gut Weight - Total, Proximal and Distal

Total Small Intestine Weight

Total small intestine weight was affected by diet at gd20, pd15 and 21 when unadjusted for pup weight (**Table 21** and **Figure 14**). When pup weight was included in the model as a covariate, only during peak lactation, at pd15, did the effect of diet on gut weight remain significant (p=0.0229). At this timepoint, gut weights were significantly heavier in pups fed 12%-restricted diets than in controls.

Proximal Small Intestine Weight

There was no main effect of diet on proximal gut weight at any timepoint when pup weight was included as a covariate in the model (**Table 23** and **Figure 15**).

Distal Small Intestine Weight

As for total gut weight, only during peak lactation (pd15) did diet continue have a significant effect of distal gut weight when pup weight was included in the model as a covariate (**Table 24** and **Figure 16**).

Table 20: Effects of Diet, Diet and Pup Weight on Small Intestine Length[1] (cm)

						MAIN EFFECT[2]		
						Model gut length = diet	Model gut length = diet, pupwt	
		Diet Group						
Time	**n[3]**	**0%**	**12%**	**24%**	**60%**	**Diet**	**Diet**	**Pup Wt**
birth	26	18.7±0.6 (14)	17.8±0.2 (86)	18.0±0.2 (89)	20.1±0.2 (90)	0.0016	ns	0.0001
12-24h	25	17.4±0.4 (20)[a]	18.9±0.2 (71)[b]	19.7±0.2 (86)[b]	19.9±0.3 (76)[b]	ns	**0.0147**	0.0001
pd7	28	-	31.2±0.4 (39)	32.0±0.3 (41)	30.9±0.4 (44)	ns	ns	0.0001
pd15	26	-	44.4±0.6 (16)	43.1±0.7 (18)	41.7±0.6 (18)	0.0206	ns	0.0001
pd21	26	-	59.5±1.4 (16)	57.5±0.8 (18)	54.2±1.0 (18)	ns	ns	0.0001
pd28	26	-	75.4±2.1 (8)	69.7±0.9 (9)	69.7±2.2 (9)	ns	ns	0.0001
pd35	26	-	76.9±4.1 (4)	78.2±1.4 (9)	72.7±3.7 (7)	ns	ns	0.0001
pd49	26	-	90.0±3.3 (8)	87.3±2.4 (9)	87.5±2.5 (9)	ns	ns	ns
adult	21	-	113.5±3.4 (7)[ab]	106.6±2.5 (7)[a]	107.8±1.5 (7)[b]	ns	**0.0367**	0.0429

[1]Values are mean±SEM. [2]P-value in analysis of variance (ANOVA). [3]Number of dams/litters. [a, b, c] Indicate significant differences between diet treatments using Scheffé's test (model **gut length=diet, pupwt**). Number of pups presented in parentheses; pups nested within dam.

Figure 13: Effects of Dietary Glucose on Small Intestine Length

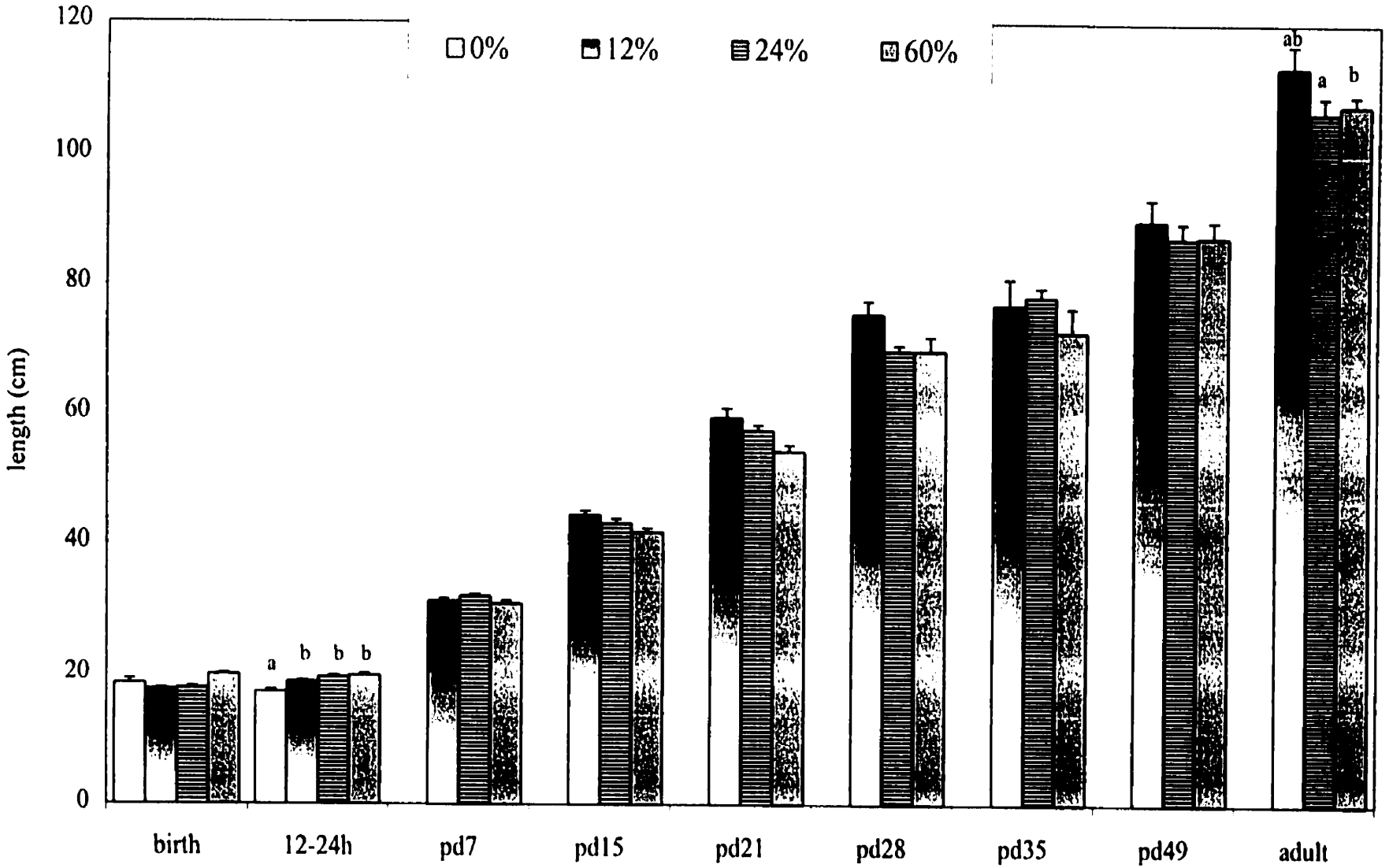
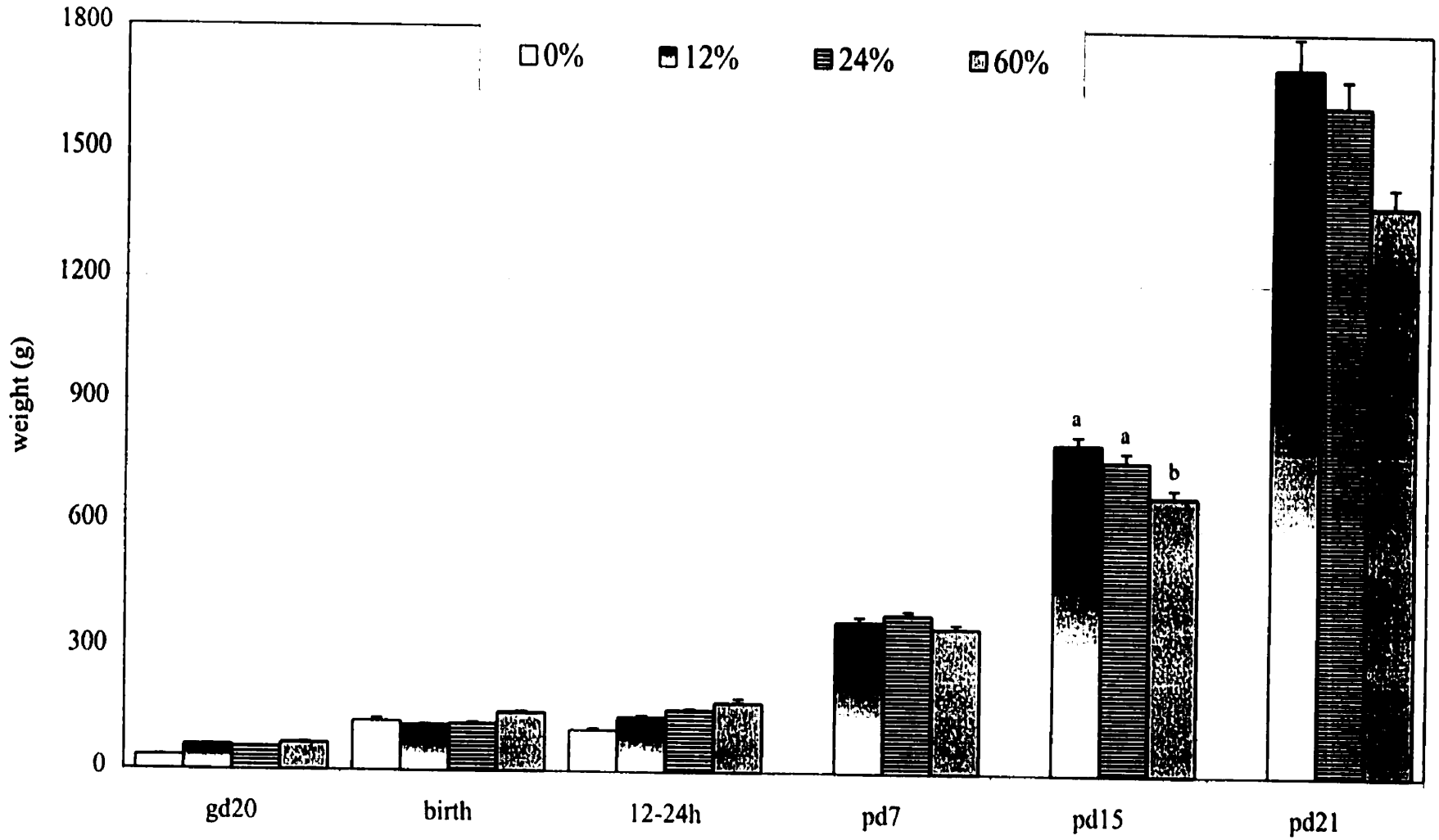


Table 21: Effects of Diet, Diet and Pup Weight on Total Small Intestine Weight[1] (mg)

						MAIN EFFECT[2]		
						Model gut weight = diet	Model gut weight = diet, pupwt	
		Diet Group						
Time	**n[3]**	**0%**	**12%**	**24%**	**60%**	**Diet**	**Diet**	**Pup Wt**
gd20	24	34.3±1.4 (61)	61.9±1.3 (70)	57.4±1.0 (78)	67.8±1.3 (73)	0.0001	ns	0.0001
birth	26	122.4±7.8 (14)	113.4±2.2 (84)	117.6±2.8 (79)	146.0±3.0 (90)	ns	ns	0.0001
12-24h	25	102.4±5.0 (20)	135.5±4.4 (68)	153.6±3.3 (75)	172.2±10.3 (75)	ns	ns	0.0001
pd7	26	-	371.7±12.1 (38)	389.4±9.1 (39)	357.4±9.1 (43)	ns	ns	0.0001
pd15	26	-	805.5±20.5[a] (16)	763.6±23.1[a] (18)	677.8±18.9[b] (18)	0.0034	**0.0229**	0.0001
pd21	26	-	1715.8±73.6 (16)	1626.5±63.1 (18)	1390.7±45.1 (18)	0.0237	ns	0.0001

[1]Values are mean±SEM. [2]P-value for main effects from ANOVA. [3]Number of dams/litters. [a, b, c] Indicate significant differences between diet treatments using Scheffé's test for the model **gut weight=diet, pupwt**. Number of pups presented in parentheses; pups nested within dam.

