
# AN INVESTIGATION INTO THE EFFECT OF GLUCOSAMINE ON REPRODUCTIVE OUTCOMES IN THE MOUSE

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# Contents

## Abstract

It is well established that conditions experienced in utero by the developing fetus can elicit permanent effects on the post natal period. Although not as well understood, a growing body of research also suggests that this can also occur in response to peri-conceptional insult. Glucosamine (GlcN) is a popular dietary supplement that is also used experimentally as a hyperglycaemic mimetic. The work contained in this thesis tests the hypothesis that pre and peri-conceptional exposure to GlcN has adverse effects on reproductive outcomes in the mouse.

Preliminary experiments (Chapter 2) confirmed that the inclusion of GlcN into the in vitro maturation (IVM) media used for mouse cumulus oocyte complex (COC) maturation, reduced oocyte developmental potential. Subsequent experiments (Chapter 3) demonstrated that the inhibition of O-linked glycosylation of unknown proteins reversed the effects of GlcN. It was also shown that GlcN exposure during IVM altered Pentose phosphate pathway (PPP) activity within the oocyte.

As predicted, preliminary in vivo experiments performed in Chapter 4 showed that maternal, peri-conceptional GlcN administration compromised fetal development. This was seen by a decreased mean implantation rate and litter size as well as an increase in the proportion of fetal resorptions on gestational day 18 (d18), and provided the impetus to examine the in vivo effects of GlcN exposure more carefully.

It was subsequently hypothesized that these adverse effects would be heightened if given to mice with overweight-induced metabolic pathologies. In contrast to Chapter 4 outcomes, GlcN elicited no effects on d18 implantation, resorption or litter size parameters, but did reduce fetal weight. Furthermore, birth defects were higher in mice given GlcN and maintained on a low fat (LF) diet. An additional cohort of mice was allowed to give birth, and offspring were assessed for 16 weeks. There was an unexpectedly high death rate in the offspring of mice maintained on a high fat (HF) diet but not given GlcN, therefore preventing optimally controlled post natal analyses to occur. Of the remaining mice, a number of physiological differences were detected within GlcN-exposed groups.

Since the principle difference between mice in Chapters 4 and 5 was maternal age, an addition experiment investigating the effects of peri-conceptional GlcN exposure in 8 week and 16 week old mice was undertaken (Chapter 6). Consistent with previous results, GlcN treatment reduced mean implantation rate and litter size only in 8 week old mice and reduced fetal weigh and length solely in 16 week old mice. Increased birth defects were also detected in the HF group given GlcN.

Collectively these results provide important insights into the importance of optimal conditions during the peri-conceptional period to facilitate successful subsequent development. They also provide evidence that GlcN is a simple but effective tool that can be used to further elucidate the impact of hyperglycaemic exposure during the early developmental period. This is of key importance given the escalating instances of diabetes and obesity in current day Western society, and the associated complications that these conditions elicit on reproductive parameters.

## Declaration

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- Schelbach, C. J., Robker, R. L., Bennett, B. D., Gauld, A. D., Thompson, J. G. & Kind, K. L. (2012). "Altered pregnancy outcomes in mice following treatment with the hyperglycaemic mimetic, glucosamine, during the periconception period." Reproduction, Fertility & Development *(in press).*

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## Publications arising from this thesis

### Papers

#### Paper arising from work presented in chapters 2 and 3

Schelbach, C.J., Kind, K.L., Lane, M. and Thompson, J.G. (2009) "Mechanisms contributing to the reduced developmental competence of glucosamine exposed mouse oocytes." Reproduction, Fertility and Development**.** **22**(5): 771–779.

#### Paper arising from work presented in chapters 4 - 6

Schelbach, C. J., Robker, R. L., Bennett, B. D., Gauld, A. D., Thompson, J. G. & Kind, K. L. (2012). "Altered pregnancy outcomes in mice following treatment with the hyperglycaemic mimetic, glucosamine, during the periconception period." Reproduction, Fertility & Development *(in press).*

### Media Releases

Schelbach, C. J. and Thompson, J. G. (2007) Interview with ABC radio "The effects of glucosamine on fertility".

Schelbach, C. J., Thompson, J. G. and Stankawicz, M. (2006) Interview with Channel 10 news "The impact of glucosamine on fertility".

## Conference Presentations

Schelbach, C.J., Kind, K. L. & Thompson, J. G. Perturbations in fetal development follow short-term, in vivo glucosamine administration in mice. Proceedings from the 41st Annual Meeting of the Society for the Study of Reproduction; 2008 May 27 -30; Kailua- Kona, Hawaii,

Schelbach, C, J., Kind, K.L., Lane M & Thompson, J. G.*The Perturbation in Fetal Outcomes that follows in vivo Glucosamine Exposure is Heightened by Obesity.* Proceedings from 1st The University of Adelaide 2007 Post Graduate Research Expo; 2007 ; Adelaide, South Australia.Adelaide, The University of Adelaide.

Schelbach, C, J., Kind, K.L., Lane M & Thompson, J. Mechanisms involved in the reduced embryo development rate that follows glucosamine exposure to eggs. Proceedings from the 2007 ASMR scientific meeting; 2007 June 6 Adelaide, South Australia

.Schelbach, C, J., Kind, K.L., Lane M & Thompson, J. G. *Improved development of murine embryos derived from COCs matured with the O-linked Glycosylation inhibitor BADGP.* Proceedings from the 2006 ESA/SRB scientific meeting; 2006 August 20 - 23, Gold Coast, Queensland.

Schelbach, C, J., Kind, K.L., Lane M & Thompson, J. G*. Abberant Murine Embryonic development following Glucosamine exposure during IVM or embryo Culture..* Proceedings from the 2005 ESA/SRB scientific meeting; 2005, August 4 - 7; Perth, Western Australia.

Schelbach, C, J., Kind, K.L., Lane M & Thompson, J. G*. Abberant Murine Embryonic development following Glucosamine exposure during IVM or embryo Culture.* Proceedings from the 2005 ASMR scientific meeting; 2005 June 2 ; Adelaide, South Australia.

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*I can no other answer make, but, thanks, and thanks.*

*William Shakespeare*

## Abbreviations

Abdo - abdominal

Akt - protein kinase B

ANOVA - analysis of variance

ATP - adenosine triphosphate

BADGP - benzyl-2-acetoamido-2-deoxy-α-D-galactopyranoside

BCB - brilliant cressyl blue

BMI – body mass index

BSA - bovine serum albumin

CAM - complementary and alternative medicine

cAMP - cyclic adenosine monophosphate

CC - cumulus complex

CDCFDA - 5,6-carboxyl-2',7'-dichlorodihydrofluorescein diacetate

CL - corporo lutea

COC - cumulus oocyte complex

D14.5 – day 14.5

D18 - day 18

DCDHFDA - 2',7'-dichlorodihydrofluorescein diacetate

eCG - equine chorionic gonadotrophin

EtOH - ethanol

FBS - fetal bovine serum

FFA – free fatty acids

FSH - follicle stimulating hormone

G6PDH - glucose-6-phosphate dehydrogenase

G-6-phosphate - glucose-6-phosphate

GFPT - glutamine fructose-6-phosphate transaminase

GlcN - glucosamine

GlcN-6-P - glucosamine-6-phosphate

GLUT - glucose transporter

GRP 78 - glucose-regulated protein 78

GSH - reduced glutathione

GSSG - oxidized glutathione

$H_2O_2$ - hydrogen peroxide

HAS - hyaluronic acid synthase

HBP - hexosamine biosynthetic pathway

hCG - human corionic gonadotrophin

HF - high fat

HF+GlcN - high fat with glucosamine

HF-GlcN - high fat without glucosamine

HSA - human serum albumin

IGF-1 - insulin like growth factor 1

IP - intraperitoneal

IRS - insulin receptor substrate

IVF - in vitro fertilisation

IVM - in vitro maturation

JNK - c-Jun N-terminal kinase

LF - low fat

LF+GlcN - low fat with glucosamine

LF-GlcN - low fat without glucosamine

MCT proteins - $H^+$-monocarboxylate cotransporter proteins

mRNA - messenger ribonucleic acid

NADH - nicotinamide adenosine dinucleotide

NADPH - nicotinamide adenine dinucleotide phosphate

NADPH - nicotinamide adenine dinucleotide phosphate

NEFA - non-esterified fatty acids

O-GlcNAcase - β-N-acetylglucosaminidase

OGT - β-linked-O-GlcNAc transferase

PA – palmitic acid

PAI - plasminogen activator inhibitor

PBS - phosphate buffered solution

PEP - phosphoenolpyruvate

PI3-kinase - phosphatidylinositol 3-kinase

PPP - Pentose Phosphate Pathway

PRPP - 5-Phosphoribosyl-1-Pyrophosphate

RDI - recommended daily intake

Retro - retroperitoneal

RIA - radioimmunoassay

ROS - reactive oxygen species

SAS - statistical analysis system

SEM - standard error of the mean

SPSS - statistical package for the social sciences

T2DM - Type 2 Diabetes Mellitus

TQEH - the Queen Elizabeth Hospital

UDP-N-GlcNAc - N-acetylglucosaminyl-1-phosphotransferase

# 1.0 Chapter 1

## GlcN-induced alterations in glucose metabolic pathway activity are detrimental for reproductive outcomes

## 1.1 Summary

The link between hyperglycemia and adverse reproductive outcomes has been well documented. However, relatively little is known about the impact of hyperglycemia during the peri-conceptional phase of pregnancy. Similarly, there is a pronounced absence of information available regarding the effects of many complementary and alternative medicines (CAMs) on reproduction. Glucosamine (GlcN) is a popular dietary supplement commonly taken to alleviate the symptoms associated with joint tissue degeneration. In addition, it is used experimentally as a hyperglycemic mimetic. This review will focus on the to-date recognised impact of peri-conceptional hyperglycemic exposure on reproductive performance and developmental competence, as well as the role that GlcN may have in inducing such effects.

## 1.2 Hyperglycemia and pregnancy

Prior to the introduction of insulin therapy early in the 20th century, instances of diabetic pregnancies were few and far between, with the first reported pregnancy being described by Beddewitz in 1824 (in: Skipper, 1933). Over time there has been a progressive reduction in the rates of complications and abnormalities associated with diabetic pregnancies (Small et al. 1986; McElvy et al. 2000; Johnstone et al. 2006; Bell et al. 2008). Regardless, many studies show that despite a general reduction in adverse outcomes, complication rates are still greater than in normoglycemic pregnancies (Dunne et al. 2003; Penney et al. 2003; Jensen et al. 2004; Macintosh et al. 2006). This is a significant concern, given that the prevalence of diabetes in many populations is escalating *(*King et al. 1998), thereby increasing the number of women at risk of experiencing a diabetes-related pregnancy complication (Bell et al. 2008).

While early research papers primarily focussed on the immediately observable effects of diabetic pregnancy on the mother, fetus and newborn (Walker, 1928; Bigby and Jones, 1945; White, 1948), there is now a growing focus on the long term effects for the offspring of exposure to hyperglycemia in utero. These long term effects include, diabetes (Dabelea et al. 2000(a); Dabelea et al. 2000(b); Clausen et al. 2008; Dabelea et al. 2008), impaired glucose tolerance (Pettitt et al. 1993; Silverman et al. 1995; Silverman et al. 1998; Cho et al. 2000; Malcolm et al. 2006), obesity (Pettitt et al. 1983; Pettitt et al. 1987; Rizzo et al. 1997; Silverman et al. 1991; Pettitt et al. 1993; Rizzo et al. 1997; Silverman et al. 1998; Vohr et al. 1999; Cho et al. 2000; Kostolova et al. 2001; Gillman et al. 2003; Malcolm et al. 2006; Hillier et al. 2007), poorer intellectual performance (Silverman et al. 1991; Silverman et al. 1998), reduced psychomotor development (Silverman et al. 1998), elevated blood pressure (Cho et al. 2000; Tam et al. 2008) and renal disease (Nelson et al. 1998).

There has also been some focus on the long term consequences of a diabetic pregnancy on maternal outcomes. There is a growing body of evidence linking gestational diabetes with the later onset of maternal Type 2 Diabetes Mellitus (T2DM) (as reviewed by Ratner, 2007). According to an analysis performed by Cheung and Byth (2003), approximately one third of parous women with diabetes have had a gestational diabetic pregnancy. Hence it is apparent that gestational diabetes has long term detrimental consequences on the health of both the offspring and mother.

Another stream of research is examining the importance of the degree of glucose elevation on outcomes. Maternal and offspring outcomes have been shown to be poorer in women that have blood glucose levels that are not high enough to be classified as gestationally diabetic,

but are in the upper spectrum of the normoglycemic range (Jensen et al. 2001; Ju et al. 2008). This is consistent with the notion that criteria for the diagnosis of diabetes may fail to capture all pregnancies at risk of suffering from hyperglycemic complications (Magee et al. 1993; Deerochanaowong et al. 1996; Bonomo et al. 1998; Bevier et al. 1999; Ferrara et al 2002; Gokcel et al. 2002; Hedderson et al. 2003; Ertunc et al. 2004; Ferrara et al. 2007).

In addition, the effects of the timing of hyperglycemic exposure require consideration. It has been found that outcomes are worse in pregnancies defined by pre-gestational diabetes than in those in which diabetes develops during the gestational period (Casson et al. 1997; Nelson et al. 1998; Shand et al. 2008). This is likely to be attributed to, at least in part, aberrant glycemic activity during the pre and early pregnancy period (Miller et al. 1981; Hanson et al. 1990; Steel et al. 1990; Rosenn et al. 1991; Kitzmiller et al. 1991; Hawthorne et al. 1997; Nielsen et al. 1997; McElvy et al. 2000; Temple et al. 2002). In fact, a study conducted by Suhonen et al. (2000) found that even slight elevations in blood glucose levels at the beginning of pregnancy in women with Type 1 diabetes also increases adverse pregnancy outcomes. As the body of research surrounding hyperglycemia during pregnancy and in particular the pre- and early pregnancy period broadens, it is becoming increasingly apparent that glucose-stimulated modifications that occur throughout development can lead to long term modifications in both the mother and her progeny.

## 1.3 The effect of diabetes on oocyte and pre-implantation embryo development

Growing evidence suggests that exposure of the developing oocyte and pre-implantation embryo to an altered environment can have consequences during fetal, neonatal and adult life. It has become increasingly apparent that diabetes alters the environment of the oocyte, and elicits an adverse impact on oocyte development. Relative to the oocyte, there is less information regarding the impact of diabetes on embryonic development. Outlined in table 1 are the experimentally determined, to-date, recognised effects of exposing oocytes and/or pre-implantation embryos to hyperglycaemic conditions.

| Structure | Effect |
|---|---|
| **Oocyte** | **Inhibited ovulation** (Kirchick et al. 1978; Colton et al. 2002), **Reduced ovulation** (Cheiri et al. 1969; Jawerbaum et al. 1996*)*, **Impaired oocyte maturation** (Diamond et al. 1989; Colton et al. 2002; Colton et al. 2003; Chang et al. 2005*;* Ratchford et al. 2007***),* Increased follicular atresia** (Chang et al. 2005) **Reduced follicular size** (Chang et al. 2005), **Reduced oocyte size** (Chang et al. 2005), **Reduced communication between the oocyte and cumulus cells** (Colton et al. 2003; Ratchford et al. 2008*),* **Reduced Connexin-43 *(cumulus cells)*** (Chang et al. 2005), **Reduced Cx26** (Ratchford et al. 2008), **Increased apoptosis** (Chang et al. 2005), **Abnormal metabolic activity** (Colton et al. 2003; Ratchford et al. 2007; Wang et al. 2009) **Meiotic defects** (Wang et al. 2009). |
| **Embryo** | **Impaired development** (Moley et al. 1991; Colton et al. 2002), **Abnormal metabolic activity** (Moley et al. 1996), **Reduced intracellular glucose concentrations in 2-cell embryos and blastocysts** (Moley et al. 1998(b)), **Increased apoptosis** (Moley et al. 1998(a); Keim et al. 2001)**, Increased fetal congenital malformations** (Wyman et al. 2008), **Fetal growth retardation** (Wyman et al. 2008*)* |

**1.3.1 Table 1:** The to-date recognised effects of exposing oocytes or pre-implantation embryos to diabetic conditions

Of important note is the novel work performed by Wyman et al. (2008). Here, the authors transferred both zygotes and blastocysts from streptozotocin- induced diabetic mice to normoglycemic mice. Rates of growth retardation and developmental defects, including neural tube, abdominal wall and limb defects, were increased in fetuses developed from oocytes or embryos from diabetic mice. In vitro exposure from the 2-cell through to blastocyst stage to hyperglycaemia, followed by embryo transfer into normoglycemic recipients also increased fetal growth retardation. Although there was no accompanying increase in instances of abdominal wall and limb defects following in vitro exposure of the pre-implantation embryo to hyperglycaemic conditions, the implantation rate of the embryos was reduced, and the rates of embryonic resorption increased. Taken together, these results provide evidence that transient exposure during the peri-conceptional period to hyperglycemic stimuli has long- term developmental consequences.

## 1.4 Distribution of GLUT transporters

Also of importance from the findings of Wyman et al. (2008), was the observation that developmental defects were different when exposure had occurred under in vivo vs in vitro conditions. While it is possible that these differences may be reflective of differences between in vivo vs in vitro exposure, they may in part be attributed to by development-associated changes in the expression of glucose transporters (GLUTs), membrane bound proteins that passively transport glucose molecules. As GLUT expression varies throughout oocyte and various stages of pre-implantation embryo development, this may contribute to the differences observed in outcomes following exposure to elevated glucose. A list of GLUTs in association with the reproductive cells or tissues that they have been identified in is provided in table 2, and their corresponding biological functions have been outlined in table 3.

| Structure | Transporter | Species |
|---|---|---|
| Ovary | GLUT 1 | **Rat** (Kol et al. 1997), **Human** (Rudlowski et al. 2004) |
| | GLUT 3 | **Rat** (Kol et al. 1997; Ballester et al. 2007), **Human** (Rudlowski et al. 2004) |
| Follicle | GLUT 1 | **Bovine** (Nishimoto et al. 2006), **Ovine** (Williams et al. 2001), **Rat** ***(granulosa cells)*** (Kodaman & Behrman, 1999), **Mouse** (Zhou et al., 2000) |
| | GLUT 3 | **Bovine** (Nishimoto et al. 2006) |
| | GLUT 4 | **Bovine** (Nishimoto et al. 2006), **Ovine** (Williams et al. 2001) |
| Oocyte | GLUT 1 | **Human** (Dan-Goor et al. 1997), **Mouse** (Morita et al. 1992; Aghayan et al. 1992; Zhou et al. 2000), **Ovine** (Leoni et al. 2007) **Bovine** (Schultz et al. 1992; Lequarre et al. 1997; Augustin et al. 2001; Brevini *et* al. 2002; Lonergan et al. 2003) |
| | GLUT 3 | **Bovine** (Augustin et al. 2001) |
| | GLUT 4 | **Bovine** (Augustin et al. 2001; Lonergan et al. 2003) |
| | SGL 1 | **Bovine** (Augustin et al. 2001) |
| Cumulus cells | GLUT 1 | **Mouse** (Zhou et al. 2000) |
| | GLUT 4 | **Human** (Robker et al. 2009) |
| Embryo | GLUT 1 | **Bovine** ***(1 cell)*** (Lequarre et al 1997), **Bovine** ***(morula)*** (Niemann & Wrenzycki et al. 2000; de Oliveira et al. 2005), **Bovine** ***(blastocyst)*** (Wrenzycki et al 1998; Lazzari et al. 2002; Rief et al. 2002; Harvey et al. 2004; de Oliveira et al. 2005; Pedersen et al. 2005; Oliveira et al. 2006; Rho et al. 2007; Rizos et al. 2007; Sagirkaya et al. 2007; Palasz et al. 2008), **Human** ***(2 cell)*** (Dan-Goor et al. 1997); **Mouse** ***(2 cell)*** (Hogan et al. 1991; Aghayan et al. 1992; Moley et al. 1998(b)); **Mouse** ***(8 cell)*** (Hogan et al. 1991), **Mouse** ***(morula)*** (Hogan et al. 1991; Pantaleon et al. 1997; Moley et al. 1998(b)); **Mouse** ***(blastocyst)*** (Hogan et al. 1991; Smith and Gridley 1992; Pantaleon et al. 1997; Moley et al. 1998(b); Carayannopoulos et al. 2000; Kind et al. 2005; Rieger et al. 2007), **Rat** ***(blastocyst)*** (Takao et al. 1993); **Rabbit** ***(blastocyst)*** (Navarrete Santos et al. 2004) |
| | GLUT 2 | **Mouse** ***(8 cell)*** (Schultz et al. 1992; Hogan et al. 1991) **Mouse** ***(morula)*** (Hogan et al. 1991; Moley et al. 1998(b)); **Mouse** ***(blastocyst)*** (Hogan et al. 1991; Aghayan et al. 1992, Moley et al. 1998(b); Leppens-Luisier et al. 2001; Kind et al. 2005) |
| | GLUT 3 | **Mouse** ***(morula)*** (Pantaleon et al. 1997; Moley et al. 1998(b)); **Bovine** ***(blastocyst*****)** (Lazzari et al. 2002; Morton et al. 2007), **Mouse** ***(blastocyst)*** (Smith and Gridley 1992; Pantaleon et al. 1997; Moley et al. 1998(b)), **Rat** ***(blastocyst)*** (Takao et al. 1993); **Rabbit** ***(blastocyst)*** (Navarrete Santos et al. 2004) |
| | GLUT 4 | **Bovine** ***(blastocysts)*** (Lazzari et al. 2002); **Rabbit** ***(morula)*** (Navarrete Santos et al. 2004); **Rabbit** ***(blastocyst)*** (Navarrete Santos et al. 2004) |
| | GLUT 5 | **Bovine** ***(blastocyst)*** (Palasz et al. 2008) |
| | GLUT 8 | **Mouse** ***(blastocysts)*** (Carayannopoulos et al. 2000); **Rabbit** ***(blastocyst)*** **(**Navarrete Santos et al. 2004) |

**1.4.1 Table 2:** Distribution of glucose transporters in select reproductive structures

| Type | Family | Transporter | Function |
|---|---|---|---|
| Facilitative glucose transport | Class 1 | Glut 1 | Passively transports glucose (Mueckler et al, 1985), mannose and galactose (Gould et al. 1991) |
| | | Glut 2 | Passively transports glucose (Fukumoto et al. 1988), mannose, galactose and fructose (Gould et al. 1991). Involved in glucose sensing (Marty et al. 2005). |
| | | Glut 3 | Passively transports glucose (Kayano et al. 1988), mannose and galactose (Gould et al. 1991). |
| | | Glut 4 | Passively transports glucose in response to insulin signalling (James et al. 1988). |
| | Class 2 | Glut 5 | Passively transports fructose (Burant et al. 1992) |
| | Class 3 | Glut 8 | Passively transports glucose (Doege et al. 2000) |
| Sodium coupled glucose transport | SGLT (Sodium dependant glucose transporter) | SGL1 | Uses active transport to co-transport glucose and sodium (Wu & Lever, 1987). |

**1.4.2 Table 3** Biological functions of the glucose transporters that have been identified in reproductive structures.

## 1.5 Metabolism of glucose in COCs and pre-implantation embryos

Biggers et al. (1967) initially established that the oocyte does not metabolize significant amounts of glucose. Their work demonstrated that while denuded mouse oocytes do not undergo spontaneous maturation when cultured in media containing glucose, maturation can be initiated by the incorporation of pyruvate or oxaloactetate into media with maturing, denuded oocytes or alternatively by the incorporation of glucose only with oocytes with intact cumulus vestments. This provided early evidence for the role of cumulus cells as the principal consumers of glucose, which in turn is metabolized into usable substrates for the oocyte, and this has been subsequently confirmed (Donahue & Stern, 1968; Leese and Barton, 1985).

Similarly, the early embryo has a minimal yet essential requirement for glucose, with the zygote relying heavily on pyruvate for its metabolic needs. It was found that oxaloactetate also supported development to the 2-cell stage, albeit to a lesser extent then pyruvate (Biggers et al. 1967). Early work conducted on the energy requirements of 2-cell mouse embryos also established that the incorporation of glucose into simple medium was inadequate for supporting subsequent embryo development. Hammond (1949) showed that the incorporation of glucose in simple medium was inadequate for supporting development from the 2 cell stage, but does promote blastocyst development when exposure occurred to the 8 cell embryo.

Subsequent research showed that development from the 2 cell stage proceeded when pyruvate, lactate DL, oxaloacetate or phosphoenolpyruvate (PEP) were included in the media (Brinster, 1965). Furthermore, it confirmed by measuring radiolabelled $CO_2$ from isotopic-labelled glucose, that very little glucose was metabolised by the early embryo, with progressively more being utilised from the 8-cell stage in the mouse. By the time the embryo reached the late blastocyst stage of development, however, glucose was being utilised as an energy source at approximately equivalent rates as pyruvate and lactate. Although markedly higher than glucose, lactate production was initially lower and markedly increased from the morula stage. The predominant energy source of the early embryo was found to be pyruvate (Brinster, 1967(a); Brinster, 1967(b)).

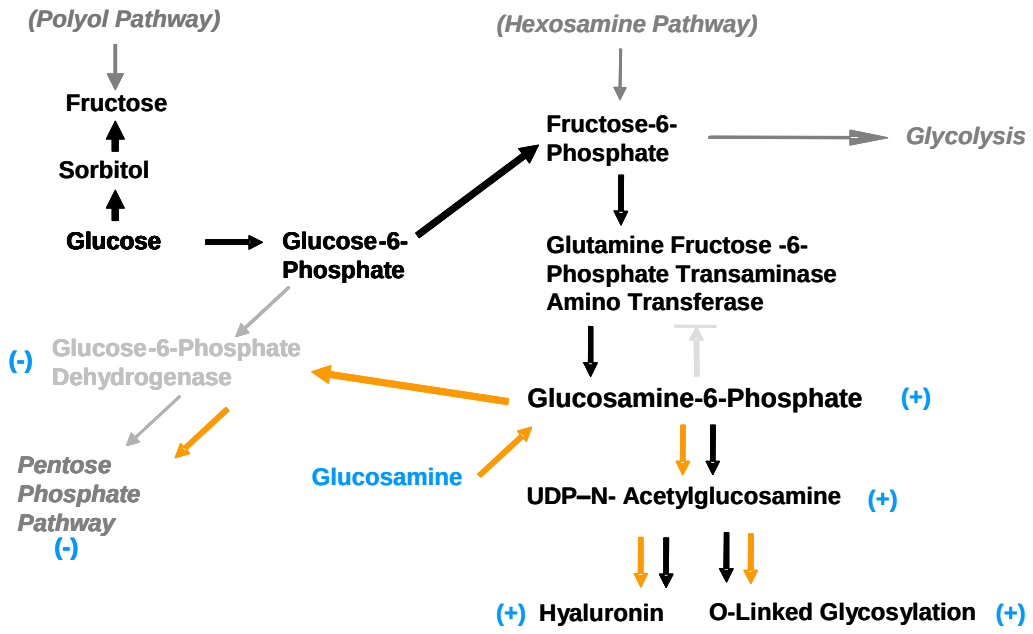
Although a negligible amount of glucose is metabolised by the early embryo, glucose exposure is none-the-less an integral part of embryo culture to ensure subsequent development. This has been demonstrated by Brown and Whittingham (1991) who found that whilst mouse embryos deprived of glucose but given lactate and pyruvate could develop into morula, blastocyst development only occurred when glucose was included in the culture medium in either the first, second or third 24-hour period of culture. Chatot et al. (1994) then demonstrated that even a one minute exposure of glucose, was sufficient to reverse the observed pattern of failed blastocyst development. In agreement, Pantaleon et al. (2010) demonstrated that whilst mouse zygotes cultured in the absence of glucose failed to reach the blastocyst stage, this phenomenon could be reversed by either continual or a 1 - 3 hour pulse of glucose exposure via its incorporation into embryo culture media from the zygote stage. Furthermore, Jansen et al. (2006) showed that embryos deprived of glucose did not express mRNA of $H^+$-monocarboxylate cotransporter (MCT) proteins, which are involved in the facilitated diffusion of pyruvate/lactate. An inversely proportional relationship between MCT protein immunoreactivity and embryo development to the morula stage was observed. Such perturbed effects were not observed in embryos that had received short term exposure

to glucose. These are important findings as they provide evidence for a role of glucose as a signalling molecule rather than a substrate during early embryo development.

Hence it is apparent that glucose is an integral factor for cumulus-oocyte complex (COC) and embryo development. Furthermore, it is also evident that the needs of COCs and embryos for glucose are static and stage dependent, given that the expression of GLUT transporters and the metabolic requirements for glucose varies throughout oocyte and embryo development.

## 1.6 Glucose Metabolic Pathways in COCs and Pre-implantation Embryos

Glucose can be metabolised by several different metabolic pathways. These pathways are depicted in Figure 1 and are outlined below.




**1.6.1 Figure 1:** Glucose metabolic pathways affected by GlcN. The introduction of GlcN leads to an increase in GlcN-6-Phosphate. In turn, this induces an increase in the production of Hexosamine metabolic pathway intermediates, as well as a reduction in PPP product formation.

### 1.6.2 Glycolysis

In glycolytic reactions, the conversion of glucose to pyruvate yields adenosine triphosphate (ATP) and reduced nicotinamide adenosine dinucleotide (NADH), energy molecules that are both integral for a variety of metabolic processes. Glycolysis is the dominant glucose metabolic pathway found in COCs. Downs and Utecht (1999) demonstrated that most glucose is metabolised via glycolysis in equine chorionic gonadotrophin (eCG) -primed mouse COCs. By assessing the production of the glycolytic end product L-lactate, it has also been shown that most glucose is metabolised by glycolysis in bovine COCs undergoing in vitro maturation (IVM) (Sutton et al. 2003; Sutton-McDowall et al. 2006). However, it has also been demonstrated that during the final stages of bovine IVM, an increasing amount of glucose is utilized by an alternative pathway(s) (Sutton et al. 2003). These authors suggested that this is likely the Hexosamine pathway (discussed below), for the generation of hyaluronin for the expanding cumulus matrix.

In contrast, glycolytic activity in the early embryo is very low. It has been repetitively shown that hexokinase, the enzyme which converts glucose to glucose-6-phosphate at the start of the glycolytic pathway, initially has low levels of activity during early embryonic development but that this increases substantially from the morula stage (Ayabe et al. 1994; Saito et al. 1994; Moley et al. 1998(b)). This is not surprising, given that glucose is not a significantly metabolised substrate during early embryo development (Hammond et al. 1949; Brinster, 1967(a); Brinster, 1967(b)).

### 1.6.3 The Polyol Pathway

The Polyol (or Sorbitol) pathway becomes activated when glucose is converted to sorbitol via aldose reductase, which in turn is converted to fructose by sorbitol reductase. Fructose can be transported to the glycolytic pathway via the conversion of fructose to fructose-6-phosphate by the enzyme hexokinase. However, under hyperglycemic conditions Polyol pathway activity surpasses fructose conversion and the resultant imbalance ultimately leads to reductions in REDOX reactions, the importance of which is discussed in the Pentose Phosphate Pathway (PPP) section below. Although little attention has been paid to the effects of hyperglycemia on the Sorbitol pathway in reproductive cells and tissues, a series of experiments performed by Colton and Downs (2004) provides important information regarding the Sorbitol pathway in oocytes of diabetic mice. Briefly, it was shown that activation of the Sorbitol pathway resulted in reduced de novo purine synthesis as well as

cell-cell communication between the oocyte and the cumulus cells, and compromised FSH-mediated cyclic adenosine monophosphate (cAMP) production. Furthermore, it was also shown that the suppression of FSH-induced meiotic maturation that occurs in oocytes of diabetic mice could be inhibited by the introduction of inhibitors of aldose reductase (Colton and Downs, 2004), an intermediate in the Polyol pathway that is generated in the presence of excess glucose and which converts glucose to sorbitol (Gabbay, 1973; Oates, 2002). This led the authors to conclude that the reduced rates of FSH-stimulated meiotic maturation that characterises diabetes- affected oocytes may be contributed to by elevated levels of glucose fluxing through the Sorbitol pathway.

The effect of hyperglycemia on the Sorbitol pathway in embryos has also been examined. Moley et al. (1996) exposed one-cell mouse zygotes to one of several glucose concentrations for 96 hours, and intra-embryonic Sorbitol levels were subsequently determined at the completion of culture. It was found that embryos cultured under hyperglycemic conditions (52 mM glucose) had significantly greater levels of Sorbitol than their control counterparts (matured with 2.8 mM glucose). The authors also demonstrated that there were glucose concentration-dependent, developmental stage differences in the embryos. The embryos exposed to 52 mM glucose, and that had the highest levels of Sorbitol, also arrested at a very early (2-cell) stage of development. Collectively, these studies demonstrate that embryonic development can be perturbed by hyperglycemia via stimulation of the Sorbitol pathway.

### 1.6.4 The Hexosamine Pathway

The flux of glucose through the Hexosamine pathway is non-reversible, owing to the fact that the intermediate Glutamine Fructose-6-Phosphate Transaminase (GFPT) can only metabolise substrate in a unidirectional way. The Hexosamine pathway is thought to be an energy sensing pathway through which approximately 1 - 3% of glucose typically fluxes (Marshall et al. 2005(b)). However, Gutnisky et al. (2007) demonstrated that the incorporation of an inhibitor of hyaluronic acid synthase (HAS) (which inhibits the production of hyaluronin) into bovine oocyte in vitro maturation (IVM) media results in approximately 23% less utilization of glucose by COCs. This finding provided further support for the claim by Sutton-McDowall et al. (2004) that during the final stages of COC maturation a significant proportion of glucose is used for the production of hyaluronin, a principal component of the expanding cumulus cell matrix (Eppig 1979; Ball et al. 1982; Talbot 1984; Suchanek et al.

1994). Therefore it appears that the metabolism of glucose through the Hexosamine pathway seems to be significantly higher in maturing COCs than in the majority of somatic tissues.

The end-product of the Hexosamine metabolic pathway is UDP-*N*-acetylglucosamine, which is increased by high glucose stimulation (Sayeski and Kudlow, 1996; Kolm-Litty et al. 1998; Du et al. 2000; Han et al. 2000; Goldberg et al. 2006). In turn, UDP-*N*-acetylglucosamine serves as a substrate for several physiological functions including the synthesis of matrix compounds (e.g. hyaluronic acid), glycoproteins (Chen and Lennarz, 1977; Waetcher and Harford, 1977), glycolipids (Waetcher and Harford, 1977) as well as the N-linked and O-linked glycosylation of proteins (Torres and Hart, 1984).

In 1984 Torres and Hart described O-linked glycosylation as a post-translational form of protein modification that occurs on serine and threonine residues of nucleocytoplasmic proteins. The number of proteins known to be O-link glycosylated is constantly expanding, and to date is known to include transcription factors, metabolic enzymes as well as signalling and cytoskeletal components (as reviewed by Wells and Hart, 2003). Furthermore, O-link glycosylated proteins are known to be involved in a number of pathologies including various cancers (Hull and Carraway, 1988; Nawrocki et al. 1988; Boland and Deshmukh, 1990), Alzheimer's disease (Refolo et al. 1989; Yao and Coleman, 1998) and hyperglycaemic conditions (Nishio et al. 1995; Patti et al. 1999; Akimoto et al. 2001; Kaneto et al. 2001; Vosseller et al. 2001).

Recently, it has become apparent that O-linked glycosylation of proteins is also important for embryo development (Pantaleon et al. 2008, 2010). Azaserine, an inhibitor of GFPT, was added to the culture media of mouse zygotes. At the completion of embryo culture a reduction in blastocyst production as well as a reduction in the expression of GLUT 3 in the blastocyst was observed, indicating that retarded Hexosamine activity is detrimental for developing mouse embryos (Pantaleon et al. 2008). Subsequently Pantaleon et al. (2010) showed that this was, at least in part, mediated by suppression of O-linked glycosylation. When mouse zygotes were cultured with an inhibitor of the O-linked glycosylation enzyme regulators OGT (β-linked-O-GlcNAc transferase) and O-GlcNAcase (β-N-acetylglucosaminidase), a significant reduction in blastocyst development was observed. In an alternative study, these authors also went on to show that excess flux through the Hexosamine pathway in embryos, induced via the incorporation of excess glucose into the culture media, also led to reduced blastocyst development and cell number (Pantaleon et al. 2010). Thus, these studies suggest that successful development of the preimplantation embryo is dependent upon an optimal level of activity of the hexosamine pathway**.**

### 1.6.5 The Pentose Phosphate Pathway

The PPP is an important glucose metabolic pathway for several reasons. It is important for the REDOX balance of NADPH, a metabolic intermediate that is required for the reduction of oxidised glutathione (GSSG) to its reduced form GSH, a reductant for reactive oxygen species (ROS). In addition, the end products of PPP metabolism – purines and nucleic acids – play important roles in the acquisition of oocyte meiotic maturation (Downs et al. 1989; Downs, 1997; Downs et al. 1998; Downs and Utecht, 1999; Downs, 2000; Colton et al. 2003). Previously it has been shown that the first rate limiting enzyme of the PPP, glucose-6-phosphate dehydrogenase (G6PDH), is inhibited by the Hexosamine intermediate GlcN-6-Phosphate (Bessell and Thomas, 1973; Kanji et al. 1976), thereby implicating hyperglycemic stimulation as a perturbing influence on PPP activity.

In 2003 Colton et al. demonstrated that PPP activity was reduced in oocytes from diabetic mice. This followed on from earlier work which demonstrated that the presence of elevated levels of glucose in media during in vitro maturation of bovine COCs, reduced GSH levels, increased ROS and reduced oocyte developmental competence (Hashimoto et al. 2000). In the porcine model, denuded oocytes exposed to ROS undergo higher instances of meiotic arrest, degeneration and apoptosis (Tatemoto et al. 2000). In human ovarian follicles, elevated production of ROS within granulosa cells is associated with a reduced number of oocytes present in follicular aspirate (Jancar et al. 2007). Furthermore, both the in vivo and in vitro introduction of antioxidants, which counter the effects of ROS, has been shown to improve reproductive outcomes. Chromosomal abnormalities that present in the mouse oocyte following exposure to oxidative stress can be reduced in vitro by the incorporation of antioxidants into IVM media, and in vivo by daily consumption of antioxidants from weaning (Tarin et al. 1998).

Similarly, embryos can be negatively affected by oxidative stress and ROS. In vitro produced bovine and mouse embryos that arrest have higher intracellular ROS levels, compared to normally developed embryos (Nasr-Esfahani et al. 1990; Noda et al. 1991; Goto et al. 1993; Favetta et al. 2007). In addition, oxidative stress induced by heat shock in bovine embryos following IVF, increases levels of ROS and decreases rates of blastocyst development *(*Sakatani et al. 2004; Sakatani et al. 2008). In agreement, the culture of mouse 2-cell embryos in hydrogen peroxide ($H_2O_2$) containing media has also been linked to reduced blastocyst development along with increased rates of perturbed morphological development (Cebral et al. 2007). Alternatively, reduced embryo development (at the 2-cell and blastocyst stages) and elevations in intraembryonic concentrations of $H_2O_2$ can be

generated by the incorporation of elevated glucose concentrations on day one in porcine embryo culture media (Karja et al. 2006). Furthermore, elevated ROS levels have been implicated in embryonic apoptosis, with increased $H_2O_2$ concentrations found in apoptotic, fragmented human embryos *(*Yang et al. 1998). Thus, it is apparent that sub optimal patterns of PPP activity can contribute to poor developmental outcomes in embryos as well as oocytes.

## 1.7 GlcN as a hyperglycemic mimetic

GlcN is a simple amino sugar that is used therapeutically for functionally compromised joint tissues (as reviewed by Reginster et al. 2007) as well as in experimental systems as a hyperglycemic mimetic (Rossetti et al. 1995; Hawkins et al. 1997; Horal et al. 2004; Yki-Jarvinen et al. 1998; Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Rumburger et al. 2003; Marshall et al. 2004). Under experimental conditions, the role of GlcN as a hyperglycemic mimetic is well acknowledged. However, despite the vast amount of literature, reports regarding its therapeutic efficacy are quite varied (Reginster et al. 2007; Sawizitzke et al. 2008). Significantly, however, there is very little data available regarding the effects of GlcN on reproduction from either experimental models or retrospective studies. This is despite the fact that this GlcN has been shown to directly modify glucose metabolic activity.

### 1.7.1 The effects of GlcN on Hexosamine activity

GlcN is converted to GlcN-6-phosphate (Marshall et al. 2005(a)), an intermediate of the Hexosamine metabolic pathway. Flux through this GFPT is non-reversible. Hence, the introduction of GlcN leads to an increase solely in the substrate level and activity of the Hexosamine pathway (Rossetti et al. 1995; Hawkins et al. 1997; Yki-Jarvinen et al. 1998; Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Rumburger et al. 2003; Marshall et al. 2004).

Of particular importance are the findings of Sutton-McDowall et al. (2006). In this study, the authors demonstrated that bovine and porcine blastocyst development was reduced when COCs were matured in vitro with GlcN prior to fertilisation. Kimura et al. (2008) subsequently

confirmed that the incorporation of GlcN into bovine in vitro maturation (IVM) media reduced developmental competence. Sutton-McDowall et al. (2006) also demonstrated that the effects of GlcN exposure during IVM on embryo development could be reduced by the addition of an inhibitor of O-linked glycosylation, BADGP (benzyl-2-acetoamido-2-deoxy-α-D-galactopyranoside) to GlcN-containing IVM medium. Essentially, these results demonstrate that GlcN induces an elevation in O-linked glycosylation which has adverse effects on the maturing COC.

GlcN also has a significant influence on embryo development. While exposure of the bovine embryo to GlcN from the one-cell to 8-cell stage elicited no measurable developmental effects, inclusion of GlcN in the culture media from the 8-cell to blastocyst stage reduced blastocyst development. Of the surviving blastocysts, an increased proportion of male embryos were observed. Addition of BADGP into GlcN- containing culture media from the 8-cell to blastocyst stage corrected the skewed sex ratio in the bovine embryos, implicating GlcN-stimulated elevations in O-linked glycosylation in abnormal embryo development (Kimura et al. 2008). Using the mouse model, Pantaleon et al. (2008) showed that both continuous as well as short term incorporation of GlcN into glucose-free embryo culture media, corrected the blocked embryo development that was observed when embryos were glucose-deprived. This is an important finding, as it demonstrates that a complete lack of Hexosamine pathway activity during embryo development has a perturbing impact. These authors also demonstrated that elevations in Hexosamine activity achieved by the addition of GlcN into mouse embryo culture media reduced blastocyst development and cell number (Pantaleon et al. 2010). While it appears that there are species-specific differences in the developmental stages at which embryos are affected by GlcN, it is apparent that mouse and bovine embryos experience developmental perturbations if sufficiently exposed in vitro to GlcN.

### 1.7.2 The effects of GlcN on PPP activity

As previously stated, the first rate limiting enzyme of the PPP, glucose-6-phosphate dehydrogenase (G6PDH), is inhibited by GlcN-6-phosphate (Bessell and Thomas, 1973; Kanji et al. 1976). This implicates the introduction of GlcN as leading to an increase in levels of GlcN-6-phosphate (a HBP intermediate), which in turn, reduces G6PDH, and ultimately inhibits PPP activity. However, there is no data available on the effects of GlcN on PPP activity in reproductive cells and tissues. Given that exposure of embryos to ROS has been linked to perturbed outcomes (Nasr-Esfahani et al. 1990; Noda et al. 1991; Goto et al. 1993;

Sakatani et al. 2004; Karja et al. 2006; Cebral et al. 2007; Favetta et al. 2007; Sakatani et al. 2008; Yang et al. 2008), effects on the PPP is a potential pathway through which the effects of GlcN on embryo development may be mediated.

### 1.7.3 The effects of GlcN on glucose transport

According to work performed by Uldry et al. (2002), GlcN can be transported into cells by GLUT1 and 4 (which have a higher affinity for glucose) as well as GLUT 2 (which has a higher affinity for GlcN). It is possible that GlcN could, therefore, enter cells that contain GLUT 1, 2 or 4. As can be seen from the distribution of GLUTs outlined in table 2, this includes the COC and pre-implantation embryo.

GlcN has been shown to have an inhibitory effect on GLUT 4 translocation (Robinson et al. 1993; Chen et al. 1997; Filippis et al. 1997; Nelson et al. 2000; Kralik et al. 2002). Since GLUT 4 has been detected in blastocysts (bovine) (Lazzari et al. 2002), it is possible that GlcN could affect COC and post compaction embryo development by interfering with glucose transport. This also applies to ovine (Williams et al. 2001) and bovine (Nishimoto et al. 2006) ovarian follicles exposed to GlcN. In contrast to GLUT 4, GLUT 1 translocation has not been shown to be affected by GlcN (Robinson et al. 1993; Filippis et al. 1997).

## 1.8 GlcN entry into the cell

A small number of studies have confirmed that GlcN label does enter ovarian and follicular tissue as well as the COC, and have demonstrated that this uptake is influenced by the accompanying hormonal environment. When [$^3$H] glucosamine is administered to mice in combination with equine chorionic gonadotrophin (eCG), GlcN label is localised to the follicular fluid of both large (Fowler and Guttridge 1987; Fowler, 1988) and small antral follicles and the zona pellucida of oocytes within these follicles (Fowler and Guttridge 1987). However, when mice are treated with labelled GlcN, eCG and hCG, GlcN labelling can additionally be observed in the thecal layers of the ovary, the oocyte of pre- ovulatory follicles (Fowler and Guttridge 1987; Fowler 1988) pre-antral and pre-ovulatory follicles, interstitial layers of the ovary, (Fowler and Guttridge 1987), granulosa cells and cumulus cells (Fowler et al. 1988). This suggests that that uptake of GlcN may be increased in mature cumulus cells, when compared to earlier maturational time points. Importantly, however, Fowler and Guttridge (1987) have demonstrated that labelling can also be detected in the zona pellucida

and follicular fluid of large antral follicles of immature, non-stimulated mice. This demonstrates that follicles can take up GlcN label regardless of the presence or absence of hormonal stimulation, suggesting that the COC can be affected by GlcN at multiple points of development.

In a follow up to previous work, Fowler and Barratt (1989) injected [$^3$H] Glucosamine into female mice at the time of mating and confirmed that GlcN label could be detected within the ovary and perivitelline space of the oocyte. However, radio-labelled GlcN was also detected in zygotes and to a limited extent in 2-cell embryos, despite the presence of negligible quantities of labelled GlcN in the oviducts. It was concluded that label was being passed from cumulus cells into the oocyte.

In agreement with the findings from mice, porcine oocytes flushed from eCG and hCG treated pre-pubertal donors and incubated in vitro in [$^3$H] glucosamine have also stained positive for GlcN label. More specifically, cumulus cell enclosed oocytes demonstrate label in the ooplasm, perivitelline space, corona cells and zona pellucida projections, whilst denuded oocytes display cytoplasmic and perivitelline space labelling *(*Fléchon et al. 2003). Collectively, the work from Fowler and Barratt, (1989) and Fléchon et al. (2003) respectively suggests that GlcN may elicit its perturbing effects on oocyte developmental competence directly or by entering the oocyte via cumulus cells.

