TRANSGENERATIONAL INHERITANCE OF DNA METHYLATION ALTERATIONS AT THE *H19* IMPRINTING CONTROL REGION FOLLOWING MATERNAL ETHANOL EXPOSURE IN MICE

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree in Master of Science (Medicine) in the Division of Human Genetics

Declaration

I, Michelle Ungerer, hereby declare this dissertation to be my own, unaided work. It is being submitted for the degree of Master of Science (Medicine) at the University of the Witwatersrand, Johannesburg. It has not been previously submitted for any degree or examination at this, or any other University.

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Michelle Ungerer

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Date

To: My Parents

Abstract

Foetal Alcohol Syndrome (FAS) is characterised by growth retardation, craniofacial dysmorphology and neurodevelopmental deficits. Whilst, not all alcohol exposed offspring display alcohol-related developmental anomalies, the percentage of affected offspring is greatly underestimated. Common behavioural disorders, such as ADHD and anxiety, are likely to be linked to the transgenerational effects of *in utero* alcohol exposure. Epigenetics has been highlighted as a potential mechanism in the aetiology of alcohol teratogenesis due to alcohol's disruptive effects on the folate pathway, and subsequently DNA methylation. The imprinted *H19/Igf2* domain is critical in foetal growth and development. The locus is regulated by the methylation-sensitive CTCF binding protein which binds to the *H19* imprinting control region (ICR) upstream of the *H19* locus. CTCF binding allows for the reciprocal expression of *H19* and *Igf2* in an allele-specific parent of origin manner. Due to the monoallelic expression of imprinted genes, DNA methylation changes within their control regions can lead to altered gene expression and possibly disease. Furthermore, if these alterations occur in the germline, disease states or susceptibility to disease may be transmittable to future generations.

A mouse model was used to investigate the potential transgenerational effects of F_0 chronic maternal ethanol exposure on parturition, growth, locomotor activity and anxiety. Furthermore, the transgenerational inheritance of *H19* ICR DNA methylation was investigated and its possible contribution to the aforementioned phenotypes was determined. Phenotypic analysis revealed significantly reduced F_1 fertility following alcohol exposure (*P = 0.003*) but no other significant perturbations in parturition. Although not significant at all generations, alcohol's effects on growth and behaviour were apparent. DNA was extracted from tail biopsies, bisulfite modified and the CTCF1 and CTCF2 regions of the *H19* ICR amplified. DNA methylation quantification via Pyrosequencing revealed significantly reduced mean methylation profiles at CTCF1 and CTCF2 within the F_1 EtOH exposed group (*P = 0.021*), with CpG sites 1, 2, 4 and 6 of CTCF1 and CpG sites 1, 2, 3 *(P = 0.021)* and 5 *(P = 0.043)* of CTCF2 displaying statistically significant differences. In contrast, the EtOH group of

the F_2 generation showed an increase in CTCF1 mean methylation that trended towards significance (*P = 0.083*) suggesting a potential recovery or compensatory mechanism within the epigenetic machinery. The F_3 generation EtOH exposed group displayed decreased CTCF1 mean methylation levels (*P = 0.083*). The F_2 and F_3 generations showed no significant difference in CTCF2 methylation levels between treatment groups. The significant change in CTCF1 methylation at the F_1 generation and the trend towards significance in the F_2 and F_3 generations indicated potential transgenerational inheritance of altered *H19* ICR DNA methylation. Correlations between DNA methylation at the *H19* CTCF1 and CTCF2 binding sites with growth rate and behaviour measures revealed no significant relationships.

This dissertation supports the involvement of epigenetic mechanisms in alcohol teratogenesis. In addition it contributes to the growing field of transgenerational epigenetic inheritance, with implications for the treatment of those with Foetal Alcohol Syndrome and/or Foetal Alcohol Spectrum Disorders and their progeny.

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Contents

List of Tables

List of Figures

Abbreviations

µg	Micro gram
µl	Micro litre
˚C	Degree Celsius
%	Percentage
5-CH_3THF	5-methyl tetrahydrofolate
A	Adenine
ADH	Alcohol-dehydrogenase
ADHD	Attention Deficit-Hyperactivity Disorder
ALDH	Aldehyde Dehydrogenase
ARBDs	Alcohol Related Birth Defects
ARNDs	Alcohol Related Neurodevelopmental Disorders
AS	Angelman Syndrome
BAC	Blood Alcohol Concentration
bp	Base pairs
C	Cytosine
CBP	CREB binding protein
CHD	Congenital heart disease
CpG	CpG site
cm	Centimetre
CO_2	Carbon dioxide
CTCF	CCCTC-binding factor
ddH_2O	De-ionised distilled water
dH_2O	Distilled water
dL	Decilitre
DMR	Differentially methylated region
DNA	Deoxyribose nucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleic acid triphosphate

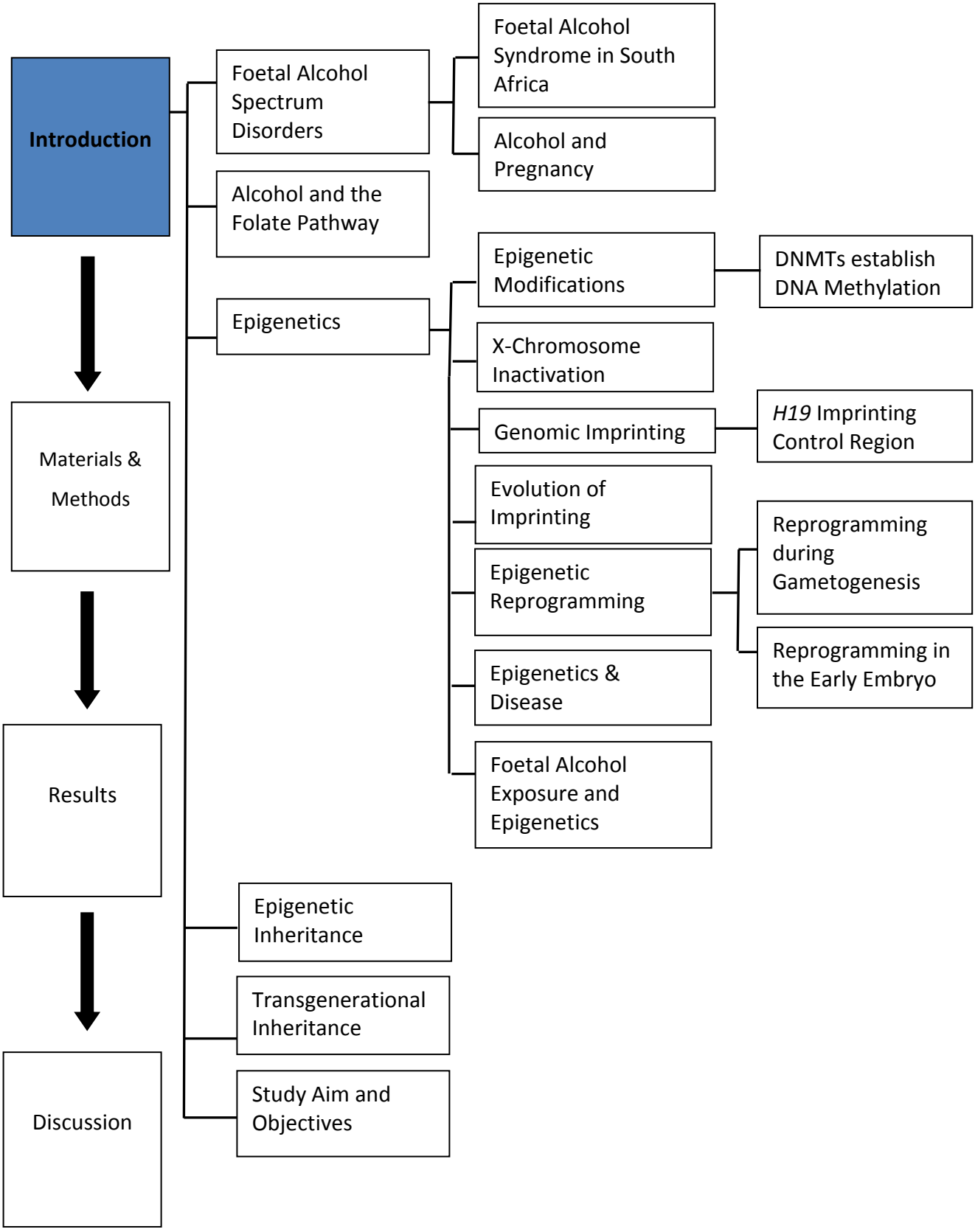
EDTA	Ethylenediamine Tetra-Acetic Acid
EtOH	Ethanol-exposed
EM	Embryonic lineage
ED	Embryonic Day
ES	Embryonic stem cells
EX	Extraembryonic lineage
F	Filial generation
F_0	Generation Zero
F_1	Generation One
F_2	Generation Two
F_3	Generation Three
FASD	Foetal Alcohol Spectrum Disorders
FAS	Foetal Alcohol Syndrome
G	Guanine
g	Gram
GD	Gestational day
GWAS	Genome Wide Association Study
H	Histone
HCl	Hydrochloric acid
IAP	Intracisternal A-Particle
ICR	Imprinting control region
ICM	Inner cell mass
Kb	Kilobase
kg	Kilogram
LTR	Long terminal repeat
MAT	Methonine adenosyltransferase
MBP	Methyl-CpG binding protein
mg	Milligram
miRNA	MicroRNA

min	Minute
ml	Millilitre
mm	Millimetre
ncRNA	Non-coding RNA
ng	Nanogram
PCR	Polymerase chain reaction
PD	Postnatal Day
PGC	Primordial germ cell
PWS	Prader-Willi Syndrome
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Revolutions per minute
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
sec	Second
SNP	Single nucleotide polymorphism
T	Thymine
Taq pol	*Thermus aquaticus* polymerase
TBE	Tris-Boric Acid-EDTA Buffer
TE	Tris-EDTA Buffer
THF	Tetrahydrofolate
TSS	Transcription start site
U	Uracil
XCI	X-Chromosome Inactivation
XIC	X-inactivation centre
Xist	X-inactive specific transcript

Chapter 1

Introduction

1.0 Introduction



1.1 Chapter Outline

The effects of *in utero* alcohol exposure are devastating. Foetal Alcohol Spectrum Disorders (FASD), specifically Foetal Alcohol Syndrome (FAS), are the most preventable causes of birth defects and intellectual disabilities worldwide. Despite this little is known about the molecular and developmental mechanisms underlying these effects. The introduction below incorporates a review of current literature and assesses an epigenetic mechanism, specifically DNA methylation and imprinted genes, in the actions of alcohol. There is increasing evidence for the introduction of germline epimutations and the potential for gestational ethanol exposure to transmit altered epimutations to not only the directly exposed offspring but also to subsequent generations. This hereditary transmission of epimutations without further exposure to alcohol is a cause for great concern and highlights the need for this investigation into the transgenerational inheritance of the FASD phenotype.

1.2 Foetal Alcohol Spectrum Disorders

Alcohol (ethanol), the organic compound produced by the fermentation of fruits and grains, is the active ingredient in many beverages and has been imbibed by various cultures throughout history. The adverse effects of alcohol exposure on a developing foetus have been recognised for centuries. Evidence of the ill effects of alcohol appear in the early writings of Aristotle who stated, *"Foolish, drunken, and harebrained women, most often bring forth children like unto themselves, morose and languid",* and in the scriptures of the Bible where the angel of the Lord warned Manoah's wife, *"Behold now, thou art barren, and bearest not: but thou shalt conceive, and bear a son. Now therefore beware, I pray thee, and drink not wine nor strong drink…"* (Judges, 13: 3 – 4, King James I Holy Bible) (Abel, 1990). More recently, during England's 'Gin Epidemic' (1720 – 1751), English physicians depicted children who were born to alcoholic mothers as *"weak, feeble, and distempered"* (Royal College of Physicians of London, 1726). However, it was not until 1973 that the first detailed account of offspring malformations associated with prenatal alcohol exposure was described (Jones & Smith, 1973; Jones *et al.*, 1973).

Prenatal alcohol exposure leads to a wide range of developmental abnormalities that include, but are not limited to, prenatal and postnatal growth retardation, abnormal growth and development of the central nervous system, mental disability and distinct craniofacial dysmorphology (such as a thin upper lip, smooth philtrum and flat nasal bridge) (Jones *et al.*, 1973; Sampson *et al.*, 1997). However, the majority of children affected by prenatal alcohol exposure display significant neurodevelopmental problems with severe learning difficulties, including attention deficit-hyperactivity disorder (ADHD) and disruptions in both fine and gross motor coordination (Sampson *et al.*, 1997). Certain regions of the brain show more sensitivity to the oxidative stress induced by alcohol exposure than others (Maier & West, 2001). Regions such as the cerebellum and the hippocampus show very low levels of the endogenous antioxidant compound Vitamin E. The cerebellum is responsible for motor performance whereas the hippocampus plays a major role in memory. These regions were shown to suffer most when exposed to alcohol and not surprisingly motor and cognitive deficits are the most common consequences of alcohol intoxication (Dursun *et al.*, 2006). The wide literature on neurodevelopmental delays in children prenatally exposed to alcohol has shown that in addition to the above mentioned difficulties a number of secondary effects in other life domains can occur. Depression and anxiety disorders are among the most commonly reported problems in children and adults with Foetal Alcohol Spectrum Disorders (FASD) [as reviewed by (Hellemans *et al.*, 2010)]. Following a 30-year follow-up of their original 1968 cohort Lemoine (1992) noted that mental health problems were the most severe manifestation of Foetal Alcohol Syndrome (FAS) in adulthood.

FASD is the umbrella term for those affected by alcohol exposure *in utero*. According to the Institute of Medicine's revised classification system there are currently six recognized clinical diagnoses: FAS, with and without confirmed maternal alcohol exposure; partial FAS, also with and without confirmed maternal alcohol exposure; alcohol related birth defects (ARBDs); and alcohol related neurodevelopmental disorders (ARNDs) (Hoyme *et al.*, 2005). FAS is considered to be the most severe clinical outcome resulting from prenatal alcohol exposure. Features similar to those observed in humans with FASD have also been observed

in several animal models of prenatal alcohol exposure (Anthony *et al.*, 2010; Chernoff, 1977; Parnell *et al.*, 2009; Sulik, 1984; Webster *et al.*, 1983).