Figure 14: Effects of Dietary Glucose on Total Small Intestine Weight



7.3 Gut Weight per Unit Length

Pup weight explained the differences in gut weight per unit length, as no diet effects were seen when pup weight was included as a covariate in the analysis (**Table 25**).

7.4 Small Intestine Histology

A qualitative evaluation of muscle and mucosal development was done in order to compile an overall developmental profile of small intestine histology for the study timepoints. Further qualitative assessment by diet group and timepoint included assessment of villus branching, #crypts/3villus as a measure indicative of possible blunting of the mucosal surface. For each timepoint, the samples, blinded for diet group, were compared based on of these criteria as assessed by Dr. Cecile Logan, pathologist. Results of this qualitative evaluation are presented in **Table 22**.

Table 22: A Qualitative Assessment of the Development of Small Intestinal Histology

Time	Description of Small Intestine Structure
gd20	• epithelium has an overall primitive appearance • mitosis present along the length of villi • villi present, but wide at base and mix of short and tall • crypts not yet developed • no Paneth cells present • high nucleus:cytoplasm ratio (large nuclei) • brush border well developed • muscle layers well differentiated (circular and longitudinal) • lamina propria thick, extending well up into villi
birth	• crypt region not mature; no paneth cells present • mitosis progressing down villi into primordial crypts • villus branching present • muscle as for gd20 • lamina propria thinner than gd20, but still somewhat diffuse • lymphocytic aggregates present
12-24h	• large supranuclear vacuoles at tips of enterocytes from primordial crypts to villus tip • crypts still beginning to form; no Paneth cells present yet • mitosis moving lower into 'crypts'; fewer mitoses visible • muscle as for gd20 • lamina propria becoming thinner than at gd20, birth
pd7	• supranuclear vacuoles along villus length and in crypts • goblet cells present along villus length • crypts present; still somewhat immature (no Paneth cells present yet) • mitosis is limited to crypts • muscle as for gd20 • lymphoid aggregates present
pd15	• supranuclear vacuoles seen only in crypt area, not along villus length • goblet cells present along villus length • Paneth cells present in crypts • villus branching abundant • muscle as for gd20 • lymphoid aggregates present
pd21	• supranuclear vacuoles no longer present; adult-type enterocytes present • more abundant goblet cells than at earlier timepoints • crypts mature, adult; abundant granules in Paneth cells • lymphoid aggregates present
pd28 pd35 pd49 adult dams	• samples for remaining timepoints did not differ significantly in development from pd21 when adult developmental profile was achieved

Table 23: Effects of Diet, Diet and Pup Weight on Proximal Small Intestine Weight[1] (mg)

						MAIN EFFECT[2]		
		Diet Group				Model gut weight = diet	Model gut weight = diet, pupwt	
Time	n[3]	0%	12%	24%	60%	Diet	Diet	Pup Wt
birth	26	77.9±5.2 (14)	71.1±1.5 (84)	74.2±1.8 (79)	89.6±1.8 (90)	ns	ns	0.0001
12-24h	25	66.1±3.2 (20)	87.2±3.0 (70)	101.9±2.3 (77)	108.2±2.6 (75)	0.0115	ns	0.0001
pd7	26	-	217.0±9.4 (38)	236.7±5.2 (39)	219.6±5.5 (43)	ns	ns	0.0001
pd15	26	-	445.7±12.7 (16)	420.0±12.7 (18)	378.6±11.4 (18)	0.0045	ns	0.0001
pd21	26	-	858.3±37.8 (16)	814.5±27.9 (18)	714.4±27.8 (18)	ns	ns	0.0001

[1]Values are mean±SEM. [2]P-value in ANOVA. [3]Number of dams/litters. Number of pups presented in parentheses; pups nested within dam.

Table 24: Effects of Diet, Diet and Pup Weight on Distal Small Intestine Weight[1] (mg)

						MAIN EFFECT[2]		
		Diet Group				Model gut weight = diet	Model gut weight = diet, pupwt	
Time	n[3]	0%	12%	24%	60%	Diet	Diet	Pup Wt
birth	26	44.5±3.1 (14)	42.2±0.8 (85)	44.0±1.3 (80)	56.5±1.4 (90)	0.0358	ns	0.0001
12-24h	25	36.3±2.0 (20)	48.8±1.6 (69)	52.1±1.3 (75)	63.9±8.9 (76)	ns	ns	0.0001
pd7	26	-	154.7±3.9 (39)	152.7±4.5 (39)	137.4±4.1 (44)	ns	ns	0.0001
pd15	26	-	359.8±10.1[a] (16)	343.6±13.1[ab] (18)	299.3±9.4[b] (18)	0.0115	**0.0418**	0.0001
pd21	26	-	857.5±41.3 (16)	812.0±38.6 (18)	676.2±24.9 (18)	0.0261	ns	0.0001

[1]Values are mean±SEM. [2]P-value in analysis of variance (ANOVA). [3]Number of dams/litters. [a, b, c] Indicate significant differences between diet treatments using Scheffé's test (model **gut wt dist=diet, pupwt**). Number of pups presented in parentheses; pups nested within dam.