## 1.9 Effects of GlcN on blood glucose and insulin levels

There is a large body of literature surrounding the effects of GlcN on glucose and insulin metabolism. The addition of GlcN into a variety of cell culture media has been shown to elicit a broad spectrum of effects, including altering a host of functions relating to glucose and insulin activities (refer to Table 4). Similarly, GlcN infusion has been shown to elicit perturbing effects on glucose and insulin metabolic profiles. Most of these studies have been performed in murine models and are summarised in Table 5. However, a study in humans also demonstrated that a 5 hour infusion of 5 µM GlcN resulted in a significant reduction in the responsiveness of insulin to glucose (Monouni et al. 2000). Whilst these infusion-based studies are useful for demonstrating the metabolic effects of GlcN and Hexosamine pathway up regulation, the supraphysiological levels of GlcN involved renders the relevance of these findings to physiological conditions as questionable.

Despite the large volume of work demonstrating an effect of GlcN on metabolic parameters, a range of conflicting data exists. For example, Rossetti et al. (1995) demonstrated that infusion of GlcN induced insulin resistance in normoglycemic, but not chronically hyperglycemic rats. In contrast, Ciaraldi et al. (1999) reported that the effects of GlcN on responsiveness and sensitivity of skeletal muscle cells to insulin were equivalent, regardless of whether the cells were derived from normoglycemic or T2DM (Type 2 Diabetes Mellitus) sources. It is possible that these differences are reflective of model based differences (i.e. GlcN administered via in vivo infusion or during cell culture) or different severities of hyperglycemic status.

In addition, many studies have failed to show any effect of glucosamine on metabolic parameters in both animal and human models. Nine weeks of oral administration of GlcN at a dose estimated to be approximately 3 - 5 that of the human RDI (1500 mg) failed to modify blood glucose measurements (Echard et al. 2001). An early study in humans showed that the consumption of 500 mg GlcN per day for 6-8 weeks elicited no changes on blood glucose parameters (Pujalte et al. 1980). Similarly, an alternative study utilizing the full RDI of 1500 mg p/day in patients with osteoarthritis also showed a lack of effect on blood glucose levels (Drovanti et al. 1980). Since then it has been found that the consumption of the RDI of GlcN by both lean and obese people induces no change in serum glucose, lipoprotein, insulin or insulin sensitivity after 4 weeks (Yu et al. 2003), or in insulin resistance after 6 weeks (Muniyappa et al. 2006). In a longer term study (3 years), the daily consumption of 1500 mg GlcN had no effect on fasting glucose levels (Reginster et al. 2001). Furthermore, in contrast to the findings of Monouni et al. (2000), Pouwels et al. (2001) reported that a 5 hour infusion of GlcN does not affect insulin-stimulated glucose uptake (Pouwels et al. 2001). Hence it is apparent that GlcN - induced metabolic effects cannot be predicted in a simple manner by the duration or mode of exposure.

| Cell line origin | Effect |
|---|---|
| **Adipocytes** | **Decreased glucose transport** (Marshall et al. 1991; Chen et al. 1997; Thomson et al. 1997; Heart & Sung, 2002), **Insulin resistance** (Marshall et al. 1991; Heart et al. 2000; Nelson et al. 2000; Nelson et al. 2002), **Increased cell surface O-linked glycosylation** (Chen et al. 2003), **Decreased GLUT4 levels** (Chen et al. 1997; Thomson et al. 1997), **Decreased insulin stimulated GLUT4 translocation** (Heart et al. 2000; Nelson et al. 2000; Chen et al. 2003), **Activation of PI3-kinase** (Filippis et al. 2002), **Impaired insulin stimulation of Akt** (Heart et al. 2000), **Impaired insulin stimulation of P70s6 kinase** (Heart et al. 2000), **Increased G6PDH levels** (Marshall et al. 2004), **Increased UDP-N-GlcNAc levels** (Marshall et al. 2004), **Inhibited glucose uptake** (Marshall et al. 2005(b)), **Decreased ATP concentration** (Hresko et al. 1998; Marshall et al. 2004) |
| **Astroglial** | **Increased Akt phosphorylation** (Matthews et al. 2007), **Increased expression of endoplasmic reticulum stress marker GRP 78** (Matthews et al. 2007) |
| **Diaphragm** | **Insulin resistance** (Robinson et al.1993; Han et al. 2003), **Impaired GLUT4 translocation** (Robinson et al. 1993; Han et al. 2003), **Decreased ATP concentration** (Han et al. 2003) |
| **Epitrochlearis** | **Decreased insulin responsiveness to glucose transport** (Heart et al. 2000) |
| **Fibroblast** | **Insulin resistance** (Robinson et al. 1993), **Impaired GLUT4 translocation** (Robinson et al. 1993) |
| **Liver** | **Decreased IGF-1 receptor phosphorylation** (Sakal & Clemmons, 2003), **Decreased insulin stimulated glucose uptake** (Bailey & Turner, 2004) |
| **Pancreatic Islets** | **Decreased insulin stimulated glucose uptake** (Balkan & Dunning, 1994), **β cell dysfunction** (Yoshikawa et al. 2002) |
| **Skeletal muscle** | **Decreased glucose transport** (Ciaraldi et al. 1999), **Insulin resistance** (Ciaraldi et al. 1999), **Decreased glucose phosphorylation** (Ciaraldi et al. 1999), **Increased JNK activity** (Srinivasan et al. 2009), **Impaired insulin stimulated glucose uptake** (Srinivason et al. 2009), **Increased UDP- GlcNAc levels** (Srinivason et al. 2009), **Increased expression of endoplasmic reticulum chaperone proteins (Viz, GRP 78, Calcireticulum, Calnexin)** (Srinivason et al. 2009) |

**1.9.1 Table 4:** The effects of the incorporation of GlcN in the culture media on a variety of cell lines.

| Physiological outcome | Reference |
|---|---|
| **Insulin resistance** | Balkan & Dunning, 1994; Rossetti et al. 1995; Miles et al. 1998; Baron et al. 1999; Kim et al. 1999; Patti et al. 1999; Virkamaki & Yki Jarvinen, 1999; Yoshino et al. 1999; Stampinato et al. 2003; Wallis et al. 2005; Einstein et al. 2008 |
| **Impaired insulin receptor signalling** | Patti et al. 1999; Stampinato et al. 2003 |
| **Decreased insulin secretion** | Balkan & Dunning, 1994; Giaccari et al. 1995; Shankar et al. 1998; Virkamaki and Yki Jarvinen, 1999; Akimoto et al. 2006 |
| **Decreased insulin stimulation of glycolysis** | Giaccari et al. 1995 |
| **IRS (Insulin receptor substrate) 1 & 2 modification** | Patti et al. 1999 |
| **Decreased glucose uptake** | Hawkins et al. 1996; Virkamaki et al. 1997; Shankar et al. 1998; Hawkins et al. 1999; Holmang et al. 1999; Patti et al. 1999 |
| **Decreased exercise-induced glucose uptake** | Miles et al. 2001 |
| **Decreased glucose-6-phosphate** | Giaccari et al. 1995 |
| **Decreased lactate** | Holmang et al. 1999 |
| **Increased UDP-N-acetylglucosamine** | Wang et al. 1998; Patti et al. 1999 |
| **Perturbed GLUT4 translocation &/or trafficking** | Baron et al. 1999 |
| **Enhanced transcription factor glycosylation** | Akimoto et al. 2006; Einstein et al. 2008 |
| **Impaired PI3- kinase activation** | Hawkins et al. 1999; Kim et al. 1999; Miki et al. 2002 |
| **Increased adipose tissue gene expression of fat- derived peptides (PAI, angiotensinogen, leptin, resistan and adiponectin)** | Wang et al. 1998; Einstein et al. 2008 |
| **Decreased hind limb muscle blood flow** | Holmang et al. 1999 |
| **Inhibited insulin stimulated increases in femoral artery blood flow and capillary recruitment** | Wallis et al. 2005 |

**1.9.2 Table 5:** A list of the effects of GlcN infusion in rats.

However, examination of the effects of GlcN exposure in people with pre-existing blood-glucose related perturbations reveals some interesting findings. Tapadinhas et al. (1982) found no adverse effects of GlcN in diabetic patients. Importantly, however, the outcome measured related to perceptions of pain, and did not involve routine clinical laboratory testing such as blood glucose or insulin tests. In contrast, an earlier study found that in diabetic patients, glucose levels during a glucose tolerance test did not approach baseline values following an injection of GlcN (Weiden and Wood, 1958*).* More recently, GlcN consumption has been associated with significantly elevated levels of blood glucose in patients with impaired glucose tolerance (Biggee et al. 2007). In addition Pham et al. (2007) have shown that in people with pre-existing insulin metabolism abnormalities, short-term exposure to the RDI of GlcN can induce insulin resistance. It is therefore possible that GlcN may elicit no short-term effects on glucose metabolism in healthy people, but may exacerbate the pathologies of pre-existing blood glucose and/or insulin related abnormalities.

## 1.10 Similarities and differences between the effects of GlcN and high glucose

Some reports have questioned the validity of the use of GlcN as a hyperglycaemic mimetic, and suggest that GlcN only mimics select metabolic aspects of hyperglycaemia. For example, when Marshall et al. (2004) exposed rat adipocytes to either glucose or GlcN, it was found whilst UDP GlcNAc was elevated 2 fold by glucose, it was elevated 4-5 fold by GlcN. Furthermore, while GlcN exposure rapidly induced a marked increase in G6PDH, levels were undetectable with glucose treatment. Conversely, however, G-6-phosphate was quickly elevated by glucose, and unchanged by GlcN treatment. Perhaps most importantly though, adipocytes that had been exposed to GlcN showed pronounced reductions in ATP levels, a phenomenon which had previously been attributed to Hexosamine stimulated elevations in G6PDH production (Hresko et al. 1998; Han et al. 2003). Furthermore, Nelson et al. (2000) reported that whilst both high concentrations of glucose and GlcN cause insulin resistance of glucose transport, this is achieved with GlcN via inhibition of GLUT 4 translocation and with high glucose by impairing GLUT 4 activity. In a follow up study, it was also shown that whilst the pre-incubation of adipocytes with GlcN elicited no effects on Akt translocation, this was reduced by 25% or 50% with pre-incubation with low or high levels of glucose respectively (Nelson et al. 2002). Finally, Han et al. (2003) noted that actinomycin D had no effect on GlcN-induced insulin resistance in cultured rat muscle cells. This contrasts with the findings of Kawanaka et al. (1999), who demonstrated that inclusion of actinomycin D in culture media prevented high glucose induced insulin resistance in muscle cells.

Differences in the effects of GlcN and glucose have also been identified in in vivo models. At the completion of a 9-week study involving oral GlcN or glucose administration in rats, it was found that unlike instances of glucose-induced insulin resistance, rats that had been given GlcN had a significantly lower systolic blood pressure than control animals (Echard et al. 2001). In another study the administration of Troglitazone, a recognised hypoglycemic agent, was able to prevent the onset of hyperglycemia induced insulin resistance but not GlcN induced insulin resistance in rats (Miles et al. 1998).

Differences in the efficacy of glucose and GlcN may be attributed to compensatory mechanisms that are activated by the glucose stimulation of pathways that are not affected by GlcN. This does not mean that GlcN is a poor mimetic of high glucose, but rather, that it may only stimulate select components of glucose metabolic pathways. Therefore, the hyperglycemic mimetic potential of GlcN should be considered within the context of its known effects on the Hexosamine pathway and PPP. Likewise, additional insights into potential metabolic similarities and differences between glucose and GlcN may prove useful in further understanding the molecular nature of clinical glucose pathologies.

### 1.11 Effects of GlcN on reproductive outcomes

The limited range of data that has been collected on the impact of GlcN on reproductive outcomes provides credence for performing further investigations. As previously stated, exposure of maturing bovine (Sutton-McDowall et al. 2006; Kimura et al. 2008) and porcine (Sutton-McDowall et al. 2006) COCs to GlcN prior to fertilisation has been shown to reduce blastocyst development. Similarly, exposure of mouse (Pantaleon et al. 2008; Pantaleon et al. 2010) and bovine (Kimura et al. 2008) embryos to GlcN had adverse effects on blastocyst development. In an alternative study, pregnant mice given a subcutaneous injection of GlcN on days 7.5 - 10.5 of gestation increased the number of fetuses with neural tube defects, which affected the closure of both the midbrain and hindbrain (Horal et al. 2004).

In contrast, two other studies failed to find evidence for an effect of GlcN on pregnancy outcomes. In the first, no adverse impact of intraplacental or intra-amnionic GlcN administration to pregnant mice on days 11 - 13 of gestation were observed (Didock et al. 1956). However, outcomes measured in this study were restricted to maternal parameters, such as vaginal bleeding and abdominal palpitations, and not embryo development. To date only one report has been published that looks at the effect of GlcN on human pregnancy. Although this study did not show an effect of GlcN exposure on pregnancy outcomes, it was

conducted on a very small sample size and with women taking various doses of GlcN (Sivojelezova et al. 2007). However, further investigation of the effects of GlcN exposure on reproductive outcomes is warranted given the documentation of adverse outcomes.

## 1.12 Patterns of consumption of GlcN

There is an incomplete understanding of the number of people that take GlcN as a complementary medicine. Kaufman et al. (2002) estimated GlcN consumption in the USA between 1988 - 1989 to be as follows: total proportion of population = 1.9%, (Men) 18 - 44 years of age = <1%, 45 - 64 years of age = 2%, 65+ years of age = 4%, (Women) 18 - 44 years of age = <1%, 45 - 64 years of age = 5 % and 65+ years of age = 4%. In a follow up study, data collected over a 5 year period ending in 2002 in the US showed that GlcN consumption amongst a representative population of adults over the age of 18 had steeply increased, and was estimated to be 4.7% for men and 8.2% for women (Hopman et al. 2006). A subsequent study, compiled with data collected in 2005, showed that, overall, 4% of the population consumed some form of GlcN. This figure was calculated from the following gender and age distributions: (Men) 18 - 44 years of age = 2.5%, 45 - 64 years of age = 6.1%, 65+ years of age = 8.7%, (Women) 18 - 44 years of age = 1%, 45 - 64 years of age = 5.4% and 65+ years of age = 7.7% (A Report from the Slone Survey, 2005).

Consumption patterns are known to be greater in more defined populations. In a study conducted in the UK it was estimated that up to 15.9% of the population over the age of 55 and suffering from osteoarthritis of the knee consumed some form of GlcN (Jordan et al. 2004). Another study which reviewed the use of complementary medicine by people aged 60-99 in the USA revealed that GlcN was the most commonly consumed of dietary supplements (Wold et al. 2005). An Australian study that looked at dietary supplement use amongst 49 - 89 year olds found that 24% took GlcN (Wilkinson and Jelinek 2009). However it is not only age that is a predictive factor for increased frequency of GlcN consumption. Amongst people that frequently consume multiple supplements, GlcN has been reportedly taken by 57.3% of men and 59.3% of women (Block et al. 2007). In another survey that looked at non-vitamin dietary supplement use in conjunction with prescription medication, it was found that between 3.1% and 16% of people took GlcN (Gardiner et al. 2006).

## 1.13 Trends in the utilization of other supplements

There is currently limited data pertaining to the consumption of dietary supplements by women that are pregnant or attempting to become pregnant. Small selections of studies have demonstrated a high rate of CAM (complementary and alternative medicine) utilization by this group. Gadsby et al. (1993), for example, reported that 30% of respondents residing in Britain had sought out and utilized some form of CAM in the absence of physician approval for the alleviation of morning sickness symptoms. In Canada, usage may be considerably higher, with one study showing that 61% of pregnant women had used some form of CAM for the alleviation of morning sickness. Perhaps most alarmingly, it was found that only 8% had consulted their physician or pharmacist (Hollyer et al. 2002). Utilization in Australia also appears to be high, with one study finding that 40% of pregnant women used at least one CAM during pregnancy. This figure was largely constituted by vitamin use (24%) and herb use (12%) (Pinn & Pallett, 2002). Subsequently, Stankiewicz et al. (2007) reported that 66% of patients attending a fertility centre in South Australia reported using supplements at initial consultation. An alternative study also conducted in Australia found that 68% of obstetricians and 78% of midwives had recommended patients seek out CAMs (Gaffney & Smith, 2004). This might suggest that perceptions that CAMs are safe and pose a relatively minor risk (Allaire et al. 2000; MacLennan et al. 2002; Koh et al. 2003; Naidu et al. 2005; MacLennan et al. 2006) are held by the general public as well as healthcare providers.

There is good reason to be suspicious of potential adverse effects of dietary supplements on reproductive outcomes, given that several have already proven to yield toxic consequences. According to data gathered from human and animal studies, gestational exposure to high doses of Vitamins A (Cohlen et al. 1953(a); Cohlen et al. 1953(b); Cohlen et al. 1954; Rothman et al. 1995), D (Friedman and Roberts, 1966; Konety et al. 1999; Norman et al. 2002), E (Martin and Hurley 1977; Viana et al. 2003; Boskovic et al. 2005) and E and C combined (Poston et al. 2006; Aris et al. 2008) increases the prevalence of developmental defects.

However, it is not just exposure to supraphysiological doses of compounds which yields adverse effects on pregnancy. For example Blue Cohosh, a plant extract which contains agents capable of stimulating uterine contraction (Ganzera et al. 2003) has been associated with infantile cardiovascular complications (Jones and Lawson, 1998; Finkel and Zarlengo, 2004) and multi-organ hypoxia (Gunn and Wright, 1996). Additionally, liquorice consumption has been demonstrated to increase rates of shorter gestational periods (born between 37-40 weeks gestation) (Strandberg et al. 2001) and premature birth (born before 37 weeks

gestation) (Strandberg et al. 2002). Furthermore, a Norwegian study reported that 39% of pregnant women had consumed herbal supplements that were classified as either harmful to, or having an incomplete level of understanding surrounding, pregnancy (Nordeng and Havnen, 2004). This is probably not an isolated phenomenon, as a study looking at the use of complementary medicines in pregnant Italian women found that only 15.2% of respondents were able to accurately name all complementary products used, and perhaps more alarmingly, 59.1% were unable to recall any (Lapi et al. 2008). Hence it is apparent that an increased level of understanding is required on the effects of CAMs on reproductive outcomes, including GlcN.

## 1.14 Conclusion

Gaining further understanding of the effects of manipulating glucose metabolic pathways in reproductive processes would potentially prove to be beneficial for two important reasons. In the first instance, further GlcN-based research on the oocyte and pre-implantation embryo would also be useful for providing additional insight into the immediate as well as longer term consequences of peri-conceptional alterations in glucose metabolic pathway activity. This is important given the increasing number of people that are developing diabetes (King et al. 1998) as well as the increasing amount of data available regarding the perturbing effect of high glucose exposure on the oocyte and pre-implantation embryo. In turn, this may also serve a secondary function of contributing to the current knowledge gap that has been generated regarding the impact of dietary supplements on reproductive outcomes

## 1.15 Hypotheses and aims

### 1.15.1 General Hypothesis

Peri-conceptional GlcN exposure perturbs reproductive outcomes in the mouse.

### 1.15.2 Specific hypotheses and aims

This general hypothesis was tested through the execution of both in vitro and in vivo experiments which were based on the following hypotheses and aims:

**Hypothesis 1**

GlcN exposure to mouse COCs during in vitro maturation (IVM) reduces developmental competence.

**Aims**

- To assess the impact of various concentrations of GlcN exposure during IVM on rates of embryo development.
- To determine the role of glucose in GlcN-mediated perturbations that occurs during IVM.
- To develop an appropriate culturing system to assess the effects of GlcN exposure during IVM on subsequent embryo development.

**Hypothesis 2**

The perturbing effects on mouse embryo development that follow GlcN exposure during IVM, occurs via both structural and metabolic processes in the COC.

**Aims**

- To assess the impact of GlcN on the HBP by inhibiting O-linked glycosylation.

- To determine the role of the cumulus cells and the oocyte in GlcN-mediated changes in O-linked glycosylation.
- To assess the impact of GlcN on the PPP by examining G6PDH activity and ROS production.

**Hypothesis 3**

In vivo peri-conceptional GlcN exposure perturbs rates of successful fetal development.

**Aims**

- To assess the effects of different doses and durations of exposure of in vivo, peri-conceptionally administered GlcN on pregnancy rates, litter size, fetal size, placental size and abnormalities on gestational d18.

**Hypothesis 4**

The perturbing effects of in vivo, peri-conceptional GlcN exposure on fetal development are heightened in the presence of maternal metabolic perturbations induced by excessive body mass.

**Aims**

- To assess the effects of a HF (22%) diet on maternal weight as well as insulin and glucose levels.
- To assess the effects of in vivo, peri-conceptional GlcN that is administered to overweight mice on pregnancy rates, ovulation rates, litter size, fetal size, placental size and abnormalities on gestational d18.
- To assess the effects of in vivo, peri-conceptional GlcN that is administered to overweight mice on the post natal development of offspring.

**Hypothesis 5**

In vivo, peri-conceptional GlcN exposure in mice of different ages elicits non-consistent effects on subsequent fetal development.

**Aims**

- To assess the role of maternal age on GlcN- mediated perturbations in pregnancy outcomes including pregnancy rates, litter size, fetal size, placental size and abnormalities, on gestational d18.
- To assess the impact of in vivo, peri-conceptional GlcN exposure on oocyte G6PDH activity.

# 2.0 Chapter 2:

# The reduced developmental competence of mouse oocytes following in vitro GlcN exposure is mediated by accompanying levels of glucose

## 2.1 Summary

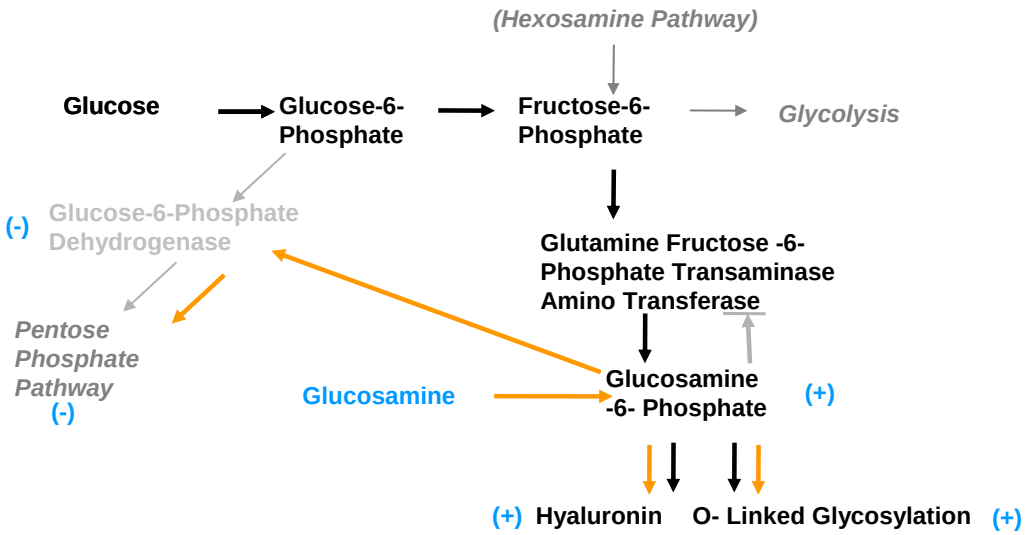
GlcN has recently been shown to reduce the developmental competence of in vitro maturing bovine (Sutton-McDowall et al. 2006; Kimura et al. 2008) and porcine oocytes (Sutton-McDowall et al. 2006). Here, it has been assessed whether various doses of GlcN also elicit perturbing effects on in vitro maturing mouse oocytes by assessing proportions of 2 cell embryos and blastocysts formed. Addition of 1.25 mM or 5 mM GlcN to IVM media containing 5 mM or 5.56 mM glucose had no effect on subsequent development when oocyte maturation occurred at a ratio of 10 µl IVM media/ COC. In subsequent experiments, however, it was shown that embryo development was impaired when levels of available glucose were increased. Addition of 1.25 mM or 5 mM GlcN to IVM media containing 20 mM glucose, in a volume of 10 µl IVM media/ COC, caused a reduction in blastocyst development (when expressed as a proportion of oocytes matured or as a proportion of 2-cell embryos). Furthermore IVM was performed using media with a glucose concentration of 5 mM, but at a ratio of 100 µl IVM media/ COC and with the addition of 1.25 mM or 5 mM GlcN. These conditions also reduced blastocyst development (as a proportion of oocytes matured or of 2-cell embryos). Finally, it was shown that when glucose levels were raised even higher, by the use of media with a glucose concentration of 25 mM and a ratio of 100 µl IVM media/ COC, there was an additive deleterious effect of glucose in the presence of GlcN. Taken together, these results confirm that in vitro exposure of maturing mouse COCs to GlcN does have a detrimental effect on subsequent development, and also show that this effect is dependent upon accompanying levels of glucose in the culture media.

## 2.2 Introduction

The approaches in performing IVM of immature COCs have undergone minimal change over the past six decades. This is reflective of a limited increase in the understanding of the requirements of the maturing COC, and is evidenced by the fact that in vivo matured COCs still prove to be of a higher quality and exhibit greater developmental success when compared to their in vitro matured counterparts (van de Leemput et al. 1999; Enright et al. 2000; Rizos et al. 2002; Khatir et al. 2007(a); Khatir et al. 2007(b)). One of the few contributing factors examined so far is the elevated concentration of glucose that is present in most historical IVM media formulations relative to the follicle (Orsi et al. 2005; Harris et al. 2005; Sutton-McDowall et al. 2004; Iwata et al. 1998; Nandi et al. 2008). It has been documented that in vitro matured COCs consume significantly more glucose than their in vivo matured counterparts (Harris et al. 2007).

Glucose availability for individual COCs in culture environments is influenced by the concentration of glucose in the media, and the volume of media provided per COC. However, there is still a great deal of variation between different systems in terms of the amount of glucose that is available for each COC. For example, although mouse IVM commonly occurs in a glucose concentration of 5 - 5.56 mM, the number of COCs cultured per drop can vary considerably (Roberts et al. 2004; Roberts et al. 2005; Dunning et al. 2007; Pries et al. 2005; Pries et al. 2007; Banwell et al. 2007). This occurs despite the fact that developmental outcomes have proven to be inconsistent when glucose concentrations (Downs 1994; Sutton-McDowall et al. 2005) as well as culture volumes (Sutton-McDowall et al. 2004) during IVM are varied.

One of the metabolic routes that glucose may flux through is the Hexosamine biosynthetic pathway (HBP) (Figure 1), which has been speculated to be an integral energy-sensing pathway in the metabolism of many tissues (Marshall et al. 1991(a)). Somatic cells typically metabolize 1 - 3% of glucose through the Hexosamine pathway (Marshall et al. 1991(b)). However, Sutton et al. (2003) showed that bovine COCs flux differential glucose concentrations through the HBP during maturation, in response to the ovulatory signaling pathway (Sutton et al. 2003; Sutton-McDowall et al. 2004), with a peak flux estimated to be around 24% of glucose uptake (Gutnisky et al. 2007). This additional flux is required for the expansion of the cumulus matrix (Sutton McDowall et al. 2004), specifically for the production of hyaluronin, a major end product of the hexosamine pathway (Eppig 1979; Ball et al. 1982; Talbot 1984; Suchanek et al. 1994).




**2.2.1 Figure 1:** The effect of GlcN on Glucose metabolic pathways. GlcN is converted to the Hexosamine intermediate, GlcN-6-Phosphate. An elevation in GlcN-6-Phosphate has the dual role of increasing the flux of metabolic activity through the Hexosamine pathway, as well as inhibiting the rate limiting enzyme Glucose-6-Phosphate Dehydrogenase, and therefore depressing, the Pentose Phosphate Pathway.

The aminosaccharide GlcN has been shown to be transported into cells via glucose transporters (Uldry et al. 2002), and converted to the HBP intermediate, glucosamine-6-phosphate (GlcN-6-P) (Marshall et al. 2005(b)). GlcN-6-P is positioned downstream of the non-reversible, rate-limiting enzyme GFPT (Figure 1). This in turn leads to an increase in (non-reversible) flux through the HBP and subsequently an increased formation of end products of the Hexosamine pathway.

Sutton-McDowall et al. (2004) proposed that addition of GlcN to bovine IVM media would stimulate an elevation in Hexosamine pathway activity, and potentially permit a reduction in the glucose concentration of the media. It was subsequently found that maturation, fertilization and early embryo development in porcine and bovine models proceeded as normal, but that ensuing development was significantly hindered (Sutton-McDowall et al. 2006). These findings served as the first evidence that exposure of the maturing COC to GlcN had a detrimental impact on the subsequent developmental competence of the oocyte.

The experiments outlined in this chapter have been designed to test the hypothesis that GlcN exposure during in vitro maturation will reduce the developmental competence of mouse oocytes. In addition, the relationship between glucose and glucosamine and their effects on

developmental competence of mouse oocytes have been examined by assessing GlcN exposure in conjunction with varying levels of glucose.

## 2.3 Methods and Materials

Except where otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St Louis, USA).

### 2.3.1 Animals

Pre-pubertal (21-23 day old) CBA x C57Bl/6 F1 female mice (CBAB6F1) were obtained from Laboratory Animal Services at the University of Adelaide or from an in-house breeding colony maintained at The Queen Elizabeth Hospital (TQEH), Woodville, South Australia. Mice were caged on a 14:10 hour light: dark cycle in the animal housing facilities at TQEH and maintained on a standard mouse chow diet.

### 2.3.2 COC Collection

Mice received a single 0.1 ml intraperitoneal (IP) injection of 5 IU eCG (Folligon; Intervet, Boxmeer, Holland). Forty-six h after eCG injection, mice were killed via cervical dislocation and ovaries were dissected out and placed into 37ºC handling media. In experiments one and two, ovaries were collected in 1 ml 37ºC Hepes- buffered αMEM (Invitrogen, Carlsbad, USA) supplemented with 50 µg/ml streptomycin sulphate, 75 µg/ml penicillin G and 5% FBS (Invitrogen). In experiments 3 - 7, ovaries were collected into 1 ml 37ºC MOPS G2.2 (Lane and Gardner, 2004) supplemented with 1 mg/ml fetuin. Two to four ovaries were placed into 35 mm Falcon 1008 culture dishes (Becton-Dickinson Labware, Franklin Lakes, USA) in 1- 2 ml warmed handling media (αMEM or MOPS G2 as indicated), and COCs were obtained by rupturing swollen follicles with a 30 gauge needle. Immature COCs surrounded completely by cumulus cells were washed in fresh, 37ºC handling media three times.

### 2.3.3 In Vitro Maturation

Washed COCs were placed into IVM media. For experiments one and two, the IVM media used was αMEM supplemented with 50 µg/ml Streptomycin, 75 µg/ml Penicillin G, 5% FBS and 50 mIU/ml recombinant human Follicle Stimulating Hormone (FSH) (Puregon; Organon, Sydney, Australia). Since αMEM is a generic tissue culture media, media designed for in vitro oocyte maturation of mouse oocytes (modified G2.2) was used for experiments three – seven. Modified G2.2 was supplemented with 50 mIU/ml recombinant human FSH and 1 mg/ml fetuin. For all experiments, media was overlaid with mineral oil and equilibrated in 35 mm Falcon dishes (37°C in 6% $CO_2$:5% $O_2$:89% $N_2$) for 4 – 18 h (Lane and Gardner, 2004). All COCs were incubated for 17 h prior to fertilization and embryo culture unless otherwise stated.

Across all experimental groups COCs were matured with exposure to varying levels of glucose availability, achieved by altering either the glucose concentration or the volume of IVM media that maturation occurred in. These parameters are described in Table 1. Reference to increasing levels of glucose availability is relative to experiments 1 and 2, where maturation occurred in a volume of 10 µL/ COC with a glucose concentration of 5 – 5.56 mM.

| Experiment | Glucose Concentration (mM) | Volume of media per COC (µL) | GlcN Concentration (mM) |
|---|---|---|---|
| **1** | 5.56 | 10 | 0, 1.25, 5 |
| **2** | 5 | 10 | 0, 1.25, 5 |
| **3** | 20 | 10 | 0, 1.25, 5 |
| | 5.56 | 10 | |
| **4** | 5 | 100 | 0, 1.25, 5 |
| **5** | 5 | 100 | 0, 1.25, 2.5 |
| **6** | 25 | 100 | 0, 1.25, 2.5 |
| **7** | 5 | 10 | 0, 1.25, 5 |
| | 5 | 50 | |
| | 5 | 100 | |

**2.3.3.1 Table 1:** Glucose availability during IVM in Experiments 1 - 7.

### 2.3.4 Developmental competence of oocytes matured ± GlcN

#### *2.3.4.1 Experiment 1*

αMEM media (with a basal glucose concentration of 5.56 mM) was prepared and placed into 100 µL drops supplemented with a) 0 mM GlcN, b) 1.25 mM GlcN or c) 5 mM GlcN. Ten washed COCs were placed into each drop, at a ratio of 10 µL media per COC. Three 100 µL drops were prepared per treatment, so that a total of 30 COCs were matured per treatment, per replicate. A total of six replicates of IVM were performed.

#### *2.3.4.2 Experiment 2*

Modified G2.2 media (with a basal glucose concentration of 5 mM) was prepared and placed into 100 µL drops supplemented with a) 0 mM GlcN, b) 1.25 mM GlcN or c) 5 mM GlcN. Thirty washed COCs were placed in each drop, at a ratio of 10 µL media per COC. Three 100 µL drops were prepared per treatment, so that a total of 30 COCs were matured per treatment, per replicate. A total of five replicates of IVM were performed.

### 2.3.5 Developmental competence of oocytes matured ± GlcN ± greater glucose availability by way of increasing the glucose concentration in the media

#### *2.3.5.1 Experiment 3*

αMEM media (with a basal glucose concentration of 5.56 mM) was prepared and placed into 100 µL drops supplemented with a) 0 mM GlcN + 14.44 mM Glucose, b) 1.25 mM GlcN + 14.44 mM Glucose, c) 5 mM GlcN + 14.44 mM Glucose or d) 5 mM GlcN. The addition of 14.44 mM glucose elevated the glucose concentration of the IVM media to 20 mM. 20 mM glucose is supraphysiological relative to mouse follicular glucose concentrations (0.01 – 2.4 mM) (Harris et al. 2005), although it is still lower than the 23 mM glucose concentration of the commonly used Waymouth culture media. Ten washed COCs were placed in each drop, at a ratio of 10 µL media per COC. Three 100 µL drops were prepared per treatment, so that a total of 30 COCs were matured per treatment, per replicate. A total of five replicates of IVM were performed.

### 2.3.6 Developmental competence of oocytes matured ± GlcN with greater glucose availability by way of an increase in the volume of maturation media

#### *2.3.6.1 Experiment 4*

Modified G2.2 media (with a basal glucose concentration of 5 mM) was prepared and placed into 3000 µL drops supplemented with a) 0 mM GlcN, b) 1.25 mM GlcN or c) 5 mM GlcN. Thirty washed COCs were placed in each drop, at a ratio of 100 µL media per COC. A total of 30 COCs were matured per treatment, per replicate and one replicate of IVM was performed.

#### *2.3.6.2 Experiment 5*

Modified G2.2 media (with a basal glucose concentration of 5 mM) was prepared and placed into 3000 µL drops supplemented with a) 0 mM GlcN, b) 1.25 mM GlcN or c) 2.5 mM GlcN. Thirty washed COCs were placed in each drop, at a ratio of 100 µL media per COC. A total of 30 COCs were matured per treatment, per replicate. A total of 6 replicates of IVM were performed.

### 2.3.7 Developmental competence of oocytes matured ± GlcN with greater glucose availability by way of increased glucose concentration in the media and an increase in the volume of maturation media

#### *2.3.7.1 Experiment 6*

Modified G2.2 media (with a basal glucose concentration of 5 mM) was prepared and placed into 3000 µL drops supplemented with a) 0 mM GlcN, b) 2.5 mM GlcN, c) 0 mM GlcN + 20 mM glucose or d) 2.5 mM GlcN + 20 mM glucose. Where glucose supplementation occurred (groups c and d), a total glucose concentration of 25 mM was achieved. Thirty washed COCs were placed in each drop, at a ratio of 100 µL media per COC. A total of 30 COCs were matured per treatment, per replicate. A total of 4 replicates of IVM were performed.

### 2.3.8 A direct comparison of the developmental competence of oocytes matured ± GlcN with varying levels of glucose availability

#### *2.3.8.1 Experiment 7*

Modified G2.2 media (with a basal glucose concentration of 5 mM) was prepared and supplemented with either 0 mM GlcN or 2.5 mM GlcN. These two IVM media were distributed in 300 µL, 1500 µL or 3000 µL drops, so that maturation occurred in ratios of 10 µL, 50 µL or 100 µL of media per COC. Thirty washed COCs were placed in each drop per replicate, and a total of five replicates of IVM were performed.

### 2.3.9 IVF

Eight- 24-week-old CBAF1 males of proven fertility, and who had not mated within the previous 48 hours, were killed via cervical dislocation 16 hours after the initiation of IVM. Both epididymides and vas deferens were immediately removed, and placed into 1 ml αMEM supplemented with 50 µg/ml streptomycin, 75 µg/ml penicillin G (Sigma Chemical Co. (St. Louis, MO)) and 3 mg/ml bovine serum albumin (BSA) (ICPbio, Auckland, New Zealand), overlaid with mineral oil. Sperm was released by squeezing the epididymides and vas deferens with fine, sterile forceps. Sperm was allowed to capacitate for one hour. Dishes containing 90 µL drops of αMEM supplemented with 3 mg/ml BSA equilibrated for 12-18 hours, were used for IVF. At the completion of the one hour capacitation period, which corresponded to the completion of the 17 hour IVM period, 10 µL sperm was added to each 90 µL drop of αMEM supplemented with 3 mg/ml BSA, and overlaid with mineral oil.

Matured COCs were assessed at the completion of the 17-hour IVM period, and viable COCs were removed and quickly washed through approximately 0.5 ml 37ºC handling media. In experiments 1 and 2, the handling media was Hepes-buffered αMEM, and in experiments 3 - 5 the handling media was MOPS supplemented with 1 mg/ml fetuin. COCs were then quickly moved to a second wash drop comprised of 90 µL αMEM supplemented with 3 mg/ml BSA. Following the second wash, COCs were quickly transferred to the 90 µL drops of αMEM supplemented with 3 mg/ml BSA with 10 µL sperm and overlaid with mineral oil. Thirty COCs were added to each IVF drop. Dishes were then transferred back to the incubator for 4 hours to allow COCs to be fertilized.

### 2.3.10 Embryo Culture

All embryo culture was performed at 37°C in 6% $CO_2$, 5% $O_2$, 89% $N_2$. All media drops were equilibrated for 4-18 h before use.

#### *2.3.10.1 Experiments 1 & 3*

Following fertilization, presumptive zygotes were transferred to a 40 µL drop of KSOM culture media that was overlaid with mineral oil. Zygotes were then quickly transferred to 10 µL drops of KSOM, at a ratio of 5 zygotes per drop and were cultured for 20 hours. Embryos were assessed for rates of 2-cell development, as indicated by the presence of 2-cell embryos. Cleaved embryos were then transferred to fresh 10 µL drops of KSOM (5 per drop), overlaid with mineral oil. Seventy-two hours later embryos were assessed for rates of blastocyst development. When the embryos could not be added to drops at exact ratios of 5 per drop, compensation was made by adding either one less or one more to drops.

#### *2.3.10.2 Experiments 2 & 4 - 7*

Following fertilization, presumptive zygotes were transferred to a 40 µL drop of G1 (Lane and Gardner (2004)) culture media that was overlaid with mineral oil. Zygotes were then quickly transferred to 10 µL drops of G1 in the same dish as the wash drop, at a ratio of 5 zygotes per drop. Twenty h later, embryos were assessed for rates of 2-cell development, as indicated by the presence of 2-cell embryos. Cleaved embryos were then transferred to fresh 10 µL drops of G1 (5 per drop), overlaid with mineral oil. Twenty-four h later embryos were assessed for rates of 4-cell development. They were then transferred to 10 µL drops of G2.2 (Lane and Gardner, 2004) culture media that was overlaid with mineral oil. Forty-eight h later embryos were assessed for rates of blastocyst development.

## 2.4 Statistical Analysis

Data was arcsine transformed to allow statistical testing utilizing tests (such as analysis of variance) associated with normally distributed data, rather than proportional data, and analyzed with Statistical Package for the Social Sciences (SPSS) 13.0. A One-way analysis of variance (ANOVA) was used to analyze rates of 2-cell development, blastocyst

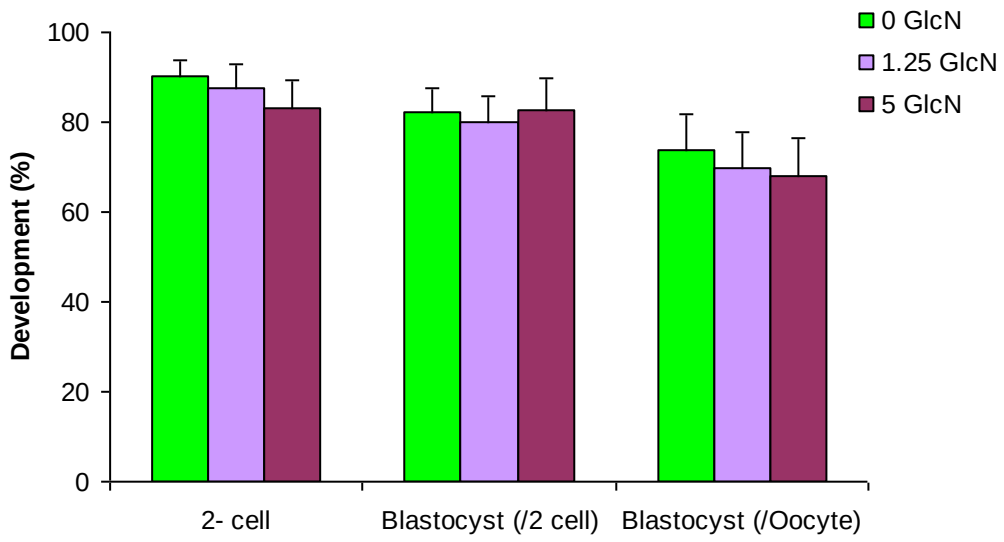
development from 2-cell embryos and blastocyst development from oocytes matured for experiments 1, 2, 3 and 5. A Two-way ANOVA was used to analyze rates of 2-cell development, blastocyst development from 2-cell embryos and blastocyst development from oocytes matured for experiments 6 and 7. Post hoc testing was performed with the Bonferroni correction owing to the test's high resistance to Type 2 errors.

## 2.5 Results

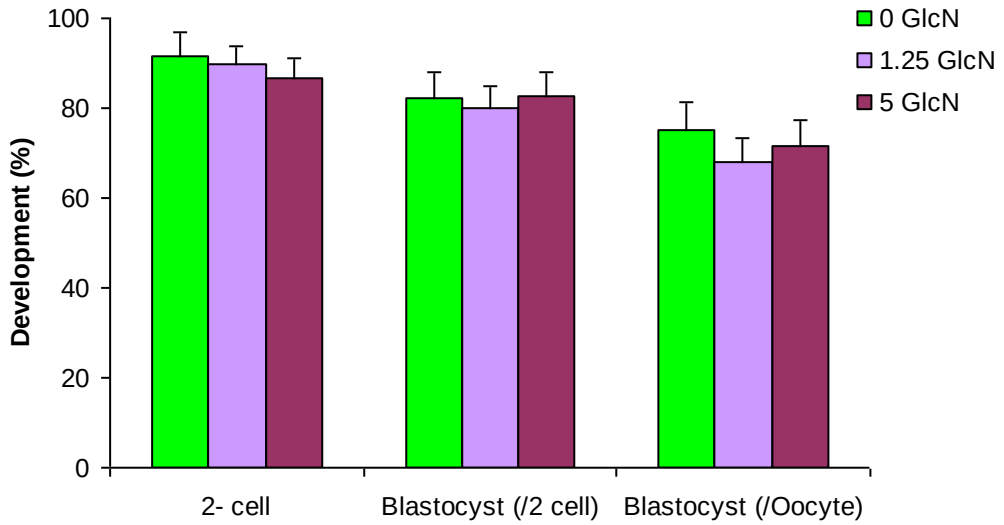
### 2.5.1 Developmental competence of oocytes matured ± GlcN

#### *2.5.1.1 Experiments 1 and 2*

For COCs matured in the presence of 5.56 mM glucose (with αMEM as the base medium) and in a ratio of 10 µL media per COC, the incorporation of either 1.25 mM or 5 mM GlcN did not alter the rates of development of 2 cell embryos or blastocysts (from 2-cell embryos or from oocytes matured), when compared to oocytes matured without GlcN (Figure 2). Similarly, no effect on embryo development was observed for oocytes matured with either 0 mM, 1.25 mM or 5 mM GlcN in a ratio of 10 µL media per COC and with mG2.2 (glucose concentration = 5 mM) as the base media (Figure 3). Collectively these results reveal that the addition of GlcN to IVM systems with a relatively low amount of available glucose does not affect developmental competence of oocytes.




**2.5.1.1.1 Figure 2:** Experiment 1: 2-cell and blastocyst development rates from oocytes exposed to 0 mM, 1.25 mM or 5 mM GlcN in conjunction with 5.56 mM glucose (αMEM) during in vitro maturation (mean ± SEM). IVM was performed in a ratio of 10 µL media per COC. Blastocyst rates are expressed as a proportion of 2-cell embryos or oocytes

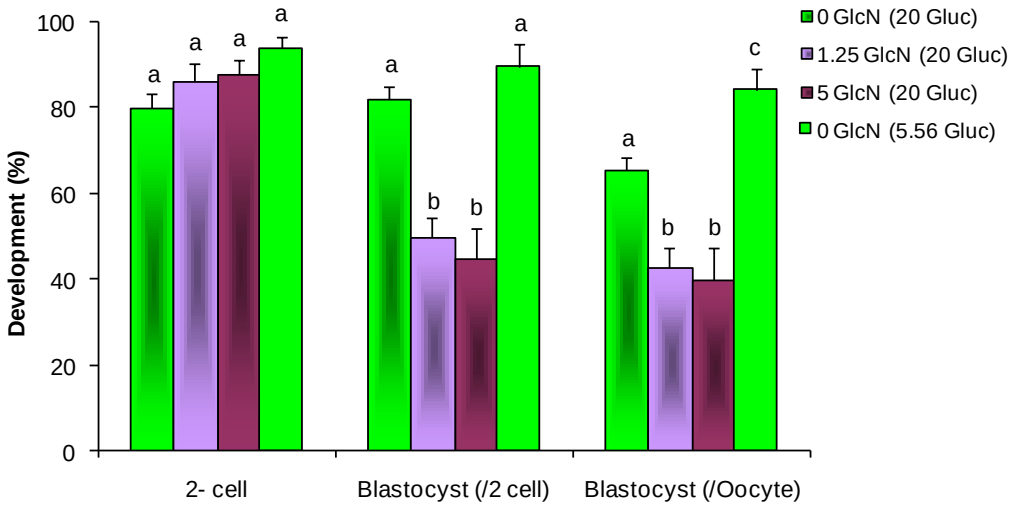



**2.5.1.1.2 Figure 3:** Experiment 2: 2-cell and blastocyst development rates from oocytes exposed to 0 mM, 1.25 mM or 5 mM GlcN in conjunction with 5 mM glucose (MG2.2) during in vitro maturation (mean ± SEM). IVM was performed in a ratio of 10 µL media per COC. Blastocyst rates are expressed as a proportion of 2-cell embryos or oocytes.