1.2.1 Foetal Alcohol Syndrome and South Africa

Given that alcohol consumption is voluntary, FASD is the most preventable cause of birth defects and mental retardation, yet FASD remains a global health problem (Abel & Hannigan, 1995). FAS has been identified in all racial and ethnic groups (Abel & Hannigan, 1995; May & Gossage, 2001). This abnormal foetal development results in lifelong mental, physical and behavioural disabilities that incur high costs to the individuals, their families and society as a whole. In the United States of America there are reports of costs of up to US$2 million per FAS individual (Lupton *et al.*, 2004). Despite continued education about the dangers associated with gestational drinking the global prevalence of FAS ranges between 0.5-2.0 per 1000 live births (May & Gossage, 2001). More alarmingly the highest rate worldwide occurs in mixed ancestry communities in the Western Cape of South Africa. Within this South African population there are between 68.0 and 89.2 per 1000 children of school-going age displaying FAS symptoms (May *et al.*, 2007). The reason for this high rate of FAS is not fully understood but is likely due to a devastating combination of maternal drinking, malnutrition, and high levels of infection, for example TB and HIV. Croxford and Viljoen have reported that in the Western Cape, almost 34% of urban women and 46% - 51% of rural women consume alcohol during pregnancy in a binge-like pattern (Croxford & Viljoen, 1999).

1.2.2 Alcohol and the Foetus

Alcohol moves easily through the placental barrier during pregnancy resulting in blood alcohol concentrations (BACs) in mother and foetus that are approximately equal (Guerri & Sanchis, 1985). The lack of hepatic alcohol dehydrogenase (ADH), the enzyme primarily responsible for the catabolism of alcohol, within the foetus results in the foetus being unable to metabolise alcohol. As a result an accumulation of alcohol occurs in the foetus and amniotic fluid as the removal of alcohol from the foetus occurs only via passive diffusion across the placenta and then through maternal elimination (Waltman & Iniquez, 1972).

Furthermore, the foetus is expected to be exposed for longer time periods than the mother as the rate of elimination of alcohol from amniotic fluid is half that from maternal blood (Brien *et al.*, 1983). Despite this, only between 5% and 10% of offspring prenatally exposed to alcohol display alcohol-related developmental anomalies, with dose, time and duration of exposure being critical determinants (Klein de Licona *et al.*, 2009). This relatively low penetrance highlights that genetic predisposition of both the mother and the foetus in conjunction with other factors, such as gender, diet and social environment, play an important role in the manifestation of the disease (Chernoff, 1980; Ogawa *et al.*, 2005). However, the number of offspring estimated to be affected by *in utero* exposure is probably greatly underestimated with common behavioural disorders, such as ADHD, likely to be linked to the transgenerational effects of alcohol exposure in preceding generations [as reviewed by (Ramsay, 2010)].

1.3 Alcohol and the Folate Pathway

Eukaryotes are unable to synthesise folate or its metabolites and subsequently obtain the vitamin through dietary intake. Folate plays a critical role in one-carbon metabolism, purine and amino acid synthesis, and DNA methylation reactions.

Methyl groups required for methylation reactions are produced by the folate-dependent pathway. The pathway involves folate being converted from 5-methyl tetrahydrofolate (5-CH_3THF) to tetrahydrofolate (THF) via B12-dependent methionine synthase (MS). MS transfers the methyl group to homycysteine generating methionine. Methionine, an essential amino acid, is then converted to S-adenosylmethionine (SAM) by methionine adenosyltransferase (MAT). Following the conversion of methionine to SAM, S-adenosylhomocysteine (SAH) is produced and the methyl groups generated are used to methylate DNA by use of DNA methyltransferases (MT) (Hobbs *et al.*, 2005) **(Figure 1.1).**

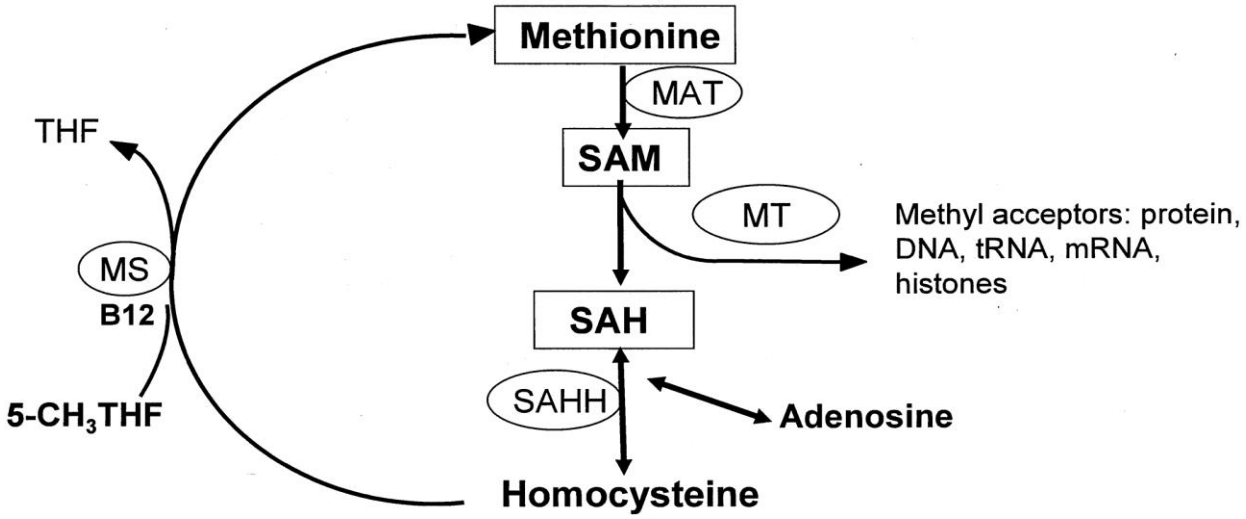


Figure 1.1: The Folate-Dependent Methionine and Homocysteine Metabolic Pathway. The folate-dependent Methionine pathway involves the conversion of 5-methyl tetrahydrofolate (5-CH_3THF) to tetrahydrofolate (THF) via B12-dependent methionine synthase (MS). The newly available methyl group is transferred to homocysteine, which generates methionine. Methionine is converted to S-adenosylmethionine (SAM) by methionine-adenosyltransferase (MAT). SAM is converted to S-adenosylhomocysteine (SAH) and the methyl groups produced are used to methylate DNA by use of methyltransferases (MT). SAH is hydrolysed to Homocysteine by SAH hydrolase (SAHH) releasing adenosine. Obtained from Hobbs *et al.* (2005).

Alcohol appears to interfere with the folate-methyl metabolic pathway by inhibiting MS and MAT (Halsted *et al.*, 2002). In addition to interfering with the folate dependent pathway, alcohol alters the normal bioavailability and metabolism of folate. Folate deficiency is a common clinical sign of chronic alcohol abuse and has been implicated in the development of alcoholism-related complications, such as alcoholic liver disease (Eichner *et al.*, 1971). Halsted *et al.* (1971) investigated the bioavailability of folate in a group of chronic drinkers and noted that, despite consuming the recommended amounts of folate, this group continued to display low levels of the nutrient. The low levels of the nutrient were seen to be due to interruptions in the processing of folate and its subsequent absorption across the luminal membrane of the enterocyte.

Folic acid (natural occurring or synthetic folate) is vital for growth and development. Folic acid and its various coenzymic forms, such as 10-formyl-THF and 5-CH_3THF are involved as

cofactors in the nucleic acid biosynthesis pathway. Due to its role in DNA synthesis and methylation large amounts of folic acid and its coenzymic forms are required to be transported from the mother to the child. Alcohol impedes development by reducing folic acid transport to the foetus (Hutson *et al.*, 2012). A study involving gestational exposure in rats altered the total folate levels and the distribution of coenzymes within maternal (liver and placenta) and foetal tissues (liver and brain). The most notable effect occurred in the foetal brain where the reduced folate levels and altered distributions were associated with a 10% decrease in foetal weight, a classic symptom of FAS (Lin *et al.*, 1992).

Furthermore, during pregnancy the protective properties of folic acid are well documented (Milunsky *et al.*, 1989; Shaw *et al.*, 1995; Werler *et al.*, 1993)}. Folic acid is critical to the rate in which formic acid, the toxic metabolite produced from the metabolism of alcohol, is detoxified. Although folic acid is able to reverse the oxidative stress induced by the metabolism of alcohol; ethanol exposure greatly reduces the antioxidant benefits of folic acid. Researchers have also found disruption of the folate pathway to be associated with neurological abnormalities and birth defects, such as, craniofacial malformations and neural tube defects. Several studies in humans and mice have linked maternal folic acid supplementation to decreased risks of the above mentioned birth effects (Cano *et al.*, 2001; Milunsky *et al.*, 1989; Werler *et al.*, 1993). However, supplementation is not effective in reducing risk in all individuals (Finnell *et al.*, 1998; Serrano *et al.*, 2010).

Although it is tempting to assume that alcohol simply produces a functional deficiency of folate, the lack of ‘folate-rescue’ in all individuals suggests that the interruption in folate metabolism may lead to irreversible downstream effects. Due to the role of folate metabolism in the establishment of DNA methylation, alcohol may alter the pattern of this epigenetic mark and subsequently alter gene expression.

1.4 Epigenetics

Epigenetics allows for the differentiation of an organism's cells into a variety of cell types despite identical genetic sequence. This tissue–specific expression ensures functional heterogeneity and subsequent survival of the organism. Epigenetics, first defined by Waddington as the *"branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being"* (Goldberg *et al.*, 2007), has more recently been redefined by Skinner *et al* as the *''molecular factors and processes around DNA that regulate genome activity independent of DNA sequence and that are mitotically stable''* (Skinner *et al.*, 2010). The epigenetic mechanisms responsible for these external modifications include DNA methylation and histone modifications (collectively termed the 'epigenome'). An additional layer of the epigenome involves non-coding RNAs that help regulate and maintain epigenetic states. The epigenome provides an interface between the environment and genome where epigenetic signatures can be influenced and modified by environmental exposures. These epigenetic modifications occur in response to the cellular environment and can have long-lasting effects in the organism and potentially in subsequent progeny and generations (Anway *et al.*, 2005). Mitotic inheritance of epigenetic marks is well accepted, however, inheritance across meiotic divisions and to future offspring is still under debate due to the 'resetting' of epigenetic marks during epigenetic reprogramming.

1.4.1 Epigenetic Modifications

Within eukaryotes DNA is wound around histones and packaged in the cell in the form of chromatin. Chromatin is further divided into two different levels, silent heterochromatin and active euchromatin. Heterochromatic DNA, including telomeres and pericentric regions, are associated with a high number of repetitive sequences and low gene content. In contrast, euchromatin is considered to contain a high number of genes and is said to be transcriptionally active. The structural building block of chromatin is the nucleosome. The nucleosome consists of 146 base pairs of DNA wrapped around a core of eight histone proteins. The octamer core consists of two copies of each histone protein: H2A, H2B, H3 and

H4. The nucleosomes are connected by a stretch of a linker DNA and linker histone H1, which offers stability to the packaged structures (**Figure 1.2**).

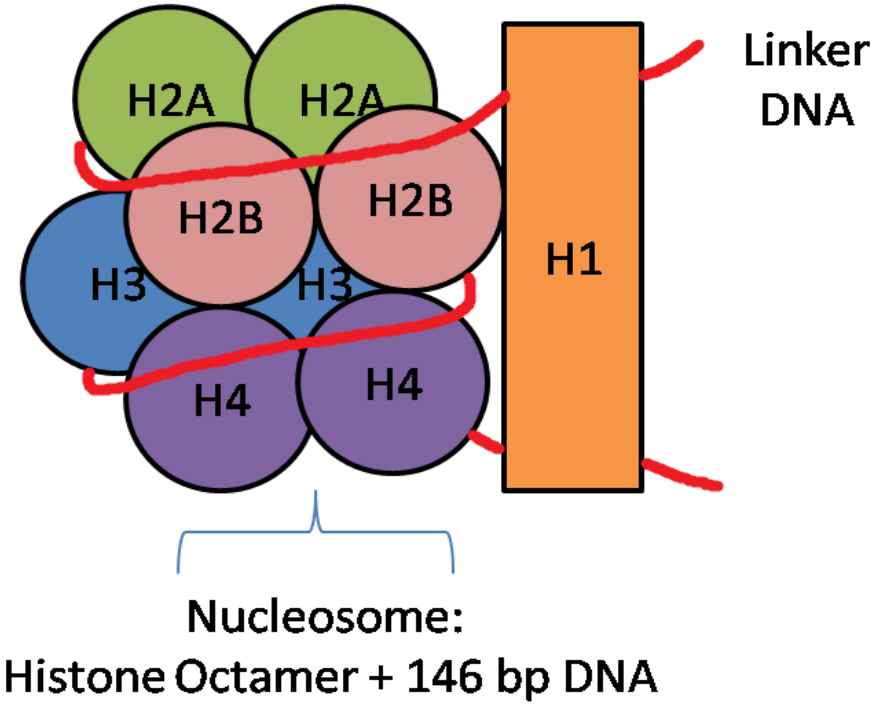


Figure 1.2: The Nucleosome Structure: A schematic representation of the structural units of chromatin known as a nucleosome. Each nucleosome is comprised of an octamer core of histones. The octamer consists of two copies of each histone H2A, H2B, H3 and H4: 146 base pairs of DNA are wrapped around the octamer. Attached to the nucleosome is the linker histone H1, the nucleosomes are connected by linker DNA.

Each histone has an N-terminal 'tail'. This 'tail' region projects out from the histone core and is the site where the majority of epigenetic histone modifications occur. The various modifications that can occur include methylation, acetylation, phosphorylation and ubiquitylation (Bartova *et al.*, 2008; Berger, 2002).