Figure15: Effects of Dietary Glucose on Proximal Small Intestine Weight

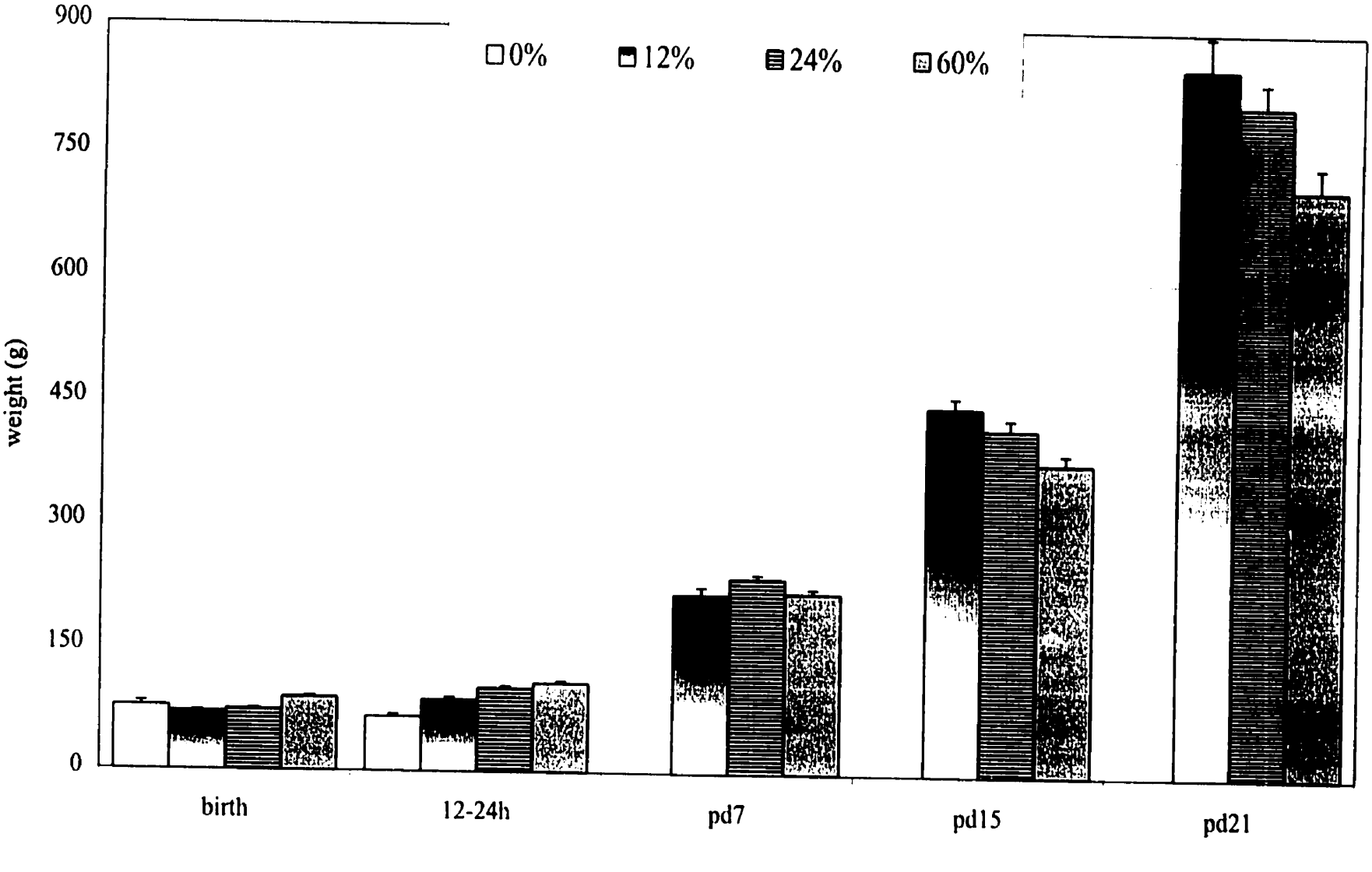


Figure 16: Effects of Dietary Glucose on Distal Small Intestine Weight

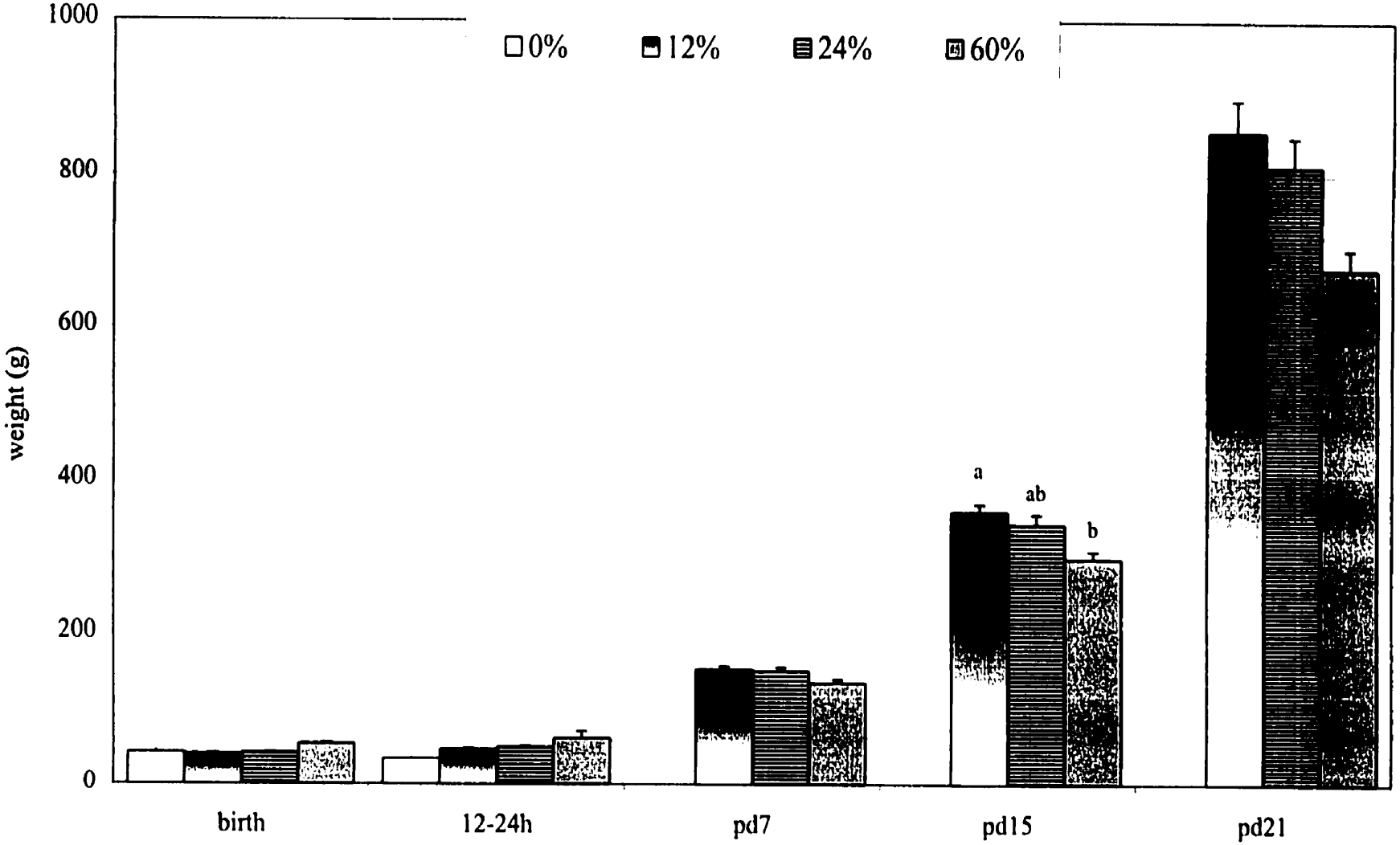


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Table 25: Effects of Diet, Diet and Pup Weight on Small Intestine Weight per unit Length (mg/cm)[1]

						MAIN EFFECT[2]		
						Model gut weight = diet	Model gut weight = diet, pupwt	
		Diet Group						
Time	**n[3]**	**0%**	**12%**	**24%**	**60%**	**Diet**	**Diet**	**Pup Wt**
birth	26	6.5±0.3 (14)	6.3±0.1 (84)	6.5±0.1 (77)	7.2±0.1 (90)	ns	ns	0.0001
12-24h	25	5.9±0.2 (20)	7.1±0.2 (68)	7.8±0.1 (75)	8.6±0.4 (75)	0.0329	ns	0.0001
pd7	26	-	11.9±0.3 (38)	12.3±0.3 (39)	11.5±0.2 (43)	ns	ns	0.0001
pd15	26	-	18.1±0.3 (16)	17.7±0.4 (18)	16.2±0.4 (18)	0.0226	ns	0.0001
pd21	26	-	28.7±0.9 (16)	28.2±0.8 (18)	25.6±0.6 (18)	ns	ns	0.0001

[1]Values are mean±SEM. [2]P-value in analysis of variance (ANOVA). [3]Number of dams/litters. [a, b, c] Indicate significant differences between diet treatments using Scheffé's test (model **gut weight/length=diet, pupwt**). Number of pups presented in parentheses; pups nested within dam.

8. SMALL INTESTINE FUNCTIONAL DEVELOPMENT

Measures of functional development were made for gd 20 on pooled litter total gut for all outcomes, and on proximal and distal pooled gut for all other timepoints. The only exception is that for glycogen analysis, total intestine was used for analysis at gd20, birth and 12-24h; postnatal analysis was by small intestine region.

8.1 Gut Glycogen Concentration

Perinatal small intestine glycogen reserves were determined for tissue samples representing whole gut (**Table 26** and **Figure 17**). No significant effects of dietary glucose intake on total small intestine glycogen concentration were found at either gd20, birth, or 12-24h.

Table 26: Effects of Dietary Glucose Intake on Total Small Intestine Glycogen Concentration (mg/g tissue)[1]

	Diet Group			
Time	**0%**	**12%**	**24%**	**60%**
gd20	2.70±0.98 (7)	1.86±0.64 (4)	2.70±1.04 (7)	1.59±0.25 (6)
birth	1.98±0.87 (3)	2.48±0.47 (6)	1.70±0.60 (4)	1.50±0.26 (3)
12-24h	-	3.15±0.62 (3)	2.32±0.22 (4)	3.06±0.47 (4)

[1]Values presented are mean±SEM. Number of samples presented in parentheses.

Tissue glycogen concentration for postnatal days was also analyzed for proximal and distal segments separately to determine whether a gradient of glycogen concentration existed at that time. Results are presented in **Table 27** and **Figure 17**.

At both pd7 and pd15 there was a significant effect of region on the glycogen content of the small intestine (pd 7: $p=0.0002$; pd15: $p=0.0132$). In both cases a gradient in glycogen content was present, with significantly higher glycogen levels in the distal segments than in the proximal gut. Glycogen concentrations for proximal gut were comparable to those found at earlier timepoints, however the glycogen concentration in the distal segments were generally higher than for early timepoints. There was no significant effect of diet or any interaction between diet and region on glycogen concentration for these timepoints. At pd28, a significant interaction between diet and region was present ($p<0.04$). When individual regions were tested for diet effects, there

was a significant impact of diet on distal SI deposition; deposition was greater in animals fed the 12%-restricted diet than in controls (p=0.0052). There were no other diet differences. At pd35 there was no significant effect of intestine region on glycogen concentration, however glycogen reserves were significantly lower in the proximal SI of the 12% group than in the 60% animals (p=0.0349). A trend towards higher glycogen levels in the 24% group versus the 12 and 60% groups was seen at pd49. By adulthood there was no significant difference between intestinal regions or diet groups.

8.2 SGLT1 Protein Expression

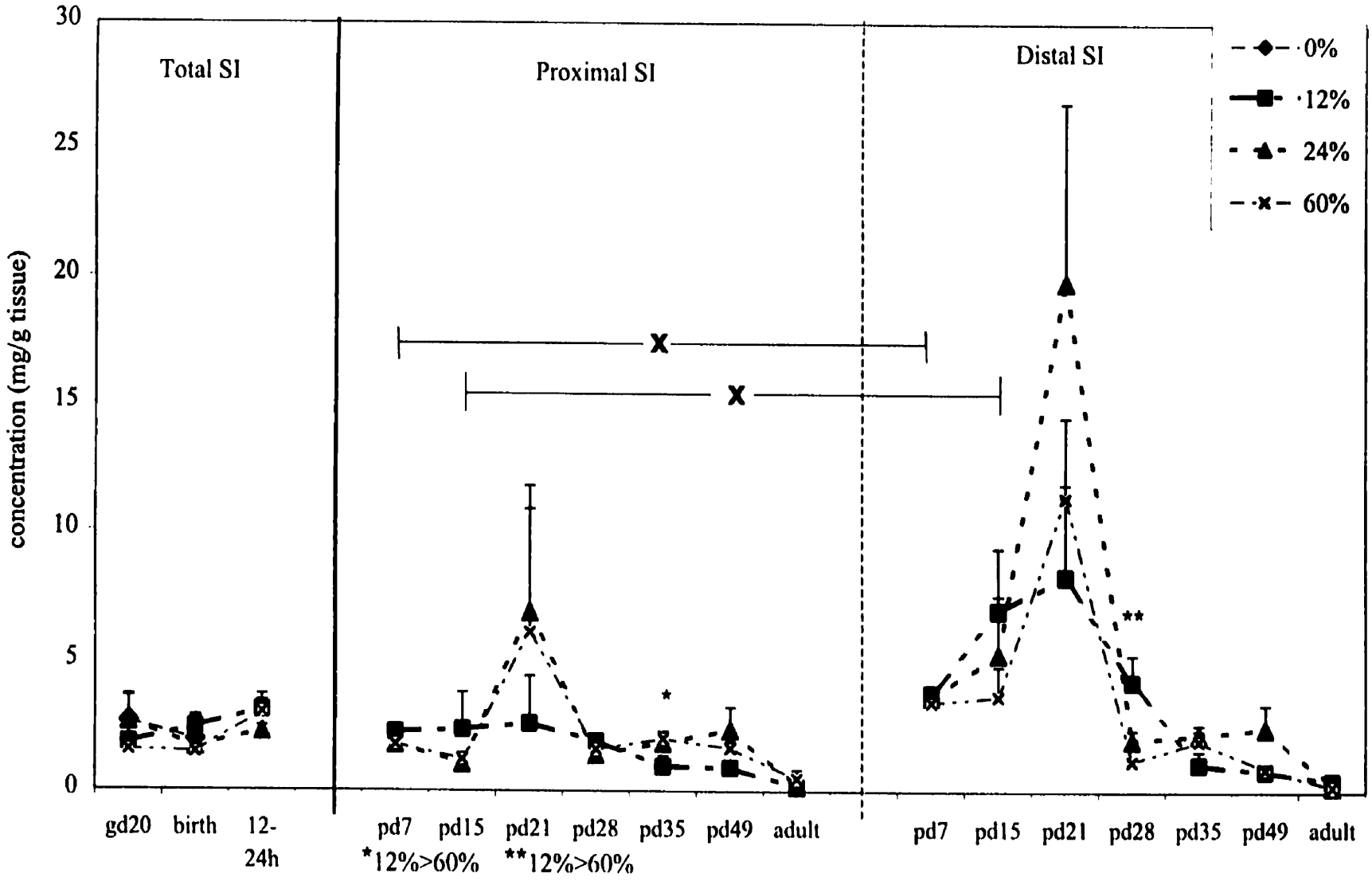
The results of statistical analyses performed on relative band density for samples as compared to a control (adult, distal SI, 60% dietary glucose) run on each blot are presented in **Table 28** and **Figure 18**. Level of dietary glucose influenced the expression of SGLT1 protein in the developing small intestine at gd20, pd15 and pd21, while significant effects of SI region were found at pd7, pd 15 and pd28. At gd 20, the relative expression of SGLT1 in the fetal SI was significantly lower for those animals in the 0% glucose deficient group that for all other diet groups (p=0.0166). There were no other significant differences between diet groups. At birth and 12-24h, there was a trend towards lower levels of SGLT1 protein with lower levels of dietary glucose for proximal segments of the small intestine, however this was not significant. At pd 7, SGLT1 protein expression was greater in distal segments than in proximal segments. This regional pattern was still present at pd15 (distal greater than proximal); a significant effect of diet in the distal segment was found by one-way ANOVA, where expression in the 12% group exceeded that of the 60% group (p=0.050). At pd21, only a diet effect was detectable, with SGLT1 expression greater in the proximal SI of animals receiving the 60%-glucose adequate diet than those fed the 12 or 24%-glucose restricted diet. By pd28 no diet effect was detected, however the expected regional difference (proximal>distal) in SGLT1 expression was significant.