### 2.5.2 Developmental competence of oocytes matured ± GlcN ± greater glucose availability by way of increasing the glucose concentration in the media

#### 2.5.2.1 Experiment 3

There were no effects of GlcN or glucose (20 mM vs 5.56 mM) on rates of 2-cell development (Figure 4). However, significantly fewer blastocysts developed from 2 cell embryos that were derived from COCs matured with either 1.25 mM or 5 mM GlcN and 20 mM glucose, relative to those matured without GlcN, irrespective of glucose concentration ($P<0.05$). GlcN treatment also reduced blastocyst development rates when expressed as a proportion of oocytes matured. However, when rates of blastocyst development were assessed as a proportion of oocytes matured an effect of glucose, in the absence of GlcN, was observed, with higher development rates seen when oocytes were matured in 5.56 mM glucose, compared to 20 mM glucose ($P<0.05$). This could show that GlcN addition to IVM media does perturb developmental potential when levels of accompanying glucose are elevated. The results also suggest that development (i.e. blastocyst development as a proportion of oocytes matured) is reduced in the presence of higher levels of glucose, irrespective of GlcN treatment.

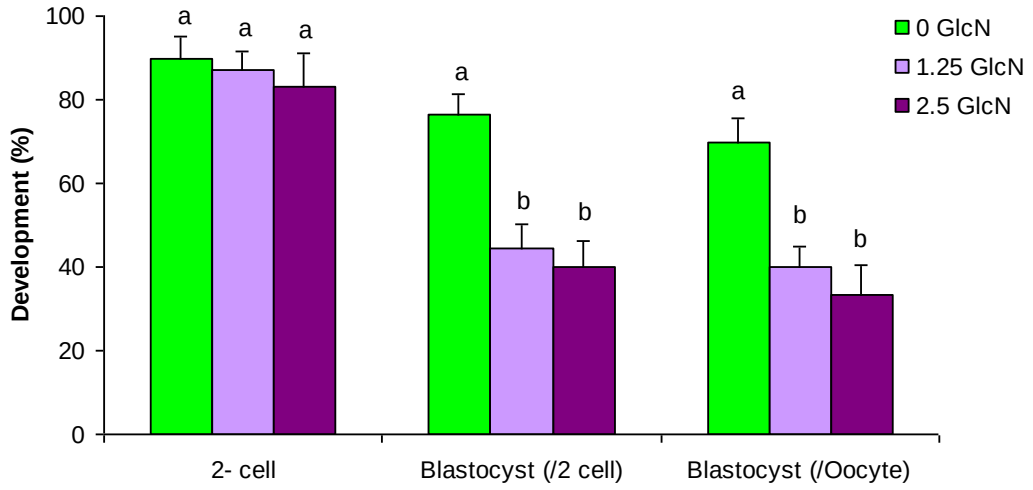



**2.5.2.1.1 Figure 4:** Experiment 3: 2 cell and blastocyst development rates from oocytes exposed to 0 mM, 1.25 mM or 5 mM GlcN in conjunction with 20 mM glucose (glucose supplemented αMEM ) or 0 mM GlcN and 5.56 mM glucose (base αMEM) during in vitro maturation (mean ± SEM). IVM was performed in a ratio of 10 µL media per COC. Blastocyst rates are expressed as a proportion of 2-cell embryos or oocytes. Different letters represent significant differences ($P<0.05$) between groups, within each developmental stage.

### 2.5.3 Developmental competence of oocytes matured ± GlcN with greater glucose availability by way of an increase in the volume of maturation media

#### *2.5.3.1 Experiments 4 and 5*

At the completion of one replicate of IVM, it was determined that the exposure of oocytes to media containing 5 mM GlcN and 5 mM glucose at a ratio of 100 µL IVM media/ COC was toxic (experiment 4, data not shown). Cumulus cells exposed to this treatment were dark, sticky and clumped in dark patches, suggestive of apoptosis. Many oocytes had also lysed. As a result, the effects of 5 mM GlcN were not investigated further and a concentration of 2.5 mM GlcN was used as the highest dose in the subsequent study (experiment 5). Here, it was shown that GlcN exposure did not alter rates of 2- cell development. However, exposing oocytes to GlcN significantly reduced blastocyst development from 2-cell embryos (P<0.001) as well as from oocytes matured (P<0.001) (Figure 5). This shows that when levels of glucose become elevated, not by increasing the glucose concentration but instead by increasing the culture volume, GlcN elicits a detrimental effect on developmental outcomes.

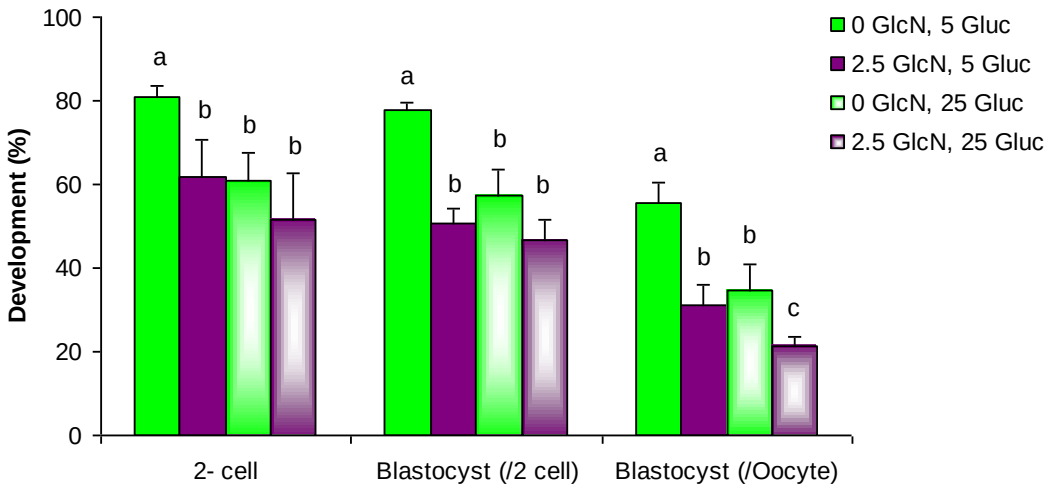



**2.5.3.1.1 Figure 5:** Experiment 5: 2-cell and blastocyst development rates from oocytes exposed to 0 mM, 1.25 mM or 2.5 mM GlcN in conjunction with 5 mM glucose (MG2.2 media) during in vitro maturation (mean ± SEM). IVM was performed in a ratio of 100 µL media per COC. Blastocyst rates are expressed as a proportion of 2-cell embryos or oocytes. Different letters represent significant differences (P<0.05) between groups, within each developmental stage.

### 2.5.4 Developmental competence of oocytes matured ± GlcN with greater glucose availability by way of increased glucose concentration in the media and an increase in the volume of maturation media

#### 2.5.4.1 Experiment 6

When glucose levels became further elevated in the presence of 2.5 mM GlcN, there were concomitant further reductions in development competence (Figure 6). Rates of 2-cell development were reduced by a high concentration of glucose (25 mM) ($P<0.05$) as well as by GlcN regardless of the concentration of glucose (either 5 mM or 25 mM), although there was no interactive effect of GlcN and glucose. Similarly, 25 mM glucose ($P<0.04$) and GlcN in the presence of both 5 mM and 25 mM glucose ($P<0.05$) significantly reduced blastocyst development from 2-cell embryos, but there was no interaction between GlcN and glucose treatments. However, blastocyst development from oocytes was shown to be reduced by GlcN ($P<0.02$), 25 mM glucose ($P<0.05$) as well as an interaction between GlcN and glucose treatments, regardless of the concentration ($P<0.05$).




**2.5.4.1.1 Figure 6:** Experiment 6: 2-cell and blastocyst development rates from oocytes exposed to 0 mM or 2.5 mM GlcN in conjunction with 5 mM or 25 mM glucose (MG2.2 media) during in vitro maturation (mean ± SEM). IVM was performed in a ratio of 100 µL media per COC. Blastocyst rates are expressed as a proportion of 2-cell embryos or oocytes. Different letters represent significant differences ($P<0.05$) between groups, within developmental stages.

### 2.5.5 A direct comparison of the developmental competence of oocytes matured ± GlcN with varying levels of glucose availability

#### 2.5.5.1 Experiment 7

From Table 2, the volume of incubation medium per COC alone had no effect on early cleavage or blastocyst development from 2-cell embryos. In contrast, the presence of 2.5 mM GlcN had detrimental effects on development from 2-cell to blastocyst, but only when the volume of medium per COC was increased to 50 or 100 µL/COC, in comparison to 10 µL/COC. Furthermore, early cleavage was also reduced when oocytes were matured in 100 µL/COC in the presence of GlcN ($P<0.04$). Collectively, these results (shown in Table 2) would suggest that the effects of GlcN may be heightened with greater availability of glucose.

| Treatment | % 2 cell/ Oocyte | % Blastocyst/ 2 cell | % Blastocyst/ Oocyte |
|---|---|---|---|
| **10 µL, 0 GlcN** | 89.7 ± 4.0 | 78.6 ± 4.1 | 70.3 ± 5.1 |
| **10 µL, 2.5 GlcN** | 83.6 ± 3.3 | 72.1 ± 6.6 | 60.5 ± 7.3 |
| **50 µL, 0 GlcN** | 88.2 ± 4.2 | 83.5 ± 6.5 | 73.2 ± 6.4 |
| **50 µL, 2.5 GlcN** | 83.4 ± 2.9 | **46.2 ± 11.2 *** | **37.6 ± 10.3 *** |
| **100 µL, 0 GlcN** | 87.4 ± 5.6 | 72.2 ± 7.8 | 63.8 ± 8.1 |
| **100 µL, 2.5 GlcN** | **68.7 ± 8.2 *** | **38.8 ± 12.3 *** | **27.9 ± 12.7 *** |

**2.5.5.1.1 Table 2:** Development rates following exposure to 5 mM glucose + either 0 mM or 2.5 mM GlcN during IVM. Maturation was performed in a ratio of either 10, 50 or 100 µL media per COC. Blastocyst rates are expressed as a proportion of 2-cell embryos or oocytes. * denotes significant differences to the "10 µL, 0 GlcN" treatment ($P < 0.05$) for each column.

## 2.6 Discussion

To date, there is an incomplete understanding of the effects of exposing mouse COCs to elevated levels of Hexosamine activity during IVM. Hexosamine activity has been shown to be up-regulated by the addition of GlcN (Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Nelson et al. 2000; Marshall et al. 2004; Marshall et al. 2005a; Marshall et al. 2005b). Initial studies in the mouse performed by Chen et al. (1990; 1993) showed that exposure of the maturing COC to GlcN did not affect embryo development, although these studies were limited to analysis of 2-cell development. Subsequently, Sutton-McDowall et al. (2006) demonstrated that this lack of effect on 2-cell development was also consistent in the bovine and porcine models, but that increased rates of embryo death were noted during the post-compaction period.

Although the outcomes from experiments 1 and 2 failed to show an effect of GlcN on mouse COC developmental competence, it cannot be concluded that GlcN does not have a perturbing effect on embryo development. Rather, it seems that GlcN-mediated developmental perturbations are dependent upon accompanying levels of glucose in the culture media. This is evidenced from the finding in Experiment 3 where blastocyst production was significantly reduced when COCs were matured in 10 µL/ COC media containing 20 mM glucose relative to COCs matured in 10 µL/ COC media containing 5.56 mM glucose. In addition, developmental competence was further reduced when either 1.25 mM or 5 mM GlcN was added to the 20 mM glucose containing media, thereby implicating an essential role of glucose in GlcN-mediated perturbations.

The role of glucose in this phenomenon was further suggested by the findings of experiments 6 and 7. In experiment 6, it was found that culturing COCs at a ratio of 100 µL/ COC reduced embryo development when IVM media was either supplemented with additional glucose to elevate glucose concentrations from 5 mM to 25 mM, or by adding 2.5 mM GlcN. Importantly, the combination of 25 mM glucose and 2.5 mM GlcN (matured in media ratios of 100 µL/ COC) had a synergetic effect and reduced embryo development to levels significantly below either perturbing treatment on its own. Subsequently, experiment 7 showed that the incorporation of 2.5mM GlcN into IVM media containing 5 mM glucose only reduced blastocyst production when COCs had been cultured in media at ratios of 50 µL/ COC or 100 µL/ COC, but not 10 µL/ COC. It does need to be noted however that several factors need to be considered in interpreting these findings. These factors do not change the validity of the outcomes, but could potentially impact upon the mechanistic influences at play.

Firstly, it would have been useful to assess the culture media for various paracrine factors that may have been secreted by the COCs during culture. This is because it is possible that the greater level of dilution of paracrine factors may have contributed towards the additive effects of GlcN addition to a larger culture drop. It is unlikely that this would be the sole contributing factor as it was shown that the combination of GlcN with higher concentrations of glucose led to reduced blastocyst development when culturing occurred in both 10 µL/COC (experiment 3) and 100 µL/COC (experiment 6). However, an analysis of embryo culture medium could identify potential signaling differences between the variously sized environments which may contribute to the effects observed.

Secondly, in light of the findings of perturbed development in response to increased concentrations of glucose (Experiments 3 and 6), it has been have inferred that when COCs are matured in larger volumes but with uniform glucose concentrations, there is an increased glucose availability because COC metabolism does not significantly decrease glucose levels in the medium (Experiment 7). It has previously been shown that glucose levels in "spent" maturation medium (following IVM of bovine COCs) is affected by density of COCs per volume of media; when maturation occurred in the presence of 5 mM glucose but in either 5 µL/COC or 10 µL/COC (Sutton-McDowall et al. 2005), much less glucose remained in the more dense maturation conditions. Although these results were derived from different species and utilized different IVM volumes to the ones outlined in the current set of experiments, they do none-the-less provide evidence that culture volume can impact upon glucose availability. Specific testing of glucose uptake from different volumes of IVM media outlined in this chapter are never the less warranted.

Such testing could also be extended to examine the effects of GlcN on glucose uptake, since the outcomes of these studies point towards dependence of GlcN on glucose for perturbations to be manifested. When Sutton-McDowall et al. (2005) analyzed the post-IVM glucose concentrations of bovine media, it was found that glucose uptake was reduced by the co-incorporation of GlcN.

Mouse cumulus cells contain Glut 1 (Zhou et al. 2000) which transports both glucose and GlcN (Uldry et al. 2002), and has a negative correlation expression pattern with glucose concentration (Koranyi et al. 1991; Tal et al. 1992). The concentration of glucose in the base media used in the experiments outlined in this chapter were 5.0 mM (MG2.2) and 5.6 mM (αMEM), markedly higher than the 0.1 - 2.4 mM glucose concentration typically found in mouse follicular fluid (Harris et al. 2005) but necessary to prevent glucose depletion in in vitro cultured cells that do not have access to an ongoing supply of substances from the

reproductive tract (Downs et al. 1998). That increased rates of developmental failures were observed in response to GlcN and higher levels of glucose (mediated in the very least by increased glucose concentrations) suggests an increased level of activity in the only known glucose transporter present in mouse cumulus cells - Glut 1. This contrasts the previously recognised pattern of down regulation of Glut 1 in response to elevated glucose concentrations. Thus, it is apparent that current understandings of GLUT activity in response to glucose concentration gradients are incomplete. Activity could be influenced by tissue specific distribution and/or differences in degrees of glycemic exposure.

Thirdly, performing IVM in larger volumes would potentially increase the availability of other substrates apart from glucose. The relative increase in availability of substrates within MG2.2 base media that follows culturing in larger volumes are not likely candidate confounders, owing to the fact that MG2.2 is an optimized mouse IVM media, and culturing in ratio of 100 µL/ COC lies within the optimized range (Lane and Gardner, 1994). It is however possible that additional GlcN is available in larger culture volumes, and such increased levels of GlcN availability may have been involved in reducing blastocyst development following IVM that was performed in media volumes of 50 µL/ COC and 100 µL/ COC (Experiment 7). It is however unlikely that increased GlcN availability would be the sole contributor to these outcomes, given that an additive effect of 2.5 mM GlcN and 20 mM glucose were observed in COCs matured in 100 µL media/ COC (Experiment 6). However, specific testing such as measuring IVM glucose concentrations throughout maturation or post maturation or assessing labeled glucose and/or GlcN incorporation within the COC, would have been useful.

It has long been known that exposure of somatic systems to GlcN can be mimetic of hyperglycemic conditions (Coulson & Hernandez 1962; Martin & Bambers 1965). To date there has been limited research examining the effects of exposure of the COC or early embryo to hyperglycemic conditions. Studies have shown that ovarian follicles from streptozotocin – induced diabetic mice are smaller and contain a greater number of apoptotic cells (Chang et al. 2005a). Similarly, COCs from diabetic mice exhibit delayed maturation (Diamond et al. 1989; Chang et al. 2005a; Ratchford et al. 2007), less gap junctional communication (Ratchford et al. 2008), perturbed metabolism (Ratchford et al. 2007) and contain oocytes that are smaller in size (Chang et al. 2005a). Recently, detrimental effects of exposing bovine and porcine oocytes to GlcN have also been observed (Sutton-McDowall et al. 2006; Kimura et al. 2008). Therefore, the use of GlcN to examine the effects of elevated Hexosamine flux on oocyte developmental competence should prove to be a very useful tool.

## 2.7 Conclusion

The results of these studies indicate that an elevated glucose level elicits a perturbing effect on blastocyst development. Furthermore, they also demonstrate that this effect can be further exacerbated by the introduction of GlcN. In the experiments outlined in this chapter levels of Hexosamine activity were not directly measured. However, GlcN is known to cause an up regulation in HBP activity in somatic tissues (Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Nelson et al. 2000; Marshall et al. 2004; Marshall et al. 2005a; Marshall et al. 2005b). We can therefore predict that the effects of exposing the COC to GlcN are, at least in part, contributed to by an up regulation in HBP activity in the COC. Additional experiments are required, however, to elucidate whether effects are mediated through the oocyte, the cumulus cells, or both and to determine the mechanisms involved.

# 3.0 Chapter 3

# Contributing mechanisms involved in the adverse outcomes that follow exposure of COCs to GlcN during IVM

## 3.1 Summary

Previously, it has been demonstrated that exposure of cow and pig COCs to GlcN during in vitro maturation results in reductions in oocyte developmental competence. In Chapter 2 this was also shown to occur in mouse COCs when higher levels of glucose were also present. Using the mouse model, some of the mechanisms contributing to the decrease in developmental competence have been elucidated. This study has confirmed that exposure of mouse COCs to GlcN during in vitro maturation perturbs developmental competence. As with cattle oocytes, inhibition of O-linked glycosylation of unknown proteins within the mouse COC significantly reversed the influence of GlcN. This was evidenced by the improvement in blastocyst development that occurred when IVM was performed with 2.5 mM GlcN and either 1 mM or 2 mM of the O-linked glycosylation inhibitor Benzyl-2-acetamido-2-deoxy-α-d-galactopyranoside (BADGP). Furthermore, it was also determined that the influence of GlcN is likely mediated by the cumulus cell vestment. When oocytes were matured in 0 or 2.5mM GlcN ± cumulus cells (CC), blastocyst development was significantly greater when IVM had occurred with cumulus cells and without GlcN. Development was reduced equivalently by performing IVM in 2.5 mM GlcN + CC, 0 mM GlcN – CC or 2.5 mM GlcN – CC indicating that there was no additive effect of GlcN exposure and cumulus cell removal. Within these same groups, significantly fewer oocytes matured with cumulus cells but without GlcN were observed to have blue cytoplasms when visualised following treatment with the G6PDH marker Brilliant Cressyl Blue, suggesting that they underwent more efficient PPP metabolism. Rates of staining were comparable between the remaining three groups, suggesting that not only may GlcN inhibit the pentose phosphate metabolic pathway within the oocyte, but also that this effect could be mediated by cumulus cells in intact COCs. Finally, when oocytes underwent IVM ± CC ± 2.5 mM GlcN ± 2 mM BADGP, it was shown that the highest rates of blastocyst development from oocytes occurred when IVM was performed in 0 mM GlcN + CC or 0 mM GlcN + CC + BADGP. In contrast performing IVM either with GlcN or without cumulus cells yielded consistently poor outcomes with the exception of maturation that was performed with 2.5 mM GlcN + CC + 2.5 mM BADGP. That improvements in developmental outcomes following the addition of BADGP to GlcN-containing media only occurred in the presence of cumulus cells, implicates cumulus cells in altered O-linked glycosylation processes that occur within COCs in response to GlcN exposure, Collectively, these results demonstrate that GlcN inhibits the developmental competence of in vitro matured mouse oocytes, and suggest that this occurs by cumulus cell mediated mechanisms.

## 3.2 Introduction

In many different types of somatic cells, GlcN has been shown to affect glucose metabolic pathways. Commonly, this has been shown to occur by stimulating the production of GlcN-6-phosphate and thereby up regulating the HBP (Virkamaki & Yki-Jarvinen 1999; Nelson et al. 2000; Marshall et al. 2004; Marshall et al. 2005(a); Marshall et al. 2005(b)). Still other studies have shown that GlcN has an inhibitory effect on PPP activity, due to GlcN-6-phosphate (the hexosamine intermediate that GlcN is converted to) inhibiting the first rate limiting enzyme of the PPP, glucose-6-phosphate dehydrogenase (G6PDH) (Bessell & Thomas, 1973; Kanji et al.1976; Horal et al. 2004).

The metabolic requirements of the maturing COC (which is comprised of one germ cell and thousands of somatic cells) are distinctive from a range of other somatic cells. Unlike somatic cells that flux 1 - 3% of glucose through the HBP (Marshall et al. 1991(a)), bovine COCs flux differential concentrations through the hexosamine pathway at different stages of maturation (Sutton et al. 2003; Sutton-McDowall et al. 2004), with a peak flux estimated to be around 24% (Gutnisky et al. 2007). This is to enable COC expansion (Sutton-McDowall et al. 2004) as expanding COCs have an increasing need for hyaluronin, an end product of the Hexosamine pathway (Eppig 1979; Ball et al. 1982; Talbot 1984; Suchanek et al. 1994). Given that such cellular-specific patterns of glucose metabolism exist within the COC, it is apparent that the COC's metabolic response to GlcN exposure cannot be simply inferred from the response patterns of other somatic cells or tissues.

It is likely that GlcN elicits its effects on mouse COCs during IVM by multiple mechanisms. The first probable mechanism involves an up regulation in protein O-linked glycosylation, which is a type of protein modification that manifests as changes to serine and threonine residues (Torres and Hart, 1984). O-linked glycosylation is emerging as a key feature of many proteins associated with perturbed health states, including conditions characterized by aberrant glucose and insulin metabolism (Patti et al. 1999; Akimoto et al. 2001; McClain et al. 2002; Federici et al. 2002; Vosseller et al. 2002; Fulop et al. 2007). Protein O-linked glycosylation has been identified in numerous models (Patti et al. 1999; Chen et al. 2003; Champattanachai et al. 2007; Park et al. 2007; Champattanachai et al. 2008; Madsen-Bouterse et al. 2008; Xing et al. 2008), including bovine in vitro matured COCs (Sutton-McDowall et al. 2006).

A second potential mechanism relates to potential reductions in PPP activity. Activation of the PPP has been shown to be essential for the maturation of oocytes in multiple species (Downs et al. 1996; Downs et al. 1998; Downs & Utecht 1999; Cetica et al. 2002; Herrick et

al. 2006). The mechanisms at play are poorly understood, but are thought to relate to the synthesis of the PPP products - purines (Downs 1994; Downs 1997), 5-Phosphoribosyl-1-Pyrophosphate (PRPP) as well as nicotinamide adenine dinucleotide phosphate (NADPH) and NADPH- dependent enzymes (Downs et al. 1998). There is also evidence that PPP-mediated lipid metabolism is essential for oocyte maturation (Cetica et al. 2002). Since it is known that GlcN-6-phosphate has an inhibitory effect on the first rate-limiting enzyme of the PPP, G6PDH (Bessell & Thomas, 1973; Kanji et al.1976; Horal et al. 2004), it is therefore likely that GlcN could also reduce PPP activity in COCs.

The experiments performed in this chapter have been designed to elucidate some of the mechanisms involved in perturbing the developmental competence of mouse COCs exposed to GlcN. Specifically, this has been approached by assessing the impact of inhibiting GlcN-mediated increases in O-linked glycosylation, ascertaining the role of cumulus cells in this pathology and assessing the impact of exposing the COC to GlcN on the PPP.

## 3.3 Methods and materials

Except where otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St Louis, USA)

### 3.3.1 Animals

Twenty-one to 23-day-old CBAB6F1 mice were obtained from Laboratory Animal Services at the University of Adelaide, or from an in-house breeding colony maintained at The Queen Elizabeth Hospital (TQEH). They received an IP injection of 5IU eCG (Folligon; Intervet, Boxmeer, Holland). Mice were caged for a further 46 hours on a 14:10 hour light: dark cycle, kept in animal housing facilities at either the Queen Elizabeth Hospital (TQEH) in Woodville, South Australia, or at the University of Adelaide Medical School, South Australia.

### 3.3.2 COC Collection and In Vitro Maturation

Forty-six hours after eCG injection, mice were sacrificed by cervical dislocation and ovaries were placed into 37ºC handling medium (MOPS-G2) (Lane and Gardner (2004)) supplemented with 1 mg/ml fetuin. Two to four ovaries were placed into 35 mm Falcon 1008

culture dishes (Becton-Dickinson Labware, Franklin Lakes, USA) with 1 - 2 ml warmed handling medium, and COCs were obtained by rupturing swollen follicles with a 30 gauge needle. COCs with normal morphology and surrounded completely by cumulus cells were washed in fresh, 37ºC handling media three times, then 30 oocytes (± cumulus cells, as indicated per experiment) per treatment group were placed into 3000 µL IVM media (MG2.2) (Lane and Gardner (2004)) with a glucose concentration of 5 mM and that was supplemented with 50 mIU/ml recombinant human FSH (Puregon; Organon, Sydney, Australia) and 1 mg/ml fetuin, equilibrated in 35 mm Falcon dishes (37°C in 6%$CO_2$:5%$O_2$:89%$N_2$) for 4 - 18 hours) and matured for 17 hours, except where otherwise indicated. Where specified, denuded oocytes were obtained by vortexing unexpanded COCs for consecutive 30 second intervals until cumulus cells were removed.

### 3.3.3 IVF

Matured COCs or oocytes were quickly washed twice in αMEM (Invitrogen, Carlsbad, USA) supplemented with 50 µg/ml streptomycin, 75 µg/ml penicillin G (Sigma Chemical Co. (St. Louis, MO)) and 3 mg/ml fatty acid free BSA (ICPBio, Auckland, New Zealand). COCs or oocytes (30 per treatment) were then transferred to 100 µL drops of the same αMEM media prepared as above containing capacitated sperm from a CBAB6F1 male of proven fertility, overlaid with mineral oil and cultured for 4 hours (37°C in 6%$CO_2$:5%$O_2$:89%$N_2$).

### 3.3.4 Embryo culture

Presumptive zygotes were placed into approximately 2 ml MOPS-G1 and residual cumulus cells were removed by gentle and repetitive pipetting, before being washed through a subsequent MOPS-G1 drop. Zygotes were placed into G1.2 (prepared according to Lane and Gardner (2004) and supplemented with 5% HSA (Vitrolife, Gothenburg, Sweden)) at a concentration of 5 zygotes/ 10 µL (media overlaid with mineral oil and equilibrated for 4-18 hours at 37°C in 6%$CO_2$:5%$O_2$:89%$N_2$)) for 20 hours. Two cell embryos were then transferred to fresh G1.2 drops (prepared as above) at a concentration of 4 - 5 embryos per 10 µL media drop. Twenty-four hours later, embryos were transferred to G2.2 (prepared according to Lane and Gardner (2004) and supplemented with 5% HSA) at a concentration of 4-5 embryos per 10 µL drop under mineral oil, equilibrated as described above, and cultured to the blastocyst stage for 48 hours.

#### *3.3.4.1 Experiment 1 – Examining the contribution of O-linked glycosylation following GlcN treatment to perturbed developmental competence*

Benzyl-2-acetamido-2-deoxy-α-d-galactopyranoside (BADGP), an inhibitor of O-linked glycosylation, was dissolved in grade 1 ethanol (Sutton- McDowall et al. 2006). COCs were then assigned to one the following seven groups: 0 mM GlcN; 0 mM GlcN + 2 mM EtOH; 0 mM GlcN + 2 mM BADGP; 2.5 mM GlcN; 2.5 mM GlcN + 2 mM EtOH; 2.5 mM GlcN + 1 mM BADGP; 2.5 mM GlcN + 2 mM BADGP. At the completion of maturation and IVF, embryo culture was performed, and rates of 2 cell and blastocyst development were assessed. Five replicates were performed.

#### *3.3.4.2 Experiment 2 – Determining the roles of the cumulus cells and the oocyte in the GlcN- mediated depression of developmental competence*

IVM was performed in the presence or absence of 2.5 mM GlcN, using oocytes with or without cumulus cells.

##### 3.3.4.2.1 Part A: Development

Rates of 2 cell and blastocyst development were assessed. Six replicates were performed.

##### 3.3.4.2.2 Part B: Examining the effects of exposing the oocyte to GlcN on activity of the first rate limiting enzyme of the PPP, G6PDH.

IVM was performed for 15.5 hours using the following 4 groups: 0 mM GlcN ± cumulus cells, 2.5 mM GlcN ± cumulus cells. After 15.5 hours of IVM, oocytes or COCs were transferred into IVM media containing 26 µM brilliant cresyl blue (BCB) and matured for the remaining 1.5 hours of IVM. BCB is reduced by G6PDH, the first rate limiting step of the PPP. High levels of cellular G6PDH permit BCB to be metabolised efficiently, meaning the blue stain is not retained in the cytoplasm. G6PDH levels in mouse oocytes were determined using an adaptation of BCB methods described by Pujol et al. (2004). At the completion of IVM, cumulus- enclosed oocytes were denuded with a 100 µL pipette. Denuded oocytes were washed 4 - 6 times in 200 µL drops of warmed MOPS medium until the media was clear of

blue stain. Oocytes were immediately visualized using a stereomicroscope (x400 magnification), and were scored as being either deficient of or containing a blue coloured cytoplasm. Three replicates of this assay were performed.

#### 3.3.4.2.3 Part C: Measurement of Reactive Oxygen Species (ROS)

IVM was performed on the following 4 groups: 0 mM GlcN ± cumulus cells, 2.5 mM GlcN ± cumulus cells. At the completion of IVM, intact COCs were denuded and oocyte ROS activity was determined via a 2',7'-dichlorodihydrofluorescein diacetate (DCDHFDA) fluorescence assay previously described by Wakefield et al. (2008) which has been widely used to measure intracellular levels of $H_2O_2$. Briefly, per replicate and treatment group, 5 (control) oocytes were incubated in the dark at 37ºC with 5,6-carboxyl-2',7'-dichlorodihydrofluorescein diacetate (CDCFDA) (Sigma Chemical Co.). After 20 minutes, oocytes were washed quickly in 37ºC MOPS-G1, transferred to microscope slides and fluorescence spectroscopy was used to measure the fluorescence of CDCF product. The average fluorescence per treatment and per replicate was then calculated. Remaining oocytes from each treatment were simultaneously incubated under the same conditions with 1 µM 2',7' dichlorofluorescein diacetate (DCDHFDA) for 30 minutes. They were then washed quickly in 37ºC MOPS-G1 and fluorescence spectroscopy was used to measure the fluorescence of DHF product. Ratios for individual fluorescence values for each oocyte relative to the mean value of control oocytes were then determined. Fluorescence was expressed as mean fluorescence units, and a total of six replicates were performed.

### 3.3.4.3 Experiment 3: Determining the roles of the cumulus cells and the oocyte in the BADGP – mediated improvement in oocyte developmental competence following exposure to GlcN

IVM was carried out in the following twelve groups: 0 mM GlcN ± 2 mM EtOH / 2 mM BADGP/ cumulus cells, 2.5 mM GlcN ± 2 mM EtOH / 2 mM BADGP/ cumulus cells. At the completion of maturation IVF and embryo culture was performed, and rates of 2 cell and blastocyst development were assessed. Six replicates were performed.

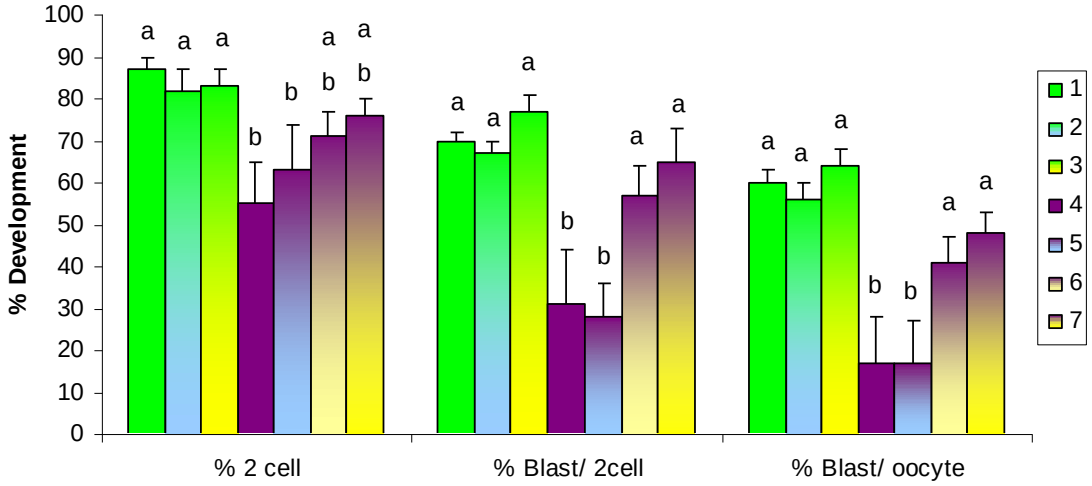
## 3.4 Statistical Analysis

All data were arcsine transformed and analyzed with SPSS (version 13.0). A One-way ANOVA was used to analyze rates of 2 cell and blastocyst (from 2 cell embryos as well as from oocytes) development for experiments 1 and 3. A Two-way ANOVA was used to analyze rates of 2 cell and blastocyst (from 2 cell embryos as well as from oocytes) development for experiment 2a, as well as to compare G6PDH and ROS activity in experiments 2b and 2c respectively. Bonferroni post-hoc tests were used for all analyses.

## 3.5 Results

### 3.5.1 Experiment 1: Examining the contribution of O-linked glycosylation following GlcN treatment on perturbed developmental competence

Since O-linked glycosylation is an end product of Hexosamine metabolism, and GlcN up regulates Hexosamine metabolism, it was sought to be determined whether inhibiting O-linked glycosylation would improve developmental outcomes following exposure of COCs to GlcN (Figure 1). The results reveal that EtOH, the vehicle that BADGP was dissolved in, had no influence on outcomes. It was shown, in agreement with results generated from experiments in Chapter 1, that the incorporation of GlcN into IVM media had a detrimental effect on 2-cell development ($P = 0.002$). There was a trend ($P<0.08$) for a reduction in the effect of GlcN on 2-cell development following the incorporation of BADGP with GlcN, although this was not significant. Also consistent with results from chapter 1, GlcN had a negative effect on blastocyst development, which was largely reversed by the incorporation of both 1 mM and 2 mM BADGP in regards to blastocyst development from 2 cell embryos ($P<0.001$) as well as from oocytes matured ($P = 0.017$). Interestingly, there was also a trend ($P<0.066$) for improved rates of blastocyst development following the incorporation of 2 mM BADGP in the absence of GlcN, although this was not significant. Collectively, these results provide evidence that the detrimental effects of GlcN exposure on developmental potential of COCs is, at least in part, mediated by an increase in O-linked glycosylation.

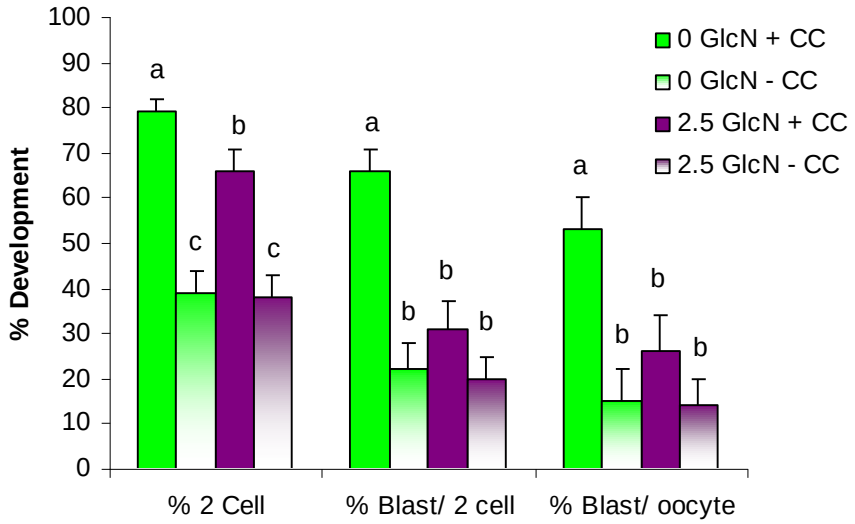



**3.5.1.1 Figure 1:** Rates of 2 cell. blastocyst development from 2 cell and blastocyst development from oocytes, following COC maturation involving exposure to GlcN and the O-linked glycosylation inhibitor, BADGP. (Groups: **1** = 0 mM GlcN, **2** = 0 mM GlcN + 2% Et OH, **3** = 0 mM GlcN + 2 mM BADGP, **4** = 2.5 mM GlcN, **5** = 2.5 mM GlcN + 2% Et OH, **6** = 2.5 mM GlcN + 1 mM BADGP, **7** = 2.5 mM GlcN + 2 mM BADGP). Different letters represent significant differences ($P<0.05$) between groups, within developmental stages. Values expressed as mean ± SEM

### 3.5.2 Experiment 2: Determining the roles of the cumulus cells and the oocyte in the GlcN- mediated depression of developmental competence

#### 3.5.2.1 Part A: Development

Highest rates of development to 2-cell and to blastocyst were observed in the control treatment groups (0 mM GlcN + cumulus cells) (Figure 2). Oocyte developmental competence was reduced by GlcN ($P<0.05$ for % 2 cell development, $P<0.0001$ for % blastocyst development from 2 cell embryos and for % blastocyst development from oocytes) as well as cumulus cell absence (all $P<0.0001$) (Figure 2). However, there was no additive effect of GlcN and cumulus cell removal. This lack of additive effects when oocytes void of cumulus cells are matured with GlcN, indicates that the perturbing effect on oocyte developmental competence that follows GlcN exposure occurs via modifications to cumulus cells.



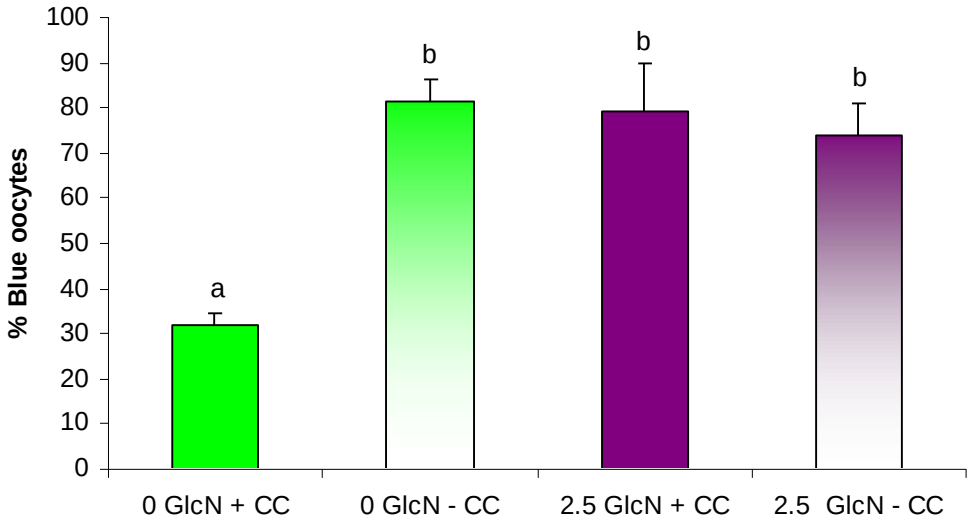

**3.5.2.1.1 Figure 2:** Rates of 2 cell, blastocyst from 2-cell and blastocyst from oocyte development following oocyte maturation ± intact cumulus cells (CC), ± 2.5 mM GlcN. Different letters represent significant differences ($P<0.05$) between groups, within developmental stages. Values are expressed as mean ± SEM.

### *3.5.2.2 Part B: Examining the effects of exposing the oocyte to GlcN on activity of the first rate limiting enzyme of the PPP, G6PDH.*

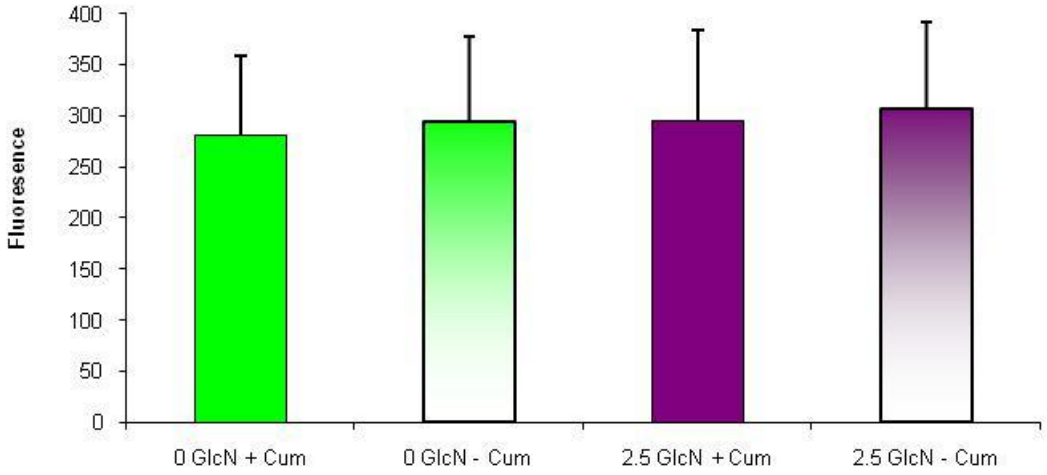
Increased numbers of oocytes presented with a blue cytoplasm when cumulus cells were removed, or GlcN was added during IVM, compared to the control group matured with cumulus cells in the absence of GlcN ($P<0.002$) (Figure 3). This indicated that the control oocytes had a higher level of G6PDH activity, and hence were able to metabolize BCB more efficiently. Equivalent effects on G6PDH activity were induced by either performing IVM in the absence of cumulus cells, or in the presence of cumulus cells + GlcN, thereby suggesting that GlcN perturbs the PPP activity of the oocyte via eliciting perturbations to the cumulus cells, which then mediates effects on G6PDH activity in oocytes.

### *3.5.2.3 Part C: Measurement of the PPP end product, Reactive Oxygen Species (ROS)*

Since either cumulus cell removal or GlcN exposure modified G6PDH activity, levels of reactive oxygen species (ROS), a further downstream product of the PPP, were assessed. There were no differences in ROS production, measured as fluorescence intensity, between oocytes matured either intact or denuded, or in the presence or absence of GlcN (Figure 4).




**3.5.2.2.1 Figure 3:** Proportion of oocytes staining positive for the G6PDH marker, BCB following oocyte maturation ± intact cumulus cells (CC), ± 2.5 mM GlcN. Different letters represent significant differences (P<0.05) between groups. Values are expressed as mean ± SEM.



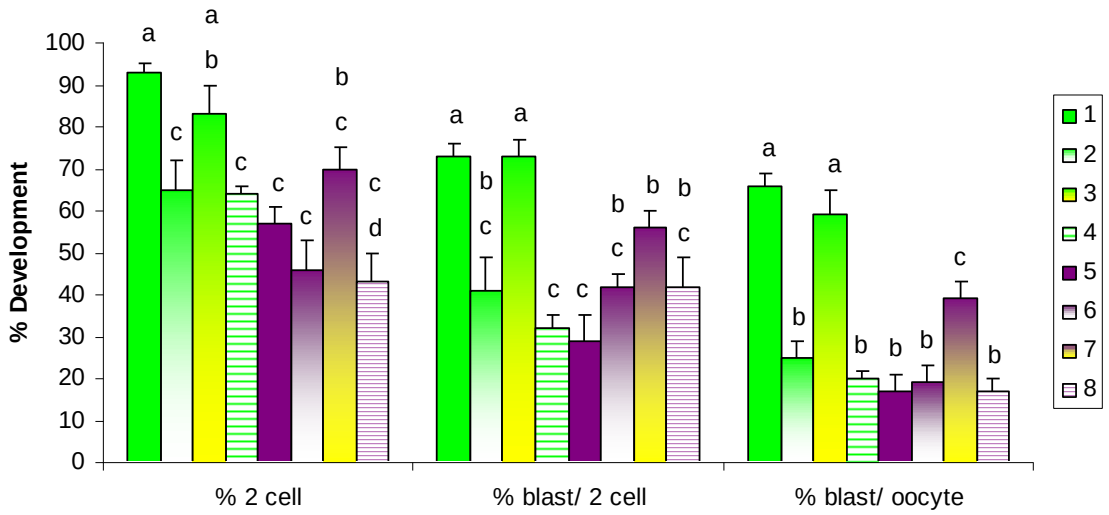

**3.5.2.3.1 Figure 4:** ROS activity of oocytes matured ± GlcN, ± cumulus cells. Values are expressed as mean ± SEM.

### 3.5.3 Experiment 3: Determining the roles of the cumulus cells and the oocyte in the BADGP - mediated improvement in oocyte developmental competence following exposure to GlcN

In experiment 3, it was shown that a contributing factor to the adverse outcomes that are experienced by COCs exposed to GlcN is the elevation in O-linked glycosylation. Since it had been shown that GlcN probably exerted its effects on cumulus cells (experiment 2), it was sought to be tested whether GlcN- perturbing effects on O-linked glycosylation were occurring in cumulus cells and/ or the oocyte.

Relative to the groups matured with cumulus cells and without GlcN (0 mM GlcN + cumulus cells (group 1), and 0 mM GlcN + cumulus cells + BADGP (group 3) most other treatments had significantly lower rates of 2 cell development. Only the group that underwent IVM in 2 mM GlcN + 2 mM BADGP in the presence of cumulus cells (group 7) showed signs of improvement, with rates of 2 cell development only being lower than that of group 1 (i.e. the positive control, $P<0.05$). However, when rates of blastocyst development from 2 cell embryos were assessed, this same group (group 7) was only significantly better than the 0 mM GlcN - CC + 2 mM BADGP (group 4) and the 2.5 mM GlcN + CC groups (group 5). No groups had development rates as high as groups matured with cumulus cells and without GlcN ($P<0.05$) (groups 1 and 3). A clear picture emerged from analysis of results for blastocyst development from matured oocytes. Here, it was shown that, although still significantly lower than rates for groups matured with cumulus cells but without GlcN (groups 1 and 3), the development rate in the 2 mM GlcN + 2 mM BADGP + CC group (group 7) was significantly higher than all other remaining groups ($P<0.05$) (Figure 5).

In agreement with the findings of experiment 3, these results show that the elevation in O-linked glycosylation that follows GlcN exposure is a likely cause for poor embryonic development. Furthermore, they also demonstrate that it is primarily the O-linked glycosylation occurring within cumulus cells, and not the oocyte, that mediates this effect.