The epigenetic modification associated with DNA is methylation. DNA methylation involves the addition of a methyl group (CH_3) to the fifth carbon of cytosine. Modification occurs predominantly at cytosines within a CpG dinucleotide context but can occur on cytosine residues elsewhere. DNA methylation is able to directly regulate gene expression by preventing the interaction between regulatory elements and their target sequences. In

addition, methyl-CpG binding domain proteins (MBDs), such as methyl-CpG-binding protein MeCP2, recognise methylated CpGs and recruit transcriptional silencers. DNA methylation and histone modifications work together to regulate gene expression by remodelling the structure of the protein–DNA complex into active and/or silenced chromatin states (Bartova *et al.*, 2008). In general, the extent of DNA methylation corresponds to the level of gene expression. Hypomethylation is typically associated with an active domain as the open conformation allows for interactions between genes and transcription elements. In contrast, hypermethylation results in a closed conformation generally silencing the genes (Li, 2002).

1.4.1.2 DNMTs establish DNA Methylation

The most frequently studied epigenetic mark is DNA methylation. Within the mammalian genome 60-90% of the cytosine residues within CpGs are modified via DNA methylation (Bird, 1986). The remaining unmethylated cytosines are unevenly distributed throughout the genome and are often found in clusters within the promoter regions of many active genes. These clusters of unmethylated cytosines are referred to as CpG islands (Shukla *et al.*, 2008).

DNA methyltransferases (DNMTs) are the enzymes responsible for both the establishment of DNA methylation patterns in early development and their maintenance through subsequent cell divisions. To date four DNMTs have been identified, DNMT1, DNMT2, DNMT3a and DNMT3b [as reviewed by (Zhang & Jeltsch, 2010)].

DNMT1, a major maintenance methyltransferase, preferentially transfers methyl groups to the cytosines of hemi-methylated strands of DNA post DNA replication. DNMT1 activity ensures the propagation of specific methylation patterns after each round of cell division, including those of imprinted regions (Leonhardt *et al.*, 1992). Dnmt3a and Dnmt3b are referred to as *de novo* methyltransferases as they are involved in the methylation of cytosine residues of previously unmethylated CpG sites allowing for the establishment of new methylation patterns during development (Okano *et al.*, 1999).

Several studies in mice have highlighted the importance of DNMTs in embryonic development (Li *et al.*, 1992; Trasler *et al.*, 1996). Li *et al.* (1992) showed significantly reduced methylation levels when targeting the *Dnmt1* gene in embryonic stem cells (ES) and in mouse embryos, with the loss of Dnmt1 increasing embryonic lethality in the embryos (Li *et al.*, 1992). Trasler *et al.* (1996) showed that $Dnmt^{n}/Dnmt^{n}$ mice failed to develop beyond the 25-somite stage. The $Dnmt^{n}/Dnmt^{n}$ 9.5dpc embryos showed evidence of developmental delay and neural tube defects (Trasler *et al.*, 1996). Studies performed by Okano *et al.* (1999) involving Dnmt3a and Dnmt3b-deficient mice, determined the importance of these Dnmts in development. Dnmt3a-deficient mice developed to term, but later became runted and died at about four weeks of age, while *Dnmt3b*$^{-/-}$ embryos did not develop to term and showed multiple developmental abnormalities, including growth impairments and neural tube defects. Dnmt3a and Dnmt3b double mutant mice displayed abnormal morphology and died shortly after gastrulation proving that both Dnmt3a and Dnmt3b are essential for development (Okano *et al.*, 1999).

In contrast, Dnmt2 showed weak DNA methyltransferase activity *in vitro.* A targeted deletion study of the *Dnmt2* gene in embryonic stem cells caused no detectable effect on global DNA methylation suggesting that the enzyme is not essential for global *de novo* or maintenance DNA methylation (Okano *et al.*, 1998).

From these studies it can be seen that these enzymes are crucial for the generation and maintenance of methylation profiles, and subsequent gene expression programs in cells. Furthermore, they allow for the transmittance of methylation patterns to progeny cells. This epigenetic inheritance allows for both tissue and temporal-specific gene expression required for cellular differentiation. Methylation plays a major role in several other important genetic phenomena, such as X-Chromosome inactivation and genomic imprinting.

1.4.2 X-Chromosome Inactivation

X-Chromosome inactivation (XCI), as hypothesised by Mary Lyon in 1961, is the mechanism in mammals responsible for compensating for X chromosome differences between males (XY) and females (XX). Females contain twice the number of X chromosomes and subsequently have the potential for twice the amount of gene product as males. Through the process of XCI the additional X chromosome becomes heterochromatic and the genes largely silent. This in effect provides equal dosage levels for most X chromosome-linked genes between the two sexes, with some notable exceptions.

The XCI process involves an initial recognition event of the two X chromosomes, the paternal or maternal X chromosome is then chosen at random to be inactivated with a probability of approximately 0.5. As the inactivation event is random some cells may inactivate the maternally derived X-chromosome, whilst others inactivate the paternally derived X-chromosome. From this stage on only one X-chromosome is active in directing protein synthesis in each cell and this is the only active X-chromosome in the entire clone of cells produced from this precursor cell. Thus, the normal human female may be considered to be a mosaic.

On the mammalian X chromosome lies a *cis*-acting region termed the 'X-inactivation centre' (Xic), which controls the inactivation process. Within this region lie several regulatory elements and four genes. One such gene, *Xist* (X-inactive specific transcript) transcribes a non-coding RNA that recruits methyl groups necessary for DNA methylation and induces histone modifications. These epigenetic marks alter the chromatin conformation to a highly heterochromatic state, now known as a Barr body, and subsequently silence the X-linked genes on one of the X chromosomes [as reviewed by (Biliya & Bulla, 2010)].

The expression of *Xist* is highly complex and requires the presence of an additional RNA, *Tsix*. *Tsix,* transcribed in the antisense orientation relative to *Xist,* is required to initiate inactivation as well as to ensure that the inactivation process occurs at the right time during

development. When *Tsix* is expressed within embryonic stem cells the *Xist* promoter acquires a heterochromatin-like organisation. As these cells begin to differentiate Tsix levels gradually decline, resulting in the activation of the *Xist* promoters and subsequent increase in *X*ist expression required for X-chromosome inactivation (Navarro *et al.*, 2006).

1.4.3 Genomic Imprinting

Genomic imprinting refers to genes that are reciprocally expressed, where one copy is expressed and the other suppressed, based on the parental origins of the gene (Vu *et al.*, 2000). The phenomenon of genomic imprinting in mammals was discovered in the mid-1980s following several studies in mice (McGrath & Solter, 1984; Surani *et al.*, 1984).

Surani *et al.* (1984) formed diploid parthenogenetic embryos (exclusively maternal genetic material) and noted that these embryos failed to develop to term despite possessing the correct number of chromosomes. Additional experiments by McGrath and Solter (1984) made use of pronucleus exchange where fertilized zygotes contained either two male (androgenones) or two female (gynogenones) pronuclei. This experiment satisfied any possible non-chromosome contributions from the sperm and it was therefore expected that both androgenones and gynogenones would develop normally. This was not the case and in both instances the embryos failed to develop beyond mid-gestation (McGrath & Solter, 1984). Both studies suggested that diploidy alone was not sufficient for normal embryonic growth and development. The maternal and paternal genome contributions are therefore functionally non-equivalent and possession of both parental genomes are essential for development. McGrath and Solter proposed that specific genes are inherited in such a way that one form is functional, while the other non-functional and that this is conditioned during gametogenesis. Surani and colleagues called this “imprinting”, which has remained the term used to refer to the process in which two parental alleles are functionally different (Surani *et al.*, 1984).

This parent-of-origin functionality results in either the paternal or maternal copy of the gene being expressed while the other is suppressed (Spencer *et al.*, 1999). Maternally imprinted genes involve the maternal allele of the gene being transcriptionally silenced, whilst the paternal copy is expressed. The opposite is true for paternally imprinted genes where the maternal copy of the gene is active and the paternal allele is silenced [as reviewed by (Swales & Spears, 2005)]. These parental-specific imprints are generally maintained in all somatic tissues but can occasionally be cell-type or tissue specific (Choufani *et al.*, 2011).

The regulation of this parental-specific differential expression occurs via DNA methylation within or around genes at differentially methylated regions (DMRs). Regulatory signals from these DMRs act in *cis* on surrounding imprinted genes and have 50% methylation as either the paternal or maternal allele may be hypermethylated (Choufani *et al.*, 2011). DMRs can be divided into two classes, namely primary and secondary DMRs. Primary DMRs, also known as imprinting control regions (ICRs), are those responsible for establishing an imprint within an imprinting domain. Secondary DMRs are imprinted via DNA methylation through signals associated with the established primary DMR. Within the genome imprinted genes often occur in clusters. One such cluster harbours the most studied imprinting domain of *Igf2/H19.*

1.4.3.1 H19 Imprinting Control Region

The Igf2 and *H19 genes* are vital for embryonic growth and development. *Igf2* is paternally expressed and encodes for insulin growth-like factor 2, a protein that promotes foetal and placental growth. *H19* is expressed from the maternal allele only and encodes a noncoding RNA which moderates growth in the foetus (Engel *et al.*, 2006).

Within mice, *Igf2* lies 80kb upstream from *H19* with a 2kb ICR located 4kb upstream of the transcription start site (TSS) of *H19* (Tremblay *et al.,* 1997). The ICR is responsible for establishing monoallelic expression of *H19* and *Igf2* via the preferential binding of the CCCTC (CTCF) binding factor. CTCF binds to the four highly conserved CTCF binding repetitive

elements within the ICR. The activity of the ICR is mediated by its methylation and the subsequent interactions with CTCF as depicted in **Figure 1.3**.

The maternal ICR is hypomethylated and allows for the binding of CTCF. This binding of CTCF blocks the interaction between the maternal *Igf2* promoter and the downstream enhancers thus silencing the maternal *Igf2* and allowing for maternal *H19* transcription. In contrast, on the paternal chromosome the ICR is methylated and thus prevents the binding of CTCF. This lack of association allows for the paternal *Igf2* promoter to interact with the downstream enhancers and directs post zygotic silencing of the *H19* promoter in *cis* (Phillips & Corces, 2009*;* Tremblay *et al.,* 1997). The *H19* promoter becomes hypermethylated and packaged into a closed chromatin structure (**Figure 1.3).**

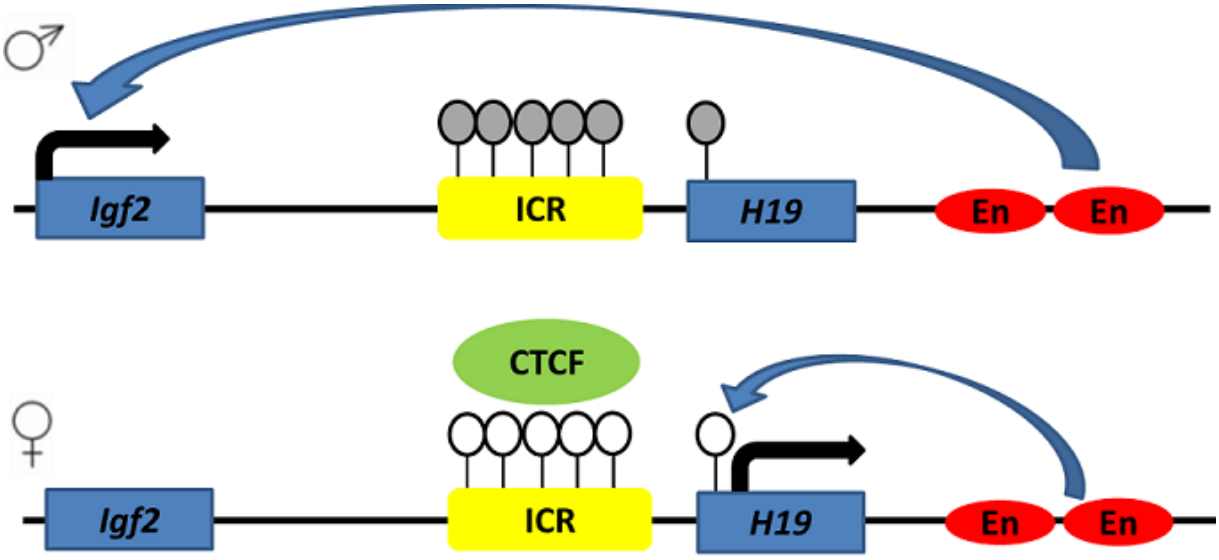


Figure 1.3: The *H19/Igf2* Imprinting Domain: Filled and unfilled lollipops represent methylated and unmethylated CpG dinucleotides, respectively. Thick black arrows represent transcription start sites of the *Igf2* and *H19* genes. When CTCF binds to the unmethylated maternal allele it blocks access of maternal *Igf2* to downstream enhancer sequences (En) allowing for monoallelic expression of maternal *H19.* Conversely, the hypermethylation on the paternal allele blocks CTCF binding, allowing the paternal *Igf2* access to the enhancers. Thus, paternal *Igf2* is expressed and maternal *Igf2* is silenced. Figure modified from Engel *et al.* (2006).

CTCF is an evolutionary conserved zinc finger phosphoprotein that binds to a number of target sites throughout the genome. Binding occurs through combinatorial use of its 11 zinc-

fingers to recognition sites spanning approximately 50 base pairs (bp). The importance of CTCF in genome regulation and development has been displayed in several mouse studies. CTCF knockouts exhibited embryonic lethality prior to implantation (Heath *et al.*, 2008; Splinter *et al.*, 2006). CTCF depletion in oocytes prior to fertilization significantly disrupted normal development of the blastocyst (Fedoriw *et al.*, 2004). Sequential deletion of the zinc fingers to create a panel of mutant CTCF proteins revealed that certain sets of zinc fingers are necessary for binding to one target sequence but are dispensable for another. CTCF has a variety of regulatory functions. Within the *Igf2/H19* domain CTCF is responsible for allele-specific insulation of the maternal *Igf2* promoter from downstream enhancers, initiation of *H19* transcription, maintenance of allele-specific imprints, and organization of chromatin modifications. CTCF's regulatory function at this locus is based on an insulator function principle. Its recognition sequence binds the insulator protein, which in turn isolates downstream enhancers from upstream promoters, creating a boundary element that results in transcriptional repression [as reviewed by (Phillips & Corces, 2009)].