Table 27: Effects of Dietary Glucose on Postnatal Proximal and Distal Intestine Glycogen Concentration (mg/g tissue)[1]

	Diet	Small Intestine Region		p-value[2]	p-value[3]	
		Proximal	Distal		Proximal	Distal
pd7[A]	12%	2.30±0.30 (3)	3.83±0.11 (3)	0.0001[A] (proximal<distal)	ns	ns
	24%	1.82±0.28 (5)	3.69±0.29 (4)			
	60%	1.82±0.23 (5)	3.47±.038 (5)			
pd15[A]	12%	2.41±1.45 (3)	6.99±2.35 (3)	0.0001[A, 4] (proximal<distal)	ns	ns
	24%	1.06±0.12 (6)	5.33±2.20 (6)			
	60%	1.23±0.24 (5)	3.70±1.14 (4)			
pd21	12%	2.64±1.84 (3)	8.27±3.55 (3)	ns	ns	ns
	24%	6.93±4.82 (6)	19.81±7.01 (6)			
	60%	6.14±4.70 (5)	11.28±3.20 (5)			
pd28[B,C]	12%	1.95±0.07 (3)	4.25±1.03 (3)[a]	0.0032[B, 4] 0.0399[4] (diet*region)	ns	0.0052
	24%	1.41±0.21 (6)	2.00±0.40 (5)[ab]			
	60%	1.63±0.29 (5)	1.17±0.15 (5)[b]			
pd35[B]	12%	0.95±0.42 (3)[a]	1.04±0.52 (3)	0.0203[B]	0.0349	ns
	24%	1.89±0.10 (6)[ab]	2.22±0.37 (6)			
	60%	2.05±0.29 (5)[b]	1.97±0.41 (5)			
pd49	12%	0.90±0.19 (3)	0.78±0.12 (3)	ns (0.0584 diet)	ns	ns
	24%	2.40±0.88 (6)	2.48±0.89 (6)			
	60%	1.67±0.26 (4)	0.87±0.20 (5)			
Adult	12%	0.15±0.01 (2)	0.41±0.16 (2)	ns	ns	ns
	24%	0.19±0.01 (3)	0.22±0.03 (3)			
	60%	0.47±0.35 (3)	0.18±0.06 (3)			

[1]Values presented are mean±SEM. [2]Significance of main effect (diet, region) from two-way ANOVA. [3]Significance from one-way ANOVA for diet. [4]ANOVA run on log transformed data. [A]Significant main effect of region. [B]Significant main effect of diet. . [a,b]Significant differences between diet treatments indicated with different lowercase letters; multiple comparisons between diet groups by timepoint were done using Scheffé's test. [C]Significant interaction (diet*region). Number of samples presented in parentheses.

Figure 17: Effects of Dietary Glucose on Developing Small Intestine Glycogen Concentration



8.3 Lactase Specific Activity

Data presented in **Table 29** and **Figure 19** are for lactase specific activity. Significant effects of either diet or region were found at gd20, birth, pd15, pd21 and 28. At gd20, lactase specific activity (SA) was significantly higher in animals fed 0% glucose diet than in those fed 12% diet - no other differences were seen among diet groups. At birth, both diet ($p<0.01$) and region ($p<0.001$) were significant; lactase SA was again greater in 0% glucose animals than 60% controls, and was lower for proximal than distal segments. By dividing data by region and performing a one-way ANOVA for diet within each region, we were no longer able to detect the diet effect. At pd15 only region was significantly different, where proximal SA continued to be lower than that for distal SI. At pd21, as pups began to wean, specific activity remained higher for distal segments than proximal. Diet had a significant effect on lactase SA in the proximal SI where activity was significantly lower for both 12 and 24% glucose-fed animals than in those fed the 60% glucose diet. By pd28, diet significantly effected lactase SA, with greater levels in animals fed the 60% diet than the 12%; as at birth, we were no longer able to detect diet differences when data was divided into proximal and distal segments.

8.4 Sucrase Specific Activity

A regional difference in sucrase specific activity (SA) (**Table 30** and **Figure 20**) was found at pd7, where proximal SA exceeded distal SA. At pd21, significant regional differences were again seen, however for this timepoint distal activities were greater than proximal. Pd21 was the only timepoint where a significant impact of dietary treatment was found. At this timepoint, sucrase SA was significantly higher in the distal SI of animals weaning to the 12% glucose diet than for those weaning to the 24% diet only.

Table 28: Effects of Dietary Glucose on Relative SGLT1 Protein Expression in Rat Small Intestine (relative band density)[1]

Time	Diet	Small Intestine Region		p-value[2]	p-value[3]	
		Proximal	Distal		Proximal	Distal
gd20[B]	0%	0.65±0.08 (5)[a]	(data for gd20 is for total small intestine)	0.0166[B]		
	12%	1.39±0.32 (4)[b]				
	24%	1.55±0.25 (4)[b]				
	60%	1.66±0.11 (3)[b]				
birth	0%	0.79±0.15 (2)	1.83±1.03 (3)		ns	ns
	12%	1.67±0.41 (4)	1.43±0.38 (4)	ns		
	24%	1.75±0.32 (4)	2.47±0.80 (3)			
	60%	1.94±0.43 (3)	2.16±0.99 (3)			
12-24h	0%	1.28±0.00 (1)	3.23±1.59 (3)	ns	ns	ns
	12%	1.36±0.64 (4)	1.72±0.65 (4)			
	24%	1.84±0.37 (4)	2.27±0.73 (4)			
	60%	2.08±0.88 (4)	2.46±0.29 (4)			
pd7[A]	12%	1.02±0.11 (3)	2.64±1.73 (3)	0.0001[A,3] (distal>proximal)	ns	ns
	24%	1.17±0.36 (5)	2.05±0.64 (5)			
	60%	0.73±0.19 (4)	1.85±0.95 (3)			
pd15[A,B]	12%	1.21±0.26 (2)	2.52±0.48(2)[a]	0.0159[A]	ns	0.0500
	24%	1.45±0.29 (3)	2.13±0.28 (3)[ab]	0.0050[B]		
	60%	0.91±0.08 (3)	1.19±0.14 (3)[b]	(distal>proximal)		
pd21[B]	12%	1.57±0.26 (3)[a]	1.91±0.52 (3)	0.0302[B]	0.0132	ns
	24%	1.29±0.35 (3)[a]	1.34±0.34 (2)			
	60%	2.84±0.16 (3)[b]	2.27±0.64 (2)			
pd28[A]	12%	1.63±0.23 (3)	0.54±0.03 (3)	0.0002[A] (proximal >distal)	ns	ns
	24%	2.07±0.51 (3)	0.45±0.10 (3)			
	60%	1.52±0.35 (3)	0.53±0.15 (3)			

[1]Mean±SEM. [2]Significance of main effect (diet, region) from two-way ANOVA. [3]Significance from one-way ANOVA (diet). [4]ANOVA run on log transformed data. [A]Main effect of region. [B]Main effect of diet. [a,b]Significant differences between diets indicated with different lowercase letters; Scheffé's test. Number of samples presented in parentheses.

Figure 18: Effects of Dietary Glucose on Relative SGLT1 Protein Expression

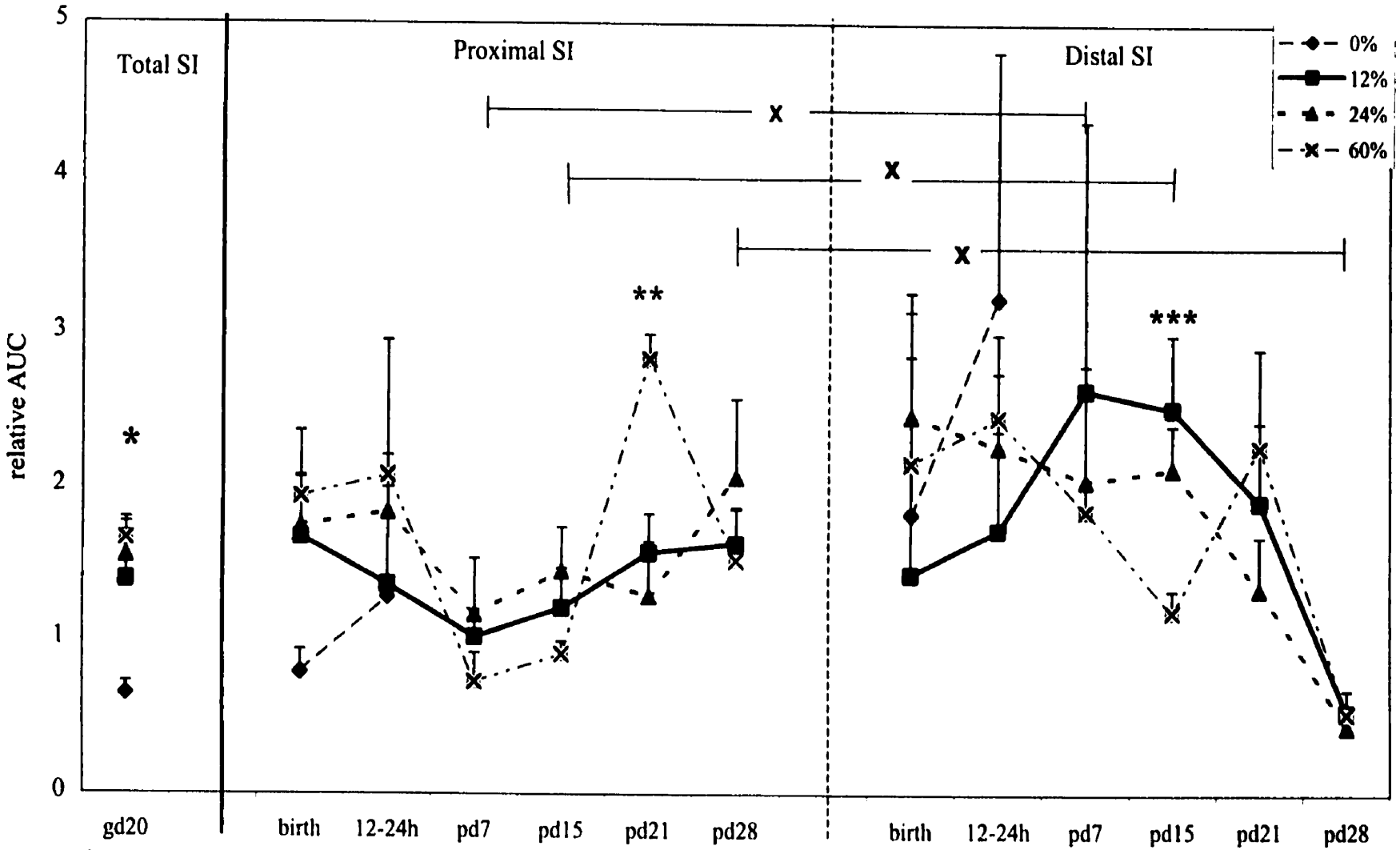


Table 29: Dietary Glucose Intake and Small Intestine Lactase Specific Activity (umol/mg protein (x 10^{-2}))[1]