**3.5.3.1 Figure 5:** Rates of 2 cell, blastocyst from 2 cell embryos and blastocyst from oocyte development following COC exposure to GlcN ± BADGP ± cumulus cell deficiency. **1** = 0 mM GlcN + CC, **2** = 0 mM GlcN – CC, **3** = 0 mM GlcN + CC + 2 mM BADGP, **4** = 0 mM GlcN - CC + 2 mM BADGP, **5** = 2.5 mM GlcN + CC, **6** = 2.5 mM GlcN – CC, **7** = 2.5 mM GlcN + CC + 2 mM BADGP, **8** = 2.5 mM GlcN CC + 2 mM BADGP. Different letters represent significant differences (P<0.05) between groups, within developmental stages. Values are expressed as mean ± SEM.

## 3.6 Discussion

The results outlined here are consistent with the findings of Sutton-McDowall et al. (2006), and Chapter 2, and have shown that exposure of the in vitro maturing COC to GlcN, has detrimental consequences on subsequent developmental competence. Also consistent with the findings of others (Chesnel et al. 1994; Chang et al. 2005(a); Luciano et al. 2005), are the findings that the developmental competence of oocytes is reduced when maturation occurs in the absence of cumulus cells (experiment 3a). These studies went further, however, and showed that cumulus cell removal and GlcN exposure significantly reduced developmental competence in an equivalent but not additive manner. This suggests a central role for cumulus cells in the pathology of GlcN effects on the COC, and that the effect of GlcN on oocyte competence is similar to the removal of cumulus cells. This is not to exclusively preclude the oocyte from any direct perturbation, given that GlcN radiolabel has been detected in several locations in mouse (Fowler and Guttridge, 1987; Fowler 1988; Fowler and Barratt (1989), and pig (Fléchon et al. 2003) oocytes, but rather suggests that cumulus cell function is likely greatly perturbed. Furthermore it should also be noted that numerous differences in gene expression between oocytes matured with and without cumulus cells have been documented (Xiang et al. 2010), thereby raising the possibility that differences in

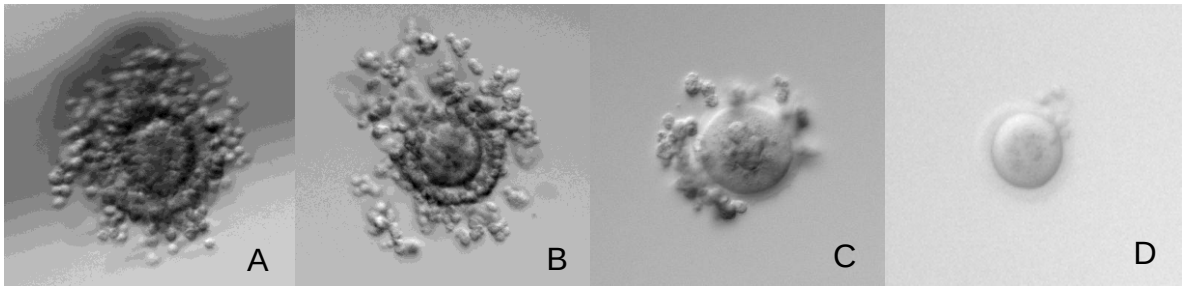
gene expression between intact COCs and denuded oocytes may have also contributed to the findings. This would not compromise the validity of the results that were obtained, but rather might impact upon the mechanistic explanation.

GlcN is known to enter cells via glucose transporters (Uldry et al. 2002). Although GLUT transporters have been found on the oocyte (Diamond et al. 1989; Moley et al. 1991; Moley et al. 1996; Moley et al. 1998(b); Keim et al. 2001; Colton et al. 2002; Colton et al. 2003; Chang et al. 2005(b); Ratchford et al. 2007; Ratchford et al. 2008; Wyman et al. 2008), and glucose metabolic pathway intermediates have been detected in the denuded oocyte (Downs and Utecht, 1999), most glucose is taken up by cumulus cells (Biggers et al. 1967). For significant quantities of GlcN to be detected in the oocyte, an alternative uptake mechanism to glucose transporters would presumably need to exist.

The effects of GlcN on developmental outcomes were inhibited by BADGP, indicating that the effects were mediated through O-linked glycosylation (experiment 1). Further experiments indicated that these effects of O-linked glycosylation modifications on oocyte developmental competence were mediated through cumulus cells (experiment 3). O-linked glycosylation is a relatively recently discovered form of protein modification (Torres and Hart, 1984), and is but one of numerous down-stream consequences of HBP up regulation. It is possible that O-linked glycosylation modifications occur in the oocyte but were not detectable by the employed methods. It is also plausible that there was negligible protein modification within the oocyte. When Sutton-McDowall et al. (2006) examined O-linked glycosylation staining in bovine COCs following GlcN exposure during IVM, they found that staining was limited to cumulus cells.

Expansion of the COC following GlcN exposure does not progress according to the stages described by van der Hyden (1990). More specifically, cumulus cells become sticky and readily separate from the complex with limited physical intervention. They also often dissociate from the complex during IVM (Figure 6). This tendency made it difficult to perform investigative procedures, such as immunohistochemistry, that require many handling stages, and therefore precluded obtaining meaningful data regarding the O-linked glycosylation status or rates of apoptosis of cumulus cells. However, such observations and the developmental data obtained from these experiments, suggest a causal role of disrupted cumulus cell-oocyte communication as a consequence of GlcN treatment, which is akin to denuding oocytes of cumulus cells. Candidate mechanisms that may be involved are both cumulus cell apoptosis and loss of gap-junction connections. Apoptosis has been shown to increase in pancreatic cells in response to elevations in O-linked glycosylation (Liu et al. 2000; Konrad et al. 2000; Park et al. 2007), and was indicated following GlcN treatment of

COCs by the tendency of cumulus cells to become sticky and clump together in dark patches. In addition, the premature loss of gap junction communication is well known to reduce developmental competence during maturation (Thomas et al. 2004).




**3.6.1 Figure 6:** COCs matured at a ratio of 100 µL IVM media (containing 5 mM glucose and 2.5 mM GlcN) per COC. **(A and B):** The outer layers of the matrix have rapidly expanded to such an extent that they are dissociating from the rest of the very modestly expanded inner cellular layers. **(C and D**): The cumulus cells have expanded so much that they have largely dissociated from the matrix, leaving few intact cells.

In developing the BCB technique, it was initially found that the BCB stain was very heavily taken up by cumulus cells, with or without GlcN exposure. Owing to the intensity of the stain, it was determined that it would not be suitable for detecting differences in PPP activity in the cumulus cells, and analysis was limited exclusively to the oocyte. This was a suitable approach, however, given that the PPP is known to be inhibited by GlcN in other models (Bessell & Thomas, 1973; Kanji et al.1976; Horal et al. 2004), and that evidence for modifications to oocyte protein O-linked glycosylation status was not found within the oocyte.

Low rates of BCB staining (indicative of high amounts of G6PDH activity, and therefore developmental immaturity (Pujol et al. 2004), were observed when maturation occurred in the absence of cumulus cells, irrespective of GlcN levels present. Oocyte PPP activity may be regulated by multiple factors, including glucose metabolic activity, as well as cumulus cells. Perturbations to either of these components may lead to impaired patterns of PPP activity and maturation, which can be detected via G6PDH analysis.

Finally, the results showed no differences in ROS activity in the COC following GlcN treatment. This was surprising, given that Hashimoto et al. (2000) found that in bovine oocytes high levels of glucose reduced rates of meiotic maturation and generated high levels of ROS. This may be reflective of inter- species differences or alternatively different effects of glucose and glucosamine. Given the high level of variability between the samples, it may

also be beneficial to explore more sensitive ROS detection methods, such as the use of the highly selective $H_2O_2$ probes.

## 3.7 Conclusion

Overall, these findings have confirmed that GlcN has a perturbing effect on oocyte developmental competence. This has been shown to be mediated by an elevation in cumulus cell O-linked glycosylation, as well as cumulus cell mediated alterations of oocyte PPP activity. Thus it would appear that the detrimental effects elicited by exposure of the maturing COC to GlcN occur through multiple mechanisms, but investigation of these was severely hampered by the hyper-expansion of the cumulus matrix in the GlcN treated oocytes.

## 4.0 Chapter 4

# In vivo GlcN exposure has a detrimental impact on reproductive outcomes in the mouse.

## 4.1 Summary

Whilst the exposure of COCs in vitro to GlcN has been associated with adverse developmental outcomes, no data has been published to date looking at the effects of in vivo GlcN exposure of the COC or early developmental period. Here, mice were administered either 0 mg/kg, 20 mg/kg or 400 mg/kg GlcN via IP injection for 4 - 7 days encompassing the peri-conceptional period. GlcN exposure, irrespective of dose or number of days of GlcN administration, reduced mean implantation number ($P<0.05$) and litter size ($P<0.05$) at day 18 of gestation, and increased the proportion of implantations that resorbed ($P<0.02$). No effect of GlcN on fetal weight and length, placental weight or incidence of birth defects was observed. These results provide the first evidence for an adverse effect of in vivo GlcN exposure during the peri-conception period on implantation and embryo development.

## 4.2 Introduction

Despite the popularity of GlcN-containing products (Kaufman et al. 2002; Wold et al. 2005; Hopman et al. 2006; Singh and Levine, 2006), only one study to date has investigated the effect of GlcN exposure during pregnancy in humans (Sivojelezova et al. 2007). This study reported no impact of GlcN exposure during pregnancy on birth weight, major malformations, gestational age at birth or pregnancy outcome (live births, spontaneous abortions, therapeutic abortions and stillbirths) (Sivojelezova et al. 2007). However, the results cannot be deemed as comprehensive, owing to the fact that a small sample size was utilized (N=54) and conditions surrounding GlcN exposure were not controlled.

Similarly, limited studies have examined the effects of GlcN administration on reproductive outcomes in animal models. An early study examining the effects of GlcN injection in pregnant mice failed to show any effect, although outcome measures were focused on maternal parameters such as vaginal bleeding and abdominal palpitations (Didock et al. 1956). Contrary to this, however, a more recent study showed that acute administration of GlcN to mice on day 7.5 of pregnancy was associated with an increase in the incidence of neural tube defects in the fetuses (Horal et al. 2004). Adverse effects on embryo development have also been observed following exposure of the bovine, porcine and murine COC to GlcN during IVM (Sutton-McDowall et al. 2006; Kimura et al. 2008, Chapters 2 and 3 of this thesis).

GlcN is used under experimental conditions as a hyperglycemic mimetic (Rossetti et al. 1995; Hawkins et al. 1997; Horal et al. 2004; Yki-Jarvinen et al. 1998; Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Rumburger et al. 2003; Marshall et al. 2004). Hyperglycemia in the mouse (Cockroft and Coppola, 1977; Phelan et al. 1997) and diabetes in humans (Soler et al. 1976; Macintosh et al. 2006) have also been linked to an increased incidence of neural tube defects, resembling the effects of GlcN exposure during pregnancy in the mouse (Horal et al. 2004). Increased neural tube defects, along with abdominal wall and limb deformities, were also observed in mouse fetuses derived from zygotes produced in vivo in a diabetic environment and then transferred into normoglycemic recipients (Wyman et al. 2008). This is an important finding as it demonstrates that hyperglycemic exposure that is limited to the peri-conceptional period is sufficient to induce congenital malformations.

Given the documented effects of GlcN exposure during pregnancy in mice (Horal et al. 2004), and the in vitro effects of GlcN on COC developmental competence in mouse (Chapters 2 and 3), bovine and porcine models (Sutton-McDowall et al. 2006, Kimura et al. 2008), the current study was designed to assess if in vivo peri-conceptional GlcN exposure

would also elicit detrimental effects on reproductive outcomes. This is the first study performed to investigate the effects of exposing maturing COCs and early embryos to GlcN in vivo, and was designed to not only document reproductive outcomes that follow in vivo GlcN exposure, but also to assess the plausibility of the use of GlcN as a novel, non-hormonal contraceptive.

## 4.3 Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, USA).

### 4.3.1 Animals

For all procedures, C57BL6 male and female mice (purchased from Laboratory Animal Services at The University of Adelaide) were used, and kept under 14:00 - 10:00 light-dark conditions in the animal housing facilities at the Queen Elizabeth Hospital (TQEH) in Woodville, South Australia. This strain was selected owing to its increased susceptibility to obesity and diabetes, both conditions in which elevated HBP activity can occur.

For each of 8 replicates, nine 7-week old naturally cycling female mice were weighed and divided into three, weight matched groups. One week later, groups were randomly allocated into one of three groups: 0 mg/kg GlcN, 20 mg/kg GlcN or 400 mg/kg GlcN. Twenty mg/kg GlcN was determined as an approximation of the standard recommended dose for adult humans (i.e. 1500 mg per day), whilst the 400 mg/kg GlcN dose was chosen as a supra-physiological dose. Mice were weighed, and volumes of either saline (0 mg/kg GlcN) or GlcN solution that needed to be administered were calculated. Mice were injected for 4 - 7 days according to the following methodology.

### 4.3.2 Treatment regime

Five µL solution per gram of body weight (Dulbecco PBS for the 0 mg/kg GlcN, 4 mg/ml GlcN (dissolved in PBS) solution for the 20 mg/kg group and 40 mg/ml GlcN for the 400 mg/kg group) was administered (IP) for 3 consecutive days at 24 hour intervals. Following the third injection, females were housed overnight with males, and overnight mating success was determined via the detection of vaginal plugs early the next morning. Successfully mated females were administered with a final injection, 24 hours after the previous injection.

Females that did not successfully mate were again injected (24 hours after the previous injection), then re-introduced to males for attempted overnight mating. This process occurred until mating was achieved (followed by a final injection 24 hours after the previous injection), or until mating had been attempted for a maximum of 4 days, with those that did not mate being excluded. Males were randomly allocated to females from different treatment groups throughout all replicates.

### 4.3.3 Pregnancy outcomes

On day 18 of pregnancy, mice were killed via cervical dislocation and post mortem examinations were performed. The number of pregnant mice from those that had successfully mated was recorded. Total fetuses and resorptions were counted in each horn. The total number of viable fetuses was used to define litter size. Resorptions were comprised of either dark clumps within the uterine horn, or conceptuses that had undergone development to a significant enough stage to render them recognizable, but that had died prematurely. Each fetus and its corresponding placenta were weighed and examined briefly for gross morphological appearance and classified as either normal or abnormal. Fetal crown-rump length and placenta length and width were recorded.

## 4.4 Statistical Analysis

Proportional data was arcsine transformed prior to analysis and all data was analysed with SAS (Statistical Analysis Software) (Version 9.2). Mean litter and fetal outcome – related data were analysed using a one-WAY ANOVA, with and without the incorporation of co-variates, (see Appendix, Table 8.11.1) and using Bonferonni Post-hoc tests. Results stated in the text or presented in the figures are least–squared means, unadjusted for covariates. Proportional data was tested with a Chi-squared analysis. Birth defect data was not analysed owing to an insufficient sample size.

## 4.5 Results

### 4.5.1 Pregnancy rates

GlcN treatment did not affect the number or proportion of mice that mated, as indicated by the presence of a vaginal plug. Similarly, there were no differences between treatment groups in the number of mice that were pregnant on day 18, or the proportion of mice that were pregnant relative to starting numbers or relative to those that successfully mated (Table 1).

| | 0 mg/kg GlcN | 20 mg/kg GlcN | 400 mg/kg GlcN |
|---|---|---|---|
| **Mated (N)** | 22 | 19 | 18 |
| **Mated (%)** | 81.5 | 70.4 | 66.6 |
| **Pregnant (N)** | 13 | 14 | 12 |
| **Pregnant from total (%)** | 54.2 | 58.3 | 50 |
| **Pregnant from plugged (%)** | 59.1 | 73.7 | 66.7 |

**4.5.1.1 Table 1:** Pregnancy data for mice administered for 4 - 7 days peri-conceptionally with 0 mg/kg GlcN, 20 mg/kg GlcN or 400 mg/kg GlcN.

Significantly more mice that received injections for 4 - 5 days had implantations on day 18 of pregnancy, when compared to mice that received injections for 6 - 7 days ($P<0.0001$). (Refer to Appendix, Table 8.12.1, for results).

### 4.5.2 Litter outcomes

Data describing the total implantation number, resorption number and the number of viable d18 fetuses is provided in Table 2. Mean implantation rate and litter size were reduced by GlcN treatment ($P<0.05$), but there were no differences in mean resorption rates per litter (Figure 1). However, a Chi-squared analysis of the total proportion of implantations that resorbed showed that this rate was significantly higher following GlcN treatment (20 mg/kg

GlcN = 29.4%, 400 mg/kg GlcN = 38.7%), then occurred in controls (mg/kg GlcN = 15.7%) (P = 0.01).

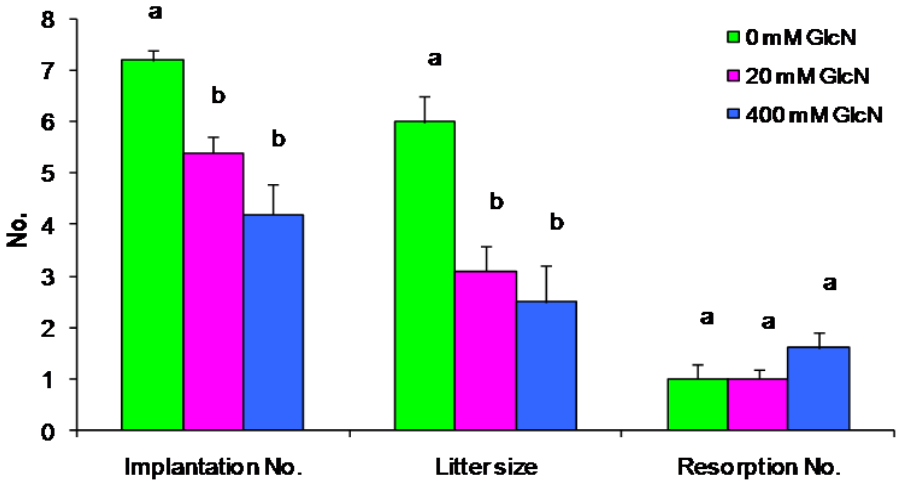
| | 0 mg/kg GlcN | 20 mg/kg GlcN | 400 mg/kg GlcN |
|---|---|---|---|
| **Total number of implantations** | 89 | 51 | 62 |
| **Total number of viable d18 fetuses** | 75 | 36 | 38 |
| **Total number of resorptions** | 14 | 15 | 24 |

**4.5.2.1 Table 2**: The total number of implantations, viable fetuses and resorptions on d18 of pregnancy (for 8 replicates) for mice administered for 4 - 7 days peri-conceptionally with 0 mg/kg GlcN, 20 mg/kg GlcN or 400 mg/kg GlcN. Different letters represent significant differences (P<0.05) between groups, within developmental

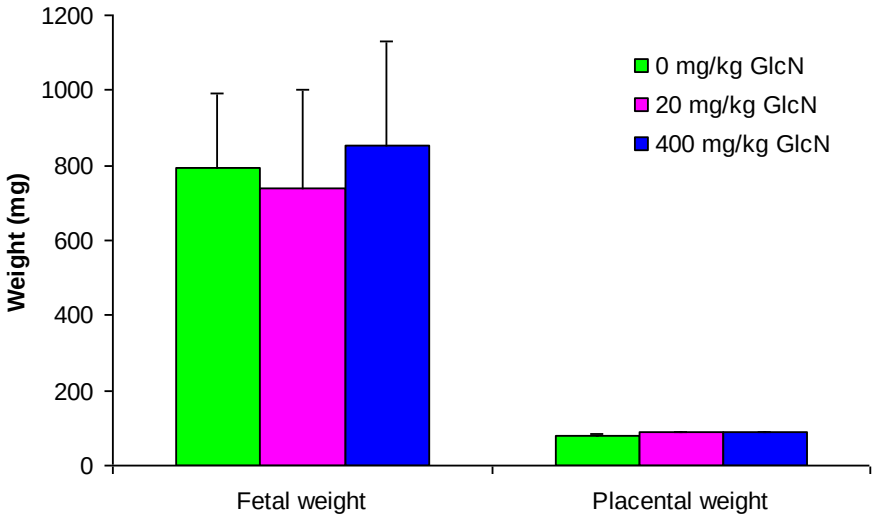
The proportions of implantations that had developed into viable fetuses at day 18 of pregnancy was also calculated from total numbers, but no differences were detected across groups (0 mg/ kg GlcN = 85.4%, 20 mg/kg GlcN = 70.6%, 400 mg/kg GlcN = 62.9%).

### 4.5.3 Fetal outcomes

Mean fetal and placental weights are shown in Figure 2. Peri-conceptional GlcN treatment did not alter fetal or placental weight. Placental weights trended towards being lower in conceptuses from control mothers (81.2 ± 2.2 mg) relative to those from mothers treated with GlcN (20 mg/kg GlcN = 88.7 ± 3.0 mg, 400 mg/kg = 88.3 ± 3.1 mg), but this was not significant (P = 0.078). Fetal length was not significantly different between groups (0 mg/kg GlcN = 18.0 ± 0.3 mm (n = 75); 20 mg/kg GlcN group = 15.9 ± 0.7 mm (n = 36) and 400 mg/kg GlcN = 19.9 ± 0.8 mm (n = 38)). Birth defects as determined by gross morphological appearance on d18 gestation were minimal. In the control group there was one fetus that presented with one very small eye. In the 20 mg/kg group one fetus had an abdominal hernia, and in the 400 mg/kg group one fetus had a grossly disproportionately large head relative to the rest of its body.




**4.5.3.1 Figure 1:** Implantation, litter size and resorption data on d18 of pregnancy for mice following peri-conception administration (IP) for 4 - 7 days with 0 mg/kg GlcN, 20 mg/kg GlcN or 400 mg/kg GlcN. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) between groups, within developmental stages.




**4.5.3.2 Figure 2**: Fetal and placental weight on d18 of pregnancy for mice following peri-conception administration (IP) for 4 - 7 days with 0 mg/kg GlcN, 20 mg/kg GlcN or 400 mg/kg GlcN. Values are expressed as mean ± SEM.

## 4.6 Discussion

In vitro studies in bovine and porcine models (Sutton-McDowall et al. 2006; Kimura et al. 2008) and the results reported in Chapter 2 and 3, have provided strong evidence for a perturbing effect of GlcN on oocyte developmental competence. The ability of GlcN to elicit hyperglycemic effects (Rossetti et al. 1995; Hawkins et al. 1997; Horal et al. 2004; Yki-Jarvinen et al. 1998; Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Rumberger et al. 2003; Marshall et al. 2004) is also a well documented phenomenon. To date, however, very little focus has been directed towards the potential for in vivo effects of GlcN on events surrounding the peri-conceptional period. GlcN label is able to be detected within the oocyte following in vitro incubation (Flechon et al. 2003). GlcN label has also been detected within the ovarian follicle and oocyte following in vivo IP injection (Fowler and Guttridge, 1987; Fowler 1988, Fowler and Barrat, 1989), and GlcN has been shown to regulate follicular development when infused (Muñoz-Gutiérrez et al. 2002; Muñoz-Gutiérrez et al. 2004). This, along with the results outlined in this chapter, provides evidence that in vivo administered GlcN may not be exclusively metabolized externally to the reproductive system.

Litter size was reduced by GlcN treatment in the current study. Whether this decrease in implantation rate and litter size is due to a reduction in ovulation rate, fertilization failure, or reduced viability of the pre-implantation embryos could not be assessed in this study. However, the reduction but not a complete abolition of litter size in the presence of supraphysiological GlcN exposure, would suggest that the potential to use GlcN as a non-hormonal contraceptive is unlikely. Multiple studies have shown an inhibitory effect of diabetes on ovulation rate (Chieri et al. 1969; Kirchick et al. 1978; Jawerbaum et al. 1996; Colton et al. 2002)**,** although the results from these studies may reflect more prolonged periods of hyperglycemic exposure than was used in our model. It is also possible that acute elevations in HBP activity, as potentially occurred from our experimental treatment, may have affected ovulatory processes, although this was not examined.

An alternative possibility is that the perturbing effects of peri-conceptional GlcN exposure are manifested at post-ovulatory events. In vitro, GlcN exposure of maturing mouse COCs altered the morphology of cumulus cell matrix formation (Chapter 2). In preliminary studies (not included), a similar morphology was also observed within in vivo matured COCs collected from females treated with GlcN. Therefore it is possible that IP administration of GlcN perturbs the process of cumulus cell expansion, which may impede sperm penetration and fertilization. In vitro experiments in the mouse (Chapters 2 and 3) as well as in bovine and porcine models (Sutton-McDowall et al. 2006; Kimura et al. 2008) show that post–cleavage embryo development is perturbed when COCs have been exposed to GlcN, which

suggests that in vivo development may also become impaired during post-fertilization events. Perturbations may also have occurred post implantation. Despite the lack of difference in resorption numbers, there were significantly greater proportions of resorptions in mothers treated with GlcN. Hence, the effects of GlcN on implantation rate and litter size on day 18 of gestation may be reflective of events at multiple developmental time points. Further studies are required to specifically test this.

Interestingly, the results outlined in this chapter differ from those obtained in a novel experiment where zygotes flushed from diabetic mice were transferred to healthy surrogates and pregnancy outcomes were assessed on day 14.5 of pregnancy (Wyman et al. 2008). Unlike the current findings, the authors noted no effect on implantation or resorption rate, but they did observe an increase in the rate of fetal malformations and growth retardation, following exposure of the oocyte and zygote to a diabetic environment. Differences between studies in the stage of pregnancy at which the outcomes were assessed could contribute to the discrepancies observed in effects on fetal growth. One possibility is that catch up growth may have occurred between day 14.5 and 18 in fetuses in the current study. However, other differences in outcomes between the studies, such as effects on implantation rates, are unlikely to relate to differences occurring in late gestation.

GlcN is routinely used as a hyperglycemic mimetic, owing to its direct up regulation of the HBP, however, it has been found that GlcN does not completely replicate all of the effects of exposure to elevated concentrations of glucose. For example, in adipocytes, translocation of GLUT 1 (Hresko et al. 1998) and GLUT 4 (Hresko et al. 1998; Nelson et al. 2000) to the plasma membrane is increased by glucose but inhibited by GlcN, despite the fact that both elevated concentrations of glucose as well as GlcN induce insulin resistance (Nelson et al. 2000). Furthermore, Akt phosphorylation in adipocytes is inhibited by exposure to high levels of glucose, but is unaffected by GlcN (Nelson et al. 2002). Although these results were obtained in adipocytes, they demonstrate differences in the mechanisms of action, and outcomes of exposure to elevated levels, of glucose and GlcN. Therefore, the effects of peri-conceptional GlcN-induced elevations in HBP activity on subsequent fetal development may differ from the effects of the metabolic perturbations associated with peri-conceptional diabetes.

## 4.7 Conclusion

Collectively, these results demonstrate that in vivo, peri-conceptional GlcN exposure has detrimental effects on pregnancy outcomes in mice by reducing mean implantation rate and litter size. The results highlight the value of GlcN as a tool to study the effects of select hyperglycemia-stimulated pathways, given that GlcN modifies only a selection of metabolic events perturbed by hyperglycemia.

# 5.0 Chapter 5

## Fetal and post natal outcomes following peri-conceptional GlcN Exposure in Lean and Overweight Mice

## 5.1 Summary

The Hexosamine metabolic pathway can be up-regulated by several factors, including hyperglycemic stimulation that accompanies conditions such as overweight/ obesity and diabetes, as well as by GlcN. In this study it was sought to be tested if the detrimental effects of peri-conceptional GlcN exposure observed in Chapter 4 would be altered in models with dietary-induced obesity. C57BL6 mice were maintained on a low fat (LF) (6%) or high fat (HF) (22%) diet for 11 weeks. At the end of this period there were significant differences in mean body weight, but no difference in glucose and insulin metabolic parameters between the groups. GlcN (0 or 20 mg/kg GlcN) was administered for 4 - 5 days, and mice were mated and then sacrificed on gestational d18 and assessed for a variety of pregnancy outcomes. Peri-conceptional GlcN exposure reduced fetal weight ($P = 0.023$). Maternal HF diet, from before and throughout pregnancy, reduced fetal weight ($P = 0.013$) and fetal length ($P = 0.02$). The number ($P = 0.001$) and proportion ($P = 0.004$) of fetuses with recognizable birth defects was increased following maternal LF + GlcN treatment, compared with all other groups. Similarly, a greater number ($P = 0.011$) and proportion ($P = 0.001$) of mothers from the LF + GlcN group carried birth defected fetuses, compared to all other groups. An additional cohort of mice was allowed to give birth, and offspring weight and health were tracked for 16 weeks. Female offspring from HF + GlcN mothers were significantly heavier from 10 weeks of age ($P<0.05$). In contrast, males, from HF + GlcN mothers were significantly lighter from 10-12 weeks of age ($P<0.05$). A greater number of offspring from the LF + GlcN group showed evidence of birth defects ($P= 0.003$), consistent with previous assessments of fetal outcomes. Collectively, these results confirm that in vivo peri-conceptional GlcN exposure has adverse effects on developing conceptuses, which manifest during the fetal as well as post natal development periods. Additionally, the results also show that maternal weight has a significant influence on developmental outcomes.

## 5.2 Introduction

To date, very little attention has been focused on the effects of GlcN exposure on reproductive outcomes. The only known study looking at the effects of in vivo GlcN exposure during the peri-conceptional period is reported in Chapter 4 of this thesis. Here, it was shown that acute GlcN exposure during the peri-conceptional period led to significant reductions in mean implantation rate and litter size. These results are complimentary to the findings that exposure of maturing mouse COCs to GlcN in vitro has deleterious effects on subsequent embryonic development (Chapters 2 and 3).

The HBP can become elevated through several means, including the introduction of GlcN (Rossetti et al. 1995; Horal et al. 2004; Yki-Jarvinen et al. 1998; Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Rumberger et al. 2003; Marshall et al. 2004) or an increased flux of glucose (Robinson et al. 1995; Hawkins et al. 1997; Han et al. 2000; Akimoto et al. 2001). Various states of health are known to be associated with elevated hexosamine activity, such as diabetes (Robinson et al. 1995; Akimoto et al. 2000; Kaneto et al. 2001; McClain, 2002; Fulop et al. 2007), abnormal insulin activity (Marshall et al. 1991a; Traxinger and Marshall, 1992; Baron et al. 1995; Giaccari et al. 1995; Hawkins et al. 1997; Rossetti et al. 1995; Patti et al. 1999; Tang et al. 2000; Federici et al. 2002; Vosseller et al. 2002; Andreozzi et al. 2004) and overweight/ obesity (Buse et al. 1997; Considine et al. 2000; Veerababu et al. 2000). Numerous studies have documented the effects of diabetes, altered insulin metabolism and obesity on reproductive success (as reviewed by Franks et al. 1996; Pasquali et al. 1997; Amaral et al. 2008; Zain and Norman, 2008). This is likely, in part, attributed to the detrimental effect of hyperglycemia on oocyte and embryonic development (Diamond et al. 1989; Moley et al. 1991; Veselá et al. 1995; Moley et al. 1996; Moley et al. 1998(a); Keim et al. 2001; Colton et al. 2002; Colton et al. 2003; Chang et al. 2005(a); Ratchford et al. 2007; Ratchford et al. 2008; Wyman et al. 2008, Wang et al. 2009). Similarly, attainment of pre-conceptional glycemic control in diabetic women has been shown to lead to reduced rates of pregnancy loss and congenital abnormalities (Pedersen and Molsted-Pedersen 1978; Kitzmiller et al. 1991; Rosenn et al 1991; McElvy et al. 2000).

Emerging evidence increasingly implicates elevated levels of fatty acids in suboptimal reproductive outcomes. Bovine studies have demonstrated that delayed maturation and decreased rates of fertilisation, cleavage and embryo development occur following exposure to non-esterified fatty acids (NEFA) (Joritsma et al. 2004) or Palmitic acid (PA) (Leroy et al. 2005), one of the most abundant free fatty acids (FFAs) in serum and follicular fluid, and which is (for serum) positively correlated with body mass index in humans (BMI) (Jungheim et al. 2011(b)). More recently, Van Hoeck et al. (2011) have reported that bovine IVM

performed in the presence of elevated levels of NEFAs leads to the production of blastocysts with reduced cell numbers but higher rates of apoptosis, along with altered metabolic function. Recent findings also provide evidence for fatty acid effects on mouse reproductive outcomes. Cultured blastocysts exposed to elevated PA concentrations were transferred to surrogates, and it was subsequently found that these fetuses were smaller on gestational d14.5. Postnatal outcomes were also assessed in an additional subset of mice, and it was found that although offspring that had been exposed to PA prior to implantation were smaller than their control counterparts at birth, they experienced rapid catch up growth to exceed the weight of the control offspring (Jungheim et al. 2011(a)). Remarkably, Wu et al. (2010) have also reported that maintaining mice on a diet comprised of 22% fat for as little as four weeks induces increased lipid deposition within oocytes. Based on the observation of not only cross species but also wide ranging effects, the effects of lipid accumulation on reproductive outcomes warrant further investigation.

Despite extensive documentation regarding the influence of singular perturbing factors on reproductive parameters, there is a pronounced lack of knowledge regarding the combined effects of such conditions. Recently, it has been shown that the combination of diabetes and hypertension (Stella et al. 2008) are associated with higher rates of adverse outcomes than a singular perturbing factor on its own. External to reproductive outcomes, diabetes has also been shown to interact with other parameters to worsen outcomes. In a recent study looking at the incidence of adenocarcinomas, it was found that people with obesity and frequent episodes of gastro-oesophageal reflux had a significantly greater chance of developing adenocarcinomas than people with obesity or frequent episodes of gastro-oesophageal reflux alone (Whiteman et al. 2008). In another study, Alokail et al. (2009) reported that pre-menopausal women have an increased chance of developing breast cancer if they are obese as well as have type 2 diabetes compared to having either condition on its own. Hence, emerging evidence suggests that the overweight/obese phenotype in combination with other pathologies leads to cumulatively worse health outcomes.

Based on the observations of perturbed reproductive outcomes following peri-conceptional GlcN treatment of healthy mice (Chapter 4), it was hypothesized that the effects of GlcN would be exacerbated in obese mice, where pre-existing exposure to elevated HBP activity could be expected to have occurred. The aim of this study was to determine the effects of peri-conceptional GlcN treatment on reproductive outcomes in mice that had been maintained on a high fat diet.

## 5.3 Methods and Materials

Unless otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, USA).

### 5.3.1 Pre – GlcN treatment

#### *5.3.1.1 Animals and Reagents*

Five-week-old C57BL6 mice were purchased from Laboratory Animal Services, The University of Adelaide. They were weighed, randomly allocated to one of two groups and received ear punctures with a sharp rodent ear punch to enable individual mouse identification. Twelve mice were allocated per treatment group. Mice were housed in the Medical School Animal House, University of Adelaide on a 14:10 hour light: dark cycle. Groups were maintained on either a low fat (LF) (6% fat, 19% protein, and 64.7% carbohydrate (SF04-057)) or high fat (HF) (22% fat 0.15% cholesterol, 19% protein and 49.5% carbohydrate (SF00-219)) diet. (The diet designated as LF has the standard recommended nutritional content of mouse chow, but has been designated as LF relative to the diet with the elevated fat content). Food was purchased from Specialty Feeds, Glen Forrest, Australia and a copy of nutritional components of the chow is included in appendix 8.16. Mouse weights were recorded weekly, and eight replicates were performed.

#### *5.3.1.2 Determination of Insulin Resistance and Glucose Clearance Rates*

Insulin and glucose profiles were initially analysed in a subset of mice to provide additional information to be used in determining the extent of time the mice would undergo dietary manipulation before being treated with GlcN. Other studies have maintained mice on HF diets for periods ranging from one to eight months, however, fat and other nutritional components of HF diets are not consistent between studies, and the response of different strains of mice to a HF diet can vary (Herberg and Kley, 1975**;** Murakami et al. 2000; Zigman et al. 2005; Jenkins et al. 2006; Messier et al. 2006; Neves et al. 2006).

#### *5.3.1.3 Insulin Analysis*

Six mice that had been maintained on the HF or LF diet for 7, 10 and 11 weeks were fasted overnight for 11 hours. Mice were weighed, anaesthetized and 400 - 600 µl blood was collected via orbital bleeds into 1.7 ml Eppendorf tubes. Blood was placed onto ice. Immediately following the completion of collection from all animals, blood was centrifuged for 10 minutes (900 x g), and plasma was frozen (-20˚C). Serum insulin levels were determined via a Rat Insulin RIA Kit (LINCO Research, Inc., St. Charles, MO), with a sensitivity of 0.1 ng/ml and an intra-assay coefficient of variation of 1.4 - 4.6 %. Insulin assays were performed by Associate Professor David Kennaway, School of Pediatrics and Reproductive Health, The University of Adelaide, South Australia. Two replicates were performed, giving a total of 12 mice that were used per dietary group. Each sample was assayed in duplicate.

#### *5.3.1.4 Glucose Clearance Rates*

Following blood collection, mice were assessed for adverse outcomes and recovery rates from the anesthesia for one hour. A small quantity of blood was then collected onto a glucometer stick following venous tail nicking, and blood glucose was measured using an Accu-Check® Advantage glucometer. Mice were then given an IP injection of 1 g/kg D-glucose, and tail blood was again collected onto glucometer strips at 15, 30, 60, 90 and 120 minutes post glucose injection. Blood glucose was immediately measured in all samples using the glucometer, and values were recorded for the construction of glucose clearance curves. Two mice were deemed to have a poor recovery from the aenaesthetic, and did not have glucose tolerance tests performed.

### 5.3.2 Pre – GlcN Treatment

#### *5.3.2.1 Glucosamine Administration Protocol*

A solution comprised of Glucosamine Hydrochloride and PBS was prepared at a concentration of 4 mg/ml and refrigerated. This concentration corresponded to 5 µL of either PBS or GlcN per gram of body weight giving rise to doses of 0 mg/kg GlcN, or 20 mg/kg GlcN, respectively.

Mice that had been maintained on the LF and HF diets were weighed and randomly assigned to receive either 0 mg/kg or 20 mg/kg GlcN. This resulted in the formation of four treatment groups 1) LF - GlcN, 2) LF + GlcN, 3) HF - GlcN and 4) HF + GlcN. The average weight of the mice that had been on the low fat diet (irrespective of assignment to 0 mg/kg or 20 mg/kg GlcN) was calculated. This was used as the weight for calculating the volume of 4 mg/kg GlcN solution to correspond to a 20 mg/kg dose. This standardized volume was given to mice on the LF diet, as well as HF diet, to avoid effects derived from non – uniformly distributed GlcN doses based on differential quantities of body fat. An equivalent volume of PBS was administered to control animals on LF and HF groups.

Mice were administered via IP injection an appropriate, (average)-weight dependent dose of PBS or GlcN solution for three days. Immediately following the third injection, mice were placed with male 16 - 24 week old C57BL6 mice overnight. The following morning, female mice were assessed for successful mating via the detection of vaginal plugs, and returned to their appropriate cages. Those mice that did not successfully mate overnight were weighed, given a subsequent injection and placed with a male overnight. Mice were allowed a maximum of two mating attempts. When successful mating occurred, mice were again weighed and administered with an appropriate dose of PBS or GlcN. Therefore, mice received injections of PBS or GlcN for a minimum of four and a maximum of five days. The date that each mouse successfully mated, as well as the number of injections that they received, was recorded. Mice were maintained on their previously assigned (LF or HF) diets throughout this process.

### 5.3.3 Outcome 1: Determining the Effect of GlcN Administration on Glucose Clearance Rates

Mice that did not mate after two attempts were fasted (with *ad libitum* water access), starting on the day of the final (fifth) GlcN injection. Glucose tolerance tests were performed as described above. A 12-hour fasting blood glucose sample as well as 15, 30, 60, 90 and 120 minutes post glucose administration samples were collected and analyzed according to the methods previously outlined.

### 5.3.4 Outcome 2: Fetal Outcomes

On day 18 of pregnancy, mice that had successfully mated were sacrificed via cervical dislocation and weighed. Dissections were performed to determine litter size, implantation rate, and resorption rate. Rates and the nature of birth defects, as ascertained by gross morphological appearance, were also recorded. Viable conceptuses were removed from uterine tracts, and fetuses and placentas were weighed and assessed for gross morphological appearance. Fetal crown-rump length and placental length and width were measured with a pair of calipers. Ovaries were removed, weighed and Corpora lutea (CL) numbers on the ovaries were counted as an indication of ovulation rate.

### 5.3.5 Contribution of the male

Since the total number of males constituted a large sample size (N = 24) it was not possible to compare outcomes in all males simultaneously. However, the proportion of females that became pregnant from those that had mated was determined for each male to ascertain if any males had failed to produce a pregnancy despite mating.

### 5.3.6 Outcome 3 - Postnatal development

Body weights of mice maintained on a LF or HF diet were recorded weekly for 11 weeks. Twenty-four mice were allocated per dietary treatment. Mice were then randomly allocated to receive GlcN or PBS, and the same treatment protocol used for the fetal outcome assessment was applied, with the exception that mice were treated for 4 - 7 days with GlcN instead of 4 - 5. Numbers and proportions of mice that mated and became pregnant were recorded as described above. A total of 12 mice per treatment were used for this experiment.

Mice that had shown evidence of successful mating were moved to separate cages between gestational days 14 - 16, and checked 2 times daily from 19 days gestation for birth. The length of gestation and number of live pups was recorded for each litter. Numbers of dead pups were also recorded wherever possible as either intact or partially consumed pups. Partially consumed pups were only recorded if it could be ascertained that the mass constituted a single corpse, such as a head or a torso with/without fully intact appendages. An individual head and individual torso found in the same cage were counted as a single

body. Readily observable defects included missing one or both eyes, or having notably small eyes. As pups aged other defects also became apparent, including overgrown teeth, non-discriminate external genitalia and shortened limbs.

Following birth, mice were checked daily for 3 days for rates of survival. Pup death was determined by either identifying carcasses directly or determining differences in litter size from the previous day(s). This approach provided no evidence for causes of death. Since there was very early evidence for high rates of maternal cannibalism, it was decided not to weigh the pups immediately to avoid the risk of heightening further maternal rejection. Hence, pup weight was recorded from 3 days of age, on a weekly basis between weeks 1 - 16 post birth. At the time of weighing, pups were assessed for state of health as well as the emergence of deformities that were not evident at birth. Such deformities related to limb and spinal deformities, stunted growth, ambiguous genitalia and eye defects. Eye deformities included small eyes or eyes with a milky coating, which only became pronounced as development proceeded.

At 21 days of age pups were weaned, ear marked and placed into gender specific cages of 5 - 6 mice in a blinded manner. Assessment of the phenotype of the offspring was also randomly performed by a person experienced in mouse development and blinded to treatment group, which confirmed the extent and the nature of abnormal development.

At 16 weeks of age, mice were weighed, assessed for appearance and then sacrificed via cervical dislocation. Body, tail and combined body and tail lengths were measured. Post mortems were performed and weights were collected for the whole body, spleen, heart, kidney, adrenal gland, pancreas, brain, lung, liver, abdominal fat (depots from reproductive organs and intestines), kidney fat, shoulder fat, retroperitoneal fat (depots from the dorsal abdominal wall), omental fat (depots from stomach and spleen), biceps, hind limbs and the remaining carcass. In addition, ovaries and uteri were weighed for females, and testes were weighed for males. Vas deferens and epididymides were removed for sperm analysis.

#### *5.3.6.1 Sperm analysis*

All preparations and analyses were kindly performed by an experienced sperm assessor (Hassan Bakos, The Research Centre for Reproductive Health, The University of Adelaide, South Australia).

Epididymides and vas deferentia were removed from carcasses and placed immediately into 1 ml 37ºC MOPS-G1 handling media (Lane and Gardner, 2004) in 35 mm dishes. Sperm from all structures was teased out with 2 pairs of tweezers. Successive serial dilutions were performed into fresh, 37ºC MOPS-G1 until a dilution of 1/50 was obtained.

#### 5.3.6.1.1 a) Determining average sperm count

Per animal, 10 µL sperm was loaded onto a polylysine coated slide. The total number of viable sperm per square was counted using a haemocytometer to calculate the average number of sperm per square mm. Viable sperm were determined as having a normal morphology. This figure was in turn used to determine the undiluted sperm concentration for each animal.

#### 5.3.6.1.2 b) Determining average sperm motility

Per animal, 10 µL sperm was loaded onto a haemocytometer slide. The total number of sperm as well as the total number of sperm with normal motility per square was counted. The proportion of sperm with normal motility per square mm was determined, and was used to calculate an overall average for each mouse.

## 5.4 Statistics

Data analysis was performed on binomial and normally distributed data with SAS (Version 9.2) following arcsine transformation.

### 5.4.1 Pre – GlcN Administration

Following allocation of LF or HF fed mice to the GlcN or PBS treatment groups, One-way ANOVAs were performed to ensure that starting weights were equivalent within the dietary groups. The effect of diet on weekly weight gain prior to mating was assessed by Repeated

Measures analysis due to each mouse being weighed multiple times, and to reduce the likelihood of generating type 1 errors.

#### *5.4.1.1 Outcome 1: Determining the Effect of GlcN Administration on Insulin Levels and Glucose Clearance Rates*

Serum insulin values for mice subjected to LF and HF diets were analyzed using a One-way ANOVA, for both preliminary as well as combined replicate data. For assessment of glucose tolerance, although there are numerous ways of determining the area under a curve, a review of methodologies by Le Floch et al. (1990) determined that using a process based on trapezoidal principals is a valid approach. Consequently, determination of glucose area under the curve during the glucose tolerance test was performed using Microsoft Excel (2003), according to trapezoidal rules. Obtained values were incorporated in a One-way ANOVA to compare glucose area under the curve for LF and HF treatments, for both preliminary and combined data.

### 5.4.2 Post – GlcN Administration

A One-way ANOVA was used to compare the weight at 11 weeks post dietary manipulation, of mice that did and did not become pregnant. This was performed within dietary groups (i.e. pregnant and non- pregnant for LF as well as for HF) as well as within treatment groups (i.e. pregnant vs non-pregnant for LF - GlcN, LF + GlcN, HF - GlcN, HF + GlcN).

The effects of 7 days treatment with 0 or 20 mg/kg GlcN on glucose tolerance in non-pregnant, LF or HF fed mice was determined by calculating area under the glucose curve during the glucose tolerance test, as described above. A Two-way ANOVA was used to analyze the obtained data.

#### *5.4.2.1 Outcome 2: Fetal Outcomes*

A Chi-squared test was used to analyze the number of mice that mated, the number of mice that became pregnant and the proportion of mated mice that became pregnant between

treatment groups. Fetal outcomes were analyzed with a Chi-squared analysis for binomial data (see Appendix, Table 8.11.2) or a 2-way ANOVA with covariates for normally distributed data (see Appendix, Table 8.11.3). Highlighted co-variates are factors that were found when included, to alter significance values of the Two-way ANOVA.

#### *5.4.2.2 Outcome 3 – Post natal development*

A Chi-squared test was used to analyze the number of mice that mated, the number of mice that became pregnant and the proportion of mated mice that became pregnant. A Repeated Measures ANOVA was used to assess weekly weight gain in the offspring, and a One-way ANOVA was used to assess organ weight, sperm motility and sperm concentration (see Appendix, Table 8.11.4). Co-variates have been highlighted if they have statistically influenced the outcomes.