Imprinted genes are highly susceptible to lethal mutations due to there being only one functional copy. Epimutations can change the methylation patterns in imprinted genes resulting in the conversion of a transcriptionally inactive allele to active or *vice versa* [as reviewed by (Biliya & Bulla, 2010)]. Furthermore, as imprinted genes often occur in domains, the disruption of a single gene may cause functional errors in a number of surrounding genes. Despite the sensitivity of these imprinting marks they are imperative to the normal development of all placental mammals.

1.4.4 Evolution of imprinting

To date it has been noted that all placental mammals, as opposed to egg laying mammals and birds, possess imprinted genes. There are several theories as to why this is so. One such theory, the 'Ovarian Time Bomb Hypothesis', suggests that imprinting acts as a defence mechanism against parthenogenesis, where spontaneous development of unfertilized oocytes can occur. Another theory associates imprints with protection against tumour

development within females by preventing excessive placental growth. However, the widely accepted hypothesis as to why imprints came about can be explained by the 'Conflict Hypothesis'. This hypothesis arose due to the role that many imprinted genes play in growth and development of the foetus and placenta as well as their directionality. It is often seen that paternally imprinted genes are involved in growth and nutrient uptake while maternally imprinted genes tend to curb foetal growth (Reik *et al.*, 2001a; Tycko & Morison, 2002).

The evolutionary principle supporting the conflict hypothesis is that each parent battles to ensure the transmission of their genetic legacy. Paternal interests seek to exert control over genes that maximise growth and survival of an individual offspring, while maternal interests try to moderate the growth of a single individual in order to distribute her resources equally for the benefit of current and future offspring (Moore & Haig, 1991). This mediation might be possible through selective methylation and demethylation of growth regulatory genes. In this regard many imprinted genes identified to date have growth regulatory functions. Evidence for this theory is strongly supported by mouse knockout models, which show dramatic over-growth and restricted-growth phenotypes (Reik *et al.*, 2001a; Tycko, 2006; Tycko & Morison, 2002). The imprinting patterns are acquired in a sex-specific manner in the developing gametes and persist throughout the developing embryo into adulthood (Reik & Walter, 2001).

1.4.5 Epigenetic Reprogramming

Epigenetic reprogramming, the erasure and re-establishment of chromatin modifications, such as, DNA methylation, was first identified by Monk *et al.* (1987). By the use of methylation-sensitive restriction enzymes the group noted that global methylation levels were much lower immediately after fertilization when compared to mature gametes and the early embryo suggesting that epigenetic reprogramming occurred in a step-wise fashion (Monk *et al.*, 1987). Epigenetic reprogramming is a bimodal event that is observed in the mammalian primordial germ cells and early embryo. The reprogramming of DNA methylation has a direct influence on genomic imprints, the regulation of pluripotency and stem cell

network, the erasure of epimutations and the transcriptional control of transposons (Reik *et al.*, 2001b; Santos & Dean, 2004).

1.4.5.1 Reprogramming during Gametogenesis

The first phase of reprogramming occurs in the primordial germ cells (PGCs), the embryonic precursor cells of the gametes (Molyneaux & Wylie, 2002). As the highly methylated PGCs enter and migrate down the genital ridge, rapid genome wide demethylation, of both imprinted and non-imprinted loci, is initiated. This demethylation process allows for the erasure of existing parental methylation patterns and is complete upon colonization of the gonad (Reik *et al.*, 2001b; Santos & Dean, 2004). In the mouse embryo, this demethylation is completed by embryonic day (ED) 13 to 14 in both female and male germ cells. Once the genomes of the male and female PGCs have been demethylated, the cells enter a mitotic (male) and meiotic (female) arrest, respectively (Reik *et al.*, 2001b). Remethylation occurs during PGC sex-specific imprintation. The exact timing of *de novo* methylation has not been firmly established but in the mouse it appears to occur earlier in the male germline, at the prospermatogonia stage, *ED15-ED16* and onwards. Remethylation in the female line takes place postnatally concurrent with oocyte growth (**Figure 1.4**) (Reik *et al.*, 2001b). Germline epigenetic reprogramming allows not only for the resetting of parental imprints in successive generations but also prevents the propagation of epimutations (Reik *et al.*, 2001b).

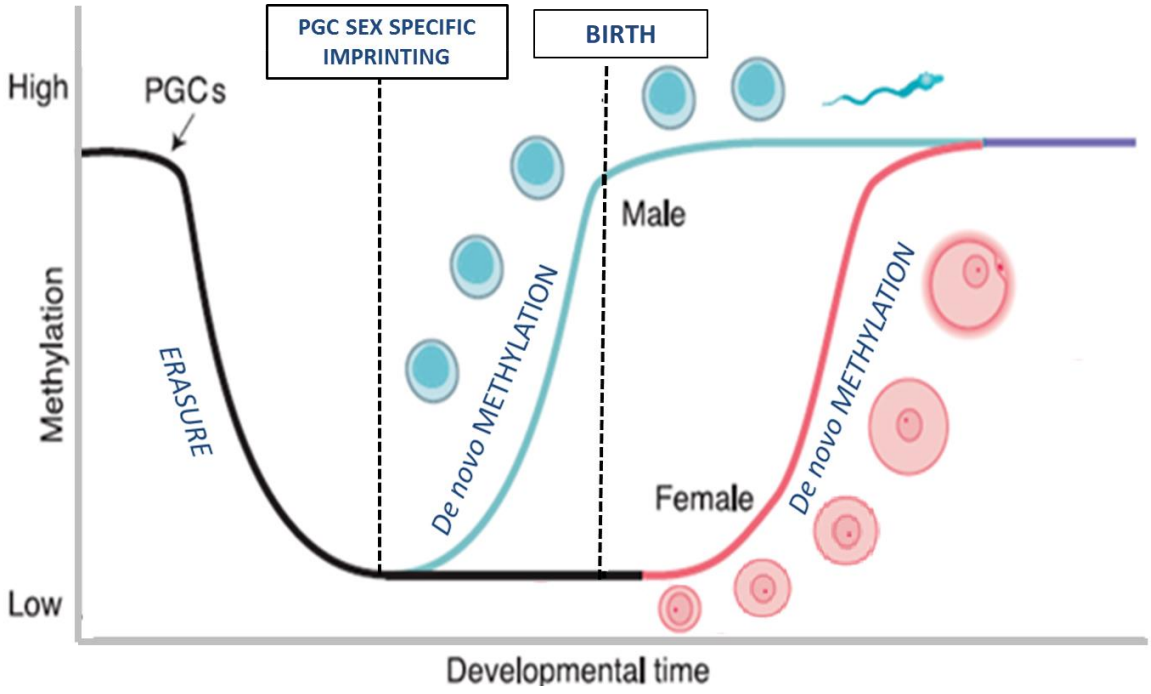


Figure 1.4: **Epigenetic Reprogramming Events in the Germline.** Primordial germ cells (PGCs) in the mouse undergo genome wide demethylaton where all methylation marks are erased. Following which PGC sex-specific remethylation is initiated in the germ cells. In the prospermatogonia of the males remethylation begins prior to birth, whilst in females remethylation occurs postnatally concurrently with growth of the oocytes. Adapted from Reik *et al.* (2001b).

1.4.5.2 Reprogramming in the Early Embryo

The second phase of methylation reprogramming occurs post-fertilisation in the preimplantation embryo between fertilization and the formation of the blastocyst. Reprogramming in the early embryo occurs in both an active and a passive manner. Upon fertilization there is a remodelling of the sperm chromatin involving the removal of protamines and their replacement with acetylated, maternally derived histones followed by active genome wide demethylation, which is completed before DNA replication commences. Thereafter, the maternal genome undergoes a step-wise decline in methylation until the morula stage. This decline occurs as a result of the absence of the primary maintenance DNA methyltransferase, DNMT1, during DNA methylation and is termed passive DNA methylation (Bestor, 2000). Several sequences, including the DMRs of imprinted genes, are protected from this demethylation (Reik *et al.*, 2001b; Santos & Dean, 2004).

Embryonic DNA methylation patterns are then established through lineage-specific *de novo* methylation that begins in the inner cell mass of the blastocyst. This remethylation is initiated at the fifth cell cycle and coincides with the first differentiative event. The methylation levels increase rapidly in the primitive ectoderm of the inner cell mass (ICM) giving rise to all tissues of the adult. Methylation is either inhibited or not maintained in the extra embryonic tissues, which are derived from the trophectoderm and give rise to the placenta (Santos *et al.*, 2002). Among the embryonic tissues that derive from the ICM there are the highly methylated PGCs. The migration of these PGCs into the germinal ridge where they will develop into mature gametes completes the cycle of epigenetic reprogramming **(Figure 1.5)**.

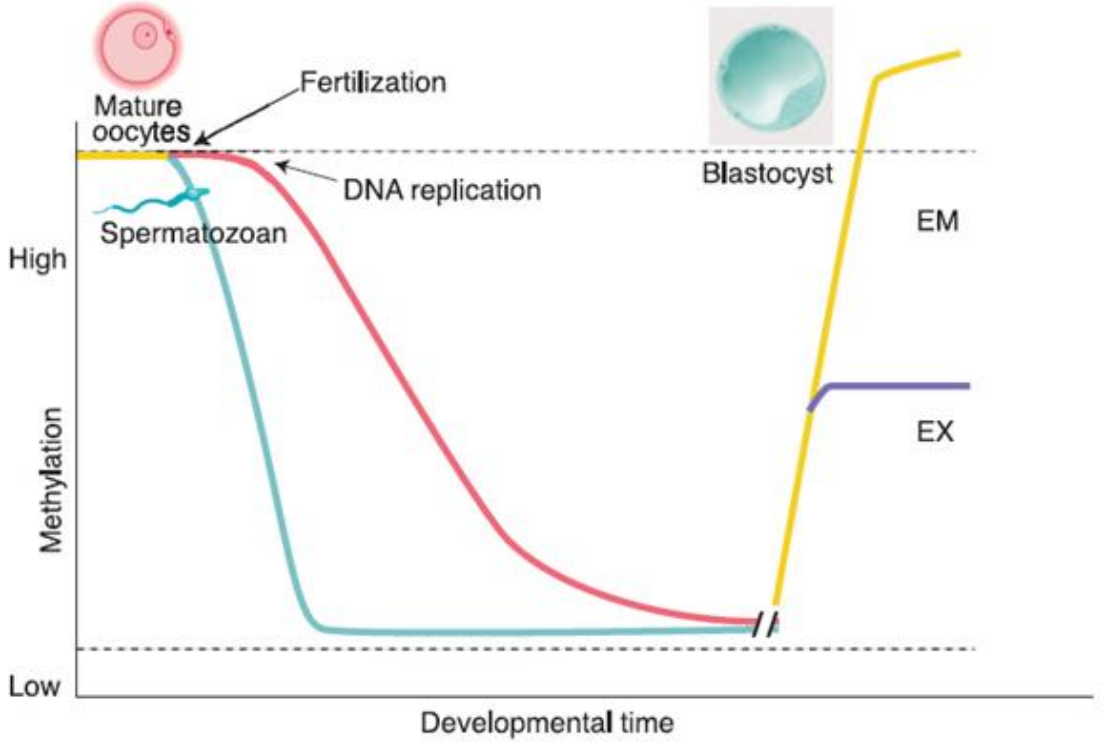


Figure 1.5: **Epigenetic Reprogramming Events in the Preimplantation Embryo**. The paternal genome (blue) is demethylated by an active mechanism immediately after fertilization. The maternal genome (red) is demethylated by a passive mechanism, dependent on DNA replication. Both are remethylated around the time of implantation to different extents in embryonic (EM) and extraembryonic (EX) lineages. Methylated imprinted genes and some repeat sequences (dashed line) do not become demethylated. Unmethylated imprinted genes (dashed line) do not become methylated (Reik *et al.*, 2001b).

The tightly controlled timing of reprogramming within the gametes and early embryo makes this system vulnerable to interference from environmental exposures and may lead to disease (Murphy & Jirtle, 2003). Furthermore, epigenetic marks are not always completely cleared between generations and subsequently the irregular epigenetic patterns may lead to disease in subsequent generations.

1.4.6 Epigenetics & Disease

Epigenetics has played an important role in the development of several disease states, especially in those of cancer and imprinting disorders. The most-characterised epigenetic effect associated with cancerous cells is the hypermethylation of promoter regions. In normal cells the CpG islands located within the promoters of most genes are generally unmethylated. However, in cancerous cells the CpG islands within promoter regions become methylated and subsequently silence the genes disrupting normal cell cycle and function (Baylin *et al.*, 2000).

Due to the monoallelic nature of imprinted genes, DNA methylation changes within these genes and their control regions can lead to disease. Such diseases include Prader-Willi Syndrome (PWS), Angelman Syndrome (AS), Silver-Russell Syndrome, Beckwith-Wiedemann Syndrome, and Albright hereditary osteodystrophy. PWS and AS are two disorders associated with the same imprinting region on Chromosome 15. The characteristic features of those affected by PWS include obesity, short stature, hypogonadism, and behavioural difficulties. AS is associated with ataxia, inappropriate laughter, and severe mental retardation. Both disorders have been linked to aberrations at the 15q11-q13 region. This region contains a large cluster of imprinted genes, as well as a non-imprinted domain. Several of the maternally imprinted (paternally expressed) genes, such as *SNURF-SNRPN*, are thought to be associated with PWS, whereas AS is caused by the lack of expression from the maternally expressed gene *UBE3A*. Paternal deletion of the 15q11-q13 region and maternal disomy 15 results in a lack of expression of the maternally imprinted (paternally expressed) genes, subsequently causing PWS. AS results from the maternal deletion of the 15q11-q13 region

and paternal disomy 15 due to the lack of expression of the maternally expressed *UBE3A* [as reviewed by (Butler, 2009)].