Time	Diet	Small Intestine Region		p-value[2]	p-value[3]	
		Proximal	Distal		Proximal	Distal
gd20[A]	0%[a]	6.35±0.62 (5)				
	12%[b]	2.48±1.01 (3)	(data for gd20 is for	0.0172[A]		
	24%[ab]	3.21±0.51 (4)	total small intestine)			
	60%[ab]	3.84±1.19 (3)				
birth[A,B]	0%[a]	4.00±0.92 (2)	6.82±0.88 (3)	0.0109[A]	ns	ns
	12%[ab]	0.96±0.23 (5)	5.17±1.25 (5)	0.0001[B]		
	24%[ab]	2.10±1.11 (5)	4.96±0.55 (5)	(proximal<distal),4		
	60%[b]	0.64±0.18 (4)	3.03±0.48 (4)			
12-24h	0%	14.87 (1)	8.29±0.96 (2)	(0.0645 diet)	ns	ns
	12%	5.67±1.51 (4)	6.66±0.67 (4)			
	24%	6.25±2.36 (4)	6.83±1.92 (4)			
	60%	3.70±1.30 (4)	6.60±1.33 (4)			
pd7	12%	7.20±2.11 (3)	4.13±0.20 (3)	ns	ns	ns
	24%	5.09±1.44 (5)	5.28±0.85 (5)			
	60%	6.97±1.74 (4)	3.97±1.23 (3)			
pd15[B]	12%	4.09±0.32 (3)	6.18±0.56 (3)	0.0004[B]	ns	ns
	24%	4.14±0.58 (4)	5.93±0.47 (4)	(proximal<distal)		
	60%	4.40±0.69 (3)	6.49±0.27 (3)			
pd21[A,B]	12%	3.48±0.12 (3)[a]	6.41±0.99 (3)	0.0105[A]	0.0159	ns
	24%	3.38±0.15 (3)[a]	4.79±0.05 (3)	0.0001[B]		
	60%[b]	4.50±0.31 (3)[b]	7.03±0.36 (3)	(proximal<distal)		
pd28[A]	12%	1.67±0.27 (3)	1.51±0.23 (3)	0.0151[A]	ns	ns
	24%	2.05±0.62 (3)	1.83±0.19 (3)			
	60%	3.31±0.31 (3)	2.26±0.32 (3)			

[1]Mean±SEM. [2]P-value in two-way analysis of variance (ANOVA; diet and region). [3]Significance in one-way ANOVA within region (diet). [4]ANOVAs on log transformed data. [A]Main effect of diet. [B]Main effect of region. [a,b]Significant differences between diets indicated with different lowercase letters; done using Scheffé's test. Number of pooled litter samples presented in parentheses.

Figure 19: Effects of Dietary Glucose on Lactase Specific Activity

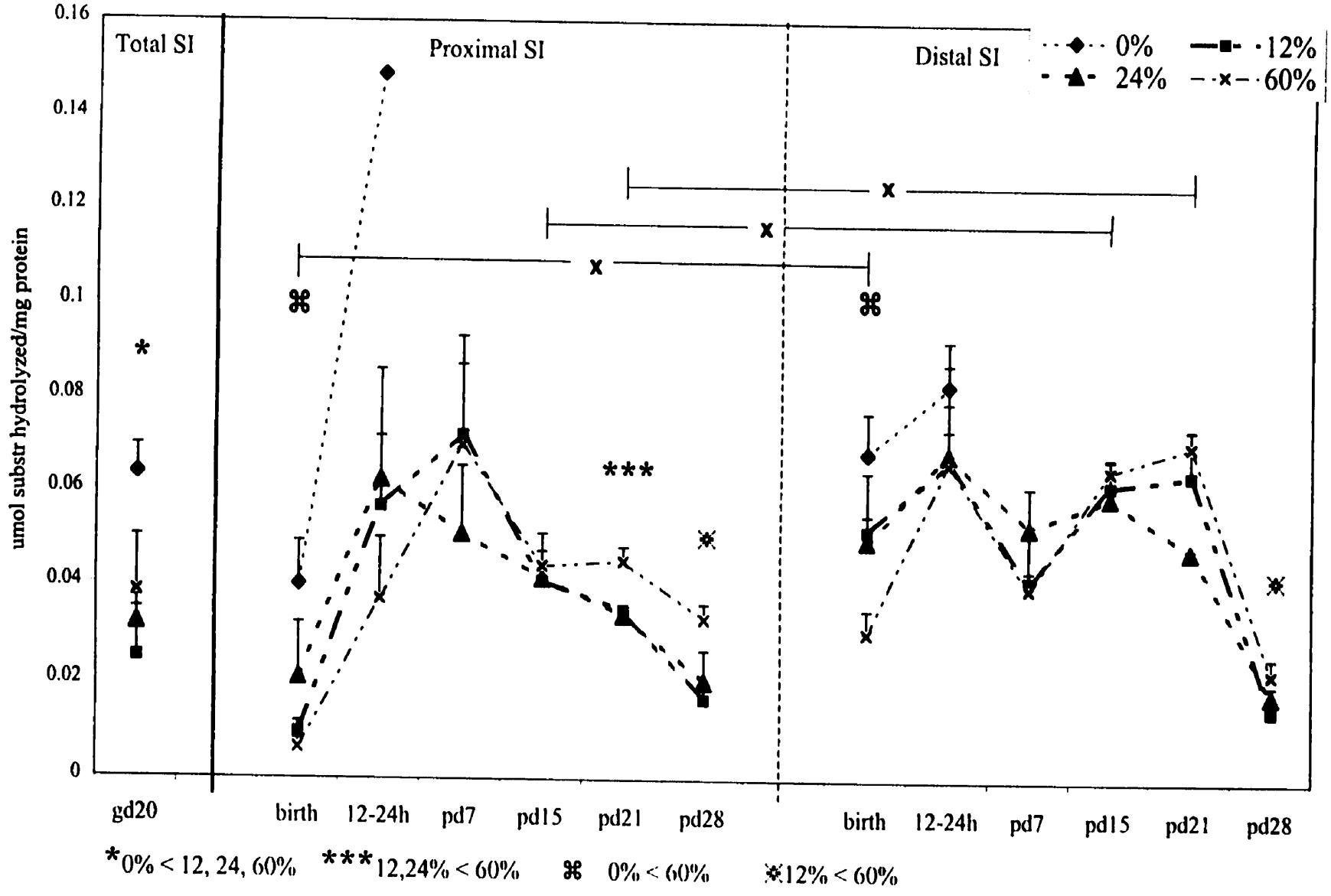
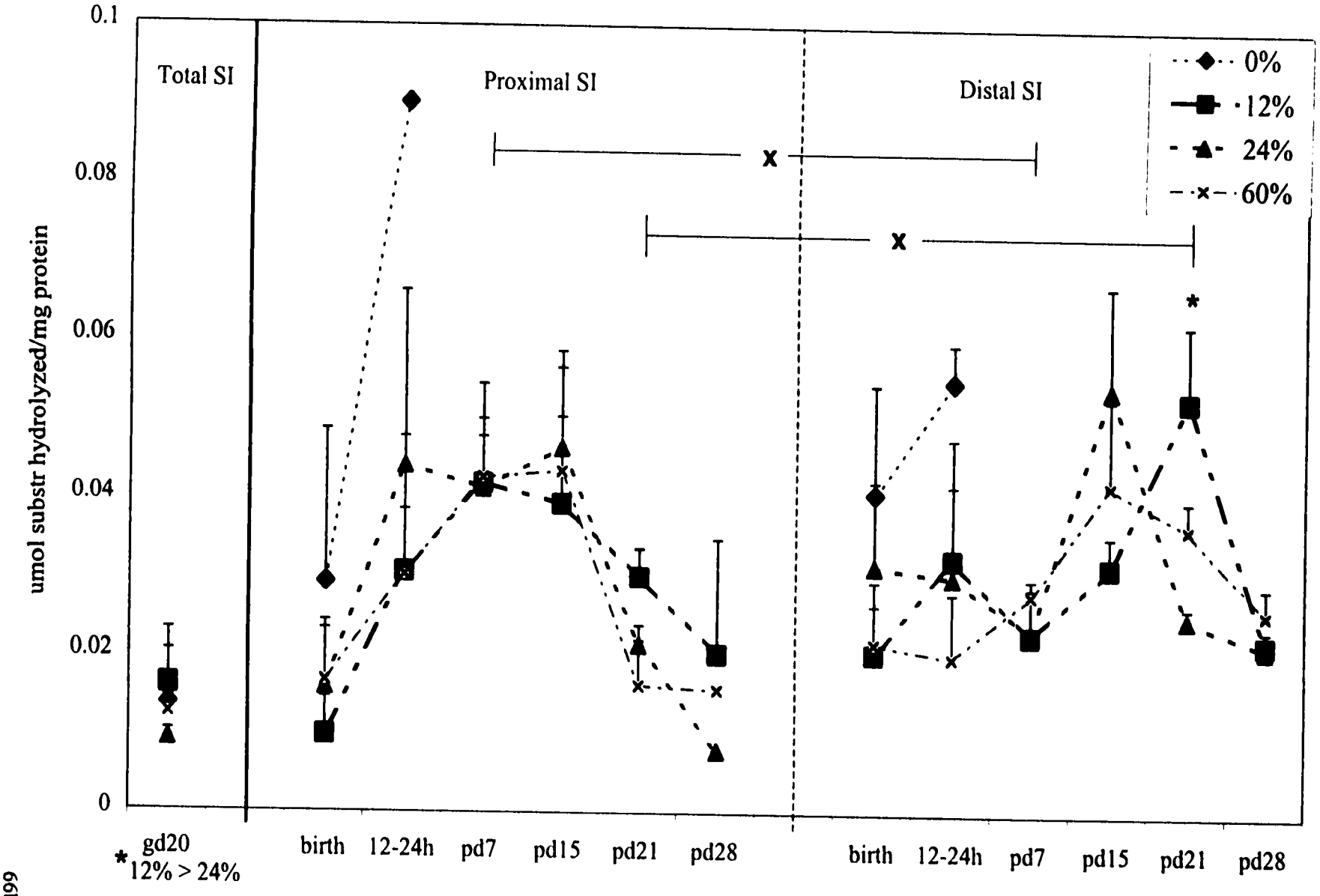


Table 30: Dietary Glucose Intake and Small Intestine Sucrase Specific Activity (umol/mg protein (x 10^{-2}))[1]

Time	Diet	Small Intestine Region		p-value[2]	p-value[3]	
		Proximal	Distal		proximal	distal
gd20	0%	1.37±0.68 (5)		ns		
	12%	1.62±0.70 (3)	(data for gd20 is for			
	24%	0.93±0.11 (4)	total small intestine)			
	60%	1.26±0.21 (3)				
birth	0%	2.93±1.95 (2)	4.09±1.39 (3)	ns	ns	ns
	12%	0.99±0.49 (5)	2.04±0.62 (5)			
	24%	1.60±0.73 (5)	3.17±1.07 (5)			
	60%	1.67±0.76 (4)	2.17±0.80 (4)			
12-24h	0%	9.03 (1)	5.52±0.46 (2)	ns	ns	ns
	12%	3.07±0.79 (4)	3.27±0.93 (4)			
	24%	4.42±2.20 (4)	3.03±1.76 (4)			
	60%	2.28±0.65 (4)	2.00±0.83 (4)			
pd7[ß]	12%	4.19±0.83 (3)	2.29±0.12 (3)	0.0148[ß] (proximal>distal)	ns	ns
	24%	4.13±0.67 (5)	2.35±0.56 (5)			
	60%	4.27±1.19 (4)	2.82±0.20 (3)			
pd15	12%	3.93±1.12 (3)	3.18±0.38 (3)	ns	ns	ns
	24%	4.65±1.02 (4)	5.44±1.28 (4)			
	60%	4.35±1.52 (3)	4.21±1.11 (3)			
pd21[^,ß]	12%	3.02±0.35 (3)	5.31±0.93 (3)[a]	0.0073[^]	ns	0.0385
	24%	2.15±0.25 (3)	2.52±0.14 (3)[b]	0.0024[ß] (proximal<distal)		
	60%	1.63±0.57 (3)	3.67±0.35 (3)[ab]			
pd28	12%	2.00±1.47 (2)	2.20±0.17 (3)	ns	ns	ns
	24%	0.81 (1)	2.14±0.11 (3)			
	60%	1.58±0.06 (2)	2.59±0.34 (3)			

[1]Values are mean±SEM. [2]P-value in two-way analysis of variance (ANOVA; diet and region). [3]Significance in one-way ANOVA within region (diet). [^]Significant main effect of diet. [ß]Significant main effect of region. [a,b]Significant differences between diet treatments indicated with different lowercase letters; multiple comparisons between diet groups by timepoint were done using Scheffé's test. Number of pooled litter samples presented in parentheses.