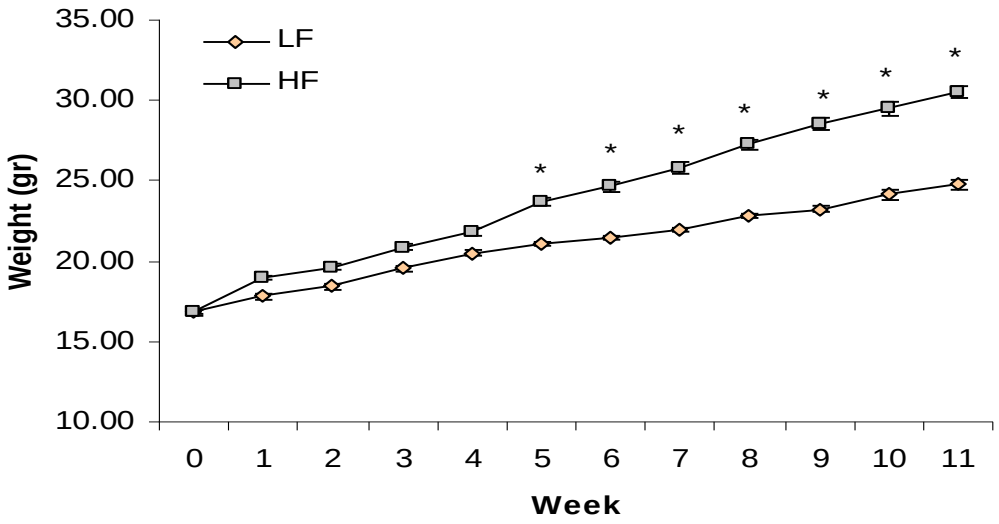
A survival table was constructed for each treatment group over the 16 week period. As gender was not determined prior to weaning, survival plots were constructed based on combined male and female data. A Chi squared analysis was performed for each time point to assess survival rates for each age assessed.

## 5.5 Results

### 5.5.1 Pre – GlcN Administration

#### *5.5.1.1 Maternal Weight Change during High Fat and Low Fat Dietary Intervention*

Weights of mice maintained on a HF or LF diet for 11 weeks were recorded weekly. Mice maintained on the HF diet were significantly heavier than their LF counterparts after 5 weeks of dietary manipulation ($P<0.023$) (Figure 1). Mice that were fasted for blood collection were not included in mean weekly weight data for one week.

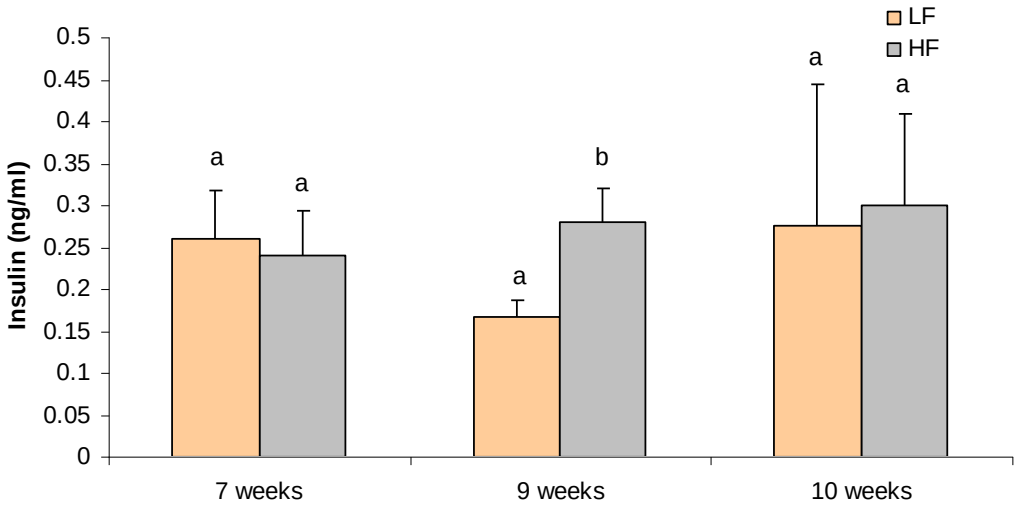



**5.5.1.1.1 Figure 1:** Weekly weights of mice maintained on a LF (low fat) or HF (high fat) diet for 11 weeks. Values are expressed as mean ± SEM. Since not all mice were weighed each week (owing to their use for blood collection) between 82 - 96 mice were used for each LF and HF weekly weight value (for the specific numbers for each week refer to Appendix, Table 8.13.1). * indicates significant difference ($P<0.05$).

### *5.5.1.2 Determination of Insulin Resistance and Glucose Clearance Rates*

#### 5.5.1.2.1 Insulin Analysis

Fasting blood samples were collected following 7, 9 and 10 weeks of feeding a LF or HF diet (12 mice per treatment/per time point) for analysis of serum insulin. Insulin levels were below the level that could be detected by the insulin assay kit that was chosen for the analyses, for a number of the animals. Although preliminary evidence from analysis of data from the first replicate indicated that serum insulin levels were significantly greater for mice maintained on a HF diet at 9 weeks (Figure 2), analysis of combined replicate 1 and 2 data showed no differences in serum insulin levels following HF feeding were detected in those animals where insulin values were obtained.




**5.5.1.2.1.1 Figure 2:** Preliminary serum insulin levels of mice undergoing LF (low fat) or HF (high fat) dietary manipulation. Values are expressed as mean ± SEM. (N: LF 7 weeks = 5, HF 7 weeks = 9, LF 9 weeks = 6, HF 9 weeks = 6, LF 10 weeks = 9 and HF 10 weeks = 4).

#### 5.5.1.2.2 Glucose Tolerance

Preliminary results from the first replicate showed that there was a significant difference in the mean area under the glucose curve during a glucose tolerance test between mice on the LF and HF diets (refer to Appendix, Table 8.14) after ten weeks of dietary intervention. However, when glucose tolerance data from the combined replicates was analyzed, no effect of HF feeding was apparent (Table 1).

| Treatment | Week 7 | Week 9 | Week 10 |
|---|---|---|---|
| **LF** | 1616.5 ± 65.1 *(N = 11)* | 1741.7 ± 73.8 *(N = 10)* | 2401.6 ± 154.4 *(N = 12)* |
| **HF** | 1752.0 ± 98.6 *(N = 12)* | 1915.4 ± 118.9 *(N = 11)* | 2662.6 ± 123.7 *(N = 12)* |
| **P** | P = 0.471 | P = 0.663 | P = 0.260 |

**5.5.1.2.2.1 Table 1:** Mean (+ St Error) area under the curve for glucose clearance rates (mM/ 120 minutes) for mice that received a LF or HF diet.

#### *5.5.1.4 Maternal Weight at the commencement of GlcN Administration*

Mice were allocated to GlcN treatment groups (0 or 20 mg/kg GlcN) following 11 weeks on LF or HF feeding, Significant differences were observed between the LF and HF groups (refer to Figure 1), but weights did not differ between GlcN treatment groups within the HF or LF groups (results not shown).

### 5.5.2 Post – GlcN Administration

#### *5.5.2.1 Maternal Weight*

There were no differences in maternal body weight at mating between mice that did and did not become pregnant within dietary and GlcN treatment groups (Appendix 8.12.2).

#### *5.5.2.2 Glucose clearance rates*

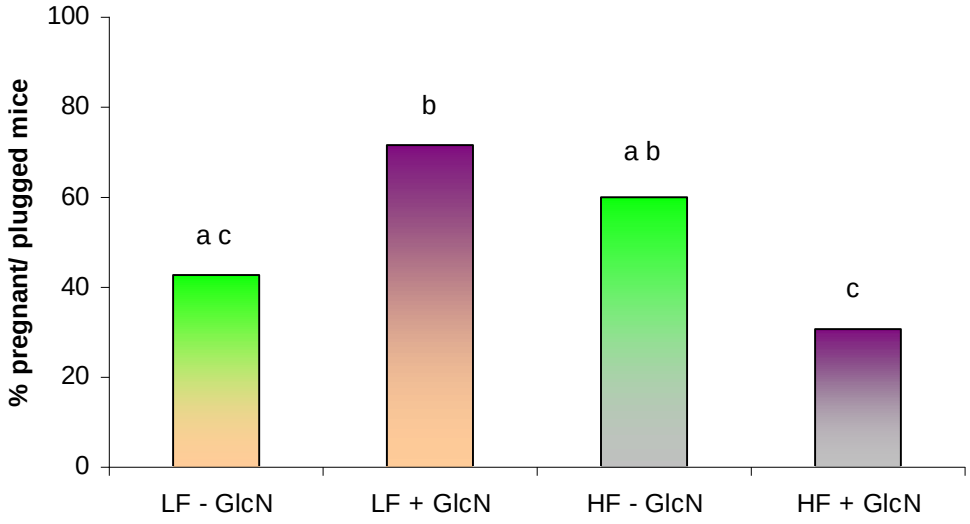
Glucose tolerance tests were performed in females that did not successfully mate to determine the effects of 7 days GlcN treatment of LF or HF fed mice on glucose tolerance. There were no differences between groups in the mean area under the glucose curve (mM/ 120 mins) (LF - GlcN = 2893.3 ± 95.2, LF + GlcN = 2807.2 ± 128.4, HF - GlcN = 3004.6 ± 112.1 and HF + GlcN = 3079.5 ± 128.4).

### 5.5.3 Outcome 2: Fetal outcomes

#### *5.5.3.1 Pregnancy rates*

There were no differences in the number of mice that had visibly detectable sperm plugs (LF - GlcN = 28, LF + GlcN = 28, HF - GlcN = 25, HF + GlcN = 26) or in the number of mice that became pregnant (LF - GlcN = 12, LF + GlcN = 20, HF - GlcN = 15, HF + GlcN = 8). However, there were significant differences between the groups in the proportion of mice that became pregnant from those that had mated (P = 0.015) (Figure 3).

Only one male was found to have failed to generate pregnancies despite having deposited sperm plugs in females. As this male had been exposed to an equal number of females from the 4 treatment groups, it was determined that male factors had no impact on the comparison of pregnancy outcomes between groups.




**5.5.3.1.1 Figure 3:** The proportion of mice that became pregnant of those that had successfully mated, following an 11 week LF or HF dietary intervention and 4 - 5 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. Values are expressed as mean ± SEM. Different letters represent significant differences ($P<0.05$) between groups.

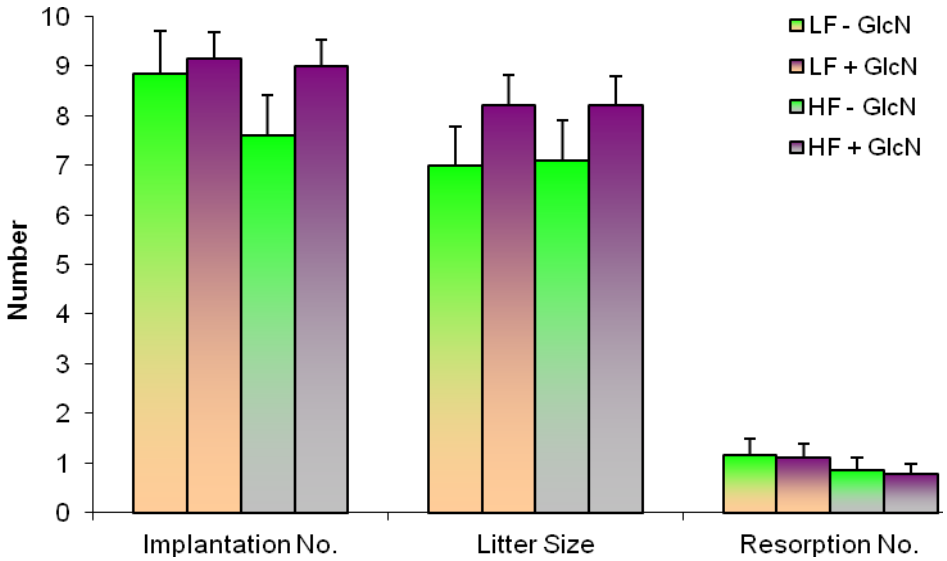
Unlike the results obtained in Chapter 4, GlcN exposure did not alter mean implantation number or litter size. There was also no difference between the groups for mean number of resorptions per litter (Figure 4). Likewise no differences were found for the proportion of viable fetuses that formed from the total number of implantations or for the proportion of implantations that resorbed (Table 2). Fetuses that were non-viable and were not counted as resorptions were not included in the analysis.

| | LF-GlcN | LF+GlcN | HF-GlcN | HF+GlcN |
|---|---|---|---|---|
| **Proportion of viable fetuses formed from total number of implantations (%)** | 85.8 | 84.3 | 95.4 | 79.6 |
| **Proportion of implantations that resorbed (%)** | 13.0 | 12.0 | 11.1 | 8.6 |

**5.5.3.1.2 Table 2:** Proportional implantation data for mice that that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating.

### *5.5.3.2 Ovulation*

There were no differences between groups in mean CL number. Results are presented in Table 3.




**5.5.3.1.3 Figure 4:** Implantation number, litter size (viable fetuses) and fetal resorption number in pregnant mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM.

| | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **Mean ovulation rate** | 10.4 ± 0.6 | 11.3 ± 1.0 | 9.1 ± 0.2 | 11.2 ± 1.1 |

**5.5.3.2.1 Table 3:** Ovulation data for mice that that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM.

### *5.5.3.3 Litter Data*

Feeding a high fat diet reduced fetal weight at day 18 gestation ($P = 0.013$). Similarly, fetal weight was reduced by peri-conception exposure to 20 mg/kg GlcN, although not to the same extent as by HF feeding ($P = 0.023$) (Figure 5a). There was no interaction between the HF feeding and GlcN treatment, regardless of the inclusion of co-variates (Appendix, Table 8.18.2). Exposure to a HF maternal diet also reduced fetal length ($P = 0.02$). GlcN exposure alone or in combination with diet did not influence fetal length (Figure 5b).

Maternal HF diet or GlcN treatment did not alter placental weight (Figure 5c) or placental volume (Figure 5d). There was a trend towards lower placental weight ($P = 0.087$) and placental volume ($P = 0.079$) in mothers fed HF diets. Inclusion of implantation ($P<0.07$) and litter size ($P<0.69$) data strengthened the trend, although significance was not achieved.

### *5.5.3.4 Birth defects*

Analyses of the prevalence of fetal birth defects are presented in Table 4. The percentage of fetuses showing visible signs of birth defects was greater in the LF + GlcN group, than any other group ($P = 0.004$). Similarly, the percentage of mothers that carried defected fetuses was higher for the LF + GlcN group relative to all others ($P = 0.0001$ respectively). The proportion of HF fed mothers that carried birth-defected pups was not affected by GlcN treatment, and was significantly greater than observed in the LF - GlcN group.

Table 5 describes the types of fetal abnormalities detected, as well as numbers of each type of abnormality for each of the four maternal treatment groups. Defects were highly variable, with eye defects including missing one or both eyes, or having one or both eyes underdeveloped (refer to Figure 6).

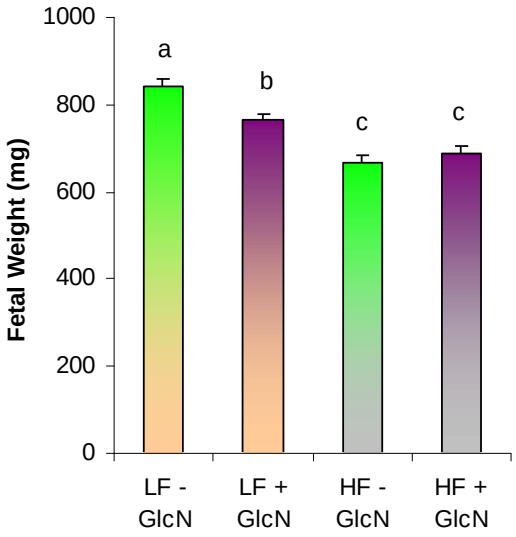



**Figure 5a:** Mean fetal weight (mg)

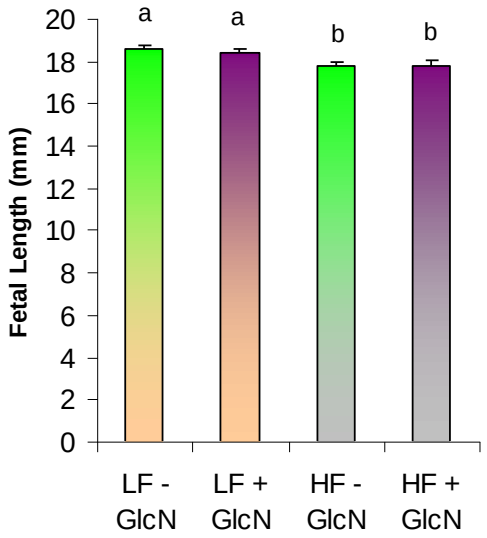



**Figure 5b:** Mean fetal length (mm)

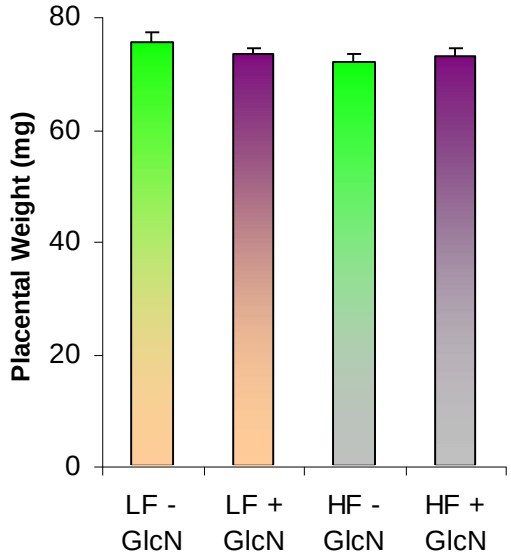



**Figure 5c:** Mean placental weight (mg)

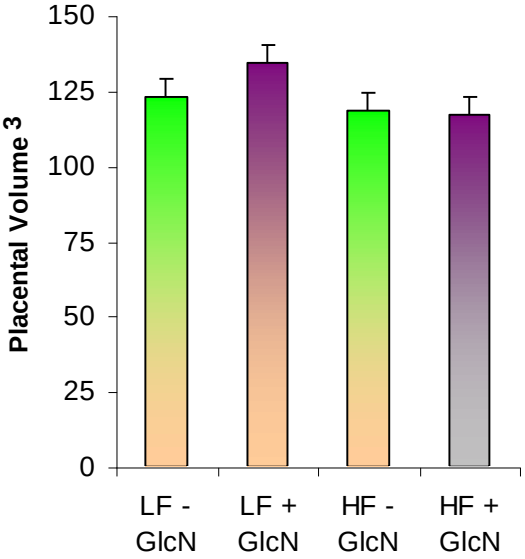



**Figure 5d:** Mean placental volume ($mm^3$)

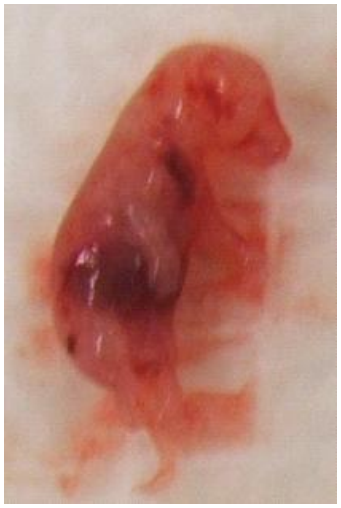
**5.5.3.3.1 Figure 5:** Fetal weight (a) and length (b) as well as placental weight (c) and volume (d) of d18 conceptuses that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences ($P<0.05$) between groups. (Fetal and placental numbers: LF - GlcN = 91, LF + GlcN = 151, HF - GlcN = 103, HF + GlcN = 74).

| | LF – GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| Fetuses (N) | 91 | 151 | 103 | 74 |
| Abnormal Fetuses (N) | 0 *(a)* | 17 *(b)* | 4 *(c)* | 3 *(a c)* |
| Abnormal Fetuses from Total (%) | 0 *(a)* | 11.25 *(b)* | 3.88 *(a)* | 4.05 *(a)* |
| Mothers (N) | 10 | 20 | 15 | 8 |
| Mothers with Abnormal Fetuses(N) | 0 *(a)* | 9 *(b)* | 2 *(a)* | 2 *(a)* |
| Mothers with Abnormal Fetuses (%) | 0 *(a)* | 45 *(b)* | 13.3 *(c)* | 25 *(c)* |

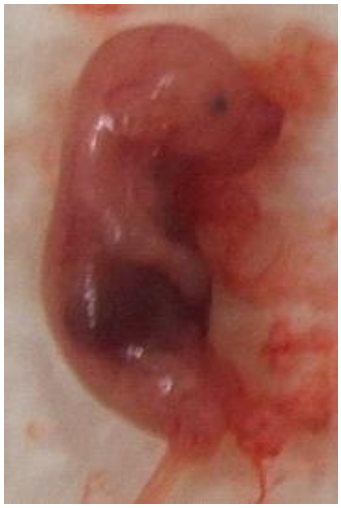
**5.5.3.4.1 Table 4:** The prevalence of fetal birth defects in mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Different letters represent significant differences ($P<0.05$) between groups for each pregnancy outcome.

| | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **Omphalocele** | 0 | 3 | 1 | 2 |
| **Eye Defects** | 0 | 7 | 0 | 1 |

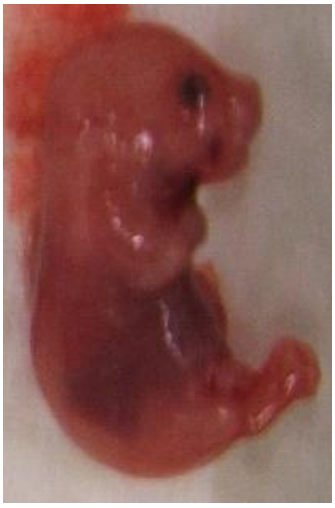
**5.5.3.4.2 Table 5:** Characterization and distribution of fetal birth defects in mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating.



Fetus 1: No eyes visible on d18 (only 1 missing eye evident from picture)



Fetus 2: 1 eye under developed on d18



Fetus 3: Normal eye development

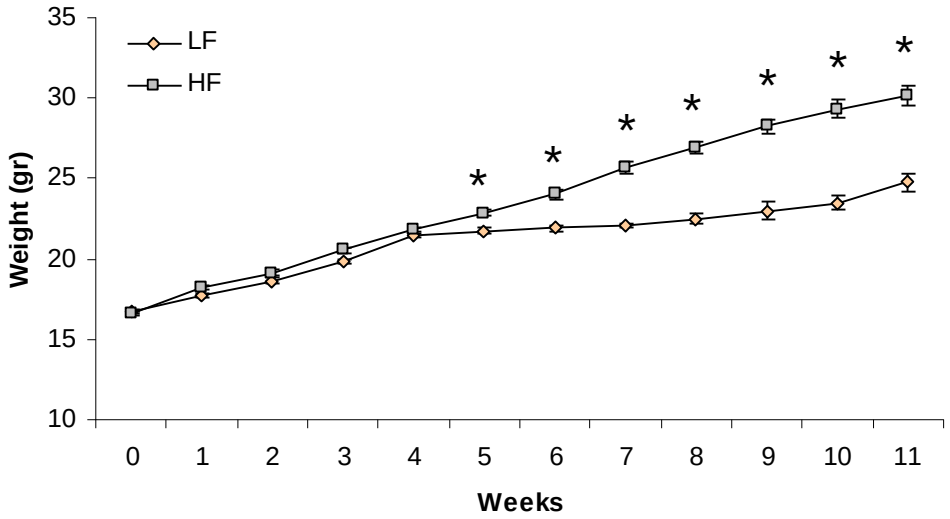
**5.5.3.4.3 Figure 6:** A representation of eyes presented in d18 fetuses derived from mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating.

### 5.5.4 Outcome 3 – Post natal development

#### *5.5.4.1 Pre- GlcN treatment*

In the cohort of mice that were allowed to deliver their litters, maternal bodyweights of mice maintained on the HF diet were higher than their LF counterparts after 5 weeks of dietary manipulation ($P<0.04$) (Figure 7).

No differences in maternal bodyweight were observed between GlcN treatment groups (within the HF or LF dietary groups), following the random allocation of mice to receive either 0 or 20 mg/kg GlcN.




**5.5.4.1.1 Figure 7:** Weekly weights of mice maintained on either a LF (low fat) (N = 24) or HF (high fat) (N = 24) diet for 11 weeks. Values are expressed as mean ± SEM. * indicates significant differences (P<0.05) between groups for each week.

### *5.5.4.2 Post – GlcN Administration*

#### 5.5.4.2.1 Maternal Weight

There were no differences in maternal body weight at mating between mice that did and did not become pregnant within dietary and GlcN treatment groups (Appendix 8.12.3).

#### 5.5.4.2.2 Pregnancy rates

There were no differences in the number of mice that had visibly detectable sperm plugs or in the number of mice that became pregnant. However, unlike mice used for the fetal outcome assessment, there were no differences between groups in the proportion of mice that became pregnant from those that had plugged. There were also no differences between any of the groups for mean litter size. Results are presented in Table 6.

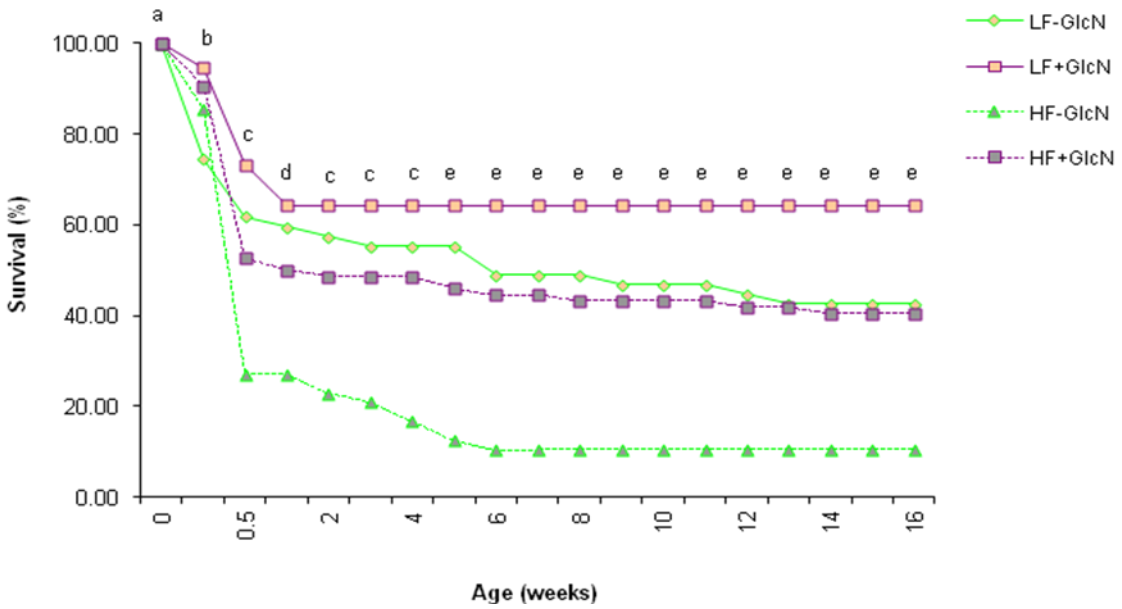
| | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **Mice per treatment** | 12 | 12 | 12 | 12 |
| **Sperm plugs post mating (N)** | 8 | 10 | 9 | 9 |
| **Pregnant (N)** | 6 | 7 | 7 | 7 |
| **Pregnant from plugged (%)** | 75 | 70 | 77.8 | 77.8 |
| **Mean litter size** | 7.8 ± 1.8 | 8.0 ±1.4 | 6.8 ± 2.3 | 10.5 ± 1.4 |
| **Total born** | 47 | 56 | 45 | 74 |

**5.5.4.2.2.1 Table 6:** Pregnancy data for mice maintained on either a LF or HF diet and administered with ± GlcN treatment.

#### 5.5.4.2.3 Post natal development

##### *5.5.4.2.3.1 Survival*

Results obtained from the completion of a survival table were used to construct a survival plot for combined male and female data (Figure 8). The highest rates of death were observed within the first week of birth for all groups. From day 3 of birth, there were consistently lower survival rates for offspring derived from mothers subjected to a HF diet without GlcN, than all other groups ($P<0.008$). Values are based on the number of live offspring observable at each time point.




**5.5.4.2.3.1.1 Figure 8:** Survival rates for offspring from mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Numbers at birth for each group are as follows: LF + 0 mg/kg GlcN = 47, LF + 20 mg/kg GlcN = 56, HF + 0 mg/kg GlcN = 45 and HF + 20 mg/kg GlcN = 74. **a)** No difference between groups; **b)** LF+GlcN = HF-GlcN = HF+GlcN, LF+GlcN> LF – GlcN, HF-GlcN = LF-GlcN; **c)** LF+GlcN = LF-GlcN, LF+GlcN> HF+GlcN> HF-GlcN, LF-GlcN = HF+GlcN; **d)** LF+GlcN = LF-GlcN = HF+GlcN> HF-GlcN; **e)** LF+GlcN> LF-GlcN = HF+GlcN> HF-GlcN. ($P<0.05$) * . Numbers of offspring for each developmental age are provided in the Appendix, Tables 8.13.2 – 8.13.4. Different letters represent significant differences between groups for each week of age ($P<0.05$).

#### *5.5.4.2.3.2 Weekly weight gain*

Owing to the small number of surviving offspring from the HF - GlcN group (N = 3 females and 2 males), subsequent analyses did not include this group.

Offspring gender was determined at 21 days of age. Male and female offspring from HF + GlcN mothers were heavier at weaning (Figure 9A and B). Between 8 - 9 weeks of age, female offspring from HF + GlcN mothers were of an equivalent weight to offspring from LF + GlcN mothers but heavier than offspring from LF - GlcN mothers ($P<0.05$). From ten weeks of age, however, HF + GlcN female offspring were consistently heavier than all other groups ($P<0.05$). In males, on the other hand, LF + GlcN offspring were heavier than both other groups during weeks 5 -6 ($P<0.03$), and heavier than HF + GlcN offspring in week 9

(P<0.05). During weeks 10 - 12, HF + GlcN male offspring were lighter than all other groups (P<0.05).

#### *5.5.4.2.3.3 Developmental Abnormalities*

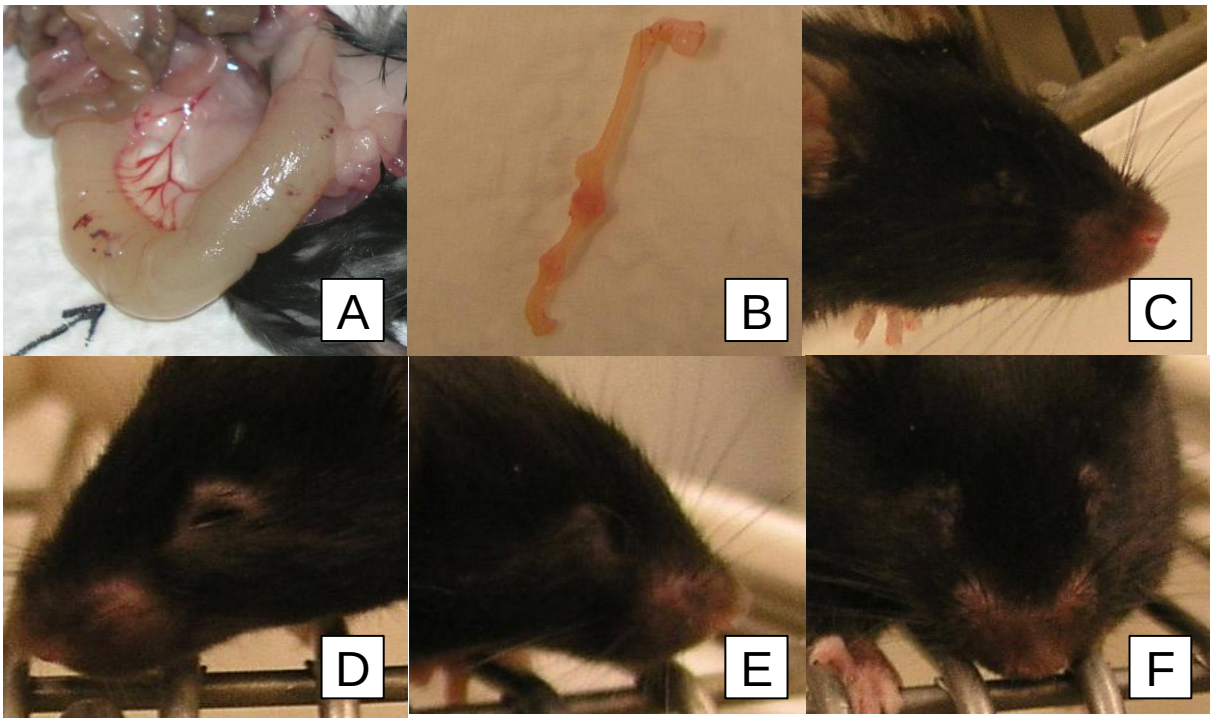
Offspring were also assessed weekly for developmental abnormalities. The number of offspring with developmental abnormalities is shown in Table 7. Gender was determined at weaning. Therefore, the values for total birth defects are greater than the sum of males and females with defects, due to deaths that occurred prior to weaning with gender unassigned. Proportions of offspring with birth defects, relative to the total number born or relative to the total alive on the day of birth are also shown in Table 7. LF + GlcN offspring had a higher proportion of birth defects for combined males and females from both the starting birth number (P = 0.003) as well as number alive on day of birth (P = 0.0012), when compared to all other groups.

| Measure | Group | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|---|
| **Number** | **Male** | 2 | 4 | 1 | 3 |
| **Number** | **Female** | 4 | 10 | / | 6 |
| **Number** | **Combined** | 6 [a] | 16 [b] | 3 [a] | 10 [a b] |
| **% (From birth No.)** | **Combined** | 12.8 [a] | 28.6 [b] | 6.3 [a] | 13.5 [a] |
| **% (From No. alive d1)** | **Combined** | 17.1 [a] | 30.2 [b] | 7.3 [a] | 14.9 [a] |

**5.5.4.2.3.3.1 Table 7:** Numbers and proportions of birth defects for offspring from mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Different letters represent significant differences (P<0.05) between groups for each measure.

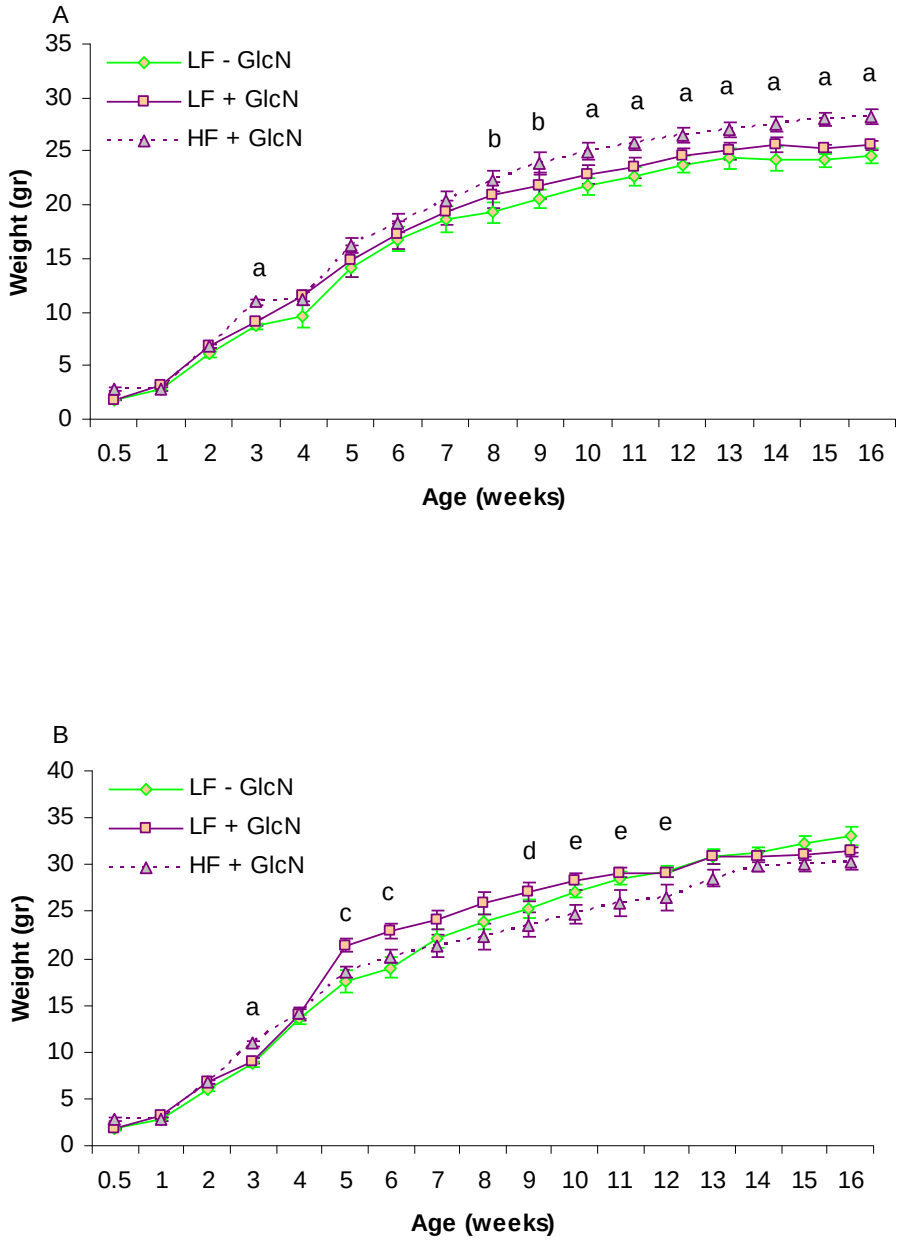
The nature of the birth defects was broad, and was detected at various developmental stages. For example, one of the most common birth defects was eye deformities (Figure 10) which presented in varying forms including: 2 eyes missing, 2 eyes small, 1 eye missing, 1 eye small and presumed blindness (eye(s) covered in a milky layer). The absence of an eye(s) was evident within the first week of birth. However, the detection of small eyes occurred between birth and week 4, and presumed blindness was detected from weeks 4 - 10. One mouse that on first appearance appeared to have no eyes subsequently had small slits detected, suggesting that eyes were potentially present but were not visible owing to

insufficiently sized eye sockets. Other birth defects included presenting with a hunch back which may represent a potential spinal defect (visible from week 2), having abnormal/overgrown limbs (detectable from 2 weeks), presenting with genitalia that could not be distinguished as male or female (evident from 3 weeks), females having bifurcated vaginae (evident from 3 weeks), and males presenting with seemingly small testicles and having no/underdeveloped penises (evident from 3 weeks).




**5.5.4.2.3.3.3 Figure 10:** Examples of birth defects and health problems identified during the post natal analysis study. Uterine horns completely swollen **(A)** or presenting with swollen nodules **(B)**, **(C)**: mouse with a very small eye that appears to be blind, **(D):** mouse with one very small eye, **(E):** mouse with fused eye socket and **(F):** mouse with 2 fused eye sockets.

Additional health problems that were not detectable/ known to be present from birth, were also identified as the offspring aged, and are presented in Table 8. Here, HF - GlcN offspring have been omitted from analysis owing to the insufficient sample size. Blindness was detected from weeks 4 - 10. However, as only mice with congenital eye defects developed blindness, blindness has been included within birth defect data. At 12 weeks of age one animal (male, from the HF + GlcN group) presented with extremely overgrown teeth and had to be euthanized. The majority of additional health problems, however, were detected on the day of autopsy.




**5.5.4.2.3.3.2 Figure 9:** Mean weekly weight of female (A) and male (B) offspring of mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. KEY: **(a):** HF + GlcN > LF – GlcN = LF + GlcN; **(b):** HF + GlcN = LF + GlcN, HF + GlcN > LF – GlcN, LF – GlcN = LF + GlcN; **(c):** LF + GlcN > LF – GlcN = HF + GlcN; **(d):** LF + GlcN = LF – GlcN, LF + GlcN > HF + GlcN, LF – GlcN = HF + GlcN; **(e):** HF + GlcN < LF – GlcN = LF + GlcN. Numbers of offspring for each developmental age are provided in Appendix Tables 8.13.3 and 8.13.4. Different letters represent significant differences (P<0.05) between groups, at each week of age.

| Gender | LF – GlcN | LF + GlcN | HF + GlcN |
|---|---|---|---|
| **Male** | • Atrophied epididymis (1 side) *(N = 1)* | • Undescended testes *(N =1)* | • Very enlarged spleen *(N = 1)* |
| **Female** | | • Very swollen uterus *(N = 3)* | • Very enlarged pancreas *(N = 1)*<br>• Very swollen uterus *(N = 1)* |

**5.5.4.2.3.3.4 Table 8:** The number and nature of health problems identified at autopsy at 16 weeks of age. Mice are the offspring of mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating.

***Post mortem weights***

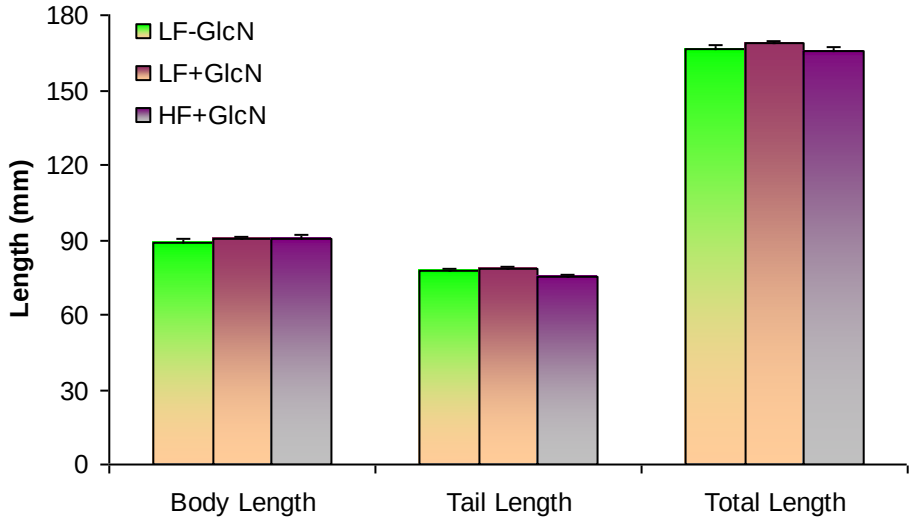
HF - GlcN offspring (N = 2 (female); N = 3 (male)) were omitted from these analyses.

**5.5.4.2.3.4.1 *Female offspring***

As can be seen from Table 9, 16 week old female offspring of the HF + GlcN mice were significantly heavier than mice from mothers maintained on a LF diet, irrespective of GlcN treatment ($P<0.05$). No differences in carcass weight (Table 9), or proportion of the body constituted by the carcass (Table 10), were found between the groups suggesting that the difference in body weight was attributable to organ weights and/or fat deposit weights. There was also no difference in body length, tail length or combined body and tail length between the three groups (Figure 11).

There were also no differences between the groups for spleen, heart, adrenal gland, brain or lung weights (Table 9) or for the weights of these organs as a proportion of body weight (Table 10) in female offspring. There was a trend towards ovarian weight being lower in mice derived from HF + GlcN mothers ($P = 0.06$). The inclusion of litter size as a co-variate strengthened the trend ($P = 0.051$). When maternal weight was included as a covariate within the analysis, ovaries of offspring from HF + GlcN mothers were significantly lighter ($P = 0.043$). However, when ovary weight as a proportion of body weight was compared, there were no differences between groups (Table 10). Furthermore, as the stage of the estrous cycle was not controlled for, it is possible that ovarian weights may be influenced by cycle-

dependent, physiological changes. Absolute weight of the kidneys, liver and pancreas was not different between groups (Table 9), but relative weights of these organs did differ between the groups (Table 10). The proportion of body weight constituted by the kidney and pancreas was reduced in 16 week old females derived from HF + GlcN mice ($P<0.05$), whilst the proportion of the body constituted by the liver was significantly greater in LF – GlcN derived mice ($P< 0.05$), when compared to both other groups.




**5.5.4.2.3.4.1.1 Figure 11:** Body, tail and combined body and tail length of female mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. (N: LF - GlcN = 13; LF + GlcN = 16; HF + GlcN = 12)

Initially, no differences were seen between groups for uterine weight ($P = 0.415$) (Table 9). The inclusion of maternal weight ($P = 0.058$) as well as number of littermates alive on day 7 ($P = 0.07$) as co-variates generated a trend towards smaller uteri in HF + GlcN offspring. However, after the inclusion of starting total litter size ($P = 0.011$) as well as the number of living littermates on day of birth ($P = 0.017$) as covariates, it was shown that HF + GlcN offspring had significantly smaller uteri. However, there were no differences between groups in uterine weight when expressed relative to body weight (Table 10). As the stage of the estrous cycle was not controlled for, it must be recognized that uterine weights may be influenced by cycle- dependent, physiological changes.

There were, however, significant differences in body fat. More specifically, female offspring from HF + GlcN had significantly greater quantities ($P<0.01$) (Figure 12) and proportions ($P<0.02$) (Table 10) of abdominal fat, greater quantities ($P<0.02$) (Figure 12) and proportions ($P<0.05$) (Table 9) of kidney fat and greater quantities ($P<0.05$) (Figure 12) and proportions ($P<0.02$) (Table 10) of retroperitoneal fat. HF + GlcN female offspring also had greater quantities of shoulder fat ($P<0.04$) (Figure 12). When shoulder fat was analyzed as a proportion of body weight, it was found that whilst LF - GlcN derived females had equivalent distributions to the other two groups, HF + GlcN offspring had a significantly greater proportion of shoulder fat than LF + GlcN offspring ($P<0.05$) (Table 10). HF + GlcN females also had greater quantities of omental fat ($P<0.02$) (Figure 12), although omental fat as a proportion of body weight was not different between the three groups.