Several decades of epidemiological research has highlighted the importance of epigenetics and environment in disease aetiology as more and more studies reveal genetics alone is not responsible for disease risk. Genome-Wide Association Studies (GWAS) investigate the associations between disease and single nucleotide polymorphisms (SNPs). Despite GWAS' success in discovering many novel disease variants, there has been some disappointment as less than 20% of the heritability of many conditions has been accounted for. This 'missing heritability' may be in part due to epigenetic inheritance. This sensitivity of the epigenome during reprogramming and the plasticity of the epigenetic factors make them particularly vulnerable to environmental exposures and dysregulation. They react to cues from the external environment and develop into a phenotype that may be propagated during cell division resulting in the maintenance of this acquired phenotype (Jirtle & Skinner, 2007).

1.4.7 Foetal Alcohol Exposure and Epigenetics

Epigenetics provides a plausible mechanism of alcohol teratogenesis due to: 1) the large heterogeneity of symptoms; 2) the effects of alcohol on the folate pathway; and 3) the timing of epigenetic reprogramming providing a sensitive period to environmental exposures. Several key studies involving gestational alcohol exposure shows the effects of alcohol on the epigenome of the developing foetus.

Garro et *al.* (1991) evaluated the effects of acute ethanol administration on pregnant mice. Pregnant mice were exposed to alcohol from the ninth to the eleventh day of pregnancy. DNA harvested from alcohol exposed foetuses displayed global hypomethylation. Furthermore foetuses of ethanol-fed dams had significantly reduced methylase activity suggesting lower levels of DNA methyltransferase activity (Garro *et al.*, 1991).

A^{vy} is a dominant mutation of the murine *Agouti* (*A*) locus caused by the insertion of an intracisternal A-particle (IAP) retrotransposon upstream of the *Agouti* gene. A^{vy} mice, despite being genetically identical, display a variety of coat colours, ranging from yellow to mottled (yellow and brown patches) to pseudoagouti (brown). If the agouti protein is overexpressed, in addition to a yellow coat colour, there are other phenotypic consequences, including glucose intolerance, obesity and increased susceptibility to tumour formation (Wolff *et al.*, 1978). A^{vy} expression is strongly correlated to the DNA methylation profile of the long terminal repeat (LTR) promoter located at the 3' end of the inserted IAP. Hypomethylation of the LTR is associated with constitutive ectopic *Agouti* expression and a yellow coat, while hypermethylation correlates with promoter silencing and a pseudoagouti coat (Dolinoy *et al.*, 2010). Kaminen-Ahola *et al.* (2010) investigated the effect of gestational ethanol exposure in A^{vy} heterozygote mice. They noted that gestational ethanol exposure increased the proportion of pseudoagouti coloured offspring. The change in offspring coat-colour proportion was linked to transcriptional silencing, which in turn correlated with hypermethylation of the A^{vy} locus. The study provided evidence that prenatal ethanol exposure altered the foetal epigenotype, which subsequently affected adult phenotype. Furthermore, microarray expression data and the occurrence of FAS-like symptoms, such as growth restriction and certain craniofacial dysmorpholgies, in wild-type siblings (to account for the pleiotropic nature of A^{vy}) highlighted other epigenetic targets of ethanol exposure (Kaminen-Ahola *et al.*, 2010).

Liu *et al.* (2009) investigated the effect of alcohol exposure on DNA methylation and gene expression. Whole-embryo cultures were exposed to alcohol at early embryonic neurulation. Methylated DNA immunoprecipitation (MeDIP) and microarray results showed altered DNA methylation profiles and associated changes in gene expression. Alcohol-induced DNA methylation changes were noted in several imprinted genes, such as *H19, Ube3a* and *Igf2r,* as well as significant methylation alterations at an additional 84 genes ($p < 0.01$) (Liu *et al.*, 2009).

More recently Stouder *et al.* (2011) evaluated the effect of pre-natal alcohol exposure on DNA methylation levels of five imprinted genes in male mice. Results indicated a decrease in *H19* ICR methylation in both F_1 sperm and F_2 whole brains. Of the five imprinted genes investigated, only *H19* methylation was affected highlighting that *H19* is a specific target for the effects of alcohol exposure during pregnancy. The altered sperm methylation profiles of the F_1 males may have been passed on to the F_2 generation and be responsible for altered brain methylation. The propagation of the altered methylation profile indicates inheritance of the epigenetic mark across several generations (Stouder *et al.*, 2011).

1.5 Epigenetic Inheritance

Epimutations may be mitotically inherited and potentially cause life-long disease within an individual. Epigenetic inheritance has been well established in plants and other non-vertebrates. One such example involves the naturally occurring mutant of *Linaria vulgaris*. The mutant displays a change in flower symmetry from bilateral to radial. This change in phenotype was associated with altered DNA methylation levels rather than DNA sequence. Hypermethylation and hypomethylation of the *Lcyc* promoter resulted in a transcriptionally silent or active domain, respectively. Epigenetic inheritance and subsequent germline transmission of the methylated allele of *Lcyc* occurred as a proportion of the F_2 plants displayed radial phenotypes (Cubas *et al.*, 1999).

Axin-fused (*Axin*^{Fu}) is a dominant gain-of-function allele in mice that provides one of the earliest pieces of evidence of mammalian germline epigenetic inheritance. The *Axin*^{Fu} phenotype manifests as a kink in the tail, and is variably expressed among *Axin*^{Fu} individuals. The mutation is similar to that of A^{vy} and is dependent on the insertion of an IAP retrotransposon into the gene, in this case at intron 6. The tail-kink phenotype is dependent on the methylation state of the LTR of the IAP retrotransposon – kinked tailed individuals displayed hypomethylation whilst normal tailed mice were hypermethylated at the locus. Breeding experiments revealed transmission of the epimutation with the majority of

offspring matching the epigenetic signature of the parent. Transmission occurs through both parental lineages (Rakyan *et al.*, 2003)

More recently Cropley *et al.* (2006) investigated whether methyl donor supplementation during midgestation could change the methylation level of the A^{vy} locus in the germline, and whether this induced epigenetic alteration could be retained in subsequent generations. The epigenetic state of the murine A^{vy} allele is highly variable. A spectrum of coat colours - yellow, mottled (yellow and brown patches) and pseudoagouti (brown) - is associated with the methylation level of an IAP retrotransposon found upstream of the *agouti* gene. Exposure shifted A^{vy} phenotypes to pseudoagouti rather than yellow in the F_1 exposed and the F_2 offspring indicating that methyl donors changed the epigenetic state of the A^{vy} allele in the germ line despite epigenetic reprogramming (Cropley *et al.*, 2006).

A reprogramming event in the germline has the potential to be inherited across successive generations. However, according to Skinner (2008) epigenetic inheritance may be multigenerational or transgenerational depending on exposure.

1.6 Transgenerational Inheritance

Transmission of an epimutation may be multigenerational or transgenerational depending on exposure (Skinner, 2008). Multigenerational inheritance involves a number of generations being affected due to direct exposure. In contrast, transgenerational inheritance requires a reprogramming event in the germline that is transmitted without direct exposure. More specifically, if post-natal or adult exposure to an environmental toxicant has occurred, the individual themselves and their gametes, those that will give rise to F_1, are directly exposed and if affected display multigenerational inheritance. In this situation if transgenerational inheritance is to be declared the F_2 generation is required to be affected.

However, if a gestating mother (F_0) is exposed to a teratogen there are different implications. Her F_1 offspring and their germ cells, the gametes that will form the F_2, are directly exposed.

Subsequently non-direct exposure of the given teratogen will only occur at the F_3 generation and thus transgenerational inheritance of an altered phenotype can only be declared if the F_3 generation display the phenotype (Skinner, 2008) **(Figure 1.6)**.

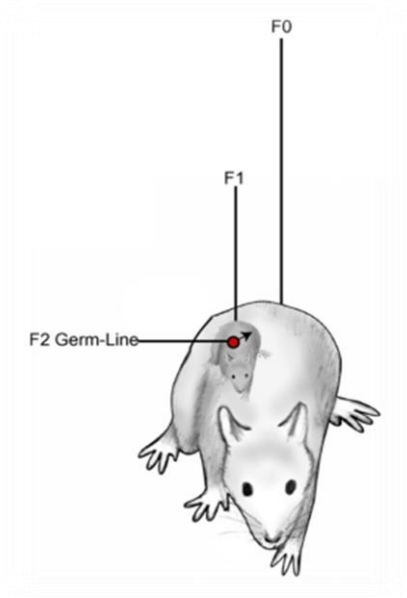


Figure 1.6: Transgenerational Inheritance vs. Multigenerational Inheritance in a Gestating Mother. A gestating mother's exposure to a teratogen results in direct exposure to the F_0, F_1 and germ cells that will give rise to the F_2 generation. Therefore, the inheritance pattern displayed within these generations is multigenerational. A phenotype in the F_3 generation is required to determine a transgenerational phenotype, as a transgenerational phenotype requires non-direct exposure and a reprogramming event in the germline. Image obtained from Skinner (2008).

It was previously believed that transgenerational epigenetic inheritance would be unlikely due to reprogramming events in the germline, however, there is increasing evidence that transgenerational epigenetic inheritance does occur. Evidence of such transgenerational inheritance following an environmental exposure was displayed by Kujjo *et al.* (2011). Here the effects of the chemotherapeutic drug doxorubicin on reproduction and behaviour were investigated across six generations in mice. A single interperitonial injection to F_0 dams induced delivery complications, despair-like behaviour and damage to a number of organs. Transgenerational effects occurred in both male and female lineages, males displayed low sperm concentrations and females had altered oocyte maturation. Females of the F_4 and F_6 generations displayed the most significant effects of neonatal death, chromosomal abnormalities and physical malformations (Kujjo *et al.*, 2011).

Another study by Anway *et al.* (2005) investigated the effect of high-dose vinclozolin (endocrine disruptor) exposure during embryo sex determination in gestating rats. Results indicated a high penetrance (>90%) transgenerational (F_1-F_4) male-specific effect in the offspring. Altered DNA methylation profiles of the male germline, which resulting from maternal exposure, were responsible for increased spermatogenic cell apoptosis, reduced sperm count, decreased sperm motility and increased incidences of infertility (Anway *et al.*, 2005). The transgenerational effects of vinclozolin were validated in mice. The DNA methylation status of five imprinted genes in various mouse tissues was assessed. Exposure affected spermatogenesis and methylation patterns of maternally and paternally imprinted genes in the offspring. The effect was transgenerational, however, the effect gradually disappeared from F_1 to F_3 (Stouder & Paoloni-Giacobino, 2010). In addition Stouder and Paoloni-Giacobino (2010) investigated the effect of an additional endocrine disruptor, methoxychlor. A decrease in methylation at *Meg3* and *H19* and a methylation increase at *Mest*, *Snrpn* and *Peg3* was found in the sperm of the F_1 offspring. Like vinclozolin, the effect was exclusively in the sperm and appeared to be transgenerational (F_1-F_3), however, the effect decreased as it moved through the generations (Stouder & Paoloni-Giacobino, 2010).

The effect of ancestral liver damage and the hepatic wound healing in response to carbon tetrachloride (CCl_4) exposure in exposed F_2 male rats was investigated. An adaptive effect was present after exposure at a single ancestral generation (F_1) but it was more pronounced following successive generations (F_0 and F_1). The mechanisms underlying this adaption included lower levels of myofibroblasts, higher hepatic expression of peroxisome proliferator-activated receptor γ (PPAR-γ) and decreased expression of the growth factor β (TGF-β1). Decreased myofibroblasts were present in the F_2 male rat following all exposure models whereas gene expression profile alterations were present only when exposure occurred at each generation suggesting both an intergenerational and transgenerational inheritance pattern of the adaptive response. Analysis revealed epigenetic effects were responsible for the altered expression levels with *TGF-β1* displaying hypermethylation and

PPAR-γ displaying hypomethylation in comparison to controls. Furthermore, the sperm of affected rats had a higher number of the histone variant H2A:Z (Zeybel *et al.*, 2012)

The wide range of morphological and physiological abnormalities associated with *in utero* alcohol exposure creates the need to investigate the mechanism of alcohol teratogenesis at a molecular level. The identification of transgenerational disease phenotypes and the effect of ethanol exposure on the developing embryo's epigenome highlight the aim of this study to investigate the transgenerational inheritance of DNA methylation alterations at the *H19* ICR following chronic maternal ethanol exposure in mice.