Figure20: Effects of Dietary Glucose on Sucrase Specific Activity



SUMMARY OF RESULTS

The following summarizes the results presented in **Chapter 5**. This section synthesizes information related to nutritional status and gut growth and development in terms of key developmental periods, rather than by individual timepoints. **Perinatal** encompass the early perinatal period: gd20, birth and 12-24h. **Suckling** timepoints include postnatal days 7, 15 and 21. The final developmental timepoints (pd28, 35, 49 and adult control) will be summarized under **post-weaning** timepoints.

1. Perinatal Timepoints

At perinatal timepoints animals were carbohydrate stressed. Maternal body weights were reduced with decreasing dietary glucose levels. The glucose deficient diet reduced plasma glucose levels and proved to be ketogenic, as evidenced by the large elevation in β-hydroxybutyrate, as would have been predicted. Plasma glucose and β-hydroxybutyrate were respectively decreased and increased in 0%-glucose deficient dams, as compared to 12% and 60% only for glucose; circulating β-hydroxybutyrate level was >15-fold higher in glucose deficient dams as compared to all other diet groups. The results of the plasma analysis indicated that the protein content of the experimental diets was adequate, even with glucose restriction or deficiency, to spare circulating albumin. No effect of diet was found on plasma albumin or urea nitrogen (**Tables 16 and 17**).

Plasma parameters were variably affected for pups at these early timepoints (**Tables 16-19**). Plasma albumin was not affected by diet at gd20, birth or 12-24h. Plasma glucose level differed only at gd20, while urea and β-hydroxybutyrate levels differed at all three perinatal timepoints. At gd20, glucose levels were lower and β-hydroxybutyrate levels were higher (~15-fold) in glucose deficient pups than for any other diet group; pup β-hydroxybutyrate levels followed the same pattern as did maternal β-hydroxybutyrate, also showing a 15-fold increase with glucose deficiency. Plasma urea levels were also significantly affected by diet at gd20, with only the 0% pups having higher urea levels than the 60% pups. At birth, urea nitrogen levels were significantly higher in 0% versus 12, 24 or 60% pups. β-hydroxybutyrate remained perturbed at birth ($p<0.003$), however the magnitude of the elevation of β-HBA in the 0% pups versus all

other diet groups was only 10-fold by this time. By 12-24 h, β-HBA remained significantly elevated in the 0% pups versus the average of the 12 and 60% pups (<3-fold higher); 24%-restricted pups did not differ from the others. At 12-24h, urea levels remained perturbed, however this was in a more stepwise manner; the 0%-glucose deficient pups still had significantly higher plasma urea than 24 and 60% pups, but the difference between 0 and 12% pups ceased to exist. Overall, pup protein status was not negatively affected by maternal dietary glucose consumption during pregnancy, as no differences were found in plasma albumin levels. Although the glucose deficient dams managed to maintain normal plasma urea levels at gd20, their pups did not at gd20 or 12-24h. This suggests that although protein status was not apparently compromised, that glucose deficient animals were experiencing an increase in protein mobilization.

Differences in small intestine growth were seen at early timepoints when only diet was considered in the analysis (**Tables 20, 21, 23-25**), but when pup weight was added as a covariate in the model, most of the differences disappeared. At gd 20, total gut weight was significantly lower in deficient pups than for all other groups. This corresponds with the differences seen in litter weight and pup weights at that time, and once pup weight was added to the analysis as a covariate, the difference in gut weight ceased to exist. Therefore the guts of these glucose deficient pups were normal in proportion to their body size. By birth, both gut length, distal gut weight and gut weight per unit length were significantly affected by diet, however these differences also ceased to exist when pup weight was included in the analysis. At 12-24 hours postpartum proximal gut weight was significantly affected by diet. When pup weight was considered in the model at 12-24h, small intestine length was found to be significantly shorter for the glucose deficient pups than for all other groups. Thus, although differences in body weight at this time were accounted for, the guts of these pups had failed to grow at the same rate as those pups whose dams were receiving some glucose in their diets. However total small intestine glycogen concentration was unaffected by the level of dietary glucose in the maternal diet for these timepoints.

Gut functional development was altered by level of maternal glucose intake during pregnancy. No effect of diet on glycogen deposition were found for these early

timepoints. At gd20 lactase specific activity (SA; **Table 29**) was significantly lower in pups of deficient dams than for pups of 12%-restricted dams. No other diet differences existed at this time. By birth, lactase SA for deficient pups exceeded that of 60%-glucose adequate pups, with no other differences present. Maternal dietary glucose intake during pregnancy significantly affected SGLT1 protein expression in utero; it was significantly lower in pups of dams fed the 0%-glucose deficient diet than for all other diet groups.

2. Suckling Timepoints

Cumulative food intake during suckling, from pd8-15 and pd16-21, which mostly represents intake by the nursing dams and not nibbling pups, was significantly higher for dams eating the 12% glucose restricted diet than for dams eating the control diet (**Table 11**). During this time interval, this increased food intake did not result in any differences in maternal weights, but at pd15 and 21 pup weight was increased in the 12% glucose group versus the 60% glucose group only. At these times, pups from the 12% dietary glucose group weighed 14 and 16% more than their 60% glucose counterparts (**Table 14**). Nutritional status indicators at these times were largely unaffected by diet (**Tables 16-19**). Albumin and urea were unaffected during suckling, indicating that our diets contained adequate protein content for lactation. At pd15, plasma glucose was significantly higher in 12% pups compared to the average of the 24 and 60% pups. β-hydroxybutyrate was unaltered by diet at pd7 and 15; this is significant as these were the only two timepoints in the entire study for which β-HBA levels were unaffected. Plasma β-hydroxybutyrate level was affected at pd21, with significantly higher levels in the 12% diet group pups than in the 24 or 60% (0.6-2.7-fold higher) dietary glucose pups.

At pd7, gut growth was unaffected by diet, with and without pup weight as a covariate in the analysis. By pd15, however, all measures of gut growth (length, total weight, proximal and distal SI weight, weight/length) were significantly increased for the 12%-glucose restricted animals as compared to the other diet groups. When pup weight was added to the statistical analysis as a covariate, total gut weight and distal SI weight continued to be significantly affected by diet. Total weight was significantly higher for both glucose restricted groups (12 and 24%) as compared to the control animals. Distal

small intestine weight was greater for only the 12% restricted group compared with the 60% glucose control group. These differences in gut weight at pd15 that remained significant when pup weight was included as a covariate suggest that some sort of compensatory small intestine growth is occurring in glucose restricted pups. At pd21, total gut weight was significantly increased with 12% glucose restriction as compared to 24% and 60% fed animals, however this difference ceased to exist when pup weight was included in the analysis.

The glycogen concentration of proximal and distal small intestine samples at both pd7 and 15 was unaffected by dietary glucose level, however glycogen content did differ by region for both timepoints; distal segments contained significantly more glycogen than did proximal segments. Distal segments contained from 39% on pd7 (24%) to 369% on pd15 (24%) more glycogen than did proximal segments. Derangements in SGLT1 expression, lactase and sucrase SA were also present during lactation. At pd15, diet and regional differences in SGLT1 expression were found; apparent upregulation of SGLT1 expression in both 12 and 24%-glucose restricted groups versus controls was found, and expression for distal segments exceeded that for proximal segments for all diet groups. By pd21, these regional differences were not detectable, and expression for 60%-glucose controls exceeded that of the 24%-glucose deficient pups; these groups did not differ significantly from the 12%-restricted group. At pd7 sucrase SA was higher in distal than proximal segments of small intestine. Regional differences in lactase SA were detected at pd15, where distal activity exceeded that of proximal SI. By pd21, this same regional difference was found for lactase as well as sucrase specific activity. Diet differences were also detected in lactase SA at pd21; activity was greater in 60%-control pups than in 24% restricted pups, with no difference between 12 and 60% or 12 and 24% pups.

3. Post-Weaning Timepoints

Food intake for the dam plus her two weaned pups present in each cage differed by diet for the week pd29-35, with 12% restricted glucose cages eating significantly more than the 60% glucose controls. In contrast, during suckling, maternal body weight was significantly affected by diet at pd28, 35, 42 and 49. At pd28 and 35 glucose restricted

dams weighed significantly less than control dams. By postnatal day 42, only the 12% restricted dams weighed significantly less than the 60% control dams. At pd49, a contrast was necessary to find the dietary difference; the average of the glucose restricted 12 and 24% dams weighed less than the 60% control dams. The only maternal plasma indicator affected by diet at pd49 was β-hydroxybutyrate, where 12% had significantly higher levels (0.83mmol/L) than the 24 and 60% dams (0.31 and 0.15mmol/L respectively).

Although food intake between pd22-28 did not differ by diet group, at pd28 pups from the 12% glucose restricted litters weighed 13% more than their 60% glucose adequate counterparts. No other differences in pup weight were found at these post-weaning timepoints. Plasma albumin, glucose and urea nitrogen levels were unaffected by diet post-weaning (**Tables 16-18**). In contrast, β-hydroxybutyrate levels were affected at all post-weaning timepoints. At pd28, the level of β-HBA was significantly higher in 12% pups than in either the 24 or 60% pups. By pd35 and 49, the 12% pups had higher levels than the 60% controls; no differences existed between the 24%-restricted group and the others. As for maternal plasma, the magnitude of these differences in plasma β-HBA levels was much smaller than for perinatal timepoints.

In adult control animals who had not received experimental diets during in utero development, gut length was found to be significantly longer in the 60%-glucose animals compared to the 24%-restricted animals. No other diet differences existed. No other significant differences in gut growth were found in post-weaned animals.

Gut functional development post-weaning was influenced by dietary glucose level. At pd28, glycogen level increased proximally-to-distally for both glucose restricted groups, however the level decreased for 60%-glucose adequate animals. Also at this first post-weaning timepoint lactase SA was significantly lower for 12%-restricted pups than for controls only. For the first time the expected adult pattern of SGLT1 expression was observed; proximal SGLT1 protein expression exceeded distal expression. At pd35 no regional differences were present, but a diet difference was found in glycogen concentration; 12%-restricted animals had less glycogen proximally and distally than both 24 and 60% pups.