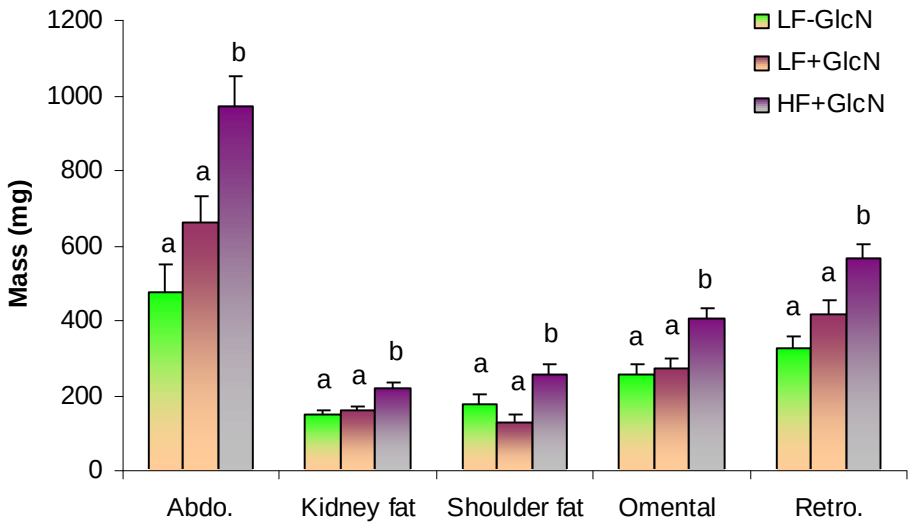
Similarly, there were also differences between groups for muscle mass. HF + GlcN female offspring had increased weights of hind limb mass, in absolute terms ($P<0.05$) (Figure 13) and relative to body weight (Table 10) ($P<0.05$). Absolute or relative biceps mass (Figure 13, Table 9) did not differ between groups. These results indicate that whilst some of the increased body weight of HF + GlcN female offspring at 16 weeks of age can be attributed to an increased muscle mass, the majority of increase in weight is due to increased adipose tissue mass

| | Female | | | Male | | |
|---|---|---|---|---|---|---|
| **Structure** | **LF-GlcN** *(N = 13)* | **LF+GlcN** *(N=16)* | **HF+GlcN** *(N =12)* | **LF-GlcN** *(N = 7)* | **LF+GlcN** *(N = 20)* | **HF+GlcN** *(N = 18)* |
| **Body weight (gr)** | **24.5 ± 0.7 a** | **25.5 ± 0.5 a** | **28.2 ± 0.7 b** | 33.0 ± 0.9 | 31.4 ± 0.4 | 30.3 ± 0.8 |
| **Adrenal gland (mg)** | 3.9 ± 0.2 | 4.2 ± 0.2 | 4.1 ± 0.3 | 2.9 ± 0.3 | 2.4 ± 0.2 | 2.4 ± 0.2 |
| **Brain (mg)** | 420.8 ± 7.0 | 423.8 ± 6.3 | 419.8 ± 7.4 | 437.1 ± 10.5 | 423.3 ± 6.2 | 412.7 ± 6.6 |
| **Heart (mg)** | 103.8 ± 3.5 | 100.0 ± 3.2 | 100.9 ± 3.7 | 132.5 ± 5.4 | 132.4 ± 3.2 | 134.9 ± 3.4 |
| **Kidney (mg)** | 132.4 ± 3.4 | 136.2 ± 3.0 | 132.1 ± 3.5 | 186.5 ± 6.7 | 182.9 ± 4.0 | 191.2 ± 4.2 |
| **Liver (mg)** | 1247.8 ± 40.6 | 1183.9 ± 36.6 | 1243.1 ± 42.2 | 1872.5 ± 62.9 | 1806.8 ± 37.2 | 1758.1 ± 39.2 |
| **Lung (mg)** | 182.3 ± 13.3 | 182.8 ± 11.8 | 203.3 ± 13.8 | 213.4 ± 15.8 | 205.1 ± 9.4 | 222.7 ± 9.9 |
| **Pancreas(mg)** | 211.1 ± 10.5 | 206.2 ± 9.51 | 191.9 ± 11.0 | 211.0 ± 7.9 | 198.0 ± 4.7 | 196.4 ± 4.9 |
| **Spleen (mg)** | 95.2 ± 5.1 | 90.6 ± 4.6 | 95.3 ± 5.3 | 211.0 ± 7.9 | 198.0 ± 4.7 | 196.4 ± 4.9 |
| **Ovary (mg)** | 2.7 ± 0.2 | 2.6 ± 0.2 | 2.1 ± 0.2 | N/a | N/a | N/a |
| **Uterus (mg)** | 86.6 ± 13.0 | 91.4 ± 11.7 | 68.1 ± 13.5 | N/a | N/a | N/a |
| **Testes (mg)** | N/a | N/a | N/a | 85.5 ± 3.1 | 86.9 ± 1.8 | 88.2 ± 1.9 |
| **Carcass (gr)** | 17.0 ± 0.5 | 17.3 ± 0.4 | 17.8 ± 0.5 | 22.4 ± 0.8 | 21.8 ± 0.4 | 21.9 ± 0.5 |

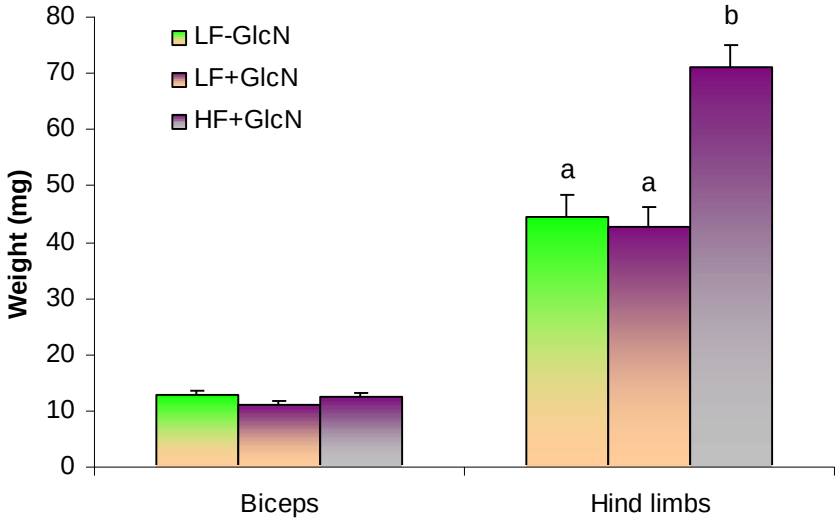
**5.5.4.2.3.4.1.2 Table 9:** Absolute organ and carcass weight of female and male mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) within genders for each outcome.

| | Female | | | Male | | |
|---|---|---|---|---|---|---|
| **Structure** | **LF-GlcN** *(N = 13)* | **LF+GlcN** *(N = 16)* | **HF+GlcN** *(N=12)* | **LF-GlcN** *(N = 7)* | **LF+GlcN** *(N = 20)* | **HF+GlcN** *(N = 18)* |
| **Adrenal gland (%)** | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 |
| **Brain (%)** | 1.7 ± 0.0 | 1.6 ± 0.0 | 1.5 ± 0.0 | 1.3 ± 0.0 | 1.4 ± 0.0 | 1.4 ± 0.1 |
| **Heart (%)** | 0.42 ± 0.01 | 0.39 ± 0.01 | 0.35 ± 0.00 | 0.40 ± 0.01 | 0.42 ± 0.01 | 0.44 ± 0.01 |
| **Kidney (%)** | **0.54 ± 0.02 a** | **0.53 ± 0.01 a** | **0.47 ± 0.01 b** | **0.56 ± 0.02 a** | **0.58 ± 0.01 a b** | **0.64 ± 0.02 b** |
| **Liver (%)** | **5.1 ± 0.2 a** | **4.7 ± 0.1 b** | **4.4 ± 0.2 b** | **5.7 ± 0.2 ab** | **5.8 ± 0.1 a** | **5.9 ± 0.2 b** |
| **Lung (%)** | 0.83 ± 0.32 | 0.86 ± 0.31 | 0.87 ± 0.32 | 0.65 ± 0.04 | 0.65 ± 0.02 | 0.74 ± 0.04 |
| **Pancreas(%)** | **0.87 ± 0.06 a** | **0.81 ± 0.02 a** | **0.68 ± 0.04 b** | 0.64 ± 0.02 | 0.63 ± 0.01 | 0.65 ± 0.02 |
| **Spleen (%)** | 0.39 ± 0.02 | 0.35 ± 0.02 | 0.34 ± 0.02 | 0.25 ± 0.04 | 0.29 ± 0.01 | 0.32 ± 0.04 |
| **Ovary(%)** | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.001 ± 0.00 | n/a | n/a | n/a |
| **Uterus (%)** | 0.36 ± 0.04 | 0.36 ± 0.07 | 0.24 ± 0.02 | n/a | n/a | n/a |
| **Testes (%)** | n/a | n/a | n/a | 0.26 ± 0.01 | 0.27 ± 0.00 | 0.29 ± 0.01 |
| **Carcass (%)** | 69.1 ± 1.5 | 67. 6 ± 1.2 | 63.3 ± 1.3 | 68.4 ± 1.2 | 69.3 ± 0.5 | 67.7 ± 0.7 |
| **Bicep (%)** | 0.05 ± 0.00 | 0.04 ± 0.00 | 0.04 ± 0.00 | 0.04 ± 0.00 | 0.04 ± 0.00 | 0.03 ± 0.00 |
| **Hindlimb (%)** | **0.18 ± 0.01 a** | **0.17 ± 0.01 a** | **0.25 ± 0.02 b** | **0.12 ± 0.01 a** | **0.08 ± 0.00 b** | **0.11 ± 0.00 a** |
| **Abdominal fat (%)** | **2.0 ± 0.2 a** | **2.6 ± 0.3 a** | **3.5 ± 0.3 b** | **1.5 ± 0.4 a** | **0.9 ± 0.2 b** | **1.5 ± 0.2 a** |
| **Omental fat (%)** | 1.2 ± 0..5 | 1.2 ± 0.4 | 1.1 ± 0.4 | **1.2 ± 0.1 a** | **0.9 ± 0.1 b** | **1.2 ± 0.1 a** |
| **Retroperitoneal fat (%)** | **1.3 ± 0.1 a** | **1.6 ± 0.1 a** | **2.0 ± 0.1 b** | **1.3 ± 0.1 a** | **0.9 ± 0.1 b** | **1.2 ± 0.1 a** |
| **Kidney fat (%)** | **0.6 ± 0.04 a** | **0.62 ± 0.05 a** | **0.78 ± 0.5 b** | **0.58 ± 0.08 a** | **0.34 ± 0.03 b** | **0.43 ± 0.3 c** |
| **Shoulder fat (%)** | **0.71± 0.1 a b** | **0.5 ± 0.05 a** | **0.91 ± 0.11 b** | **0.8 ± 0.14 a** | **0.43 ± 0.03 b** | **0.61 ± 0.05 a** |

**5.5.4.2.3.4.1.3 Table 10:** Relative organ and carcass weights as a proportion of body weight of female and male mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) within genders for each outcome.




**5.5.4.2.3.4.1.4 Figure 12:** Weight of fat deposits in female mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) between groups for each outcome. (N: LF - GlcN = 13; LF + GlcN = 16; HF + GlcN = 12).

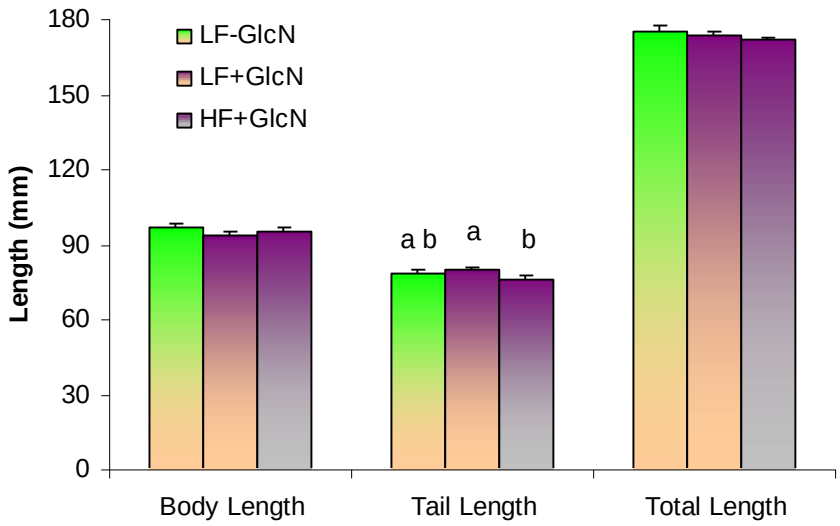



**5.5.4.2.3.4.1.5 Figure 13:** Weight of biceps and hind limb muscles in female mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) between groups for each outcome. (N: LF - GlcN = 13; LF + GlcN = 16; HF + GlcN = 12).

#### 5.5.4.2.3.4.2 *Male offspring*

In contrast to female offspring, there was no difference between the body weights at post mortem of the male offspring (Table 9). Analysis of carcass weight (Table 9) as well as the proportion of the body constituted by the carcass (Table 10) showed that carcasses were not different between the three groups.

There were no differences between groups for body length. However, offspring from the HF + GlcN group had significantly shorter tails then those from the LF + GlcN group, with the tail length of LF - GlcN offspring being equivalent to both other groups ($P<0.05$). Despite the differences in tail length, there were no differences between groups for combined tail and body lengths (Figure 14).



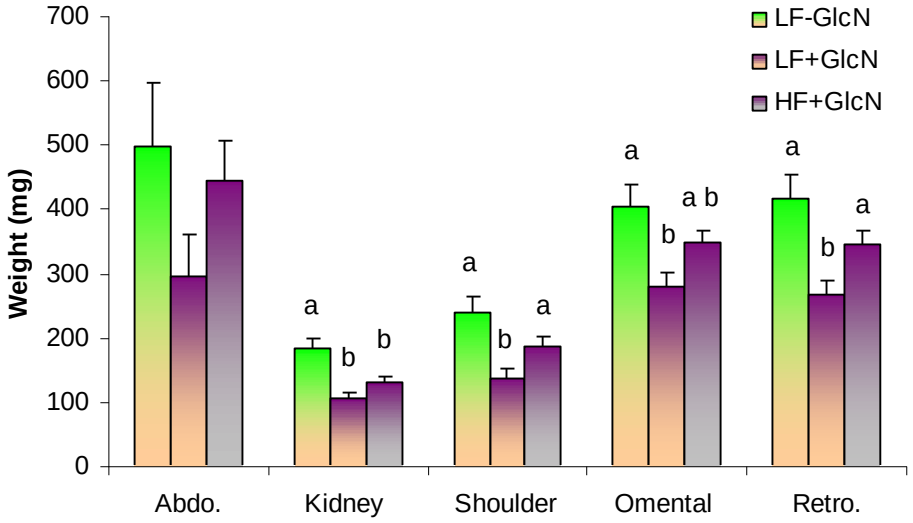

**5.5.4.2.3.4.2.1 Figure 14:** Body length, tail length and combined body and tail length of male mice. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences ($P<0.05$) between groups for each outcome. (N: LF - GlcN = 7; LF + GlcN = 20; HF + GlcN = 18).

There were no differences between groups for absolute or relative weights of the heart, adrenal gland, pancreas, spleen, or testes (Table 9, 10) in male offspring. There were also no differences in mean kidney or liver mass (Table 9). However, relative kidney weight was greater in male offspring of HF + GlcN treated mothers, when compared to those of LF -

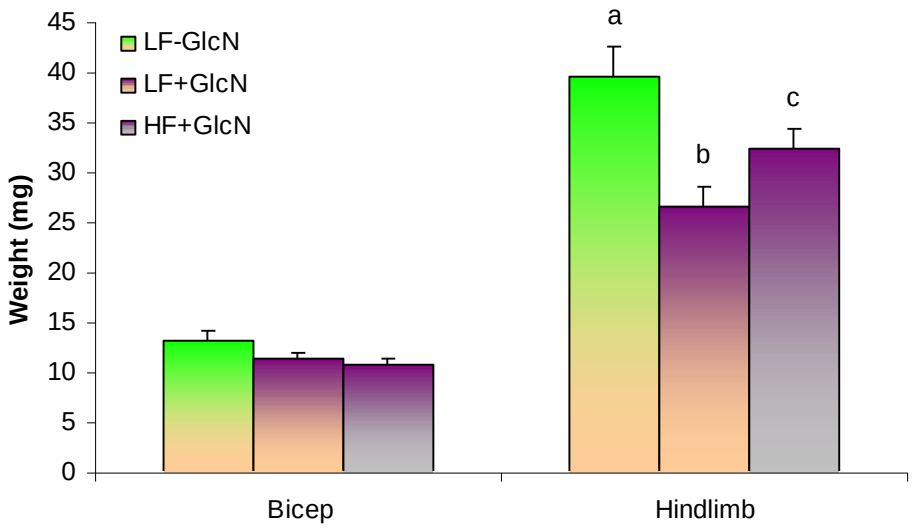
GlcN mothers (P<0.05) (Table 10). Relative liver weight was reduced in male offspring of LF + GlcN mothers, when compared to male offspring of HF + GlcN mothers (P<0.05) (Table 10). Although no differences were seen for brain weight, there was a trend observed towards smaller brain weights for HF + GlcN male offspring (P = 0.08) (Table 9). This trend was further strengthened by the incorporation of maternal weight as a co-variate (P =0.057). There was no difference between the groups in the proportion of the body constituted by the brain (Table 10).

Unlike females, although there was a trend present (P = 0.077), there were no differences in male offspring between groups for abdominal (abdo.) fat mass (Figure 15). However, the proportion of the body constituted by abdominal fat was significantly less for LF + GlcN male offspring relative to the other groups (P<0.05) (Table 10). Kidney fat weight was highest in the LF - GlcN group (P=0.026). Both LF - GlcN and HF + GlcN male offspring had greater mass of shoulder fat (P<0.05) and retroperitoneal (retro.) fat (P<0.05), when compared to the LF + GlcN group. As a proportion of body weight, kidney fat was significantly lower (P<0.05) in LF + GlcN offspring when compared to both other groups. Kidney fat, as a proportion of body weight, was also significantly higher in LF - GlcN offspring (P<0.05), when compared to both other groups. In addition, the proportion of shoulder fat (P<0.04) and retroperitoneal fat (P<0.05) was significantly lower in the LF + GlcN group than in the LF - GlcN and HF + GlcN groups (Table 9). Male offspring from the LF - GlcN group had significantly more omental fat than those from the LF + GlcN group, but offspring from the HF + GlcN group had deposits equivalent to both groups (P<0.05) (Figure 15). In contrast, male offspring from the LF + GlcN group had significantly less omental fat as a proportion of body weight relative to the other two groups (P<0.05) (Table 10).

Consistent with the results obtained from female offspring, there were no differences between groups in biceps weight (Figure 16) or proportion of body weight in male offspring (Table 10). However, hind limb muscle mass was highest in male offspring from LF - GlcN mothers, compared to both other groups. Hind limb muscle mass, in absolute (Figure 16) and relative (Table 10) terms, was lower in males from LF + GlcN mothers, when compared to both other groups




**5.5.4.2.3.4.2.2 Figure 15:** Weight of fat deposits in male mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) between groups for each outcome. (N: LF - GlcN = 7; LF + GlcN = 20; HF + GlcN = 18).




**5.5.4.2.3.4.2.3 Figure 16:** Weight of muscle deposits in male mice at 16 weeks of age. Mice are the offspring from mothers that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating. Values are expressed as mean ± SEM. Different letters represent significant differences (P<0.05) between groups for each outcome. (N: LF - GlcN = 7; LF + GlcN = 20; HF + GlcN = 18).

#### 5.5.4.2.3.4.2.1 Post mortem sperm analysis

##### *5.5.4.2.3.4.2.1.1 Sperm count*

There were no differences in mean sperm count (millions/ ml) between the three groups (LF - GlcN = 16.22 ± 2.36 (N = 6), LF + GlcN = 13.69 ± 1.31 (N =15) and HF + GlcN = 15.63 ± 1.02 (N =14)).

##### *5.5.4.2.3.4.2.1.2 Sperm motility*

There were no differences in mean sperm motility (%) between the three groups (LF - GlcN = 35.31 ± 1.63 (N = 7), LF + GlcN = 34.19 ± 1.76 (N =20) and HF + GlcN = 35.14 ± 1.0 (N =18).

## 5.6 Discussion

Significantly, the results obtained from these studies provide evidence that maternal dietary manipulation and the peri-conceptional administration of GlcN to mice induce a variety of reproductive perturbations, including alterations in fetal programming. For example, d18 fetuses from HF fed mice were significantly shorter and lighter than their LF counterparts, irrespective of GlcN exposure. In LF fed mice GlcN treatment also reduced fetal weight, although not to the same extent as occurred from the HF diet. In addition, GlcN exposure increased the incidence of birth defects detected in both late gestation and in live offspring. Unfortunately, owing to the high death rate of HF - GlcN offspring, further specific comparisons of the combined effects of dietary manipulation and GlcN could not be made. However, several gender-specific differences in the remaining treatment groups were observed, including increased adiposity, body weight and proportional hind limb mass in female offspring from the HF + GlcN group, whilst adiposity and proportional hind limb mass was increased in male offspring from the LF + GlcN group.

There were several notable differences in fetal outcomes between Chapters 4 and 5. For example, while implantation number and litter size were reduced by GlcN exposure in Chapter 4, these parameters were statistically equivalent in Chapter 5. The current experiment has utilized a much larger cohort compared to Chapter 4. However, it cannot simply be ascertained that the results from Chapter 4 were obtained in response to under-

sampling, as analysis revealed that numbers were sufficient to detect statistical relationships. Hence, it is likely that the effects observed in both studies are reflective of variations in the models, such as maternal age. In addition, whereas GlcN exposure in Chapter 4 elicited no effect on fetal size, results from Chapter 5 revealed that maternal, peri-conceptional exposure to GlcN in the presence of a normal diet leads to reduced fetal weight. Furthermore this effect was exacerbated by maternal consumption of a HF diet, irrespective of GlcN exposure. This is in agreement with the findings of Jungheim et al. (2010) who observed that the maternal consumption of a HF diet resulted in the generation of smaller offspring.

Also contrasting with Chapter 4 is the greater instance of birth defects observed in fetuses and live offspring from mothers treated peri-conceptionally with GlcN. Results from Chapter 5 showed that birth defects were increased, albeit not to the same extent, in fetuses from dietary- induced, obese mothers, irrespective of GlcN exposure. Differences observed in the number of birth defects between mice from the LF + GlcN and HF + GlcN group should not be related to the dose of GlcN received, since mice were administered with a dose determined by mean body mass. However, it is possible that GlcN was metabolized in a non-uniform manner in adipose tissue relative to other sites, which resulted in non uniform amounts of GlcN being available for reproductive structures in obese mothers.

Diabetes and obesity, both conditions in which HBP activity is known to be up regulated (Robinson et al. 1995; Buse et al. 1997; Akimoto et al. 2000; Considine et al. 2000; Veerababu et al. 2000; Kaneto et al. 2001; McClain, 2002; Fulop et al. 2007) are also associated with increased rates of birth defects (as reviewed by Chung and Myrianthopoulos, 1975; Reece, 2008). Previously, Wyman et al. (2008) showed that in vivo, peri-conceptional hyperglycemic exposure modifies developmental outcomes, by demonstrating that fetal size is reduced and instances of birth defects are increased in response to exposure of the murine zygote to hyperglycemia. GlcN has previously also been linked to perturbed reproductive outcomes. In 2004, Horal et al. observed an increase in the incidence of neural tube defects in fetuses derived from mice administered with GlcN between 7 - 10 days gestation. Although peri-conception GlcN exposure has not previously been linked to birth defects, in vitro exposure of COCs to GlcN reduces oocyte developmental competence in bovine, porcine (Sutton-McDowall et al. 2006; Kimura et al. 2008) and mouse (Chapters 2 and 3) models. Consequently, these in vivo results can be taken as further evidence that GlcN exposure elicits impairments on developmental outcomes, with the nature of such deviations potentially dependent upon the stage of exposure.

Interestingly, in addition to having the highest instances of birth defects, the LF + GlcN group also had the highest pregnancy rates, suggesting that although there were more fetuses formed in this group, they tended to be of a poorer quality. Potential reasons for the increase in pregnancy rate are not overtly obvious. It cannot be attributed to differences in mating rates or male fertility given that all groups showed evidence for mating at similar rates and were exposed comparably to the males. Rather, it seems that GlcN exposure to mice maintained on a LF diet may have elicited effects that could span the ovulatory – early post-fertilisation phase of development via unknown mechanisms. Although GlcN did not affect CL numbers, it may have caused more mice to ovulate developmentally compromised COCs. GlcN exposure in this group may also have elicited effects on the pre and post fertilization hormonal environment that assisted fertilisation to occur and/ or early pregnancies to be maintained, and the assessment of the effects of GlcN on key pregnancy hormones such as oestrogen and progesterone would be warranted.

Of key importance to the perturbed fetal and postnatal outcomes in these studies is the lack of treatment-induced modifications of maternal metabolic parameters measured at the time of mating. Exposure of mice to a HF diet has previously been shown to lead to metabolic perturbations. Minge et al. (2004) reported that 16 weeks of exposure to the same HF diet outlined in these studies led to alterations in serum insulin levels. Tortoriello et al. (2004) also found that the administration of a HF diet to mice for 16 weeks modified insulin and glucose levels. Although HF fed mice in this study did not demonstrate any detectable differences in insulin and glucose levels, the fact that they were overweight would suggest that some metabolic perturbations would be present or developing, but may have not been measurable with the methods employed. This notion is supported by the reduced fetal length and weight in pups from mothers maintained on a HF diet. That fetal differences were observed despite the absence of detectable maternal metabolic perturbations, may indicate that reproductive outcomes can be influenced by only slight metabolic perturbations.

Important findings that compliment this notion have been reported by Wu et al. (2010), with six week old mice that were maintained on the same HF (22%) diet as the mice in the studies in this chapter. After only four weeks of dietary manipulation, it was found that oocytes from mice maintained on the HF diet presented with increased quantities of lipid and decreased mitochondrial membrane potential, a marker of mitochondrial damage. The cumulus cells from pre-ovulatory follicles from such treated mice also showed significantly higher rates of apoptosis. Additionally, rates of anovulation were significantly higher in mice fed the HF diet, and of the oocytes that were collected, fewer became fertilised. Unfortunately the authors did not report mean body mass values or indicate whether metabolic assays such as serum glucose and insulin levels were assessed. Regardless however, the documentation of

numerous reproductive perturbations in mice that were started on the same dietary regime at comparable ages but for almost one third of the time duration, provides further evidence that short term exposure to a diet with 22% fat can lead to measurable disruptions in optimal reproductive functioning.

Glucose clearance rates were also not affected by short term exposure to GlcN in the current study. Mice received IP injections of GlcN, which would suggest that the complete dose of GlcN was available to participate in physiological processes. This is because the bioavailability of GlcN is dependent upon its route of administration (Setnikar et al. 1984: Setnikar et al. 1993). While significantly less GlcN is available for distribution around the body when orally administered owing to the fact that ingested GlcN is not largely absorbed (Setnikar et al. 1993; Adebowale et al. 2002; Aghazadeh-Habashi et al. 2002; Persiani et al. 2005), IP injected GlcN has been shown to have complete bioavailability (Aghazadeh-Habashi et al. 2002).

The metabolic results in this chapter complement the findings of studies that employed a wide range of GlcN doses and durations, and which showed that oral GlcN consumption in normoglycemic individuals did not modify blood glucose levels (Echard et al. 2001; Scroggie et al. 2003; Yu et al. 2003; Tannis et al. 2004; Muniyappa et al. 2006). Importantly, however, some studies have shown an effect of GlcN in subjects with abnormal glucose and insulin metabolism. Biggee et al. (2007) reported increased blood glucose levels following the consumption of 1500 mg GlcN taken during a glucose tolerance test, when patients had pre-existing, impaired glucose tolerance. In another study, Pham et al (2007) also confirmed that short term (6 weeks) exposure to the RDI of 1500 mg GlcN leads to insulin resistance in people with pre-existing insulin abnormalities. Using the rat model, Virkamaki et al. (1997) showed that the infusion for 6 hours of 30 µM per min per kg GlcN with insulin (18 mU per min per kg)) reduced insulin-stimulated glucose uptake.

Based on the results obtained for fetal outcomes, no evidence was found for the existence of additive effects of GlcN and maternal overweight/obesity. Although HF + GlcN derived offspring were in some instances significantly different to offspring from other groups, this analysis is based on a comparison of only three groups. In the absence of data from offspring from HF - GlcN treated mice, no conclusion can be formulated regarding the existence of a relationship between GlcN and maternal overweight/obesity. There are no obvious reasons why offspring from the HF - GlcN group had significantly worse survival outcomes than their dietary counterparts that received GlcN. Although peri-natal survival is known to be reduced by maternal complications including diabetes and overweight/obesity (as reviewed by Kalter, 2002), this offers no explanation as to why survival would seemingly

be improved by GlcN exposure (i.e. HF + GlcN). It is possible that this was due to random chance alone, and that the utilization of a larger cohort would generate alternative findings. Alternatively, it is also possible that the addition of GlcN served as a sufficient stimulus to activate compensatory mechanisms that assisted survival, but which ultimately heighten instances of developmental problems in surviving offspring.

At 3 weeks of age, body weight was increased in both male and female offspring of mice maintained on the HF diet and exposed to GlcN. However, these differences in body weight were not maintained. Reasons for the transient nature of the weight increase in the mice are unknown. It is possible that this could be representative of catch up growth in the HF - GlcN derived animals, consistent with the results of Jungheim et al. (2010) who demonstrated that mice born from HF fed mothers that were initially smaller, became significantly bigger than their control counterparts by post natal day 25. Unfortunately catch up growth cannot be confirmed in these studies without birth weight data, although interestingly it was shown that d18 fetuses from HF mice were significantly lighter than their LF counterparts.

Male offspring showed no long term differences between groups for body weight. In contrast, female offspring from HF + GlcN mice had consistently higher body weight from 10 weeks of age relative to both other groups. Greater hind limb mass and more importantly significantly greater fat deposits contributed to the generation of a higher body weight at 16 weeks, and were also presumably contributing mechanisms at 10 weeks of age. In agreement, other studies have also revealed gender specific differences in postnatal outcomes following exposure to a maternal HF diet. For example, female, but not male, offspring of rats maintained on a HF diet before conception as well as throughout pregnancy have elevated blood pressure between 180 – 360 days of age (Khan et al. 2003). Similarly, insulin secretion is reduced at 50 weeks of age in female, but not male, offspring of HF fed rats (Han et al. 2005). In addition, Jungheim et al. (2010) have provided evidence for gender specific effects of a HF maternal diet, by demonstrating that only males had elevated serum cholesterol levels and compromised glucose tolerance at 10 and 13 weeks respectively.

Interestingly, GlcN has also previously been shown to elicit a gender specific effect on survival. Kimura et al. (2008) reported that the addition of GlcN to bovine IVM and culture media from the 8 cell stage onwards skewed the blastocyst sex ratio towards males, and that moreover, this phenomenon was corrected by the co-incorporataion of the O-linked glycosylation inhibitor BADGP. O-linked GlcNAc transferase (OGT) is an X Chromosome linked enzyme that is used for O-linked glycosylation. Previously, it has been shown that culturing bovine embryos from the 8 cell stage in the presence of 2.5 mM glucose also increases the male sex ratio skew (Kimura et al. 2005). Based on these findings, it would

have been interesting to ascertain if GlcN also altered the fetal sex ratio of offspring derived from mothers treated with GlcN in vivo.

Measurement of hexosamine levels, such as GlcN-6-phosphate, in peri-conceptional, in vivo GlcN-exposed samples of maternal and embryonic origin would have been of interest in this study. Others have shown that HBP activity is increased under conditions of obesity (Buse et al. 1997; Considine et al. 2000; Veerababu et al. 2000) as well following GlcN treatment (Rossetti et al. 1995; Hawkins et al. 1997; Horal et al. 2004; Yki-Jarvinen et al. 1998; Patti et al. 1999; Virkamaki and Yki-Jarvinen 1999; Rumberger et al. 2003; Marshall et al. 2004). While there is no doubt that administration of GlcN would increase HBP activity, specific analyses would be required to determine the extent to which this occurred with the treatment regimen used in the current study, and in reproductive tissues. Similarly, the extent to which the obesity model used in the current study affected HBP activity would also require specific analysis. Following on from this, it would be useful to test how GlcN - induced changes to Hexosamine activity affected PPP flux. An understanding of the metabolic changes resulting from exposure to GlcN during the peri-conceptional period would enhance the understanding of the mechanisms through which GlcN affects reproductive outcomes in vivo.

## 5.7 Conclusion

The experiments outlined in this chapter have confirmed that in vivo, peri-conceptional GlcN exposure elicits detrimental effects on fetal development. Additionally, they also show that maternal weight also has measurable effects on reproductive outcomes. The finding of perturbed development following peri-conceptional GlcN exposure are complimentary to the findings of Chapters 2 and 3, where embryonic development was compromised following in vitro GlcN exposure during IVM. However, the effects of in vivo GlcN administration on reproductive outcomes contrast with those reported in Chapter 4, with a reduction in fetal weight but no effect on litter size detected in the current study. This suggests that other factors, apart from maternal weight, influence the effects of GlcN on reproductive outcomes.

# 6.0 Chapter 6

## Fetal developmental outcomes in the mouse in response to acute, peri-conceptional GlcN exposure are determined by maternal age.

## 6.1 Summary

It was noted in Chapter 5, that reproductive outcomes were inconsistent between mice maintained on a standard caloric intake described in Chapters 4 and 5 (LF diet). As the primary difference between the two models was maternal age, the current study aimed to determine whether the effects of peri-conceptional GlcN exposure are influenced by maternal age. C57BL6 mice of either 8 weeks or 16 weeks age were given 4 - 7 days 0 mg/kg (PBS) or 20 mg/kg GlcN during the peri-conceptional period, and outcomes were assessed on gestational d18. Consistent with the results from Chapters 4 and 5, GlcN treatment reduced mean implantation rate ($P<0.01$) and litter size ($P<0.01$) in 8 week old mice, but not 16 week old mice. In addition, GlcN treatment reduced fetal weight ($P<0.05$) and length ($P<0.05$) only in 16 week old mice. Within 16 week old mice, GlcN also increased the proportion of birth defects and the proportion of mothers that carried birth defected pups ($P<0.05$). These results demonstrate that the reproductive consequences of peri-conceptional GlcN exposure are influenced by maternal age.

## 6.2 Introduction

Despite the fact that the knowledge base of factors that negatively impact upon reproduction is constantly growing, there are still marked deficiencies of knowledge. One area of deficiency pertains to the interaction or cumulative effect of multiple stimuli which on their own are recognized as having a significant perturbing influence. Although it has long been known that diabetes (as reviewed by Kalter et al. 2002) as well as hypertension (as reviewed by Marshall and Carpenter, 2007) both independently perturb reproductive performance, it has recently been shown that the combined pathologies elicit significantly worse effects (Stella et al. 2008), and thereby provides evidence for the potential significance of examining outcomes of simultaneously occurring health perturbations.. In Chapters 4 and 5, it was shown that acute, peri-conceptional exposure to GlcN results in impaired reproductive outcomes. Intriguingly, however, the nature of the perturbations was found to be inconsistent. For example, GlcN exposed mice from Chapter 4 that were maintained on a standard diet and had received GlcN treatment at 8 weeks of age, had a reduced implantation rate and litter size, as well as increased proportion of implantations that resorbed. In contrast, no such effects were seen in the mice from Chapter 5, irrespective of receiving either a HF or LF (standard) diet. Relative to the mice studied within Chapter 4, the mice studied in Chapter 5 were of the same strain and given equivalent treatment, but were of an older age (16 weeks) as they were acting as controls for mice whose diets had been manipulated for 11 weeks. Furthermore, there was a reduction in fetal weight and fetal length in offspring of 16 week old mice that were maintained on a standard diet and given GlcN (Chapter 5), although no difference in these outcomes was evident in GlcN treated 8 week old mice in chapter 4. These findings have prompted an investigation into whether the effects of peri-conceptional GlcN administration are influenced by maternal age at the time of exposure. The aim of the current study was to directly compare the effects of GlcN treatment at 8 weeks or 16 weeks of age on reproductive outcomes in mice.

## 6.3 Methods and Materials

Unless otherwise stated all chemicals were purchased from Sigma Chemical Co. (St Louis, USA).

### 6.3.1 Animals

For all procedures, C57BL6 male and female mice (purchased from Laboratory Animal Services at The University of Adelaide) were used, and kept under 14:00 - 10:00 light-dark conditions. They were housed in the animal housing facilities at the University of Adelaide Medical School.

### 6.3.2 Experiment 1 – Fetal outcomes following peri-conceptional exposure to GlcN at different maternal ages

For each replicate (8 in total), 6 x 5 week old mice (young) and 6 x 13 week old mice (older) were weighed weekly for 3 weeks. All mice were first parity. At 8 and 16 weeks of age, the young and older mice groups were equally distributed into two, weight-matched subgroups and allocated to receive either 0 mg/kg GlcN or 20 mg/kg GlcN. This created 4 groups (8 wks + 0 mg/kg GlcN, 8 wks + 20 mg/kg GlcN, 16 wks + 0 mg/kg GlcN, and 16 wks + 20 mg/kg GlcN).

#### *6.3.2.1 Treatment regime*

A solution comprised of glucosamine hydrochloride, dissolved in PBS was prepared at a concentration of 4 mg/ml and refrigerated. This concentration corresponded to 5 µL of either PBS or GlcN per gram of body weight giving rise to doses of 0 mg/kg GlcN, or 20 mg/kg GlcN, respectively.

For each replicate, average weights for the 8 week old and the 16 week old mice were calculated. These average weights were used to calculate the volume of 4 mg/kg GlcN solution to correspond to a 20 mg/kg dose for each age group. An equivalent, weight-

dependent volume of PBS was also administered for control animals (0 mg/kg GlcN) in each of the age groups.

Mice were given appropriate, weight dependent doses of PBS or GlcN solution for three days via IP injection, at 24 hour intervals. Immediately following the third injection, mice were placed with an individual male, 24 - 32 week old C57BL6 mice overnight. The following morning, female mice were assessed for successful mating via the detection of vaginal sperm plugs, and returned to their appropriate cages. Those mice that did not successfully mate overnight were weighed, given a subsequent injection and placed with a male overnight. This process was continued until successful mating occurred, for a maximum of 4 mating attempts. When successful mating occurred, mice were again weighed and administered with an appropriate dose of PBS or GlcN. Therefore, mice received injections of PBS or GlcN for a minimum of 4 and a maximum of 7 days. The date that each mouse successfully mated, as well as the number of injections that they received, was recorded.

Previously it was shown that there were no differences in outcome (except for the distribution of days of GlcN exposure before mating) that were dependent upon days of GlcN exposure (Chapter 4). As non-mated mice were not going to be used for other analyses (e.g. glucose tolerance as occurred in Chapter 5), it was decided that mice would be given the opportunity to mate for a maximum of 7 days.

#### *6.3.2.2 Pregnancy outcomes*

On day 18 of pregnancy, mice that had mated were killed via cervical dislocation and weighed. Litter size, implantation rate and resorption rate were recorded, as were rates and nature of birth defects as ascertained by gross morphological appearance of conceptuses. Viable conceptuses were removed from uterine tracts, and fetuses and placentae were weighed and assessed for gross morphological appearance. Fetal crown-rump length, placental length and placental width were measured with a pair of calipers.

Ovaries were assessed for corpora lutea as indicators of ovulation rate. Ovaries were initially removed, weighed and placed individually into chilled PBS. Ovaries were then placed into a 35mm Nunc dish containing 2 ml chilled PBS, and assessed microscopically for corpora lutea observable on the ovarian surface.

### 6.3.3 Experiment 2 – Assessing PPP activity in oocytes derived from mice subjected to peri-conception GlcN exposure at different maternal ages

#### *6.3.3.1 GlcN treatment*

Five and 13 week old mice were allocated to treatment groups (8 weeks ± 20 mg/kg GlcN, 16 weeks ± 20 mg/kg GlcN) as described above. After 3 weeks and at the age of 8 and 16 weeks, they received IP injections of weight-determined doses of either PBS or GlcN for 3 days and on the third day were placed with a vasectomised male overnight. The detection of a vaginal sperm plug the following morning was taken as evidence that mating and therefore ovulation had occurred. If mating had not occurred, consecutive treatment with GlcN and vasectomised males occurred as described above. The GlcN administration procedure in experiment 2 differed from experiment 1 in one respect, however, although this most likely did not significantly alter the experimental uniformity. Instead of 24 hours after the previous dose, in experiment 2 the final GlcN dose was administered 17 hours after the previous dose, because females were killed 24 hours after receiving the second last dose of either PBS or GlcN. Three mice per treatment group and per replicate were used, and a total of four replicates were performed.

#### *6.3.3.2 COC collection*

Mice were sacrificed via cervical dislocation, and oviducts were removed and placed into 37ºC MOPS-G1 handling media (Lane and Gardner, 2004). Ovulated COCs were extracted from the oviduct, placed into 1.6 ml Eppendorf tubes and vortexed for 1 - 3 consecutive, 30 second periods to remove the cumulus cells.

#### *6.3.3.3 Glucose-6-Phosphate Dehydrogenase (G6PDH) assay*

As described in Chapter 2, denuded oocytes were briefly washed with MOPS-G1, and then transferred to MOPS-G1 drops containing 26 mM BCB overlaid with mineral oil at a ratio of one oocyte per 100 µL media, for 30 minutes. Preliminary experiments had determined that the optimal concentration, volume and exposure period (data not shown). Oocytes were washed through 4 - 6 drops of MOPS-G1 until the handling media was no longer blue. They

were immediately visualized using a stereomicroscope, and recorded as either positive or negative for presentation of a blue cytoplasm.

## 6.4 Statistics

Except where otherwise stated, all data was arcsine transformed prior to analysis with SPSS (version 13.0).

### 6.4.1 Pre – GlcN Administration

Comparison of the mean weekly weight change in mothers across all replicates was performed with a Repeated Measures ANOVA and Bonferroni corrected pairwise comparison. Per replicate, One-way ANOVAs were performed on the subdivided groups of both the 8 week and 16 week old groups. This was performed to confirm that the randomly formed groups (± 20 mg/kg GlcN) had equivalent weights within age groups.

### 6.4.2 Post GlcN administration

A One-way ANOVA was used to compare the weight of mice that did become pregnant as well as mice that did not become pregnant. This was performed within age groups (i.e. pregnant and non- pregnant for 8 weeks as well as for 16 weeks) as well as GlcN treatment groups (i.e. pregnant vs non-pregnant for 8 weeks - GlcN, 8 weeks + GlcN, 16 weeks - GlcN and 16 weeks + GlcN).

#### 6.4.2.1 *Experiment 1 – Reproductive outcomes following peri-conceptional exposure to GlcN at different maternal ages*

Outcomes were analyzed with a Chi-squared analysis (see Appendix, Table 8.11.5) or a Two-way ANOVA with covariates (see Appendix, Table 8.11.6), and a Bonferonni post hoc test. Co-variates that when included were found to alter significance values of the Two-way ANOVA have been highlighted.

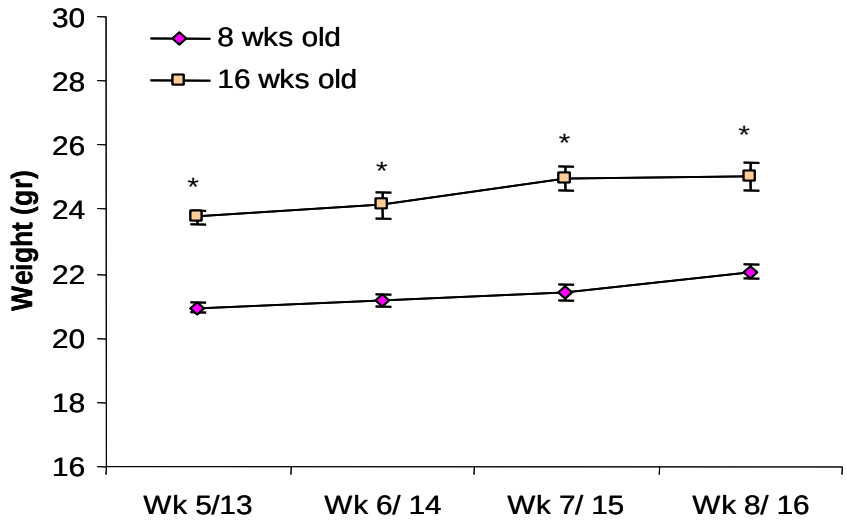
### *6.4.2.2 Experiment 2 – Assessing PPP activity in oocytes derived from mice subjected to peri-conceptional GlcN exposure at different maternal ages*

The number and proportion of mice that ovulated was analyzed with a Chi-Squared test. The proportion of oocytes with a blue cytoplasm was analyzed with a Two-way ANOVA. Co-variates including maternal weight at 8 and 16 weeks, maternal weight change from 5 - 8 weeks and 13 - 16 weeks and days of GlcN exposure were included in the analyses. The incorporation of these co-variates were not found to influence the significance levels as detected without co-variate incorporation.

## 6.5 Results

### 6.5.1 Pre-GlcN administration

Older mice (mated at 16 weeks) were consistently heavier than younger mice (mated at 8 weeks) for the 3 weeks they were housed prior to GlcN treatment (P< 0.03) (Figure 1).




**6.5.1.1 Figure 1:** Weights of mice over 5 - 8 weeks (for those given ± 20 mg/kg GlcN at 8 weeks) and over 13 - 16 weeks (for those given ± 20 mg/kg GlcN at 16 weeks) during the peri-conceptional period. Values are (mean ± SEM) * represents significant differences (P<0.05) for each week.

### 6.5.2 Post-GlcN administration

#### *6.5.2.1 Weight*

There were no differences in maternal body weight at mating between mice that did and did not become pregnant within dietary and GlcN treatment groups (Appendix 8.12.4).

#### *6.5.2.2 Pregnancy rates*

There were no differences in the total number or proportion of mice that presented with sperm plugs between age or treatment groups. Similarly, there were no differences in the number of mice that became pregnant, or the proportion of pregnant mice, relative to total starting numbers or relative to those that presented with a sperm plug (Table 1).

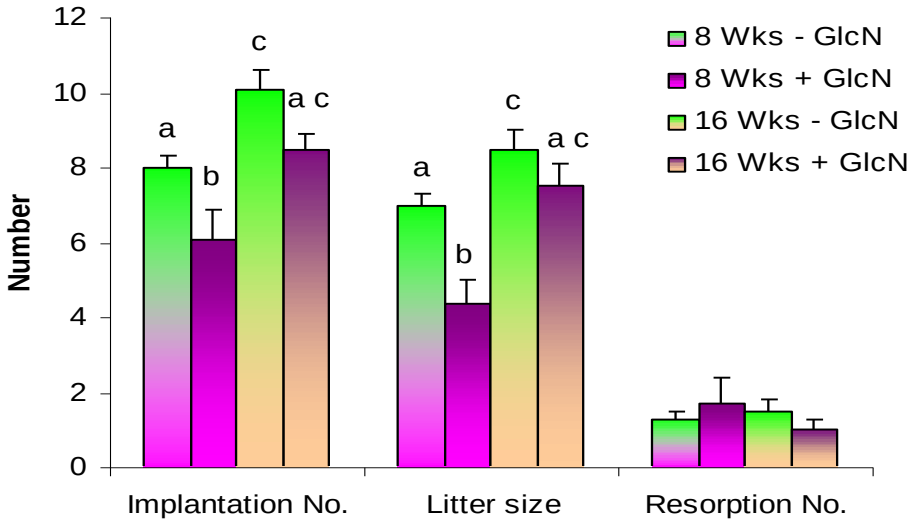
| | 8 wks - GlcN | 8 wks + GlcN | 16 wks - GlcN | 16 wks + GlcN |
|---|---|---|---|---|
| **Mated (N)** | 20 | 19 | 21 | 19 |
| **Mated (%)** | 83.3 | 79.1 | 87.5 | 79.1 |
| **Pregnant (N)** | 11 | 7 | 9 | 11 |
| **Pregnant (from total) (%)** | 45.8 | 29.2 | 37.5 | 45.8 |
| **Pregnant (from plugged) (%)** | 55.0 | 36.8 | 42.8 | 57.9 |

**6.5.2.2.1 Table 1:** Numbers and proportions of 8 and 16 week old mice that presented with sperm plugs and became pregnant after receiving 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN.

Significantly more mice mated over the first 2 days compared to the third and fourth days of GlcN treatment ($P<0.0001$). Numbers and proportions of mated mice are presented in the Appendix, Table 8.19.3.