1.7 Study Aim and Objectives

The purpose of this study was to determine the transgenerational inheritance of DNA methylation alterations at the *H19* imprinting control region following chronic maternal ethanol exposure during embryonic development in mice. The research objectives were thus;

To develop a transgenerational mouse model

To determine the phenotype of the offspring at generations F_1-F_3

To determine the DNA methylation status of the *H19* ICR in all generations (F_0-F_3)

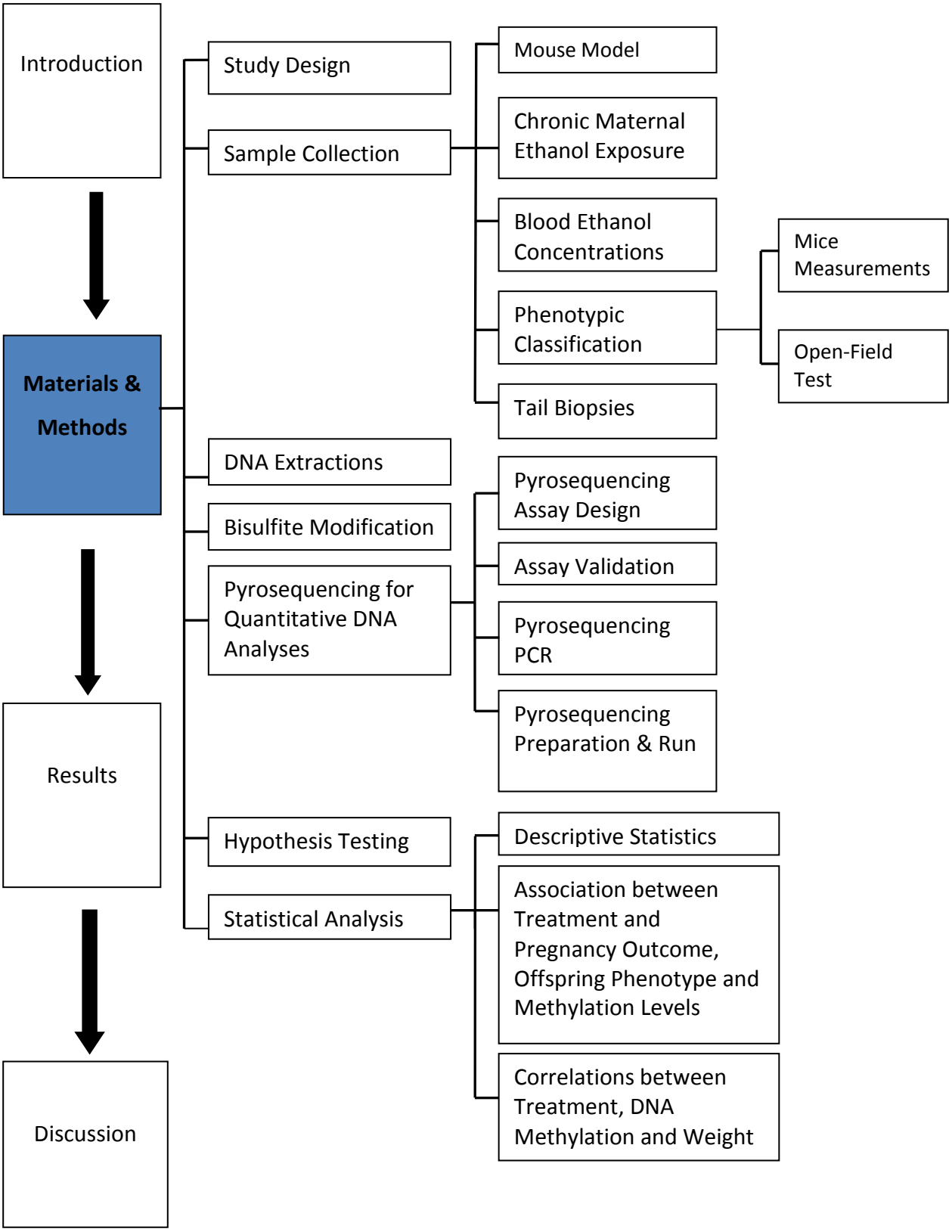
To establish the parental lineage of altered phenotypes in the F_3 generation

It was hypothesised that chronic alcohol exposure *in utero* during the period of embryo sex determination would result in DNA methylation alterations within the germ line. These alterations would therefore occur within the F_2 generation and potentially be inherited transgenerationally to the F_3 generation. Furthermore, it was predicted that these altered DNA methylation levels would result in the offspring of subsequent generations displaying perturbations in various phenotypic measures.

Chapter 2

Materials & Methods

2.0 Materials & Methods



2.1 Chapter Outline

The material and methods chapter describes the study design, the sample collection, the instrumentation and techniques used, the outcome measures, as well as the statistical techniques used to analyse the data.

2.2 Study Design

This study is a prospective, experimental study with both longitudinal and transversal aspects. The study plan is summarized in **Figure 2.1**.

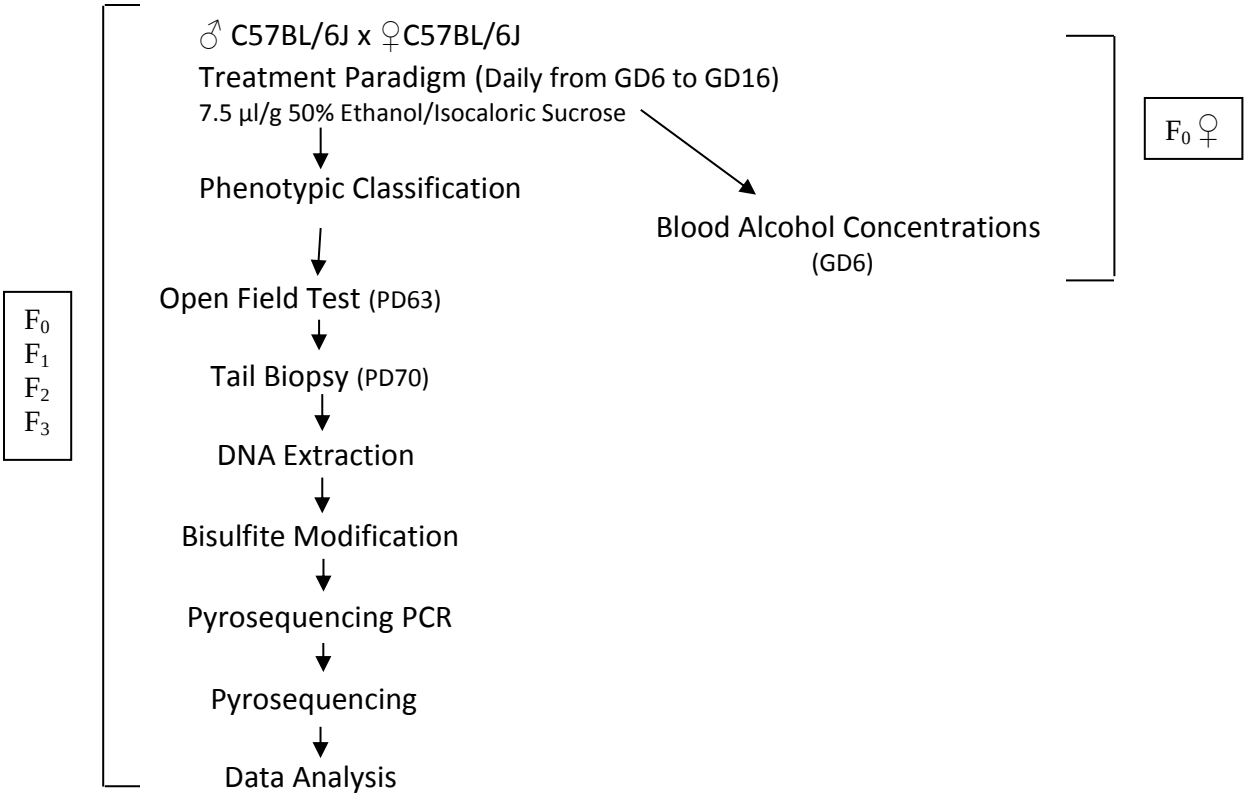


Figure 2.1 Flow Diagram of Methodology. The C57BL/6J mouse strain was used for all mating procedures. Daily from gestational day 6 (GD6) to gestational day 16 (GD16) the F_0 generation pregnant female mice received a treatment paradigm of either ethanol or calorically equivalent sucrose solution. The F_1, F_2, F_3 gestating females did not receive treatment. On GD6 blood alcohol concentrations were determined in the F_0 females. All generations (F_0-F_3) underwent phenotypic classification, the open-field test at postnatal day 63 (PD63), followed by tail biopsy at PD70. Following collection of tissue sample, DNA extraction, bisulfite modification, Pyrosequencing PCR, Pyrosequencing and data analysis were performed; see text below for details.

2.3 Sample Collection

2.3.1 Mouse Model

A group of twenty nulliparous female and eleven male C57BL/6J mice aged four to five weeks were selected for the F_0 generation. The C57BL/6J mice strain was selected as several studies have shown the strain to be susceptible to the teratogenic effects of alcohol (Boehm *et al.*, 1997; Webster *et al.*, 1983). Mice were bred at the National Health Laboratory Service (Sandringham, South Africa). The weights of the male and female mice ranged from 9.5g to 15.0g and 9.5g to 14.0g, respectively. All mice were tagged with steel ear tags with unique identification numbers (National Band and Tag Company, Newport, KY). At the sexually mature age of twelve weeks mice were housed into male and female pairs. The mice were mated for 24 hours with the presence of a vaginal plug being indicative of copulation; this was designated as gestational day one (GD1). Presumptive pregnant females were then individually housed up until parturition. Beginning on GD18, females were checked twice daily for deliveries. Parturition dates were noted and the day of birth referred to as postnatal day one (PD1). All pups were kept to allow for the mouse's accurate sexing as well as to ensure that minimum numbers required for the study were maintained. Litters remained group-housed up until weaning at PD25 and were then re-housed in groups of five to six according to sex at PD30.

Matings for subsequent generations occurred by mating F_1 ethanol-exposed (EtOH) males (i.e. male offspring from F_0 females exposed to ethanol) with F_1 EtOH females (female offspring from F_0 females exposed to ethanol) to give rise to the F_2 EtOH generation. The F_2 EtOH males were then bred with the F_2 EtOH females to generate the F_3 EtOH generation. Mice from the sucrose-exposed group (i.e. offspring from F_0 females exposed to sucrose) are referred to as the 'control' group and were bred in the same way for generations F_1 to F_3 ***(See Appendix A)***. Outcross experiments were performed to determine through which lineage, maternal, paternal or both, the transgenerational phenotype was transmitted ***(See Appendix B)***. Three EtoH F_2 generation males were crossed with three control F_2 females. In

conjunction, the reverse outcross also occurred where three EtOH F_2 females were mated with three control F_2 males. All matings occurred in such a way as to minimise inbreeding **(*See Appendix A and Appendix B*)**. Two female and two male offspring from each cross were selected at random, via the computer generated random number method, for phenotypic and methylation analysis when possible **(*See Appendix A and Appendix B*)**. The study design resulted in ten groups: the two treatment conditions (i.e. EtOH and Control) over four generations (F_0, F_1, F_2 and F_3) plus the offspring of the two outcross experiments (F_2 EtOH Female x F_2 Control Male and F_2 EtOH Male x F_2 Control Female).

The power to determine an absolute difference in methylation of at least 5% between the two treatment groups (n = 40 per group) is 99% assuming the standard deviation in methylation is 5% and an alpha value of 0.05 is applied. This power decreases to 60% with a more extreme estimate of methylation variance at 10% (G*Power 3.1.3 ®).

Mice were housed at the Central Animal Service (CAS), Medical School, University of the Witwatersrand, throughout the duration of the experiment. They were housed under both temperature and humidity controlled conditions. All health and dietary needs were attended to by the CAS veterinary and support staff. Food and water were given *ad lib* to the mice.

Ethics approval for the use of a mouse model was obtained from the Animal Ethics Screening Committee, University of the Witwatersrand – Clearance Certificate Number: 2011/02/04 ***(See Appendix C)***.

2.3.2 Chronic Maternal Ethanol Exposure

Mice were randomly assigned, by the use of a computer generated random number method, to one of two treatment groups, F_0 ethanol-exposed (EtOH) or F_0 sucrose-exposed (control). The females of the EtOH group (n = 10) and the control group (n = 9) were mated to a common group of male mice (n = 11). This was done to minimise genetic diversity in the offspring between the two groups. Originally ten females were assigned to each group but

one female assigned to the sucrose group did not survive to the time of matings and subsequent exposure. The EtOH treatment group received a single dose consisting of 7.5µl/g of 50% ethanol (3g/kg) per day for ten days from GD6-GD16 (**Figure 2.1**). This treatment paradigm, simulating chronic *in utero* alcohol exposure, was modelled on that of Parnell *et al*. (Parnell *et al.*, 2009). The ten day period was chosen as it encompasses the period of embryo sex-determination. At this time the epigenome is at its most sensitive as epigenetic reprogramming of the primordial gametes begin, allowing for the establishment of genetic imprints in the germline in a parent-of-origin manner, i.e. male embryos will begin developing male imprinted germ cells and female embryos will begin developing female imprinted germ cells (Reik *et al.*, 2001b). The control group received an isocaloric sucrose solution over the same period (**Figure 2.1**). Both the treatment and control groups received the alcohol/sucrose solution via intragastric intubation (oral gavage) with a blunt-ended stainless steel feeding needle and syringe. 96% (v/v) pharmaceutical grade ethanol was used and prepared as a 50% (w/v) solution mixed with distilled water and stored at room temperature ***(See Appendix D)***. The sucrose solution was prepared as a 0.704 g/ml solution and autoclaved before use ***(See Appendix D)*** (Knezovich & Ramsay, 2012). Generations F_1-F_3 in both treatment groups did not receive a treatment paradigm. However, offspring produced in later generations were still grouped as 'EtOH' or 'control'.

2.3.3 Blood Alcohol Concentrations

Blood alcohol concentration (BAC) is a critical factor in alcohol's effect on offspring (Pierce & West, 1986b). Ethanol readily crosses the placenta where both foetal blood and tissue have similar alcohol concentrations to those of the mother (Guerri & Sanchis, 1985). To assess the BACs of the F_0 exposed generation blood was collected and analysed from the F_0 EtOH (n = 5) and F_0 control (n = 6) gestating females (**Figure 2.1**). On GD6, thirty minutes post-gavage, blood was collected via the saphenous vein bleeding method. Collection occurred at this time based on Bielawski and Abel (2002) finding in rats, that peak BACs occurred at this time regardless of ethanol dose. The saphenous vein bleeding method involved the mouse being held head first in a restrainer so that only the rear legs and tail were free. Once this was done

the rear leg was stretched out to its natural position. Hair was removed by clippers and the area sterilised with alcohol. Petroleum jelly was applied to the area to allow for blood drop formation. An animal lancet punctured the saphenous vein and 50-80µl of blood was collected into heparinised capillary tubes (Fisher Scientific Ltd, UK) as drops formed from the vein. Bloods were placed into Micron serum filters (Merck Millipore, MA, USA) and underwent centrifugation at 10 000 rpm for 20 minutes to separate blood serum from the plasma. After separation, the serum was diluted at a dilution factor of 1:200. The blood alcohol content (mg/dL) of the samples was detected using the BioVision Ethanol Assay Kit (BioVision Research Products, CA, USA) ***(full protocol shown in Appendix E).***

2.3.4 Phenotypic Classification

2.3.4.1 Mice Measurements

Mice selected at each generation to be involved in either pregnancy analysis or phenotypic analyses (Figure 2.1) were chosen using a computer generated random number method. The outcomes measured at each pregnancy included: pregnancy outcome, the number of matings required for a successful pregnancy, the gestation length of the pregnancy, the litter size, the survival rate per litter and the percentage of male offspring.