In summary, during perinatal plasma indicators showed animals to be experiencing carbohydrate stress. The dietary model produced IUGR offspring, whose guts were proportionately shorter. Functionality was also impaired, with reduced levels of SGLT1 protein in 0%-deficient animals compared to all other groups, and the sharp rise in SGLT1 protein, lactase and sucrase specific activity between gd20 and 12-24h suggested precocious maturation when there was a nutritional stress. During lactation greater food intake in the 12%-restricted group compared to controls resulted in significantly heavier pups at pd15 and 21. As well, at peak lactation (pd15) gut growth was promoted in glucose restricted animals even when pup weight was included as a covariate suggesting a critical timepoint for catch-up growth with moderate increases in food intake even with a restricted diet. During lactation diet also significantly affected functional development; diet induced regulation of SGLT1 protein was observed for pd15 and 21 pups. Post-weaning, pup body weight continued to be greater for glucose-restricted animals at pd28, however after this timepoint no diet differences on pup weight were seen. We had expected to observe adult functionality characterized by proximal-distal gradients for SGLT1, lactase and sucrase and by the capability to adaptively regulate levels of SGLT1, lactase and sucrase to level of dietary carbohydrate by postnatal day 28. We found that only for SGLT1 was the gradient established, although there was a strong trend for lactase, and only SGLT1 exhibited diet induced regulation. There are critical timepoints that dietary glucose restriction had a significant impact on gut growth and development, however these effects were not permanent.

CHAPTER 6: DISCUSSION

DIET MODEL

This dietary model of carbohydrate restriction created animals who were carbohydrate stressed, thus allowing us to test the hypothesis that the absence of glucose, an essential nutrient, could lead to poor gut growth and development in intrauterine growth retarded offspring. In the perinatal period (gd20, birth, 12-24h), maternal as well as pup weights were reduced significantly with 0%-glucose deficiency ($p<0.001$) compared to those animals whose diets contained glucose; glucose deficiency during pregnancy created intrauterine growth retarded (IUGR) pups. This IUGR was the result of glucose deficiency and not due to the protein content of the diets. We used a dietary model in which glucose is made the most limiting nutrient. It was necessary to provide the most minimally adequate level of protein in the diets, so as not to provide extra gluconeogenic precursors in the glucose deficient diet. Thus the protein content of the diets, 11% casein plus 0.34% methionine, provided adequate protein and amino acids for pregnancy and growth. This level of protein was shown to be adequate to support normal pup growth (Koski *et al.*, 1986), and is considered adequate by the NRC (1995). The level was set at 11 g/100g dry weight, and thus provided 26.5 mg protein/kcal of diet. This level is within the range of 25-31 mg lactalbumin protein/kcal of diet which has been reported to be in accordance with the NRC guidelines (1995) to be adequate for maximum weight gain in young rats. Plasma indicators of nutritional status further supported the presence of the predicted diet induced carbohydrate stress in glucose deficient animals, but not protein deficiency. In the adult rat the half-life of albumin is 1.9-2.4 days (Reed et al., 1988). If the diets had been inadequate in protein, we would have expected to see differences in plasma albumin during the time course of this study; no differences in plasma albumin were found in either the dams or the pups at any timepoint with glucose restriction. However we know that carbohydrate deficiency and restriction stresses pups, as we observed increased urea levels. Furthermore, since β-hydroxybutyrate levels were elevated in the most glucose restricted group for almost every timepoint as compared to those animals fed higher levels of glucose, we concluded that this elevation in ketone

bodies showed that the more restricted animals were under carbohydrate stress (Cahill, 1988). Also urea levels were elevated in the 0%-deficient pups during the perinatal period only. The fact that only the glucose deficient animals showed elevations in ketones and urea supports this; had the problem been with the level of protein in the diet, we expected all groups would have been similarly affected.

During lactation and post-weaning glucose restriction did not have the same consequences as during the perinatal period where food intake and pup body weight were reduced with glucose deficiency. As litters were culled to an equal size at pd7, differences in milk intake among pups in a litter was likely negligible. Postnatally, food intake for the 12%-glucose restricted group exceeded that of the 24 and 60% groups from pd0 through 21; by postnatal day 15 the 12%-glucose restricted pups weighed significantly more that the 60%-glucose controls ($p<0.01$). This pattern of increased body growth with glucose restriction continued until postnatal day 28 ($p<0.02$ at pd21, and $p<0.05$ at pd28). Using the same diet model, Lanoue and Koski (1994) found that although milk fat level was reduced in both 12 and 24% glucose restricted dams by pd15, body growth was compromised in only the 12% group. This indicated that, although both milks were less energy dense, only in the 12% diet group was it likely that milk yield insufficient to support normal pup growth. In that study however maternal food intake was reduced in the 12% group; ours was increased. These 1994 results are internally consistent (12% animals: reduced food intake → reduced milk fat/yield → reduced pup growth), as are the results of the present study (12% animals: increased food intake → increased pup growth and gut growth), but suggested, due to adaptation of rats that food intake could markedly change the outcome of pup growth. At lower glucose intake, in other words, if the dams increased their food intake to compensate then compromised growth could be avoided. Therefore to minimize differences in pup weight, a confounder, it was included as a covariate in our statistical analyses for gut growth parameters. This allowed the direct measurement of the effects of carbohydrate deficiency on all gut growth parameters.

Plasma β-hydroxybutyrate was not elevated in glucose restricted pups at pd7 or 15, and combined with increased body growth by pd15, this suggests that during

lactation, glucose restriction did not have the capacity to compromise pup physiological state or body growth. From postnatal day 21 forward, β-hydroxybutyrate was once again elevated in the 12%-restricted group, even with pups weighing more at pd21 and 28. Thus by weaning, 12%-glucose in the diet was insufficient to prevent ketosis.

Possible limitations in the design of the study that may have affected results or limited the interpretation of results are that litters were not culled until postnatal day 7. Early differences in litter size may have resulted in increased competition for milk; differences in milk intake during this important period of early postnatal life could have affected gut growth and development. In the present study no measures of milk yield or composition were made. Previously it was found that the feeding of 20 and 40% glucose diets did not affect milk yield (A. Matsuno, M.Sc. Thesis; 1996), however 12% glucose restricted diets did result in reduced milk yield (Lanoue and Koski, 1994).

In summary, the diet model created intra-uterine growth retarded offspring. Both deficient dams and their pups were experiencing a carbohydrate stress, and not a deficiency in protein, as indicated by elevations in plasma urea and β-hydroxybutyrate, a reduction in plasma glucose level and no reduction in circulating albumin. Gut growth was compromised with glucose restriction in the perinatal period, however during lactation both pup weight and gut weight showed catch-up growth with increases in maternal food intake. These effects did not last through post-weaning. SGLT1 protein expression, lactase, glycogen and sucrase were all variably affected by level of dietary glucose throughout development, however no permanent retardation was observed in gut function. Continued consumption of a diet restricted in glucose during postnatal development resulted in periodic, but no permanent effects on gut growth and development.

GUT GROWTH AND DEVELOPMENT

Postnatal small intestine growth and development is characterized by the acquisition of a mature adult profile of rapidly turning over enterocytes, capable of the digestion and absorption of a mixed solid diet (Buddington, 1992). The adult small intestine possesses the ability to adaptively regulate levels of nutrient transporters to the

level of dietary substrate, and exhibits characteristic proximal-distal gradients for brush border disaccharidases and sodium-dependent glucose co-transporter (SGLT1) protein. To our knowledge a complete developmental profile for proximal and distal small intestine glycogen concentration, lactase and sucrase specific activities and SGLT1 protein expression has not been compiled for young animals under conditions of varying levels of dietary carbohydrate.

Pre- and postnatal malnutrition have been shown to compromise small intestinal growth and result in functional derangement of brush border associated functions (Lebenthal, 1989; Patlak *et al.*, 1981; Guiraldes and Hamilton, 1981; Butzner *et al*, 1990; Karasov *et al.*, 1985; Shanklin and Cooke, 1993). Further to this, the importance of amniotic fluid swallowing to gut growth has been demonstrated for sheep (Trahair *et al.* 1986 and 1997) and rabbits (Jacobs *et al.*, 1989; Mulvihill *et al.*, 1985, 1986). For the current study, it was postulated that the dietary model of IUGR which produces low levels of amniotic fluid glucose (Koski and Fergusson, 1992) would therefore result in reduced small intestine growth. We found that although small intestine length, total proximal and distal weights were compromised in the perinatal period (gd20, birth and 12-24h), once differences in pup body weight were accounted for the only significant difference remaining was in small intestine length at 12-24h where 0%-deficient pups had significantly shorter guts than all other diet groups ($p<0.02$).

In contrast to the literature reviewed and to our hypothesis, postnatal small intestine growth was enhanced with glucose restriction, as shown by significantly heavier distal and total SI weights for pups at pd15 above those differences observed for body weight. In our study, food intake during lactation in 12%-glucose restricted dams was significantly greater than that for 60% controls; there were no other diet differences. This suggested an unexpected compensation in food intake for the imposed dietary glucose restriction. Catch-up gut growth was the result. Distal gut weight was heavier in 12% pups than 60% pups ($p<0.04$; no other diet differences), and total SI weight for both restricted groups exceeded that for the control animals ($p<0.02$). We further suggest that the increased food intake and the heavier guts at pd15 promoted pup body growth as evidenced by significantly heavier pups at pd15, 21 and 28 in the 12%-glucose restricted

group as compared to the 60%-glucose adequate controls ($p<0.01$, $p<0.02$ and $p<0.05$ respectively). These results suggest that the early dietary insult produced no lasting negative effects on gut growth for 12%-glucose restricted animals; compensatory food intake allowed 12%-restricted animals to undergo catch-up growth.

In a short-term feeding study in adult mice, the absence of dietary carbohydrate had no effects on SI length or weight compared to controls (Diamond and Karasov, 1984), however the same investigators found that feeding the diet lacking in carbohydrate during postnatal development irreversibly reduced gut and body weight (Karasov *et al.*, 1985). In agreement with the results of Shanklin and Cooke (1993) who found that IUGR human infants had significantly shorter small intestines, we found that feeding 0%-glucose deficient diets throughout pregnancy resulted in IUGR pups with shorter guts, whereas 12%-glucose in the diet was sufficient to result in compensatory gut growth during lactation. No permanent effects of glucose restriction on body weight or gut growth were found in our study.

The potential impact of other diet constituents on gut growth and development were also considered for the present study. Levels of dietary fat and cellulose were necessarily modified along with level of dietary carbohydrate (**Table 4**). Source, but not level, of dietary fiber has been identified as a potential modifier of gut growth. Although dietary fibre supplementation, especially with pectin and/or guar gum, has been shown to increase colonic growth (Stark *et al.*, 1995), to cause ultrastructural alterations in the small intestine (Tamura *et al.*, 1997; Brunsgaard *et al.*, 1995), and potentially alter brush border digestive and absorptive processes (Muir *et al.*, 1996), the impact of cellulose feeding on gastrointestinal growth appears limited to its ability to modify colonic weight (National Academy of Sciences, 1995). For this reason, the impact of the varying levels of cellulose across diet groups in the present study was not considered causative of any differences in small intestine growth. Studies examining the impact of dietary fat on small intestine growth have focused on fatty acid composition rather than on the level of dietary fat. Fatty acid composition of the diet is readily reflected in apical membrane fatty acid composition (Thomson *et al.*, 1989; Jarocka-Cyrta *et al.* 1998), and has been shown to alter weight gain (Jarocka-Cyrta *et al.*, 1998) and galactose absorption (Lindley

et al., 1995). Fat composition in the present study was constant across diet treatments, and thus was not expected to exert any effect of gut structural or functional characteristics measured.