### *6.5.2.3 Litter outcomes*

GlcN exposure reduced mean implantation rate and litter size in young mice (P<0.02). (GlcN x age interaction, P<0.01 for both) (Figure1). Relative to 16 week old mice not given GlcN there was a trend towards reduced implantation rates and litter sizes in older mice treated with GlcN, although this was not significant. However, older mice that were not treated with GlcN had significantly higher implantation rates and litter sizes than young mice (P<0.01), and an interaction between GlcN and age was detected for implantation rate and litter size (P<0.01 for both) (Figure 1). Collectively, these results suggest that GlcN exposure has an age-dependent perturbing effect on implantation rate and litter size. No differences in mean resorption rates were detected between the four groups (Figure 1).




**6.5.2.3.1 Figure 1:** Implantation number, litter size and resorption number for pregnancies in 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. Values are (mean ± SEM). Different letters represent significant differences (P<0.05) between groups for each outcome.

Overall, the total number of implantations (P<0.0001) as well as viable fetuses (P<0.0001) was significantly less in the 8 wk + GlcN group. No differences in total resorptions were seen between the four groups (Table 2).

| | 8 Wks – GlcN | 8 Wks + GlcN | 16 Wks – GlcN | 16 Wks + GlcN |
|---|---|---|---|---|
| **Implantation No.** | 89 [a] | **43** [b] | 91 [a] | 94 [a] |
| **Pup No.** | 77 [a] | **31** [b] | 77 [a] | 83 [a] |
| **Resorption No.** | 14 [a] | 12 [a] | 14 [a] | 11 [a] |

**6.5.2.3.2 Table 2:** Total numbers of implantations, pups and resorptions for pregnancies in 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. (Litter No. = 8). Different letters represent significant differences (P<0.05) between groups for each outcome.

A greater proportion of implantations resorbed (P = 0.02) in 8 week old mice that received peri-conceptional GlcN treatment (Table 3).

| | 8 Wks – GlcN | 8 Wks + GlcN | 16 Wks - GlcN | 16 Wks + GlcN |
|---|---|---|---|---|
| **Ovulated oocytes that implanted (%)** | 86.4 [a] | 95.5 [a] | 92.8 [a] | 81.7 [a] |
| **Implantations that resorbed (%)** | 13.5 [a] | **27.9** [b] | 15.4 [a] | 11.7 [a] |

**6.5.2.3.3 Table 3:** Proportions of ovulated oocytes that formed implantations, as well as proportions of implantations that resorbed, in 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. Different letters represent significant differences (P<0.05) between groups for each outcome.

### *6.5.2.4 Ovulation data*

Mean CL number, tended to be lower in young mice that received 20 mg/kg GlcN, although this was not significant (P<0.08, Table 4).

| | 8 Wks –GlcN | 8 Wks + GlcN | 16 Wks – GlcN | 16 Wks + GlcN |
|---|---|---|---|---|
| **Mean CL No.** | 11.4 ± 1.3 | 9.0 ± 2.6 | 12.2 ± 0.6 | 12.8 ± 0.7 |

**6.5.2.4.1 Table 4:** Mean CL number of 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN.

### *6.5.2.5 Fetal outcomes*

Maternal age did not independently influence fetal weight. GlcN was found to have a significant effect on fetal weight ($P<0.03$), and there was a significant interaction between GlcN and maternal age ($P<0.01$). Fetal weight was reduced by peri-conceptional GlcN treatment in 16 week old mice ($P<0.05$) (Figure 2a), while the same treatment did not affect fetal weight in 8 week old mice.

Fetal length was reduced in pups derived from 16 week old mice given GlcN ($P<0.05$) (Figure 2b). Neither GlcN nor the mothers' age independently contributed to alterations in fetal length. However, a significant interaction was found between GlcN and maternal age ($P<0.04$), that disappeared when maternal weight was included in the analysis as a co-variate, suggesting that maternal weight is a contributing component to age-associated changes in fetal size.

Placental weight was increased in 8 week old mice treated with 20 mg/kg GlcN, when compared to all other groups ($P<0.05$) (Figure 2c). GlcN treatment had a significant effect on d18 placental weight ($P<0.05$).There was no effect of maternal age on placental weight, and there was no interaction between age and GlcN treatment. No differences were detected in placental volumes between the 4 groups (Figure 2d).

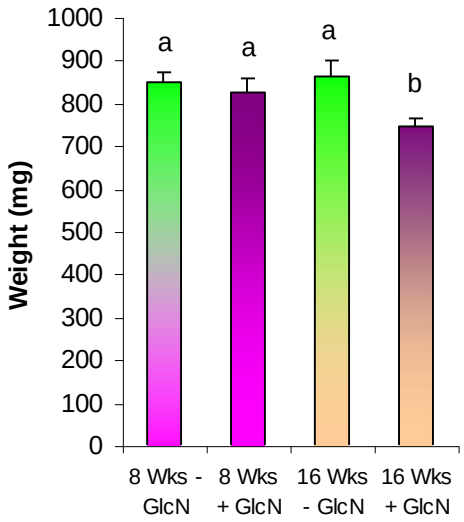



**Figure 2a:** Mean Fetal Weight (mg)

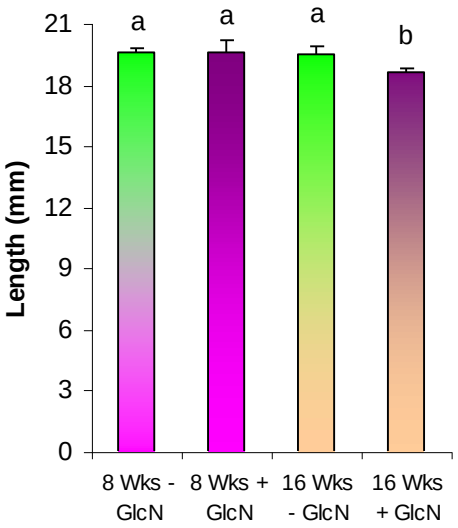



**Figure 2b**: Mean Fetal Length (mm)

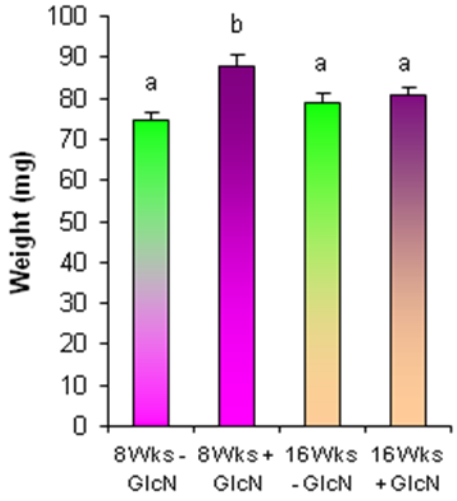



**Figure 2c**: Mean Placental Weight (mg)

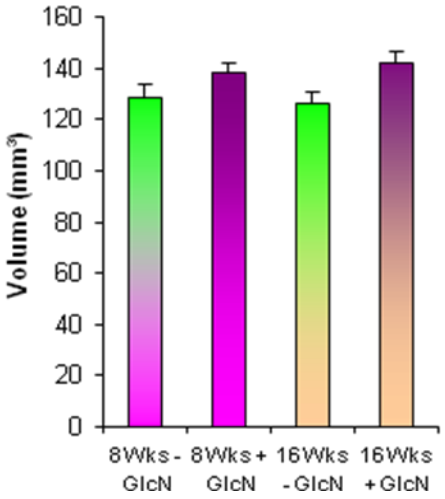



**Figure 2d**: Mean Placental Volume (mm³)

***6.5.2.5.1*** **Figure 5:** Mean fetal weight, fetal length, placental weight and placental volume for pregnancies in 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. Different letters represent significant differences ($P<0.05$) between groups and for each outcome. ((N): 8 Wks - GlcN = 76; 8wks + GlcN = 30; 16 Wks - GlcN = 76; 16 Wks + GlcN = 83).

### *6.5.2.6 Birth defects*

As not all treatment groups presented with abnormally developing conceptuses, a Two-way ANOVA was not able to be used to assess abnormalities. Instead, a Chi- squared analysis was employed, and demonstrated a significantly higher number of abnormal fetuses were present in litters of older mothers treated with GlcN as opposed to litters from younger mothers that were not exposed to GlcN ($P<0.05$) (Table 5). A higher number, and proportion, of GlcN treated older mothers had pregnancies characterized by at least one abnormally developing fetus, when compared to the remaining groups ($P<0.05$) (Table 5).

| | 8 Wks – GlcN | 8 Wks + GlcN | 16 Wks – GlcN | 16 Wks + GlcN |
|---|---|---|---|---|
| **No. Fetuses** | 76 | 30 | 76 | 83 |
| **No. Abnormal Fetuses** | 2 [a] | 0 [a] | 3 [a] | 8 [b] |
| **% Abnormal Fetuses from Total** | 2.6 | 0 | 3.9 | 9.6 |
| **No. Mothers** | 11 | 7 | 9 | 11 |
| **No. Mothers with Abnormal Fetuses** | 2 [a] | 0 [a] | 2 [a] | 5 [b] |
| **% Mothers with Abnormal Fetuses** | 18.2 [a] | 0 [b] | 22.2 [a] | 45.4 [c] |

**6.5.2.6.1 Table 5:** The prevalence of fetal and placental birth defects in 8 and 16 week old mice that received 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. Different letters represent significant differences ($P<0.05$) between groups within each outcome.

## 6.5.3 Experiment 3 – Assessing PPP activity in oocytes derived from mice subjected to peri-conceptional GlcN exposure at different maternal ages

Horal et al. (2004) previously demonstrated that GSH, an intermediate of the PPP, is reduced in fetuses that had been exposed in vivo to GlcN. To ascertain if differences in PPP

activity could be detected in the oocyte, we utilized BCB to assess G6PDH activity. No differences were detected in the proportion of oocytes that exhibited a blue cytoplasmic stain, suggesting that G6PDH levels were not significantly different between the four groups. There were also no differences in the number or proportion of mice that ovulated (12 mice per treatment). Results are presented in Table 6.

| | 8 Wks – GlcN | 8 Wks + GlcN | 16 Wks - GlcN | 16 Wks + GlcN |
|---|---|---|---|---|
| **Oocytes with blue cytoplasm (%)** | 78.3% ± 16.0 | 70.1% ± 11.4 | 85.0% ± 9.2 | 78.2% ± 9.7 |
| **Mice that ovulated (N)** | 6 | 6 | 5 | 7 |
| **Mice that ovulated (%)** | 50 | 50 | 41.7 | 58.3 |

**6.5.3.1 Table 6:** The proportion of oocytes that presented with a blue cytoplasm after exposure to BCB, as well as the number and proportion of mice that ovulated. Oocytes were ovulated from 8 and 16 week old mice that received 4 - 7 days exposure to either 0 or 20 mg/kg GlcN.

## 6.6 Discussion

Collectively, the results obtained from the investigations outlined in this chapter indicate that there are age-specific differences in reproductive outcomes in mice subjected to acute, peri-conception GlcN exposure. Firstly, this was demonstrated by the finding that fetuses from 16 week old, GlcN treated mice were lighter and shorter (in agreement with Chapter 5) compared to all other groups. Previously Wyman et al. (2008) demonstrated that fetal growth is reduced following in vivo exposure to a maternal diabetic environment limited to the zygote stage. This suggests that the GlcN-induced, reduced fetal size in this Chapter may be attributed to HBP upregulation, although provides no explanation as to why fetal size was unaffected by GlcN exposure in 8 week old mice.

16 week old mice treated peri-conceptionally with GlcN also had an increase in the total and proportion of fetuses with birth defects as well as the number and proportion of mothers that carried birth defected fetuses. While there is similarly no immediate explanation for GlcN to affect only 16 week old mice in this manner, these results can be seen to be consistent with

those of Wyman et al. (2008) who demonstrated that congenital malformations in mouse fetuses were increased following in vivo exposure to a diabetic environment limited to the zygote stage. Furthermore, the results of Chapter 6 are also consistent with the outcomes of Chapter 4, where malformations were equivalent between 8 week old mice regardless of GlcN exposure, and Chapter 5, were malformations were increased in 16 week old mice following GlcN exposure, regardless of diet.

The results of this Chapter also confirm the age-related differences observed in Chapters 4 and 5 in relation to fetal resorptions. While resorption rates were not different in the 16 week old mice from Chapter 5, regardless of GlcN or dietary treatment, differences were detected in the 8 week old mice used in Chapter 4. Specifically, there were a significantly higher proportion of pregnancies in Chapter 4 that were characterized as having at least 50% of implantations resorb in response to GlcN treatment. Previously, it has been shown that, similar to clinical observations (as reviewed by Rosenn et al. 1994; Kalter, 2002; Jovanovic et al. 2005), mouse fetuses from diabetic mothers have an increased likelihood of undergoing developmental failure (Moley et al. 1991; Chi et al. 2000). The nature of the specific embryonic defects resulting in post implantation developmental failure following GlcN treatment of young mice requires further analysis, to determine why these outcomes vary between young and older mice. Whether these effects relate to the quality of the embryo, or the implantation process is uncertain.

In addition, it was also shown that 8 week old mice administered with GlcN produced heavier placentas. This is consistent with the outcomes of Chapter 5 where placental weights were equivalent between all 16 week old mice, regardless of GlcN exposure and dietary regime, as well as Chapter 4 were a trend for increased placental weight was observed in 8 week old mice treated with GlcN. Previously, Ericsson et al. (2007) demonstrated that the IP administration of glucose to pregnant rats on gestational day 10 increased near-term placental and fetal weights (measured on day 21 of a 23 day gestation period), thereby demonstrating that mid gestational, transient glucose fluxing alters the placental growth trajectory. Based on the outcomes of Chapter 6 it also seems that placental growth may be influenced by glycemic events that occur peri-conceptionally.

While the reduced fetal weight and increased instance of birth defects demonstrates that embryo quality is perturbed by GlcN exposure in 16 week old mice, it is also important to note that these embryos implanted and developed to late gestation at equivalent rates. Concomitantly, the higher frequency of resorptions and increased placental weight demonstrates that embryo quality is also influenced by GlcN in 8 week old mice. Mechanisms involved in the generation of divergent reproductive outcomes in the different

mouse ages are unclear and require further investigation. The results of these studies do however support the findings reported in Chapters 4 and 5 that in vivo administered GlcN is not metabolized exclusively externally to the reproductive system (Fowler and Guttridge, 1987; Fowler 1988; Fowler and Barrett, 1989; Muñoz-Gutiérrez et al. 2002; Horal et al. 2004; Muñoz-Gutiérrez et al. 2004).

Due to its role as a substrate for mucins, GlcN–induced effects may potentially affect follicular function, the oocyte, and possibly the oviduct and/or uterine environment. Based on the results of the current study it could be argued that this does not involve an inhibition of ovulation, given the lack of significant difference in CL numbers between the 4 groups which is in agreement with the findings from Chapter 5, although this requires closer examination. It is, however, perhaps in contrast to multiple studies that have shown an inhibitory effect of diabetes on ovulation rate (Chieri et al. 1969; Kirchick et al. 1978; Jawerbaum et al. 1996; Colton et al. 2002), although the extent of hyperglycemic exposure that may be needed to induce this effect may be considerably higher than what would have been achieved from these experimental treatments.

An alternative possibility is that GlcN could be metabolized differentially in adipose tissue relative to other tissues. If this is the case, older mice with differential distributions of fat to younger mice may metabolize equivalent doses of GlcN differently. However, body composition was not assessed in the pregnant dams in this study. It is also possible that a compensatory response to the hyperglycemic stimulation is more active as age increases, as a result of increasing insulin resistance throughout the aging process. Indeed, Jovanovic et al. (2005) have previously speculated that adaptations may occur in the chronically hyperglycemic pregnancy which permits some degree of conceptus or maternal resistance to perturbing hyperglycemic stimulation.

The experimental design outlined in this chapter effectively included two control groups at different ages (8 wks - GlcN and 16 wks - GlcN). However, findings of differences between these two groups should not be viewed as difficult to interpret or of having compromised reliability. The experiments conducted in Chapter six were designed to confirm results obtained from Chapters 4 and 5, whereby GlcN elicited effects of different natures on reproductive outcomes in mice that varied in age.

Although both increased maternal age (as reviewed by Hansen, 1986; Friede et al. 1988; Nybo Andersen et al. 2000; Miletic et al. 2002) as well as hyperglycemic stimulation (as reviewed by Kalter et al. 2002) are recognized as independent factors that perturb reproductive success rates, little is known about the cumulative effects of these stimuli. In light of the increasing age of pregnant women (Martin et al. 2003; Martin et al. 2005) and the

increasing incidence of metabolic disorders involving perturbed glucose metabolism (Harris et al. 1998; Dunstan et al. 2001; Mokdad et al. 2003), it is surprising that this has received little attention.

It is important to remember however that the mice in this experiment were of a younger age than that classically used in aging studies and mice of the same age and maintained on the same diet in Chapter 5 showed no evidence of metabolic perturbation following GlcN treatment. Since GlcN administration has only been shown to modify serum glucose levels in models where glucose and/or insulin metabolism is already compromised (Weiden and Wood, 1958; Biggee et al. 2007; Pham et al. 2007), it would be unexpected that GlcN administration would lead to overtly measurable changes in serum glycemic parameters, in accordance with the outcomes of Chapter 5. Regardless however, the results of this chapter show that GlcN administration and increased maternal age in reproductively fit, non-classically-aged mice interact to reduce d18 fetal weight and length. It is therefore possible that acute exposure to elevated hexosamine activity, as could occur more frequently with increasing age or via acute GlcN administration, could elicit effects at a cellular level that could modify reproductive outcomes. However, specific testing of hexosamine activity would be required to assess this. .

Failure to detect differences in G6PDH activity between the 4 groups should not be interpreted to indicate that PPP activity in in vivo matured oocytes is not affected by GlcN. As previously stated, it has been demonstrated that IP administered GlcN label penetrates the ovary in the mouse (Fowler and Guttridge, 1987; Fowler 1988; Fowler and Barratt, 1989). It has also been shown that GSH, a product of PPP activity, is depressed in the tissue of fetuses derived from mothers infused with GlcN (Horal et al. 2004). It is possible that in this experiment, GlcN - induced changes may have occurred at an earlier time point, and were not detectable at the time of our collection and assay.

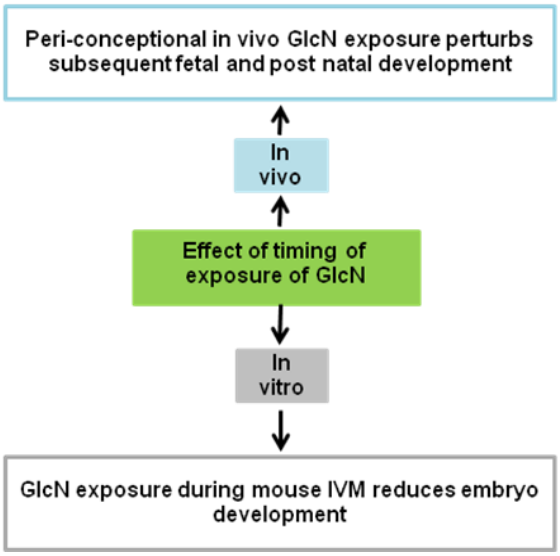
## 6.7 Conclusion

Collectively, these results demonstrate that in vivo, peri-conceptional GlcN exposure elicits detrimental effects upon fetal development. More specifically, they have provided evidence for the existence of an age – GlcN interaction which affects implantation number and litter size, as well as fetal weight and length. The findings that GlcN exposure reduced implantation rate and litter size in 8 week old mice, reduced fetal weight in 16 week old mice, and increased placental weight in 8 week old mice, suggests that peri-conceptionally administered GlcN is metabolized in a non-uniform manner in mice of different ages. It is also possible that there is an age - GlcN interaction which occurs with respect to birth defects, although owing to insufficient numbers of abnormal pups, this could not be statistically ascertained. Given that hyperglycemia elicits a broad spectrum of effects by perturbing divergent pathways, the use of GlcN as a tool to study the effects of select hyperglycemia-stimulated pathways may prove to be useful for gaining a more complete understanding of glucose pathophysiologies. In particular, it is also a likely useful tool for further analyzing the outcomes of associations between age and hyperglycemia. Concomitantly, the nature of the effects that have been outlined by these studies may be a source of concern in regards to GlcN consumption during pregnancy.

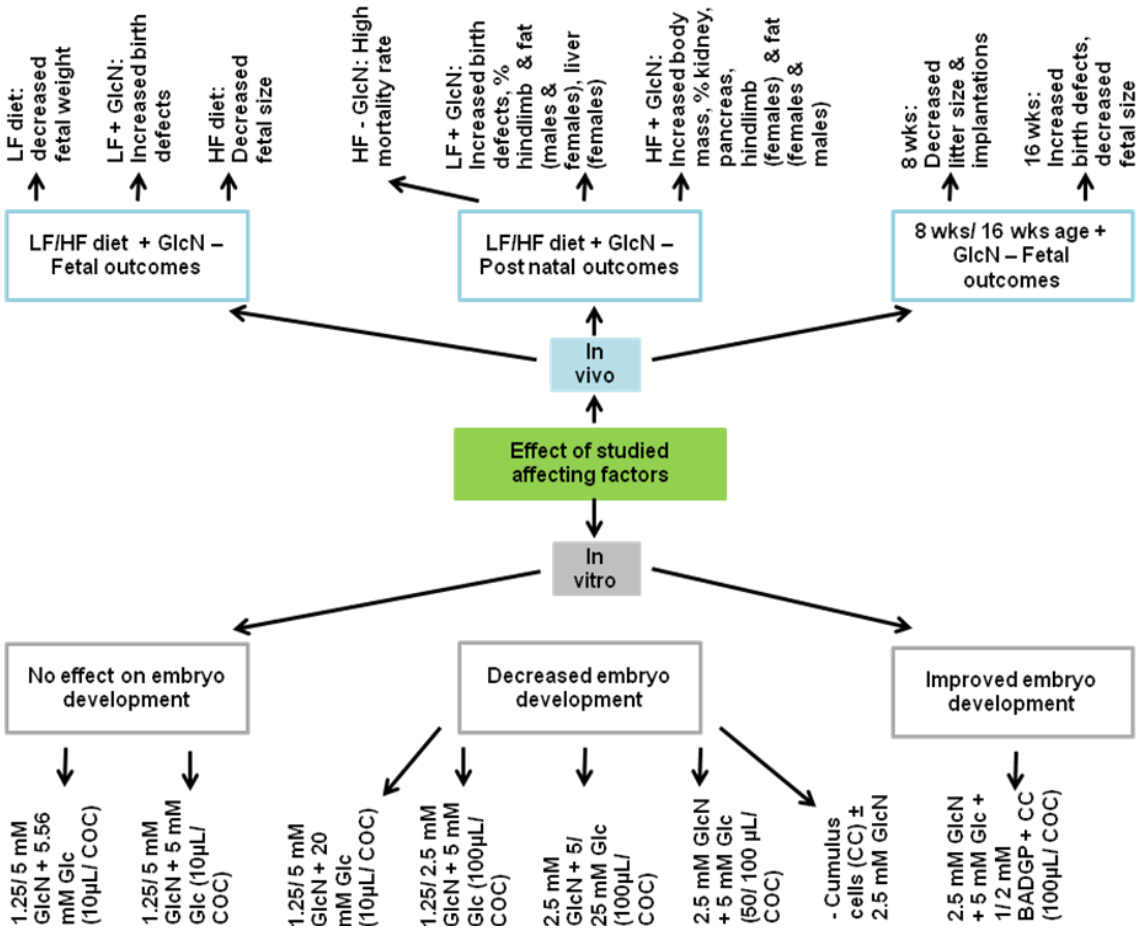
# 7.0 Chapter 7

# Summary of major results and future directions

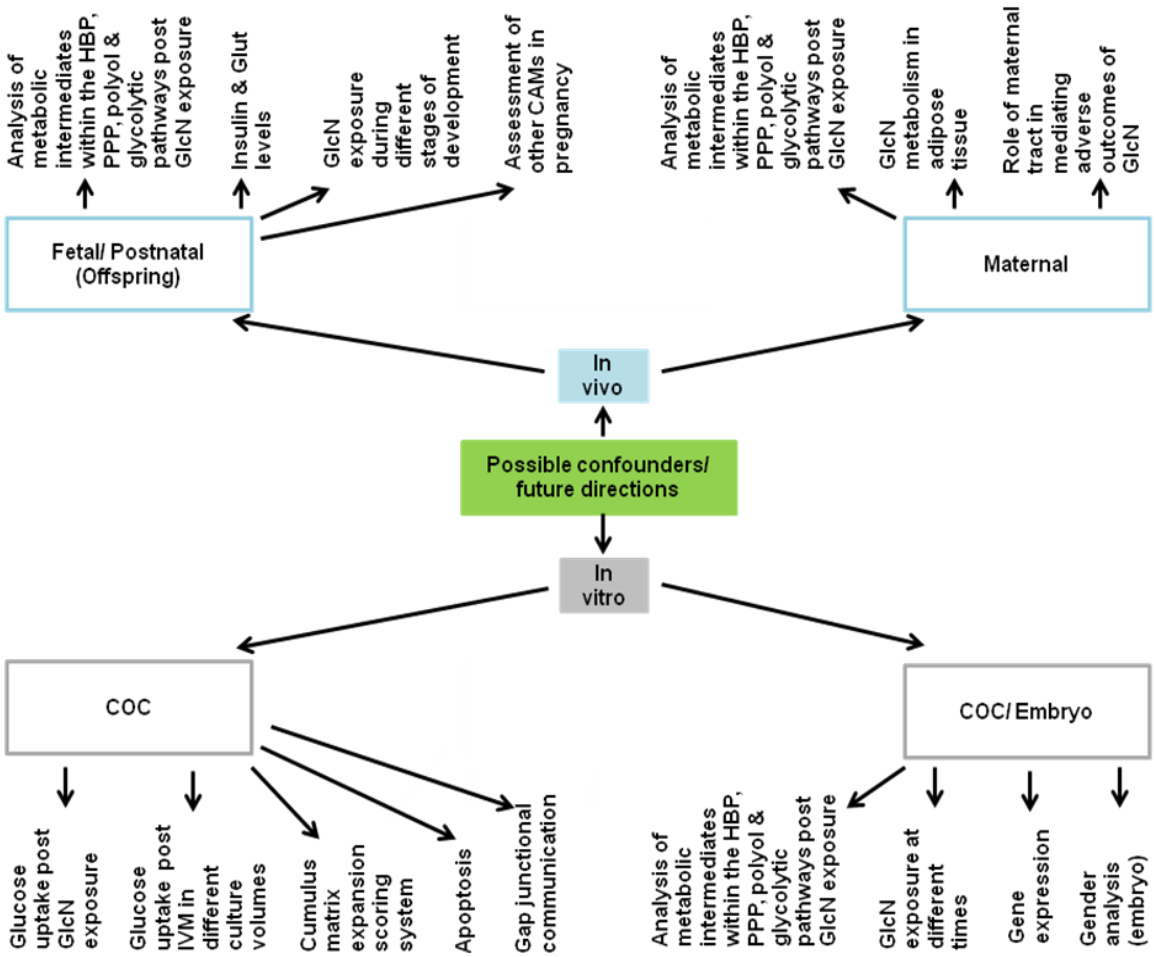
Despite the fact that high glucose levels have long been recognized to have detrimental effects on pregnancy, research surrounding the impact of high glucose exposure during pre and early pregnancy is much less understood. Emerging studies demonstrate that exposure of both COCs (Diamond et al. 1989; Colton et al. 2002; Colton et al. 2003; Chang et al. 2005(b)*;* Ratchford et al. 2007; Ratchford et al. 2008; Wang et al. 2009) as well as pre-implantation embryos (Moley et al. 1991; Moley et al. 1996; Moley et al. 1998(a); Keim et al. 2001; Colton et al. 2002; Wyman et al. 2008) to hyperglycemic conditions is detrimental to subsequent development, although the mechanisms involved and the full spectrum of long term consequences are yet to be determined. GlcN is used experimentally as a hyperglycemic mimetic owing to its ability to upregulate the HBP and its subsequent effects on O-linked glycosylation (Virkamaki & Yki-Jarvinen 1999; Nelson et al. 2000; Marshall et al. 2004; Marshall et al. 2005 (a); Marshall et al. 2005 (b)). Furthermore, it can readily be used in both in vitro and in vivo systems. The studies outlined in these chapters have been designed to 1) examine the effects of altering HBP activity via the introduction of GlcN in mouse COCs undergoing IVM and 2) examine the effect of GlcN exposure in vivo during the peri-conceptional period on subsequent pregnancy, fetal and post natal outcomes. An overall summary of the findings as well as future directions is provided below in Figure 1.




**7.0.1 Figure 1 (A):** Summarising schema of overall outcomes of GlcN exposure on reproductive outcomes




**7.0.1 Figure 1 (B):** Summarising schema outlining the effects of studied affecting factors on the efficacy of in vivo and in vitro administered GlcN on reproductive outcomes.




**7.0.1 Figure 1 (C):** Summarising schema of confounding factors and future research directions arising from assessing the impact of GlcN on reproductive outcomes.

## 7.1 The effect of exposing mouse COCs to GlcN during in vitro maturation

### 7.1.1 The importance of glucose

Studies described in Chapter 2, demonstrated that the addition of GlcN into IVM media had a perturbing effect on subsequent embryo development, but only in the presence of adequate levels of glucose. This was achieved by increasing the glucose concentration in the IVM media or alternatively, by increasing the volume of media that IVM was performed in and therefore increasing glucose availability. Glucose uptake was not measured in these experiments. However, the failure for GlcN-induced perturbations to be induced in the

presence of lower levels of glucose is complementary to the findings of Gutninsky et al. (2007), who demonstrated that a peak of 24% of glucose uptake was accounted for by the HBP in bovine COCs undergoing IVM. Specifically, the results from Chapter 2 suggest a dramatic increase in HBP activity as a consequence of FSH-stimulated cumulus expansion, which is far higher than within somatic cells, and normally accounts for 1 - 3% of glucose metabolism in the hexosamine metabolic arm (Marshall et al. 1991(b)). This increased demand for substrate by the HBP in COCs as a consequence of FSH stimulation facilitates the production of hyaluronin, an end product of the HBP and an essential component of the expanding cumulus cell matrix (Eppig 1979; Ball et al. 1982; Talbot 1984; Suchanek et al. 1994).

Future experiments aimed at investigating flux through the HBP in response to FSH or epidermal growth factor (EGF) should be conducted to examine the molecular and protein responses of key enzymes within the HBP. Significantly, this increased activity implies a rapid increase in GFPT1 and/or 2 to accommodate this increased activity. The effect of GlcN could also be investigated on downstream transcription factors such as PI3-kinase, or the glycosylation status of transcription factors, which have previously been shown to be perturbed in vivo by GlcN infusion (Akimoto et al. 2006; Einstein et al. 2008; Kim et al. 1999). Alternatively, levels of ATP could be measured, as Hresko et al. (1998), Han et al. (2003) and Marshall et al. (2004) have previously documented ATP depletion following GlcN infusion, presumably due to its use in the GlcN-stimulated increase in the production of G6PDH. Although these potential analyses have been suggested in reference to COCs matured in vitro with GlcN, they could also be performed on in vivo exposed COCs, in vitro or in vivo exposed embryos as well as maternal reproductive and metabolic samples.

### 7.1.2 Evidence for effects of GlcN on cumulus cells

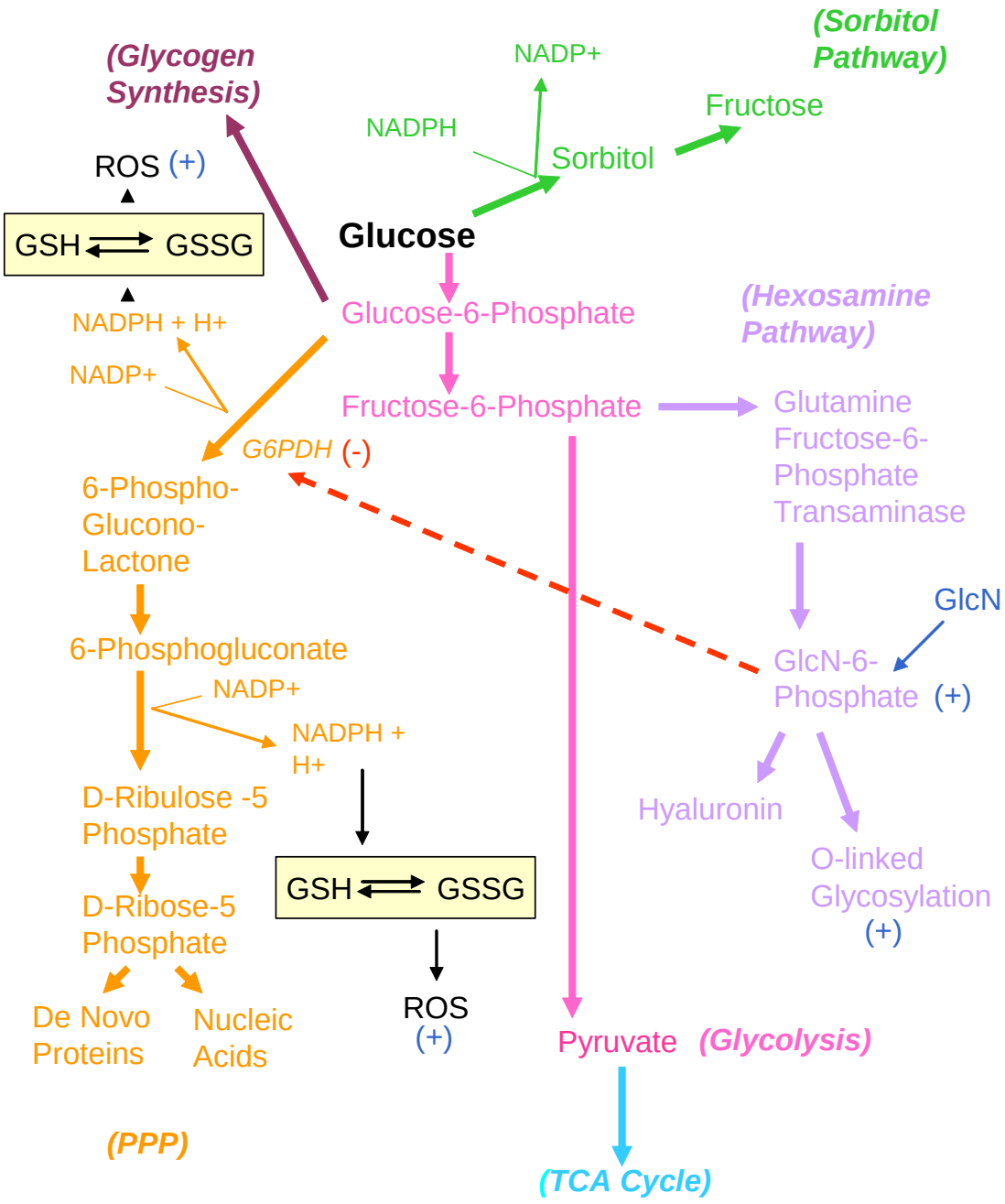
Previously, others have demonstrated that performing IVM on oocytes devoid of their cumulus cells produces oocytes with reduced developmental competence (Chesnel et al. 1994; Chang et al. 2005(a); Luciano et al. 2005), demonstrating the cross communication between the oocyte and its surrounding cumulus cells, which provides for a host of metabolic and signalling requirements (as reviewed by Buccione et al. 1990; Eppig et al. 1997; Gilchrist et al. 2008). That this result was replicated in Chapter 3 was not surprising. However, the same experiments also revealed that the extent of the reduction in rates of blastocyst development was equivalent between groups that had undergone IVM either with GlcN, or

without cumulus cells or alternatively with GlcN as well as without cumulus cells. The absence of a cumulative effect of GlcN exposure and cumulus cell removal on developmental competence suggests that the effects of GlcN on the COC are mediated through the cumulus cells. This could potentially occur through a variety of mechanisms such as changing key metabolic and structural developmental processes that are integral to successful development, reducing gap junctional communication or inducing apoptosis.

### 7.1.3 Metabolic processes

Evidence for an oocyte-localized, metabolic effect of GlcN that is mediated via the cumulus cells can also be taken from Chapter 3. When oocytes were matured either with or without cumulus cells and in either the presence or absence of GlcN, there were notable colour differences between groups of oocytes following exposure to BCB. BCB was first described by Pujol et al. (2004) as a marker of oocyte development given that the BCB stain is reduced by G6PDH, the first rate limiting step of the PPP, and that G6PDH levels change throughout maturation. Results from Chapter 3 revealed that fewer control oocytes (matured with cumulus cells and without GlcN) presented with blue cytoplasms, signifying that they had greater levels of G6PDH activity and were able to metabolize the BCB more efficiently. Equivalent rates of presentation of a blue oocyte cytoplasm occurred when maturation occurred either with GlcN, without cumulus cells or with both GlcN addition and cumulus cell removal.

Others have previously reported that GlcN inhibits G6PDH and therefore PPP activity (Bessell & Thomas, 1973; Kanji et al.1976; Horal et al. 2004), although the nature of its effects within the COC remains to be elucidated (Figure 2). Previously, purines and nucleic acids which are generated by the PPP have been shown to play important roles in the acquisition of oocyte meiotic maturation (Downs et al. 1989; Downs, 1997; Downs et al. 1998; Downs and Utecht, 1999; Downs, 2000; Colton et al. 2003), suggesting that any perturbation of the PPP may yield undesirable effects on the health of the oocyte. Such inhibitory properties of GlcN on G6PDH may explain why GlcN treatment in Chapter 3 reduced the efficiency at which BCB was reduced. However, it fails to explain why reduced suppression of BCB, suggesting inhibited G6PDH activity, was also observed in oocytes devoid of cumulus cells. One possibility is that PPP activity within cumulus cells play an integral role in successful oocyte maturation. Further investigations examining this are warranted.




**7.1.2.1 Figure 2:** Glucose metabolic pathways and the known effects of GlcN on activity through these pathways. (+) shows up regulation and (-) shows down regulation.

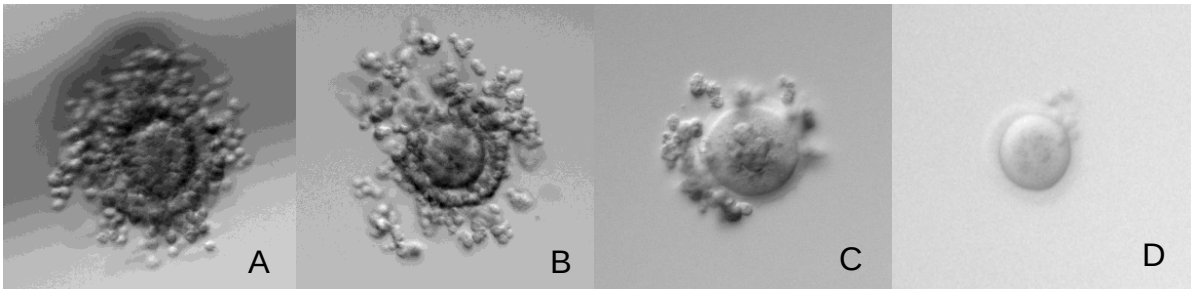
### 7.1.4 O-linked glycosylation

In a variety of cell lines and tissues exposure to GlcN is associated with an increase in levels of O-linked glycosylation (Patti et al. 1999; Chen et al. 2003; Sutton-McDowall et al. 2006; Champattanachai et al. 2007; Park et al. 2007; Champattanachai et al. 2008; Madsen-Bouterse et al. 2008; Xing et al. 2008). In Chapter 3, GlcN-mediated changes in O-linked glycosylation within the COC were identified as a mechanism contributing to the reduction in developmental competence. Preliminary results suggested that the increase in O-linked glycosylation appeared to be localized to cumulus cells in the mouse, a phenomenon previously documented in bovine COCs (Sutton-McDowall et al. 2006). It has previously been shown that the health of the oocyte is perturbed by hyperglycemic exposure (Diamond et al. 1989; Chang et al. 2005b; Ratchford et al. 2007; Ratchford et al. 2008). Since GlcN is used as a hyperglycemic mimetic owing to its ability to up regulate Hexosamine pathway activity (Patti et al. 1999; Virkamaki & Yki-Jarvinen 1999; Nelson et al. 2000; Marshall et al. 2004; Marshall et al. 2005 (a); Marshall et al. 2005 (b)), the results reported in this thesis could be taken as support that one of the mechanisms through which the hyperglycemic state perturbs the COC is via increasing levels of protein O-linked glycosylation in cumulus cells. Further studies quantifying levels of O-linked glycosylation in cumulus cells, and identifying protein targets of O-linked glycosylation, especially following up-regulation of the HBP are required.

### 7.1.5 Structural developmental processes

An additional observation that was evident throughout Chapters 2 and 3 was that mouse COCs exposed to GlcN underwent a differential pattern of cumulus cell expansion to the one described by van der Hyden (1990). For example, some COCs matured in the presence of GlcN underwent such a rapid and pronounced expansion of the outer cumulus cell layers that they started to dissociate from the rest of the inner, minimally expanded components of the matrix (Figure 3a and 3b). Another common occurrence was that expansion occurred to such an extent that no, or relatively few, cumulus cells remain attached to the oocyte by the completion of IVM (Figure 3c and 3d). In addition, it was noted that cumulus cells (either attached or detached from the COC) often presented in dark, sticky clumps after maturation, suggesting apoptosis. This hyper-expansion likely occurred due to the increased availability of HBP substrate (GlcN) for the production of hyaluronin (Figure 2), an integral component of the expanding cumulus matrix (Eppig 1979; Ball et al. 1982; Talbot 1984; Suchanek et al. 1994). An appropriate scoring system, with additional developmental options, is required to

allow assessment of the developmental stage at which cumulus cell expansion becomes perturbed following GlcN exposure.




**7.1.5.1 Figure 3:** COCs matured at a ratio of 100 µL IVM media (containing 5 mM glucose and 2.5 mM GlcN) per COC. **(A and B):** The outer layers of the matrix have rapidly expanded to such an extent that they are dissociating from the rest of the very modestly expanded inner cellular layers. **(C and D**): The cumulus cells have expanded so much that they have largely dissociated from the matrix, leaving few intact cells.

### 7.1.6 Gap junctional communication

Bi-directional communication between the oocyte and its companion cumulus cells occurs through multiple mechanisms and plays a crucial role in the process of successful oocyte maturation (as reviewed by Buccione et al. 1990; Eppig et al. 1997; Gilchrist et al. 2008). Previously, it has been shown that one such mechanism, gap junctional communication, which occurs between the oocyte and its surrounding cumulus cells, is perturbed in COCs derived from Streptozotocin - induced diabetic mice (Chang et al. 2005(a); Ratchford et al. 2008). It is therefore possible that one of the effects elicited by GlcN on the maturing COC is a perturbation of oocyte - cumulus cell gap junctional communication, mediated by the separation of the hyper-expanded cumulus matrix from the oocyte. Further studies to directly assess gap junctional communication could assess this.

Collectively, the outcomes of these manipulations of mouse COC IVM have demonstrated that GlcN elicits perturbing effects on development. Although some mechanisms have been identified, such as O-linked glycosylation and the dependence upon the concentration of accompanying glucose levels, others such as the specific effects of GlcN on cumulus cell function, require further investigation. Although these results, along with the perturbed embryonic development identified in porcine and bovine models (Sutton-McDowall et al. 2006; Kimura et al. 2008), were obtained in vitro, it was reasoned that the effects of GlcN on development would likely not be limited to in vitro systems. For one, the in vivo infusion of

GlcN radiolabel has previously been localized in multiple reproductive structures including the unfertilized oocyte (Fowler and Guttridge, 1987, Fowler 1988; Fowler and Barratt, 1989; Fléchon et al. 2003) as well as one and two cell embryos (Fowler and Barratt, 1989), indicating that GlcN is likely not metabolized exclusively externally to the reproductive system. Furthermore when Horal et al. (2004) infused GlcN into pregnant mice on day 10 of pregnancy, an increased number of fetal malformations were detected, thereby showing that in vivo post implantation development is impacted by GlcN exposure. With these results in mind, it was sought to determine if in vivo peri-conceptional GlcN exposure would also elicit undesirable effects on developmental outcomes.

### 7.1.7 Apoptosis

Increased rates of protein O-linked glycosylation are positively correlated with apoptosis in pancreatic cells (Liu et al. 2000; Konrad et al. 2000; Park et al. 2007). Given that GlcN has been shown to increase levels of O-linked glycosylation in in vitro produced bovine (Sutton-McDowall et al 2006; Kimura et al. 2008) and mouse (Pantaleon et al. 2010) embryos, an analysis of apoptosis in GlcN – exposed mouse COCs and embryos would be useful in further ascertaining the nature of the deleterious effects of GlcN during pre and early development that have been documented in this thesis. However, owing to the fragile state of GlcN exposed COCs and embryos which have a tendency to fall apart during repetitious handling, specialised techniques would need to be developed.

### 7.1.8 Gene Expression

Following the findings of GlcN – mediated developmental perturbations in in vitro matured COCs, experimental efforts were focussed on ascertaining whether such effects could also be replicated in vitro. In addition to the various additional mechanistic analyses that have been described, it would also be useful to assess GlcN - induced changes in gene expression. A number of candidate genes would be suitable for such analyses.

The expanding cumulus matrix has an increasing reliance on glucose as maturation proceeds, a phenomenon attributed to increased production of the key matrix component hyaluronin (Sutton McDowall et al. 2004). Hyaluronin synthase 2 (HAS2) is recognised as an integral enzyme involved in the production of hyaluronin within the cumulus matrix (Fulop et al. 2007). Similarly, Insulin like growth factor 1 (IGF-1) has also been implicated in enabling

FSH – induced expansion during IVM (Singh and Armstrong, 1997). Given that perturbation in cumulus cell expansion were observed following GlcN exposure in Chapters 2 and 3, an analysis of GlcN - influenced HAS2 and IGF-1 receptor gene expression throughout maturation could provide further insights into the processes at play. In addition, owing to the fact that GlcN enters cells via GLUT transporters (Uldry et al. 2002), it would be useful to measure levels of Glut 1 within oocytes (Morita et al. 1992; Aghayan et al. 1992; Zhou et al. 2000) and cumulus cells (Zhou et al. 2000).

Since a variety of GLUT transporters are also present in the mouse blastocyst, it would be useful to measure levels of Glut 1 (Hogan et al. 1991; Smith and Gridley 1992; Pantaleon et al. 1997; Moley et al. 1998(b); Carayannopoulos et al. 2000; Kind et al. 2005; Rieger et al. 2007), Glut 2 (Hogan et al. 1991; Aghayan et al. 1992, Moley et al. 1998(b); Leppens-Luisier et al. 2001; Kind et al. 2005), Glut 3 (Smith and Gridley 1992; Pantaleon et al. 1997; Moley et al. 1998(b)) and Glut 8 (Carayannopoulos et al. 2000) within blastocysts. Interestingly, Kimura et al. (2008) showed that the incorporation of GlcN into bovine IVM culture media from the 8 cell to blastocyst stage not only reduced embryo development, but also skewed the sex ratio of the remaining embryos towards males, a finding that the authors attributed to the localisation of O-linked GlcNAc transferase (OGT) - an enzyme involved in the utilisation of GlcN in O-linked glycosylation - on the X chromosome. In addition to assessing whether there are also sex differences in surviving mouse blastocysts that have been derived from COCs exposed to GlcN, it could also be useful to assess levels of OGT expression in either COCs or embryos that have been exposed to GlcN.