Pregnancy outcome was recorded as 'not pregnant' when no parturition event took place, or 'successfully pregnant', when a birthing event had occurred. The number of matings required for a successful pregnancy was only recorded in generations F_1-F_2 Outcross. If necessary, mating pairs were crossed multiple times in order to generate a pregnancy. Successive matings occurred one week apart as, if a fertilization event had taken place, the second mating would not interfere with the first (CAS Veterinary Staff, Personal communication). Survival rate was calculated as the percentage of offspring who survived past PD7. The percentage of male offspring provided a tool to compare if there were sex ratio differences within the treatment groups. Of the pups that survived the number of males within each litter was recorded and converted to a percentage. Offspring weights were recorded every day from day seven up until day thirty and weighed thereafter every five days until matings

were initiated at twelve weeks. Weight was only recorded seven days after birth as females are known to cannibalise their new-borns, which can be exacerbated following human contact with the pups (Marques-de-Araújo & Cardosa, 1999). The mice were weighed on a calibrated Sartorius scale with 0.01g readability and a SD of 0.006.

2.3.4.2 Open-Field Test

Hall (1934), the original developer of the open field test, proposed that measuring aspects of rat behaviour in a contained arena would indicate the emotional reactivity of the tested individual. He suggested that faecal count was a correlation of emotional reactivity - more specifically the higher the number of faecal boli deposited during testing the more anxious the individual. A more modern application of the test is associated with the time spent in the centre of the testing area versus the periphery. Mice will seek protection in the peripheral regions rather than enter the relatively more vulnerable centre - mice that are less anxious will spend more time in the centre. Furthermore, locomotor activity, often measured as the total distance travelled, may also be established and compared between individuals (Van Meer & Raber, 2005).

In this study all mice underwent the open-field test at nine weeks (PD63) of age in order to determine general motor activity and anxiety (**Figure 2.1**). The methodology of the test required placing a single mouse in a large white perspex square area, 600mm x 600mm with a 200mm perimeter wall, and recording, by use of an overhead video camera, the mouse's movements and the time spent in the periphery versus the centre (**Figure 2.2**). The centre, termed the inner zone, was not physically demarcated but rather an area of 300 mm x 300 mm in the centre of the testing apparatus was determined by the software used. The periphery, or outer zone, was also demarcated by use of the ANYMaze ® software.

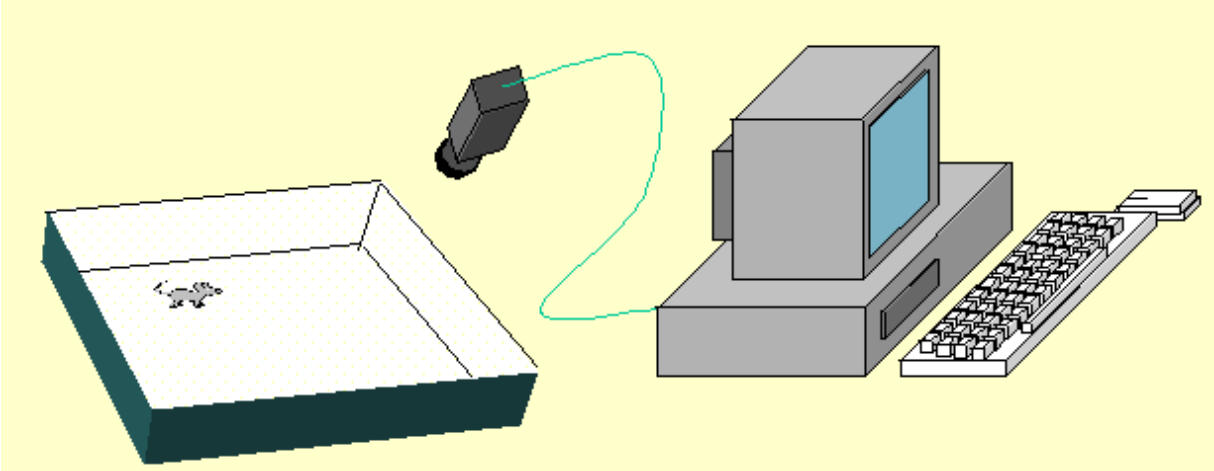


Figure 2.2 The Open-Field Test Setup. The open-field used in this study is a 600 x 600 mm square arena with walls 200 mm high. In the open-field test a mouse is placed in the centre of the arena and left to explore for 300 seconds, while being filmed from overhead. The camera records the mouse and monitors their movement. The dependent variables being recorded are those associated with total locomotor activity and anxiety. Obtained from http://www.phenotyping.com/mdigital.html

One week prior to open field testing all mice were placed in the testing room to allow for acclimation to the new surroundings. Subjects were tested during the light cycle, between 09h00 and 14h00 under normal room lighting conditions. Around the testing area poster boards were placed to shield the area from the outside environment and the experimenter so as to not disturb the mouse during testing.

At the start of each test a mouse was placed in the centre of the square and their movements recorded for 300 seconds. The software was set to automatically start recording once the mouse was within the camera's field of view. After each individual test the box was cleaned with 70% ethanol to eliminate the influence of odour cues from the previous animal on activity of the test animal. A 90 second waiting period before each test occurred to allow for the complete evaporation of the 70% ethanol between testing. The test was performed only once, as not to familiarise the mouse with the surroundings.

At the F_3 generation all conditions remained the same, however, the acclimation period was reduced from 7 days to 30 minutes. In addition, the testing room housed twenty rats. The

presence of rats may alter the mouse's performance due to the assumption that mice may find it stressful to live in proximity to rats, a potential predator (Karli, 1956).

The total distance travelled served as the performance measure for activity. The time spent in the inner zone as well as number of faecal boli and amount of urine produced during the test period provided the parameters for anxiety.

Audiovisual data were recorded by the use of the software package ANYmaze (Stoelting Co. IL, USA).

2.3.5 Mice Tail Biopsies

Tail samples were collected from all offspring at PD70 (**Figure 2.1**). Tail biopsies were used as the method is considered to be relatively non-invasive and does not affect the health of the mice and their future matings (Lander *et al.*, 1978). Using a sterile scalpel a 6mm fragment was cut from the tip of the tail. To avoid distress to the animal during tissue collection, the animal was anaesthetised via inhalation with isoflurane (8%). After the biopsy the tail tips were quaterised to avoid further bleeding. Some tail biopsies were unable to be obtained due to a shorter length of tail. If taken this would require more complex surgery as bone material would have to be removed (CAS Veterinary Staff, Personal communication). The tail biopsies were stored at -70˚C.

2.4 DNA Extraction

Frozen tail biopsies were thawed and DNA extracted using the DNeasy Blood and Tissue Preparation Kit (**Figure 2.1**) (QIAGEN, CA, USA). The protocol was based on that for 'Purification of Total DNA from Animal Tissues' ***(full protocol shown in Appendix F)***. The protocol was followed as per the manufacturer's instructions. The recommended starting sample tissue size of 6mm of adult mouse tail was used for each extraction. In addition the cells were lysed overnight as to ensure complete cell lysis. DNA concentration quantification was done via the use of the Nanodrop® ND-1000 Spectrophotometer. DNA yields ranged

from 32ng/µl to 90ng/µl for the F_0 generation, 18ng/µl to 100ng/µl for the F_1 generation, 12ng/µl to 116ng/µl for the F_2 generation and 30ng/µl to 103ng/µl for the F_3 generation. Collected DNA was eluted in 200µl AE Buffer and stored at 4˚C.

2.5 Bisulfite Modification

Bisulfite modification is used to detect and quantify the level of DNA methylation at CpG sites within the genome. The process involves the use of sodium bisulfite to preferentially deaminate non-methylated cytosines converting them to uracil. In contrast, methylated cytosines (5-methylcytosines) remain unchanged. Thus, the sequence of the treated DNA will differ from its original at the sites of unmethylated cytosines. Upon PCR amplification, uracil will pair with adenosine followed by complementation with thymine. In contrast, 5-methylcytosines will pair with guanine followed by complementation with cytosines. This allows for the differentiation between methylated and unmethylated cytosines (**Figure 2.3**) (Frommer *et al.*, 1992). Primers for downstream PCR applications are designed based on the chemically-modified sequence and assuming that non-CpG cytosines are rarely methylated.

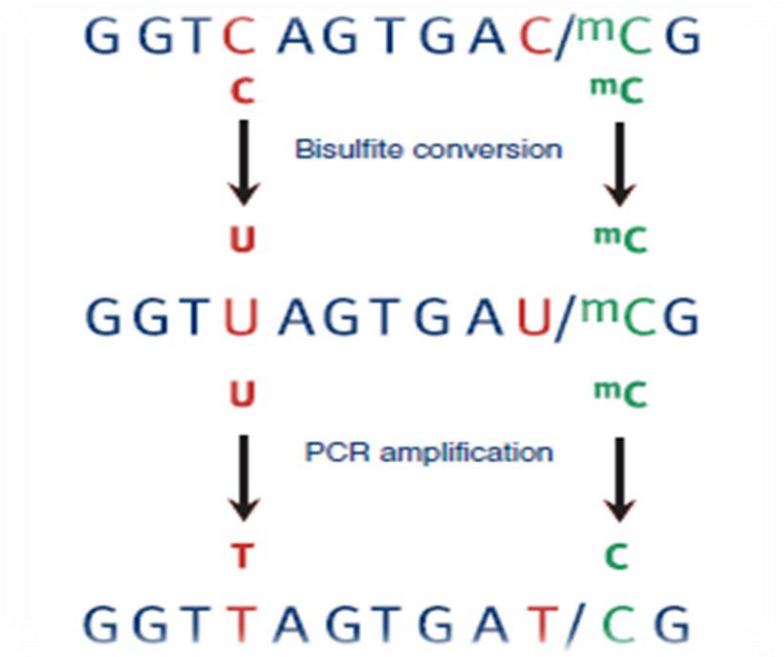


Figure 2.3: DNA Treatment with Sodium Bisulfite. Methylated cytosines (^{m}C) (green) remain as Cs, while unmethylated Cs (red) are converted to uracil (U) and subsequently to thymine (T) during PCR. Modified from England and Pettersson (2005).

Following DNA extraction 500ng of DNA was bisulfite modified (**Figure 2.1**) using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA). The protocol was carried out according to the manufacturer's specifications *(**full protocol shown in Appendix G**).* The procedure involved several key steps that included bisulfite conversion, washing, desulfonation and elution.

Exposing the DNA to sodium bisulfite in a PCR-like reaction allowed for the conversion of unmethylated cytosines to uracil. Converted single-stranded DNA was then bound to the membrane of the spin-column for purification through a wash step. Following purification desulfonation occurred. This step completed bisulfite conversion by removing the sulfonate group from uracil, which if not removed is inhibitory to downstream PCR reactions (Schumacher, 2009). A further washing step removed the desulfonation agent; which was followed by elution of the pure, converted DNA in 10µl elution buffer. Bisulfite modified DNA was stored at -20˚C.

2.6. Pyrosequencing for Quantitative DNA Methylation Analyses

Pyrosequencing allows for CpG site-specific quantitative analysis of DNA methylation based on sequencing-by-synthesis technology rather than chain termination sequencing with dideoxynucleotides. The technique is based on a light-emitting reaction technique that utilises single-stranded bisulfite modified DNA as templates to synthesise complementary strands (England & Pettersson., 2005). The process involves a sequencing primer, DNA template, four enzymes (polymerase, ATP-Sulfurlyase, luciferase and apyrase) and two substrates (adenosine 5' phosphosulfate and luciferin). An enzyme cascade is initiated with a nucleotide incorporation by polymerase, that in turn results in the release of inorganic pyrophosphate (PPi). The amount of PPi released is equimolar to that of the number of incorporated nucleotides. The released PPi is converted to ATP by ATP sulfurylase, which subsequently provides the energy to luciferase to oxidise luciferin to oxyluciferin, thus generating light. The light brightness is proportional to the number of nucleotides incorporated and as the type of nucleotide incorporated into the growing strand is known

the sequence can be determined. The light generated is sequentially displayed as peaks in a Pyrogram™. Apyrase degrades unincorporated dNTPs and stops the light-emitting reaction, and the dNTP incorporation cycle is continued (**Figure 2.4**) (Ronaghi, 2001).

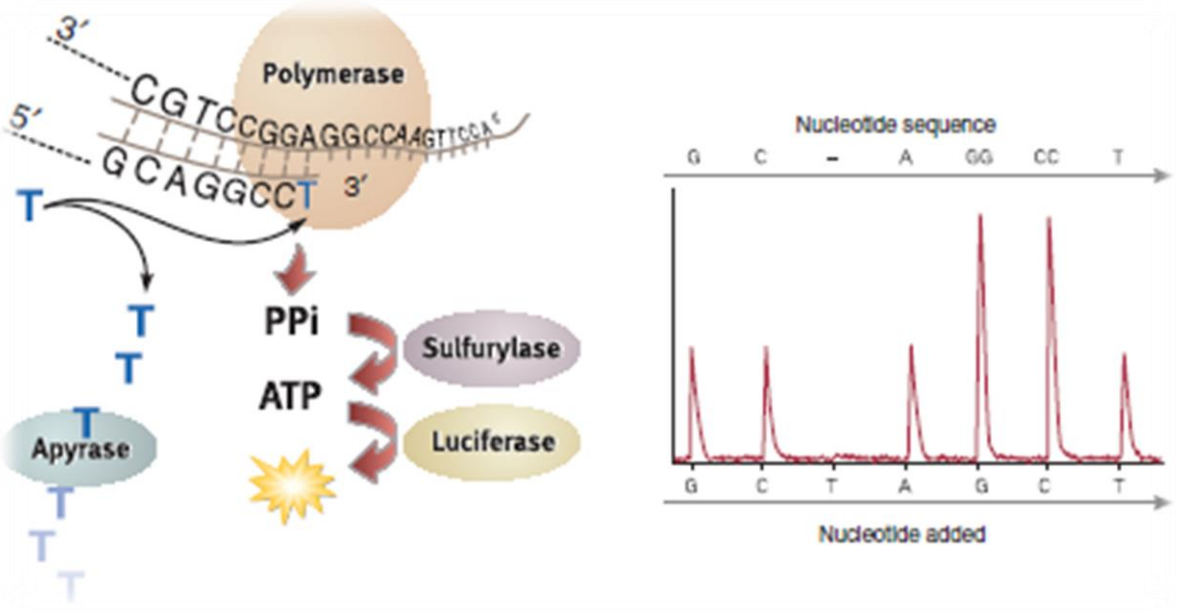


Figure 2.4: The Principle of Pyrosequencing. This technique involves the release of pyrophosphate (PPi) following a nucleic acid polymerization reaction due to nucleotide incorporation on the complementary strand to the single-strand template. The released PPi is converted to ATP by ATP sulfurylase, which subsequently provides the energy to luciferase to oxidise luciferin to oxyluciferin and generate light. The reaction produces a light signal proportional to the quantity of PPi releases. The light is captured by a CCD camera and recorded as peaks in a Pyrogram™. Obtained from England and Pettersson. (2005).