There is a paucity of studies in the literature examining the relationship between small intestine glycogen deposition and growth, and no studies were found examining the impact of diet on SI glycogen. It has been shown that glycogen deposits present in the perinatal SI are largely metabolized within the period immediately following birth (Lev and Weisberg, 1969; Hartmann and Gossrau, 1978); there was an indication of the formation of a proximal-distal gradient for glycogen by the decline in ileal glycogen levels (Hartmann and Gossrau, 1978). Others failed to describe any proximal-distal gradient for glycogen in adult rats (Várkonyi *et al.*, 1979). Previous work with this diet model had found high levels of perinatal mortality (Koski and Hill, 1990) in the glucose deficient animals, which may be linked to levels of fetal liver, heart (Lanoue *et al.*, 1992) and brain (Koski *et al.*, 1993) glycogen reserves were significantly reduced with the feeding of glucose restricted diets. We had therefore hypothesized that small intestine glycogen reserves would be likewise compromised with glucose restriction, and that the expected reduction in SI growth and glycogen reserves were in part responsible for the perinatal mortality of the pups. In contrast to these previous results in other tissues from our lab, we found that SI glycogen reserves in the perinatal period were not vulnerable to this dietary deprivation of glucose during pregnancy. The presence of adequate SI glycogen reserves did not prevent pup death, therefore its energetic contribution may be minimal or infact may be overshadowed by metabolic events occurring in other organs with this diet model. Only at pd35 were any diet induced differences detected; glycogen reserves were significantly lower in 12%-restricted animals compared to both 24 and 60% fed animals. Furthermore, we found that the proximal SI contained significantly less glycogen than the distal at pd7 and 15 ($p<0.0001$ for both timepoints). Regional differences in glycogen content of the SI were not found for any other timepoint. Thus even in more mature intestines, the proximal-distal gradient found for other gut functional measures was not established for glycogen. These results coincide with the findings of Várkonyi *et al.* (1979).

In this study, we have shown for the first time that the expression of SGLT1 protein *in utero* can be affected by maternal dietary glucose intake. We found that the relative expression of SGLT1 in the small intestine of gd20 pups of dams fed a 0%-glucose deficient diet throughout pregnancy was significantly lower ($p=0.0166$) than in 12 or 24%-glucose restricted or 60%-glucose adequate pups. It is known that luminal nutrients are required for the maintenance of SGLT1 protein in the adult human (Dyer *et al.*, 1997) and ovine (Shirazi-Beechey *et al.*, 1991) small intestine, and that supplementation of amniotic fluid with glucose or galactose increased uptake of these monosaccharides (Buchmiller *et al.*, 1992). We therefore postulated based upon reduced amniotic fluid glucose with this diet model (Koski and Fergusson, 1992) that prenatal diet could alter glucose transporter protein expression and suggest that significant reductions ($p<0.0001$) in amniotic fluid glucose in our deficient animals and thus reduced exposure of the prenatal small intestine to luminal glucose could be responsible for the reduced SGLT1 expression in 0%-glucose animals.

It has been suggested in the literature that regulation of SGLT1 protein by diet is not possible until postweaning (Buddington, 1994 review, Lynn Searles M.Sc. Thesis, 1995). The onset of diet induced regulation of SGLT1 protein during development has not been determined. David *et al.* (1995) concluded that postnatal diet could not induce glucose transport until after pd60. Butzner *et al.* (1990) found enhanced glucose uptake (SGLT1 protein was not measured) in protein-energy malnourished animals in the postnatal period. In contrast to the research that dictates that pre-weaning diet cannot modulate SGLT1 expression, we found significant effects of maternal diet on SGLT1 protein expression at pd 15 and 21. At pd15, in agreement with Butzner *et al.* (1990), 12%-glucose restriction resulted in significantly upregulated SGLT1 expression above 60%-controls in distal SI, however by pd21 SGLT1 expression was higher in the proximal SI of 60% controls than in the 24% restricted group. Given the 3-7day enterocyte life-span for suckling rats (Lebenthal, 1989), perhaps pups of 60%-glucose adequate dams had begun nibbling the 60%-glucose diet before the restricted animals started nibbling and this resulted in the apparent upregulation of SGLT1 protein at pd21. No qualitative examination of gut contents is available to determine if there were indeed

differences in the onset of nibbling between diet groups. A further consideration is the possibility of alterations of milk composition with glucose restriction resulting in these differences in SGLT1 expression, however previous work with this diet model did not find any differences in milk lactose content with the feeding of glucose restricted diets (Lanoue and Koski, 1994).

A proximal-distal gradient for SGLT1 should be established as the small intestine achieves its characteristic adult profile (Buddington, 1994); on pd7 and 15 we saw the opposite, with distal SGLT1 expression exceeding proximal, however by pd28 the adult proximal-distal gradient for SGLT1 protein was established ($p<0.0002$), and no diet induced differences were noted. Thus although SGLT1 protein was responsive to dietary glucose in the perinatal period and during lactation, by pd28 there was no detectable response of the small intestine to level of dietary glucose.

To date no developmental profile for SGLT1 protein expression has been found in the literature. This study presents for the first time a complete developmental profile from late gestation through weaning for the rat. The internal control used for all Western Blot analyses was prepared from the distal SI of adult animals fed the 60% glucose diet. It is notable from Figure 18 that in the distal SI there was a pattern across all diet groups of greater SGLT1 expression throughout the entire suckling period above that of adult distal SI (relative area under the curve > 1). We also found that the distal SI showed dramatic growth during this period. This suggests that perhaps SGLT1 expression above that of adult levels facilitated this growth.

It is well recognized in the literature that exogenous administration of hormones, especially the glucocorticoids, thyroxine or triiodothyronine, and acetylcholine are potent inducers of GI maturation (Henning, 1987; Lebenthal, 1989; Menard and Calvert, 1991). In fact serum corticosterone levels rise at the beginning of the third postnatal week, accompanying the abrupt increase in sucrase and decrease in lactase activity seen in this period of gastrointestinal maturation in rats (Henning, 1987). A serious limitation to the study of the importance of dietary composition (i.e. milk composition during weaning) has been the precocious maturation of lactase and sucrase activities due to the stress of premature weaning (Lebenthal, 1989; Henning, 1987). In this study, significantly higher

lactase activity and a pattern of higher sucrase activity for both proximal and distal SI in the guts of our 0%-glucose deficient animals at gd20 and birth was found. Also notable was a sharp rise in lactase and sucrase activity as well as for SGLT1 expression proximally and distally between birth and 12-24h after birth in these glucose deficient animals. It is therefore theorized that this macronutrient deficiency, specifically glucose deficiency, resulted in a pattern of precocious maturation of gut function similar to that noted with exogenous hormone administration. These results contradict those expected for protein and/or energy malnutrition (Patlak *et al.*, 1981; Guiraldes and Hamilton, 1981) who found that malnourished animals had significantly lower lactase and sucrase activities than control animals.

Lactase specific activity for proximal and distal segments of small intestine were reported in the range of 0.010-0.025 UE/mg protein during peak lactation, and decreased to less than 0.010 UE/mg protein around weaning, with the formation of the proximal-distal gradient at pd21 (Castillo *et al.*, 1992). In the present study, lactase activity during peak lactation was typically 0.04-0.06 UE/mg protein, with higher activities distally than proximally at pd15. As expected lactase levels appear to rise between the end of gestation and 12-24h after birth in the proximal SI, plateau until pd21 and then begin a steady decline (Henning, 1987) as the animals were exposed to a milk-based diet and then were weaned to a solid diet (Buddington, 1992). The proximal-distal gradient for lactase was expected to develop during the third postnatal week (Henning, 1987) and should be formed by decreases in ileal lactase with respect to jejunal lactase (Castillo *et al.*, 1992) Interestingly, although a regional difference was found at pd21 (proximal < distal), the characteristic adult proximal-distal gradient was not found at pd28. There was a drop in distal lactase specific activity between pd21 and pd28 and a trend toward lower levels in distal SI. Although this did not achieve significance, there was an impact of dietary glucose intake on lactase levels. At pd21 it was found that 12 and 24%-glucose restriction resulted in lower lactase SA than in controls in the proximal SI. At pd28, significantly lower lactase SA for the whole SI was found in 12%-glucose restricted animals than in controls ($p<0.01$ and $p<0.02$ respectively). While precocious weaning has been shown to result in early declines in lactase activity, delayed weaning can delay the

maturational decline of lactase activity (Henning *et al.*, 1994). As pups in the present study were not separated from their dams at weaning, continued suckling by the pups may have affected the expected maturational decline in lactase levels.

The literature has shown that sucrase is not generally detectable until postnatal day 16 for rats, when it is then expected to rise dramatically as the animal prepares to wean (Henning, 1987). Indeed proximal levels were seen to increase dramatically to approximately 0.1 UE/mg protein in normally weaned animals by pd21, with distal levels remaining significantly lower (Castillo *et al.*, 1992). In this study it was found that although sucrase levels were generally lower than those for lactase, it was easily detectable at all timepoints, and reached a plateau in the proximal SI of 0.04 UE/mg protein by 12-24h post partum. By pd7 we were able to detect a gradient in sucrase activity, with activity greater for proximal than for distal SI ($p<0.02$). Distally, levels appeared to peak for the 24% and 60%-glucose fed pups at pd15, with an apparent delay in this peak to pd21 for the 12%-restricted animals. By pd21, however the sucrase regional gradient was reversed (i.e. distal activity exceeded proximal). Ferraris *et al.* (1992a) showed that for adult mice, feeding a high sucrose diet resulted in a significant increase in proximal sucrase activity. Surprisingly, as our diets were glucose based and thus contained no sucrose, we found that sucrase activity was significantly higher with 12%-glucose restriction than for all other groups at pd21. These findings suggest that there may be a non-specific effect of dietary dextrose on luminal sucrase activity. Interestingly, the characteristic adult proximal-distal gradient was not detected at pd28 in our study.

In summary, these results show that dietary deficiency of glucose during pregnancy resulted in animals who were growth retarded and had smaller guts, and that SGLT1 protein expression *in utero* was significantly reduced as compared to animals whose maternal diet had contained some glucose. Total mortality in these glucose-deficient pups before postnatal day 7 may be the result of compromised gut growth and development. Compensatory food intake during lactation allowed 12%-restricted animals to undergo catch-up body and gut growth. Sharp increases in proximal and distal SGLT1 expression, lactase and sucrase activity in 0%-glucose deficient animals between gd20

and 12-24h post partum suggested a precocious induction of gut functionality due to a macronutrient deficient diet. During lactation, regional gradients for glycogen, SGLT1, lactase and sucrase were established; for all except sucrase at pd7, distal levels exceeded proximal levels. By pd15 dietary regulation of SGLT1 protein was observed; by pd21 diet induced differences were present for SGLT1, lactase and sucrase. Interestingly, only for SGLT1 expression was the expected adult proximal-distal gradient established by pd28, although there was a strong trend towards this gradient for lactase. Continued consumption of a diet restricted in glucose during postnatal development resulted in periodic, but no permanent effects on gut growth and development.

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