## 7.2 Reproductive consequences of In vivo, peri-conceptional GlcN administration

Chapters 4 - 6 were based on in vivo models of peri-conceptional GlcN exposure. Preliminary findings from Chapter 4, which involved 8 week old mice receiving one of 3 doses of GlcN, injected intraperitoneally during the peri-conceptional period, demonstrated that in vivo GlcN administration was also detrimental to reproductive outcomes. As increased maternal weight is known to be associated with increased Hexosamine metabolic activity (Buse et al. 1997; Hawkins et al. 1997; Considine et al. 2000; Tang et al 2000; Veerababu et al. 2000), it was hypothesized that the effects of GlcN administration would be worse in obese or overweight mice with pre-elevated hexosamine activity. Unexpectantly, although it was shown that GlcN did perturb reproductive outcomes in 16 week old mice, the nature of these defects were different to what had been observed in Chapter 4. Furthermore, they

were also largely independent of dietary exposure to a high fat diet, as many of the developmental perturbations were observed in the offspring of mice maintained on standard chow. In light of the observed differences in fetal outcomes between the experiments presented in Chapters 4 and 5, a direct comparison was made between 8 and 16 week old mice given GlcN. Interestingly, the discrepancies between reproductive outcomes in different aged mice were confirmed in Chapter 6 when an interaction was detected between GlcN treatment and maternal age, despite the fact that both of the age groups (8 weeks and 16 weeks) were within a reproductively fit range.

### 7.2.1 Pregnancy outcomes

Results from Chapter 4 revealed that peri-conceptional GlcN exposure reduced mean litter size, and that this was contributed to by a reduction in mean implantation rate as well as an increase in the proportion of implantations that resorbed. In contrast, litter size, implantation and resorption parameters were not affected by GlcN exposure in Chapter 5. Results from Chapter 6 confirmed prior results that litter size and implantation rate were reduced, and resorption rates were increased, exclusively in 8 week old mice treated with GlcN. The results outlined in Chapter 6 are particularly important, as they demonstrate that reproductive outcomes can be perturbed by a combination of increased maternal age that is still within a reproductively fit range, and glycemic fluctuations that are so mild that they were not detected via serum analysis of glucose and insulin.

### 7.2.2 Fetal outcomes

Although results from Chapter 4 showed that there were fewer viable fetuses on d18 of pregnancy following peri-conceptional GlcN exposure, these fetuses were equivalent in size. In Chapter 5, fetuses from HF fed mice (irrespective of GlcN treatment) were lighter and shorter than their LF counterparts, consistent with the findings of Jungheim et al. (2010) on embryonic day 14.5 when fetal analysis was performed following maternal HF diet exposure. Importantly however, results from Chapter 5 also showed that d18 fetuses from the LF+GlcN group were significantly lighter than those from the LF-GlcN group, contrasting results from Chapter 4. The results of Chapter 6 confirmed the existence of an age- mediated effect of GlcN on fetal outcomes, when mean fetal weight and length were reduced exclusively in 16 week old mice given GlcN. Why fetal size was reduced by GlcN treatment in low fat fed, but

not high fat fed mice in Chapter 5 is not clear, although this could be due to adipose tissue mediated differences in GlcN metabolism. Alternatively, it is possible that the increased adiposity enabled an adaptation to develop towards transient elevations in Hexosamine activity. However, fetal weight was reduced by high fat feeding, therefore, it is possible that further GlcN – induced perturbations were undetectable with the methods employed.

### 7.2.3 Birth defects

In contrast to the findings of Chapter 4, results from Chapter 5 showed that peri-conceptional GlcN exposure led to an increase in fetal birth defects. Specifically, this involved an increase in the number and proportion of fetuses with birth defects as well as the number and proportion of mothers that carried birth defected fetuses following GlcN exposure in mice maintained on a low fat diet and given GlcN. Increased rates of birth defects were also observed for post natal outcomes. Although exposure to a HF diet also increased numbers and proportions of birth defects, as well as the proportion of mothers that carried birth defected fetuses, this did not occur to the same extent as that seen in the LF+GlcN group. Results from Chapter 6 confirmed that this difference was attributable to differences in maternal age, when an increase in the prevalence of birth defects were detected exclusively in 16 week old mice treated with GlcN, and not mice that were given GlcN at 8 weeks of age.

The findings of increased rates of birth defects are complimentary to the findings of Horal et al. (2004) following in vivo GlcN exposure between days 7.5 - 10.5 of pregnancy, as well as Wyman et al. (2008) following in vivo, peri-conceptional hyperglycaemic exposure. Importantly, however, the effects documented by Wyman et al. (2008) were derived from a model with experimentally measurable diabetes and created via maternal exposure to streptozotocin. In contrast, GlcN-induced changes in serum glucose levels were not detected in mice assessed in Chapter 5. Thus, while acute GlcN exposure may not modify blood glucose levels it may none-the-less lead to cellular changes in the developing oocyte and embryo, which in turn compromise subsequent fetal development. Such potential changes may be diverse in nature, but may include metabolic alterations to the HBP, PPP, glycolytic and polyol pathways, as well as changes in levels of glucose and insulin and their respective receptors.

### 7.2.4 Post natal outcomes

The extension of the dietary manipulation study to postnatal outcomes also yielded interesting results. Although there were marked rates of early postnatal death across all groups, the majority of offspring from the HF - GlcN treated group died within the first 3 days of birth and were consequently omitted from subsequent analyses. It was found that there were a number of differences between the 3 remaining groups in outcomes at 16 weeks of age, although the nature of the differences were gender specific. For example, female offspring from the HF + GlcN mice had consistently larger fat deposits than all other groups which in turn contributed to greater body masses in this group. In male offspring, on the other hand, offspring with the greatest fat deposits were from the LF - GlcN or HF + GlcN groups, with offspring from the LF + GlcN group consistently having the smallest fat deposits.

### 7.2.5 Collective peri-conceptional GlcN outcomes

Similarly to the results of experiments performed in vitro, the peri-conceptional administration of GlcN was shown to have perturbing effects on subsequent development. Broadly speaking, these were manifest as reduced pregnancy rates, altered fetal and post natal body composition, and increased fetal and postnatal birth defects. Furthermore, an association between GlcN and maternal age was also identified. From the results obtained from these experiments, many additional questions and issues that need to be addressed have emerged. Whilst some of these have been addressed within previous paragraphs, others are outlined below.

## 7.3 Additional future investigations

### 7.3.1 Glucose metabolism

In addition to the HBP, glucose is also metabolised through other metabolic arms including Glycolysis, the PPP and the Polyol pathway (Figure 2). Previously, GlcN infusion has been shown to reduce glycolysis (Giaccari et al. 1995), owing to its ability to reduce glucose uptake (Hawkins et al. 1996; Virkamaki et al. 1997; Holmang et al. 1999; Patti et al. 1999). Reduced glucose uptake has also been documented in various cell culture systems following

the introduction of GlcN (Balkan & Dunning, 1994; Bailey & Turner, 2004; Marshall et al. 2005(b)). Therefore it may appear that GlcN exposed COCs and pre-implantation embryos could also take up reduced levels of glucose, which in turn could impact on glucose fluxing into alternative metabolic pathways.

None-the-less, it is important to measure activity and metabolic intermediate formation in each of these pathways to accurately assess the broader effects of GlcN exposure on glucose metabolism. This is important for several reasons. Firstly, it has previously been documented that COCs have atypical glucose metabolic profiles relative to somatic cells. More specifically, whilst many somatic cells flux 1 - 3% of glucose through the HBP (Marshall et al. 1991(a)), bovine COCs have been shown to have an increasing need for glucose throughout IVM (Sutton et al. 2003), with a FSH mediated peak flux estimated to be around 24% (Gutnisky et al. 2007). Secondly, Polyol pathway activity has previously been shown to be increased in the oocytes of diabetic mice (Colton and Downs, 2004), as well as embryos exposed to hyperglycemic (52 mM) conditions as zygotes (Moley et al. 1996). Furthermore, Ratchford et al. (2007) have reported that glycogen levels in the COCs of diabetic mice are significantly elevated, suggesting that glycogen metabolism should be assessed in models characterized by hyperglycemia or GlcN treatment. Thus, the determination of GlcN-stimulated glucose metabolism through additional metabolic arms to the HBP would provide further information regarding the similarities of GlcN and elevated glucose levels, and provide additional insight into the appropriateness of considering GlcN as a hyperglycemic mimetic. Potentially, glucose shunting through these pathways could be measured in COCs and embryos exposed to GlcN both in vivo and in vitro, as well as in samples of maternal origin.

### 7.3.2 Examination of the effects of GlcN exposure at different points of development

Although the experiments outlined in these chapters have shown that there is both an in vivo and an in vitro effect of GlcN, there is a lack of uniformity in the developmental stage that exposure occurs in the in vivo and in vitro models. More specifically, the maturing COC was exposed to GlcN in vitro, while a wider developmental spectrum was exposed in vivo, encompassing the pre-ovulated oocyte through to the early embryo. It would be useful, however, to examine the effects of GlcN exposure at equivalent points of exposure in vitro and in vivo. This could be achieved via several designs, including exposing the oocyte/ embryo to GlcN at several stages post maturation. It is also possible that pre-ovulated oocytes could be exposed in vivo to GlcN, and then flushed out and artificially matured,

fertilized and cultured. Alternatively, GlcN - exposed oocytes could also be fertilized in vitro and then collected for assessment at various stages of development.

Previously it has been demonstrated that in vitro exposure of mouse zygotes to GlcN perturbs subsequent development (Pantaleon et al 2008; Pantaleon et al. 2010). However, the effects of post zygote exposure have not been examined in vivo. When Kimura et al. (2008) incorporated GlcN into either bovine IVM media pre compaction or post compaction embryo culture media, it was noted that the perturbing effects of GlcN on embryo development were limited to exposure that occurred during IVM or post compaction development. Interestingly, the same authors also reported that whilst the co-incubation of the O-linked glycosylation inhibitor BADGP reversed the perturbed patterns of development, it was only successful at correcting the GlcN – induced, skewed male embryo sex ratio when exposed to the post compaction embryo. This same sex ratio skewing can also be induced by culturing bovine embryos in concentrations of 2.5 mM glucose or greater, and is attributable to the localization of OGT (a key enzyme used for O-linked glycosylation) on the X chromosome (Kimura et al. 2005). Further investigations are warranted into stage and species specific effects of GlcN exposure, which should also incorporate gender analysis studies.

Furthermore, Horal et al. (2004) demonstrated that exposure of post implantation embryos to GlcN increased the numbers of birth defects in pups. However, these birth defects largely consisted of neural tube defects, in contrast to those observed in my studies. These findings provide additional evidence for differential perturbing effects of GlcN at different developmental periods as well as evidence for in vivo effects of GlcN. It would therefore be useful to administer GlcN at various developmental time points and examine outcomes. This would increase our understanding of whether hyperglycemic exposure elicits developmental-stage specific effects on conceptuses, which in turn could lead to greater levels of understanding in regards to expected outcomes from hyperglycemia or GlcN exposure at different times in pregnancy.

### 7.3.3 Examination of the influence of the effect of GlcN on the maternal reproductive tract

When GlcN is administered via IP injection, it has the potential to elicit effects on the reproductive tract and its contents, as well as other body systems including the major tissues responsible for regulating maternal metabolism and the metabolic response to pregnancy. Hence, it is possible that effects of peri-conceptional GlcN exposure were mediated directly

by GlcN, or indirectly via stimulation from GlcN-modified maternal conditions. In order to elucidate the influence of GlcN on the maternal condition, embryos from mice that received peri-conceptional GlcN in the manner described in Chapters 4 - 6 could be flushed from donors and transferred into non GlcN treated recipients.

### 7.3.4 Assessing post natal outcomes in age controlled mice

Although it appeared that GlcN elicited no effects on the developmental outcomes of offspring derived from mice maintained on a HF diet, it was subsequently shown that female offspring became significantly heavier than other groups from 10 weeks of age, which at 16 weeks of age, was contributed to by an increase in levels of adipose tissue. This finding adds to a growing body of evidence which links later physiological outcomes in life with events experienced during early development. Results from Chapter 6 revealed that reproductive outcomes were different in mice given GlcN at different ages. Specifically, 16 week old mice treated with GlcN had d18 fetuses that were smaller in size and exhibited an increase in birth defects, whereas 8 week old mice treated with GlcN had litters of a reduced size owing to reduced implantation rates and increased resorption rates. Since differences in reproductive outcomes for the two ages of mice were detectable at the fetal stage, it is possible that even more pronounced differences may have emerged if post natal analyses were performed. Hence it would be interesting to assess post natal development in mice derived from mothers exposed to GlcN at different ages.

### 7.3.5 Further analysis of the interaction of factors that separately are known to perturb reproductive outcomes

Currently there is an extensive list of factors that are known to independently perturb reproductive outcomes. In addition to those already mentioned (i.e. hypertension, increased maternal age and perturbed maternal glucose metabolism as can occur in conditions such as diabetes and overweight/ obesity) a select few others include smoking, poor nutrition, illicit drug use and the lack of use of appropriate pregnancy associated vitamins and minerals. However, limited studies have investigated the combined effects of exposure to these factors.

Recently, Stella et al. (2008) found that pregnancy outcomes are worse for women that have both hypertension and diabetes relative to those that are afflicted with only one of these conditions. In the experiment outlined in Chapter 6, it was shown that there was a maternal age-dependent effect of GlcN on overall pregnancy outcomes. These newly emerging results provide evidence for the potential existence of additive effects of age and hyperglycaemic insult to worsen reproductive outcomes. The investigation of simultaneously presented, adverse influences warrants further investigation, either via the analysis of clinical records or through direct experimental manipulation of animal models.

### 7.3.6 A broader study of the effects of complementary and alternative medicines

CAMs are significantly represented as a component of healthcare expenditure. For example, it was found that public expenditure on CAMs was almost four times as great as expenditure on pharmaceuticals in Australia in 2000, which was extrapolated to a cost of $2.3 billion (MacLennan et al. 2002). Alarmingly, it has also been reported that rates of reporting CAM use to healthcare practitioners is, whilst rather variable, not high (Gadsby et al. 1993; Maclennan et al. 2002; MacLennan et al. 2006).

This is a potential cause of concern, given the absence of a complete spectrum of understanding of the efficacy of many CAMs. More specifically for many CAMs there is in general a paucity of knowledge regarding supraphysiological dosing, extended periods of exposure as well as interactions with other supplements, non- prescription medications and prescription medications, and with a multitude of common physiological conditions such as pregnancy, diabetes and asthma. The experiments outlined in these chapters have provided evidence for a largely unrecognized and adverse effect of a popular dietary supplement (Kaufman et al. 2002; Wold et al. 2005; Hopman et al. 2006; Singh and Levine, 2006) on reproductive outcomes. They also draw attention to the need to further understand the short term and long term outcomes of CAM use within a greater range of physiological contexts.

## 7.4 Conclusion

In the experiments outlined within the chapters of this thesis, both in vitro and in vivo GlcN exposure has been shown to elicit perturbing effects on reproductive outcomes in mice. It would be valuable to gain further insight into the mechanisms contributing to these effects of GlcN. It would also be helpful to gain further understanding of the effects of exposure to GlcN in additional physiological contexts. Although the experiments conducted have provided valuable insight into the effects of a popular dietary supplement on reproductive outcomes, they have also provided the basis for the emergence of many new questions.

# Appendix

## 8.1 General Stock Solutions

### 8.1.1 Stock A

| Substance | Quantity | Instructions |
|---|---|---|
| NaCl | 5.5 g | Dissolve reagents in water |
| KCl | 0.41g | |
| MgSO4 | 0.44g | Filter, refridgerate and store for 3 weeks. |
| NaHPO4 | 0.035g | |
| Glucose | 0.9g | |
| L-Na Lactate | 1.17g | |
| MQ H2O | 100 ml | |

### 8.1.2 Stock B

| Substance | Quantity | Instructions |
|---|---|---|
| NaHCO3 | 2.101 g | Dissolve reagents in water. |
| Phenol Red | 10 mg | Filter, refridgerate and store for 1 month. |
| MQ H2O | 100 ml | |

### 8.1.3 Stock C

| Substance | Quantity | Instructions |
|---|---|---|
| Pyruvic acid | 55 mg | Mix, filter, refridgerate and store for 1 week. |
| MQ H2O | 10 ml | |

### 8.1.4 Stock D

| Substance | Quantity | Instructions |
|---|---|---|
| CaCl2H20 | 1.176 g | Mix, filter, refridgerate and store for 3 months. |
| MQ H2O | 40 ml | |

### 8.1.5 Stock G

| Substance | Quantity | Instructions |
|---|---|---|
| D(+) Glucose | 1.080 g | Mix, filter, refridgerate and store for 3 months. |
| MQ H2O | 40 ml | |

### 8.1.6 Stock L

| Substance | Quantity | Instructions |
|---|---|---|
| Na Lactate (60% Syrup) | 2.832 g | Slowly draw the syrup up into a sterile 1 ml pippette tip (unfiltered) with the tip cut off the end. Slowly expel the syrup into the H2O. Rinse tip well. Refridgerate and store for 3 months. |
| MQ H2O | 37.168 ml | |

### 8.1.7 Stock M

| Substance | Quantity | Instructions |
|---|---|---|
| Mg.SO4.7H2O | 1.972 g | Mix, filter, refridgerate and store for 3 month. |
| MQ H2O | 40 ml | |

### 8.1.8 Fetuin (20 mg/ml Stock)

| Substance | Quantity | Instructions |
|---|---|---|
| Fetuin | 20 mg | Slowly allow fetuin to dissolve. Do not store. |
| MQ H20 | 1 ml | |

## 8.2 IVM →embryo culture system 1

### 8.2.1: αMEM

(N.B. Preparation of this stock is the first phase in the preparation of bicarbonate buffered media (for use in IVM and IVF) as well as HEPES media (for use as COC handling media). 2 x solutions used for the final preparation of the cell culture media).

Dissolve 1 x packet Gibco αMEM powder in 200 ml MQ H2O. Divide the solution equally between 2 x 250 ml volumetric flasks.

### *8.2.2: αMEM handling media (2 x stock)*

Dissolve in 200 ml MQ H2O:

252 mg NaHCO3

2380 mg HEPES

25 mg Streptomycin sulphate

\+ 37.5 mg Penicillin G

Add to the volumetric flask with αMEM (from 8.2.1). Make up to 250 ml with MQ H2O. Adjust pH to 7.2. Filter and store for 2 weeks.

### 8.2.3 αMEM handling media

| Substance | Quantity | Instructions |
|---|---|---|
| *αMEM HEPES (2 x stock)* | 23.75 ml | Gently invert, filter, refridgerate and store for 1 week. |
| MQ H2O | 23.75 ml | |
| NHT FCS | 2.5 ml | |

### *8.2.4: αMEM IVM media (2 x stock)*

Dissolve in 200 ml MQ H2O:

1100 mg NaHCO3

25 mg Streptomycin sulphate

\+ 37.5 mg Penicillin G

Add to the volumetric flask with αMEM (from 8.2.1). Make up to 250 ml with MQ H2O. Adjust pH to 7.2. Filter and store for 2 weeks.

### 8.2.5: *αMEM IVM (base) media*

| Substance | Quantity | Instructions |
|---|---|---|
| *αMEM 2xStock* | 2835.75 µl | Gently and thoroughly mix. Do not store. |
| MQ H2O | 2835.75 µl | |
| Fetuin stock (20 mg/ml) | 298.5 µl | |
| rh FSH | 30 µl | |

### *8.2.6: αMEM IVF media*

| Substance | Quantity | Instructions |
|---|---|---|
| *αMEM (bicarb buffered) (2 x stock)* | 2.5 ml | Mix *αMEM (bicarb buffered) (2 x stock) with H2O. Add BSA and allow to dissolve (do not shake). Filter.* |
| MQ H2O | 2.5 ml | |
| BSA | 15 mg | |

### *8.2.7: KSOM 10 x stock*

| Substance | Quantity | Instructions |
|---|---|---|
| NaCl | 5.552 g | Mix, filter, refidgerate and store for 3 month. |
| KCl | 0.178 g | |
| KH2PO4 | 0.0068 g | |
| EDTA | 0.0038 g | |
| MQ H2O | 100 ml | |

### *8.2.8: KSOM culture media*

| Substance | Quantity | Instructions |
|---|---|---|
| KSOM (10 x stock) | 1 ml | Add all ingredient (except for *BSA) and mix. Adjust the osmolarity to between 250 – 260 mOsm and pH to 8. |
| Stock B | 1 ml | |
| Stock C | 40 µL | Add BSA and allow to dissolve (do not shake). |
| Stock D | 84 µL | |
| Stock G | 14 µL | Filter, refridgerate and store for 1 week. |
| Stock L | 200 µL | |
| Stock M | 10 µL | |
| Glutamax | 50 µL | |
| MQ H2O | 7.602 ml | |
| *BSA | 10 mg | |

## 8.3 IVM → embryo culture system 2

### *8.3.1: MOPS handling media (protein free)*

| Substance | Quantity | Instructions |
|---|---|---|
| NaCl | 5.283 g | Dissolve the first nine chemicals in approximately 500 ml MQ H2O in a 1L volumetric flask. |
| Glucose | 0.090 g | |
| KCl | 0.410 g | Dissolve CaCl2 in a small volume of MQ H2O, and add to the other ingredients in the volumetric flask. |
| MOPS | 5.800 g | |
| Na Pyruvate | 0.035 g | |
| NAHCO3 | 0.420 g | After weighing lactate, mix with a small amount of water and add to the other ingredients in the volumetric flask. |
| Phenol Red | 0.005 g | |
| MgSO4 | 0.440 g | Add NEAAs and Glutamax, make up to 1L with MG H2O and mix well. |
| NaH2PO4 | 0.035 g | |
| CaCl2 | 0.147 g | |
| Lactate | 1.95 g | Adjust pH tp 7.3 and osmolarity to 255 ± 3 mOsm. Filter, refridgerate and store for 3 months. |
| Non essential amino acids | 10 ml | |
| Glutamax | 10 ml | |

### *8.3.2: MOPS handling media (working media)*

Add Fetuin at a concentration of 1 mg/ml to MOPS handling media. Allow to dissolve and then filter.

### *8.3.3: MG2 IVM (base) media*

| Substance | Quantity | Instructions |
|---|---|---|
| Stock A | 1.0 ml | Mix to make up to 10 mls. Do not store. |
| Stock B | 1.0 ml | |
| Stock C | 0.1 ml | |
| Stock D | 0.1 ml | |
| Glutamax (x100) | 0.1 ml | |
| Eagles non-essential amino acids (x100) | 0.1 ml | |
| Eagles essential amino acids (x50) | 0.1 ml | |
| Eagles MEM vitamins (x100) | 0.5 ml | |
| HSA | 0.5 ml | |
| MQ H2O | 6.9 ml | |

### *8.3.4: G1 culture media*

G1 Culture media was kindly donated by Dr Michelle Lane of the Research Centre for Reproductive Health, The University of Adelaide, South Australia. The composition of the G1 culture media is provided below.

| Component | Concentration (mM) | Component | Concentration (mM) |
|---|---|---|---|
| NaCl | 90.1 | EDTA | 0.01 |
| KCl | 5.5 | Alanyl – glutamine | 0.5 |
| $NaHPO_4\,2H_2O$ | 0.25 | Alanine | 0.1 |
| $MgSO_4\,7H_2O$ | 1.0 | Asparagine | 0.1 |
| $NaHCO_3$ | 25.0 | Aspartate | 0.1 |
| $CaCl_2\,H_2O$ | 1.8 | Glutamate | 0.1 |
| Glucose | 0.5 | Glycine | 0.1 |
| Na Lactate | 10.5 | Proline | 0.1 |
| Na Pyruvate | 0.32 | Serine | 0.1 |

### *8.3.5: G2 culture media*

G2 Culture media was kindly donated by Dr Michelle Lane of the Research Centre for Reproductive Health, The University of Adelaide, South Australia. The composition of the G2 culture media is provided below.

| Component | Concentration (mM) | Component | Concentration (mM) |
|---|---|---|---|
| NaCl | 90.1 | Leucine | 0.4 |
| KCl | 5.5 | Lysine | 0.4 |
| $NaHPO_4\,2H_2O$ | 0.25 | Methionine | 0.1 |
| $MgSO_4\,7H_2O$ | 1.0 | Phenylalanine | 0.2 |
| $NaHCO_3$ | 25.0 | Proline | 0.1 |
| $CaCl_2\,H_2O$ | 1.8 | Serine | 0.1 |
| Glucose | 3.15 | Threonine | 0.4 |
| Na Lactate | 5.87 | Tryptophan | 0.5 |
| Na Pyruvate | 0.1 | Tyrosine | 0.2 |
| Alanyl – glutamine | 1.0 | Valine | 0.4 |
| Alanine | 0.1 | Choline chloride | 0.0072 |
| Arginine | 0.6 | Folic acid | 0.0023 |
| Asparagine | 0.1 | Inositol | 0.01 |
| Aspartate | 0.1 | Nicotinamide | 0.0082 |
| Cystine | 0.1 | Pantothenate | 0.0042 |
| Glutamate | 0.1 | Pyridoxal | 0.0049 |
| Glycine | 0.1 | Riboflavin | 0.00027 |
| Histodine | 0.2 | Thiamide | 0.00296 |
| Isoleucine | 0.4 | | |

## 8.4 GlcN solutions

### 8.4.1: In vitro (400 mM stock)

| Substance | Quantity | Instructions |
|---|---|---|
| GlcN | 86.24 mg | Gently invert, filter, refridgerate and store for 1 week. |
| MQ H2O | 0.5 ml | |

### 8.4.2: In vivo

#### *8.4.2.1 For a dose of 20 mg/ ml (per 5 µL injected)*

| Substance | Quantity | Instructions |
|---|---|---|
| GlcN | 40 mg | Gently invert, filter, refridgerate and store for 1 week. |
| MQ H2O | 10 ml | |

#### *8.4.2.2 For a dose of 400 mg/ ml (per 5 µL injected)*

| Substance | Quantity | Instructions |
|---|---|---|
| GlcN | 800 mg | Gently invert, filter, refridgerate and store for 1 week. |
| MQ H2O | 10 ml | |

## 8.5 BADGP (100 mM stock)

| Substance | Quantity | Instructions |
|---|---|---|
| BADGP | 155.66 mg | Using a sterile instrument, dissociate clumped material to aid the dissolving process. Shake frequently. Once dissolved, freeze aliquotted stock. |
| EtOH | 5 ml | |

### 8.6 BCB stain (100 mM stock)

| Substance | Quantity | Instructions |
|---|---|---|
| Brilliant Cressyl Blue | 192.98 mg | Mix. Filter, refridgerate and store for 4 weeks. |
| MQ H20 | 5 ml | |

### 8.7 ROS stain

#### *8.7.1 5,6-carboxyl-2',7'-dichlorodihydrofluorescein diacetate (1 mM)*

| Substance | Quantity | Instructions |
|---|---|---|
| 5,6-carboxyl-2',7'-dichlorodihydrofluorescein diacetate | 2.65 mg | Mix, refridgerate and store for 3 months |
| DMSO | 5 ml | |

#### *8.7.2 2',7' dichlorofluorescein diacetate (1 mM)*

| Substance | Quantity | Instructions |
|---|---|---|
| 2',7' dichlorofluorescein diacetate | 192.98 mg | Mix, refridgerate and store for 3 months |
| DMSO | 5 ml | |

### 8.8 Avertin

#### *8.8.1 Avertin stock*

| Substance | Quantity | Instructions |
|---|---|---|
| 2,2,2-tribromoethanol | 0.5 g | Gently mix, store in thee dark at room temperature for 6 months |
| Tert-amyl alcohol | 0.5 ml | |

### *8.8.2 Avertin working solution*

| Substance | Quantity | Instructions |
|---|---|---|
| Avertin stock | 0.12 ml | Gently mix, refridgerate and store in the dark for 3 weeks. |
| Saline | 10 ml | |

## 8.9 Glucose solution (1000 mg/ml)

| Substance | Quantity | Instructions |
|---|---|---|
| D-glucose | 10 g | Gently mix, refridgerate and store for 1 month |
| PBS | 10 ml | |

## 8.10 Nutritional profile of food given to mice for LF vs HF feeding

### *8.10.1 Nutritional overview*

| | LF diet (SF04 – 057) | HF diet (SF00 – 219) |
|---|---|---|
| **Metabolizable energy** | 16.1 MJ/kg | 19.4 MJ/kg |
| **Caloric content** | 3.85 kcal/g | 4.64 kcal/g |
| **Fat** | 6% | 21% |
| **Protein** | 19% | 19% |
| **Crude fibre** | 4.7% | 4.7% |
| **Acid detergent fibre** | 4.7% | 4.7% |
| **Cholesterol** | - | 0.15% |

### *8.10.2 Ingredients*

| | LF diet (SF04 -057) (g/kg) | HF diet (SF00 – 219) (g/kg) |
|---|---|---|
| **Casein** | 195 | 195 |
| **DL Methionine** | 3 | 3 |
| **Sucrose** | 341 | 341 |
| **Wheat starch** | 154 | 154 |
| **Cellulose** | 50 | 50 |
| **Canola oil** | 60 | - |
| **Ghee (clarified butter)** | - | 210 |
| **Calcium carbonate** | 17.1 | 17.1 |
| **Sodium chloride** | 2.6 | 2.6 |
| **Potassium citrate** | 2.5 | 2.5 |
| **Potassium sulphate** | 1.6 | 1.6 |
| **Potassium dihydrogen phosphate** | 6.9 | 6.9 |
| **AIN 93G trace minerals** | 1.4 | 1.4 |
| **Choline chloride** | 2.5 | 2.5 |
| **SF00 – 219 vitamins** | 10 | 10 |
| **USP cholesterol** | 15 | 15 |
| **Etoxyquin (66%)** | 0.04 | 0.04 |

### *8.10.3 Fat content*

| | LF diet (SF04 – 057) (%) | HF diet (SF00 – 219) (%) |
|---|---|---|
| **Saturated fats C12 : 0 or less** | - | 2.4 |
| **Myristic acid 14 : 0** | Trace | 2.3 |
| **Palmitic acid 16 : 0** | 0.3 | 6.1 |
| **Stearic acid 18 : 0** | 0.1 | 1.9 |
| **Arichidic acid 20 : 0** | Trace | 0.5 |
| **Palmitoleic acid 16 : 1** | Trace | 1 |
| **Oleic acid 18 : 1** | 3.6 | 5.6 |
| **Gadoleic 20 : 1** | Trace | Trace |
| **Linoleic 18 : 3n3** | 0.6 | 0.8 |
| **Cholesterol** | - | 0.15 |
| **Linoleic acid 18 : 2n6** | 0.6 | - |
| **A linoleic acid 18 : 2n6** | 1.1 | Trace |
| **Arichodonic acid 20 : 4n6** | Trace | Trace |
| **EPA 20 : 5 n3** | Trace | trace |

### *8.10.4 Amino acids*

| | LF diet (SF04 – 057) (%) | HF diet (SF00 – 219) (%) |
|---|---|---|
| **Valine** | 1.2 | 1.2 |
| **Leucine** | 1.7 | 1.7 |
| **Isoleucine** | 0.8 | 0.8 |
| **Threonine** | 0.7 | 0.7 |
| **Methonione** | 0.8 | 0.8 |
| **Cystine** | 0.05 | 0.05 |
| **Lysine** | 1.5 | 1.5 |
| **Phenylalanine** | 0.9 | 0.9 |
| **Tyrosine** | 1.0 | 1.0 |
| **Tryptophan** | 0.3 | 0.3 |

### *8.10.5 Vitamins*

| | LF diet (SF04 – 057) (mg/kg unless otherwise stated) | HF diet (SF00 – 219) (mg/kg unless otherwise stated) |
|---|---|---|
| **Vitamin A** | 10 000 IU/ kg | 10 000 IU/ kg |
| **Vitamin D3** | 1 100 IU/ kg | 1 100 IU/ kg |
| **Vitamin E** | 62 | 62 |
| **Vitamin K** | 12 | 12 |
| **Vitamin C** | 700 | 700 |
| **Vitamin B1** | 11 | 11 |
| **Vitamin B2** | 11 | 11 |
| **Niacin** | 50 | 50 |
| **Vitamin B6** | 11 | 11 |
| **Pantothenic acid** | 33 | 33 |
| **Biotin** | 200 | 200 |
| **Folic acid** | 1 | 1 |
| **Inositol** | 55 | 55 |
| **Vitamin B12** | 18 | 18 |
| **Choline** | 2 500 | 2 500 |

### *8.10.6 Minerals*

| | LF diet (SF04 – 057) (% unless otherwise stated) | HF diet (SF00 – 219) (% unless otherwise stated) |
|---|---|---|
| **Calcium** | 0.58 | 0.58 |
| **Phosphorous** | 0.3 | 0.3 |
| **Magnesium** | 0.09 | 0.09 |
| **Sodium** | 0.11 | 0.11 |
| **Chloride** | 0.16 | 0.16 |
| **Potassium** | 0.4 | 0.4 |
| **Sulphur** | 0.22 | 0.22 |
| **Iron** | 90 mg/kg | 90 mg/kg |
| **Iodine** | 0.22 mg/kg | 0.22 mg/kg |
| **Copper** | 0.67 mg/kg | 0.67 mg/kg |
| **Manganese** | 20 mg/kg | 20 mg/kg |
| **Zinc** | 40 mg/kg | 40 mg/kg |
| **Selenium** | 0.15 mg/kg | 0.15 mg/kg |
| **Chromium** | 2 mg/kg | 2 mg/kg |
| **Lithium** | 0.1 mg/kg | 0.1 mg/kg |
| **Boron** | 0.7 mg/kg | 0.7 mg/kg |
| **Nickel** | 0.55 mg/kg | 0.55 mg/kg |
| **Vanadium** | 0.1 mg/kg | 0.1 mg/kg |

## 8.11 Supplementary statistical information

### *8.11.1 List of covariates included within statistical analysis of outcome data (Chapter 4)*

| Covariate description | Analysis covariate included within |
|---|---|
| **Maternal weight (at time of treatment commencement)** | Implantation number, litter size, resorption number, fetal weight, fetal length, placental weight |
| **Days of GlcN exposure** | Implantation number, litter size, resorption number, fetal weight, fetal length, placental weight |
| **Implantation number** | Litter size, resorption number, fetal weight, fetal length, placental weight |
| **Implantations per horn** | Litter size, resorption number, fetal weight, fetal length, placental weight |
| **Litter size** | Fetal weight, fetal length, placental weight |

### *8.11.2: A list of the reproductive outcomes analysed with a Chi squared test following an 11 week LF or HF dietary intervention prior to 4 - 5 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. (Chapter 5, fetal outcomes)*

| Parameter | Chi-Squared Analysis |
|---|---|
| **Pregnancy** | Number of mice with visible sperm plugs, number of mice that became pregnant, proportion of mice that became pregnant from those that had plugged, proportion of fetuses that had formed from total implantations |
| **Birth Defects** | Total number of abnormal fetuses, proportion of abnormal fetuses from the total number, total number of mothers carrying abnormal fetuses, proportion of mothers carrying abnormal fetuses, total number of abnormal placentas, proportion of abnormal placentas, total number of mothers with abnormal placentas, proportion of mothers with abnormal placentas |

***8.11.3: A list of the reproductive outcomes analyzed with a Two-way ANOVA plus associated covariates, following an 11 week LF or HF dietary intervention prior to 4 - 5 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. (Chapter 5, fetal development)***

| Parameter | Covariate |
|---|---|
| **Pregnancy** | |
| ***Mean Implantation Number*** | Mean ovulation number, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure |
| ***Mean Litter Size*** | Mean ovulation number, mean implantation number, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure |
| ***Mean Resorption Number*** | Mean ovulation number, mean implantation number, mean litter size, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure |
| **Ovulation** | |
| ***Mean Ovulation Rate*** | Maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure |
| **Conceptus** | |
| ***Fetal Weight*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure, placental weight, placental volume |
| ***Fetal Length*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure, placental weight, placental volume |
| ***Placental Weight*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure, fetal weight, fetal length |
| ***Placental Volume*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at completion of 11 week dietary intervention, maternal weight change from 0 – 11 weeks dietary intervention, days of GlcN exposure, fetal weight, fetal length |

***8.11.4: A list of the reproductive outcomes (post natal) analyzed with a One-way ANOVA (+ co-variates) for mice maintained on a LF or HF diet for 11 weeks before undergoing 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN.(Chapter 5, post natal outcomes)***

| Parameter | One-way ANOVA | Groups included in analysis |
|---|---|---|
| **Weekly weight gain (offspring)** | Maternal weight at 16 weeks, maternal weight change over 11 weeks, days of GlcN exposure, litter size at birth, number of living litter mates on day of birth, number of dead litter mates at birth, litter size at 1 week, number of dead litter mates at 1 week, number of cage mates at start of weaning, number of cage mates per week | 8 wks – GlcN<br>8 wks + GlcN<br>16 wks + GlcN |
| **Organ post mortems** | **Maternal weight at 16 weeks,** maternal weight change over 11 weeks, days of GlcN exposure, **litter size at birth**, **number of living litter mates on day of birth,** number of dead litter mates at birth, litter size at 1 week, number of dead litter mates at 1 week, weight (16 weeks), number of cage mates at start of weaning, number of cage mates at end of 16 weeks | 8 wks – GlcN<br>8 wks + GlcN<br>16 wks + GlcN |
| **Sperm analysis** | Maternal weight at 16 weeks, maternal weight change over 11 weeks, days of GlcN exposure, litter size at birth, number of living litter mates on day of birth, number of dead litter mates at birth, litter size at 1 week, number of dead litter mates at 1 week, weight (16 weeks), number of cage mates at start of weaning, number of cage mates at end of 16 weeks | 8 wks – GlcN<br>8 wks + GlcN<br>16 wks + GlcN |

***8.11.5: A list of the reproductive outcomes analysed with a Chi squared test for 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN (Chapter 6)***

| Parameter | Chi-Squared Analysis |
|---|---|
| **Pregnancy** | Number of mice with visible sperm plugs, number of mice that became pregnant, proportion of mice that became pregnant from those that had plugged, proportion of fetuses that had formed from total implantations |
| **Ovulation** | Proportion of fetuses that formed from ovulated oocytes *(ovulated oocytes determined from CL number)* |
| **Birth Defects** | Total number of abnormal fetuses, proportion of abnormal fetuses from the total number, total number of mothers carrying abnormal fetuses, proportion of mothers carrying abnormal fetuses, total number of abnormal placentas, proportion of abnormal placentas, total number of mothers with abnormal placentas, proportion of mothers with abnormal placentas |

***8.11.6: A list of the reproductive outcomes (fetal) analysed with a Two-way ANOVA (+ co-variates) for 8 and 16 week old mice given 4 - 7 days of peri-conceptional exposure to either 0 or 20 mg/kg GlcN. (Chapter 6)***

| Parameter | Covariate |
|---|---|
| **Pregnancy** | |
| ***Mean Implantation Number*** | Mean ovulation number, maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure |
| ***Mean Litter Size*** | Mean ovulation number, mean implantation number, maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure |
| ***Mean Resorption Number*** | Mean ovulation number, mean implantation number, mean litter size, maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure |
| **Ovulation** | |
| ***Mean Ovulation Rate*** | Maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure |
| **Conceptus** | |
| ***Fetal Weight*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure, placental weight, placental volume |
| ***Fetal Length*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, **maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks,** days of GlcN exposure, placental weight, placental volume |
| ***Placental Weight*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure, fetal weight, fetal length |
| ***Placental Volume*** | Mean ovulation number, mean implantation number, mean litter size, mean resorption number, maternal weight at 8/ 16 weeks, maternal weight change from 5-8 weeks/ 13-16 weeks, days of GlcN exposure, fetal weight, fetal length |

## 8.12 Supplementary mating results

***8.12.1: Rates of successful mating for mice that received IP administrations of 0 mg/kg, 20 mg/kg or 400 mg/kg GlcN for 4 - 5 days vs 6 - 7 days (Chapter 4)***

| | 0 mg/kg GlcN | 20 mg/kg GlcN | 400 mg/kg GlcN |
|---|---|---|---|
| **Number mated in 4 - 5 days** | 15 | 15 | 15 |
| **Number mated in 6 - 7 days** | 7 | 4 | 3 |
| **Proportion mated in 4 - 5 days** | 68.2 | 78.9 | 83.3 |
| **Proportion mated in 6 - 7 days** | 31.8 | 20.1 | 16.7 |

***8.12.2: Mean (± SEM) weight (gr) of mice that did and did not become pregnant after being maintained on a LF (low fat) or HF (high fat) diet for 11 weeks before being treated (IP) with 0 mg/kg or 20 mg/kg GlcN for 4 - 5 days. (Chapter 5, fetal outcomes)***

| | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **Pregnant** | 24.7 ± 0.3 | 25.0 ± 0.6 | 30.4 ± 0.6 | 31.2 ± 0.7 |
| **Not pregnant** | 24.5 ± 0.4 | 24.4 ± 0.5 | 30.8 ± 0.5 | 30.8 ± 0.6 |

***8.12.3: Mean (± SEM) weight (gr) of mice that did and did not become pregnant after being maintained on a LF (low fat) or HF (high fat) diet for 11 weeks being treated (IP) with 0 mg/kg or 20 mg/kg GlcN for 4 - 5 days. (Chapter 5, post natal outcomes)***

| | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **Pregnant** | 24.5 ± 0.4 | 24.6 ± 0.4 | 29.3 ± 0.5 | 30.1 ± 0.6 |
| **Not pregnant** | 24.6 ± 0.5 | 24.9 ± 0.3 | 29.9 ± 0.5 | 30.8 ± 0.4 |

***8.12.4: Mean (± SEM) weight (gr) of 8 and 16 week old mice that did and did not become pregnant after being treated (IP) with 0 mg/kg or 20 mg/kg GlcN for 4 - 5 days. (Chapter 6)***

| | 8 wks - GlcN | 8wks + GlcN | 16 wks - GlcN | 16 wks + GlcN |
|---|---|---|---|---|
| **Pregnant** | 21.0 ± 0.2 | 21.2 ± 0.3 | 25.0 ± 0.3 | 25.3 ± 0.8 |
| **Not pregnant** | 21.1 ± 0.2 | 20.8 ± 0.4 | 25.1 ± 0.4 | 24.9 ± 0.3 |

**8.13: Supplemental sample size data**

***8.13.1: Numbers of mice that were included in mean weekly weight calculations. (Chapter 5, supplemental data for figure 1: Weekly weights of mice maintained on a LF (low fat) or HF (high fat) diet for 11 weeks)***

| Week | LF (No.) | HF (No.) | Week | LF (No.) | HF (No.) |
|---|---|---|---|---|---|
| **0** | 95 | 96 | **6** | 95 | 96 |
| **1** | 95 | 96 | **7** | 83 | 84 |
| **2** | 94 | 96 | **8** | 95 | 96 |
| **3** | 95 | 96 | **9** | 82 | 83 |
| **4** | 95 | 96 | **10** | 82 | 82 |
| **5** | 95 | 96 | **11** | 94 | 93 |

***8.13.2: Number of surviving offspring (from birth to 16 weeks of age) from mice maintained on a LF (low fat) or HF (high fat) diet for 11 weeks being treated (IP) with 0 mg/kg or 20 mg/kg GlcN for 4 - 5 days. (Chapter 5, Post natal outcomes)***

| Week | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **0 *(Birth)*** | 47 | 56 | 48 | 74 |
| ***(Day)* 1** | 35 | 53 | 41 | 67 |
| ***(Day)* 3** | 29 | 41 | 13 | 39 |
| **1** | 28 | 36 | 13 | 37 |
| **2** | 27 | 36 | 11 | 36 |
| **3** | 26 | 36 | 10 | 36 |
| **4** | 26 | 36 | 8 | 36 |
| **5** | 26 | 36 | 6 | 34 |
| **6** | 23 | 36 | 5 | 33 |
| **7** | 23 | 36 | 5 | 33 |
| **8** | 23 | 36 | 5 | 32 |
| **9** | 22 | 36 | 5 | 32 |
| **10** | 22 | 36 | 5 | 32 |
| **11** | 22 | 36 | 5 | 32 |
| **12** | 21 | 36 | 5 | 31 |
| **13** | 20 | 36 | 5 | 31 |
| **14** | 20 | 36 | 5 | 30 |
| **15** | 20 | 36 | 5 | 30 |
| **16** | 20 | 36 | 5 | 30 |

***8.13.3: Total number of female offspring for developmental ages 3 - 16 weeks, of mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating***

| Week | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **3** | 15 | 16 | 4 | 16 |
| **4** | 15 | 16 | 4 | 16 |
| **5** | 15 | 16 | 3 | 15 |
| **6** | 14 | 16 | 3 | 14 |
| **7** | 14 | 16 | 3 | 14 |
| **8** | 14 | 16 | 3 | 13 |
| **9** | 13 | 16 | 3 | 13 |
| **10** | 13 | 16 | 3 | 13 |
| **11** | 13 | 16 | 3 | 13 |
| **12** | 13 | 16 | 3 | 13 |
| **13** | 13 | 16 | 3 | 13 |
| **14** | 13 | 16 | 3 | 13 |
| **15** | 13 | 16 | 3 | 13 |
| **16** | 13 | 16 | 3 | 13 |

***8.13.4: Total number of male offspring for developmental ages 5 - 16 weeks, of mice that were maintained on a LF or HF diet for 11 weeks prior to mating and throughout pregnancy, and were treated with 0 or 20 mg/kg GlcN for 4 - 5 days prior to mating.***

| Week | LF - GlcN | LF + GlcN | HF - GlcN | HF + GlcN |
|---|---|---|---|---|
| **3** | 11 | 20 | 6 | 20 |
| **4** | 11 | 20 | 4 | 20 |
| **5** | 11 | 20 | 3 | 19 |
| **6** | 9 | 20 | 2 | 19 |
| **7** | 9 | 20 | 2 | 19 |
| **8** | 9 | 20 | 2 | 19 |
| **9** | 9 | 20 | 2 | 19 |
| **10** | 9 | 20 | 2 | 19 |
| **11** | 9 | 20 | 2 | 19 |
| **12** | 8 | 20 | 2 | 18 |
| **13** | 7 | 20 | 2 | 18 |
| **14** | 7 | 20 | 2 | 18 |
| **15** | 7 | 20 | 2 | 18 |
| **16** | 7 | 20 | 2 | 18 |

**8.14: Mean (± SEM) area under the curve for glucose clearance rates (mM/ 120 minutes) for mice that received a LF or HF diet. (Chapter 5)**

| | | Week 7 | Week 9 | Week 10 |
|---|---|---|---|---|
| **Replicate 1** | **LF** | 1634.6 ± 122.4 *(N= 5)* | 1676.2 ± 131.8 *(N = 4)* | 2234.8 ± 198.0 *(N = 6)* |
| **Replicate 2** | **HF** | 1822.5 ± 196.6 *(N = 6)* | 1970.2 ± 231.6 *(N = 6)* | 2705.0 ± 213.4 *(N = 6)* |
| **P** | | P = 0.195 | P = 0.092 | **P<0.05** |
| **Replicates 1 & 2 combined** | **LF** | 1616.5 ± 65.1 *(N = 11)* | 1741.7 ± 73.8 *(N = 10)* | 2401.6 ± 154.4 *(N = 12)* |
| **Replicates 1 & 2 combined** | **HF** | 1752.0 ± 98.6 *(N = 12)* | 1915.4 ± 118.9 *(N = 11)* | 2662.6 ± 123.7 *(N = 12)* |
| **P** | | P = 0.471 | P = 0.663 | P = 0.260 |

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