The use of Pyrosequencing technology allows for the parallel analysis of all amplicons within a pooled PCR sample. This enables the accurate quantification of multiple CpG sites with high resolution and reproducibility. In addition, Pyrosequencing assays ensure the generation of reliable data as they contain a number of internal controls for bisulfite treatment to evaluate the completion of the bisulfite conversion step (England & Pettersson., 2005)

2.6.1 Pyrosequencing Assay Design

Pyrosequencing assays were designed using PSQ Assay Design software version 1.0 (Biotage, Uppsala, Sweden). This software designs an assay to amplify a region of interest that contains a number of single nucleotide polymorphisms (SNPs). In this case the SNP is comparative to the cytosine nucleotide contained within a CpG dinucleotide. Upon bisulfite modification and subsequent PCR, if methylated, the cytosine residue will remain as a cytosine (C), or if unmethylated be amplified as thymine (T). Therefore in essence, the "polymorphism" of interest is C/T. The sequence of interest was imported into the design software, with the CpG sites denoted as C/TG.

Once a target region encompassing the SNPs or CpG sites of interest has been selected the assay design software generates a series of potential primer sets based on optimum annealing temperatures, primer lengths and minimisation of non-specific binding. The software allows the user to manipulate which region and subsequently which CpG sites are covered. A target region greater than of 400 base pairs is selected, from this an amplicon of 80 to 100 base pairs is generated (this amplicon length allows for optimal Pyrosequencing runs). These primer sets include both a pair of Pyrosequencing primers, as well as a single sequencing primer, to amplify and sequence the region of interest, respectively. Generated primer sets were then sent for synthesis by Integrated DNA Technologies (Leuven, Belgium).

The *H19* ICR contains four CTCF binding sites. The region amplified in the present study contains two of these four CTCF binding sites. The first CTCF site contains six CpG sites, and the second contains five (**Figure 2.5 A**). Two assays were designed, one for CTCF1 and one for CTCF2, each with their own forward and reverse primer (used for amplification during Pyrosequencing PCR) and sequencing primer (used during Pyrosequencing). The primers used to ampify the *H19* ICR region and to sequence the specific regions of interest are shown in **Figure 2.5 B**.

A

5′-GCAACTGATGACCAGACAGTACTGAGTCTGCCTGGAGCCTGAGTTAAAACCGAGAAAATAGCCATT
GCCTACAGTTCCCGAATCACCACAAGGAAAGAAAAAGGTTGGTGAGAAAATAGAGATTCTATTTTCAT
GTCCGGGGGATGAGCGTGCAGGGCACTTACACCCAGGACTCAAAGGAACATGCTACATTCACACGAGC
ATCCAGGAGGCATAAGAATTCTGCAAGGAGACCATGCCCTATTCTTGGACGTCTGCTGAATCAGTTGT
GGGGTTTATACGCGGGAGTTGCCGCGTGGTGGCAGCAAAATCGATTGCGCCAAACCTAAAGAGCCCCC
CCACCCCTGGTATTGGAATTCACAAATGGCAATGCTGTGGGTCACCCAAGTTCAGTACCTCAGGGGGG
TCACAAATGCCACTAGGGGGGCAGGACACATGCATTTTCTAGGCTGGTACCTCGTGGACTCGGACTCC
CAAATCAACAAGGTCGGCTTACTCTCTGCAAAGAATCCTTTGTGTGTAAAGACCAGGGTTGCCGCACG
GCGGCAGTGAAGTCTCGTACATCGCAGTCCTAAAACGGATTGCAACTGATTGAGTTTTCTCCCCTATC
ACCATCTATGATCCCATAGTCATGGGCTTCATGAGGCCAGGGGTTCATGCTAGTCCTTGATAAAACGT
TCTCAAGAGCTATCTCAGGTATCTGACTTATAGGGTTCTGGGGCCATCAGCGCTATTGTGTGAAAATT
GAAGTATAGCTACTATGTGTGGTGATCATGGAATGTATTGCAGTACCATAATGCAGACCCCACTAAGC
ATGGTCCTCAAATTCTGCACATCTATGAGGACA-3′

B

5′-GTAATTGATGATTAGATAGTATTGAGTTTGTTTGGAGTTTGAGTTAAAATCGAGAAAATAGTTATT
GTTTATAGTTTTCGAATTATTATAAGGAAAGAAAAAGGTTGGTGAGAAAATAGAGATTTTATTTTTAT
GTTCGGGGGATGAGCGTGTAGGGTATTTATATTTAGGATTTAAAGGAATATGTTATATTTATACGAGT
ATTTAGGAGGTATAAGAATTTTGTAAGGAGATTATGTTTTATTTTTGGACGTTTGTTGAATTAGTTGT
GGGGTTTATACGCGGGAGTTGTCGCGTGGTGGTAGTAAAATCGATTGCGTTAAATTTAAAGAGTTTTT
TTATTTTTGGTATTGGAATTTATAAATGGTAATGTTGTGGGTTATTTAAGTTTAGTATTTTAGGGGGG
TTATAAATGTTATTAGGGGGGTAGGATATATGTATTTTTTAGGTTGGTATTTCGTGGATTCGGATTTT
TAAATTAATAAGGTCGGTTTATTTTTTGTAAAGAATTTTTTGTGTGTAAAGATTAGGGTTGTCGTACG
GCGGTAGTGAAGTTTCGTATATCGTAGTTTTAAAACGGATTGTAATTGATTGAGTTTTTTTTTTTATT
ATTATTTATGATTTTATAGTTATGGGTTTTATGAGGTTAGGGGTTTATGTTAGTTTTTGATAAAACGT
TTTTAAGAGTTATTTTAGGTATTTGATTTATAGGGTTTTGGGGTTATTAGCGTTATTGTGTGAAAATT
GAAGTATAGTTATTATGTGTGGTGATTATGGAATGTATTGTAGTATTATAATGTAGATTTTATTAAGT
ATGGTTTTTAAATTTTGTATATTTATGAGGATA-3′

Figure 2.5: CTCF1 and CTCF2 Genomic Sequences and their CpG Sites. **A**. *Mus musculus* genomic sequence spanning a region of the *H19* ICR containing CTCF1 and CTCF2. The sequence shown is the *H19* reference sequence at nucleotides 7:149767397-149768243 (GRCm38) in the Ensembl Genome Browser (http://www.ensembl.org). The positions of the CTCF1 and CTCF2 binding sites are highlighted in yellow with the CpG sites underlined. CTCF1 is closest to the 5′ end and CTCF2 to the 3′ end. **B**. The bisulfite modified sequence spanning the same region as A. The sequences highlighted in green are the CTCF1 Pyrosequencing PCR forward and reverse primer. The sequences in pink are the CTCF2 forward and reverse primer. The sequences in red and blue are the CTCF1 and CTCF2 sequencing primer, respectively.

2.6.2 Pyrosequencing Assay Validation

Before the designed CTCF1 and CTCF2 Pyrosequencing assays were carried out on collected samples; validation of the primer sets occurred to ensure that no preferential amplification towards methylated or unmethylated DNA was occurring during the PCR. In addition assay reproducibility was evaluated. Methods were based on those previously described by (McKay *et al.*, 2012) and involved the production of unmethylated and methylated controls, bisulfite modification, pre- and post PCR mixes, Pyrosequencing PCR, pyrosequencing and analysis through calibration curves.

2.6.2.1 Unmethylated and Methylated Control Preparation

The initial step of the validation process involved the generation of unmethylated (0%) and methylated (100%) controls. The 0% control was generated by carrying out a PCR with a primer pair that flanked the 5' end of CTCF1 and the 3' end of CTCF2 so that both regions were within the amplicon. C57BL/6J genomic DNA was used. The primer pair used to generate this amplicon is shown in **Figure 2.6** with the reaction occurring in the Thermoblock 96® PCR machine (SensoQuest, Germany) as per the conditions described in **Table 2.1**.

5′-GCAACTGATGACCAGACAGTACTGAGTCTGCCTGGAGCCTGAGTTAAAACCGAGAAAATAGCCATT
GCCTACAGTTCCCGAATCACCACAAGGAAAGAAAAAGGTTGGTGAGAAAATAGAGATTCTATTTTCAT
GTCCGGGGGATGAGCGTGCAGGGCACTTACACCCAGGACTCAAAGGAACATGCTACATTCACACGAGC
ATCCAGGAGGCATAAGAATTCTGCAAGGAGACCATGCCCTATTCTTGGACGTCTGCTGAATCAGTTGT
GGGGTTTATACGCGGGAGTTGCCGCGTGGTGGCAGCAAAATCGATTGCGCCAAACCTAAAGAGCCCCC
CCACCCCTGGTATTGGAATTCACAAATGGCAATGCTGTGGGTCACCCAAGTTCAGTACCTCAGGGGGG
TCACAAATGCCACTAGGGGGGCAGGACACATGCATTTTCTAGGCTGGTACCTCGTGGACTCGGACTCC
CAAATCAACAAGGTCGGCTTACTCTCTGCAAAGAATCCTTTGTGTGTAAAGACCAGGGTTGCCGCACG
GCGGCAGTGAAGTCTCGTACATCGCAGTCCTAAAACGGATTGCAACTGATTGAGTTTTCTCCCCTATC
ACCATCTATGATCCCATAGTCATGGGCTTCATGAGGCCAGGGGTTCATGCTAGTCCTTGATAAAACGT
TCTCAAGAGCTATCTCAGGTATCTGACTTATAGGGTTCTGGGGCCATCAGCGCTATTGTGTGAAAATT
GAAGTATAGCTACTATGTGTGGTGATCATGGAATGTATTGCAGTACCATAATGCAGACCCCACTAAGC
ATGGTCCTCAAATTCTGCACATCTATGAGGACA-3′

Figure 2.6: Validation Flanking PCR Reaction. The genomic sequence spanning a region of the *Mus musculus H19* ICR containing CTCF1 and CTCF2. The region maps to nucleotides 7:149767397-149768243 (GRCm38) in the Ensembl Genome Browser (http://www.ensembl.org). The positions of the CTCF1 and CTCF2 binding sites are highlighted in yellow with the CpG sites underlined. CTCF1 is closest to the 5′ end and CTCF2 to the 3′ end. The sequences highlighted in green are the flanking PCR forward and reverse primer, respectively.

Table 2.1: Flanking PCR Reaction Mix and Conditions for *H19* Amplification

Primers	**5'⟶ 3'**		**Amplicon Length**
Forward	GGAACATGCTACATTCAC		592bp
Reverse	CACCACACATAGTAGCTATAC		
Reagent	**Volume (µl)**		
HotStar® Taq Master Mix (QIAGEN)	12.5		
Forward Primer (10µM)	1		
Reverse Primer (10µM)	1		
ddH_2O	9.5		
DNA (4ng)	1		
Total volume	**25**		
PCR Cycling Conditions	**Temperature (°C)**	**Time (min:sec)**	**Cycles**
Initiation	95	15:00	
Denaturation	95	00:15	45
Annealing	50	00:30	
Extension	72	00:15	
Final Extension	72	05:00	
Hold	4	∞	

Following flanking PCR, *in vitro* methylation of an aliquot of the PCR product was carried out in order to generate a 100% control. *In vitro* methylation involved 5µl (for a DNA concentration of 2.5ng/µl) of flanking PCR product being incubated with SssI Methylase (NEB Ltd, UK) at 30°C and SAM (S-adenylmethionine) added every 3 hours for 16 hours (**Table 2.2)**. The CpG methyltransferase allows for the complete methylation of all cytosines within a CpG dinucleotide context.

Table 2.2: *In vitro* Methylation Reaction Mix and Conditions

Reagent	Volume (µl)	Conditions
Flanking PCR Product (2.5ng/µl)	5.0	**Incubate at 30˚C for 16 hours** **Add 1 µl SAM every 3 hours**
Buffer 2 (10x) (NEB)	2.0	
M.SssI (NEB)	6.0	
SAM (1:8) (NEB)	1.0	
ddH_2O	6.0	
Total Volume	**20.0**	

Following flanking PCR and *in vitro* methylation 500ng of the 0% and 100% controls underwent bisulfite modification using the EZ DNA Methylation-Gold™ Kit (Zymo Research, CA, USA) according to the manufacturer's specifications ***(full protocol shown in Appendix G)***.

2.6.2.2 Validation Mixes and PCR Amplification

In order to generate a range of methylation levels, 0, 5, 10, 25, 50, 75, 90, 95 and 100% the bisulfite modified 0% and 100% controls were mixed before and after Pyrosequencing PCR **(Table 2.3 and Table 2.5)**. The pre-PCR mixed samples underwent PCR with the designed primers to amplify the CTCF1 and CTCF2 regions. The reaction was performed in the Thermoblock 96® (SensoQuest, Germany) machine as per the conditions described in **Table 2.4**. The PCR amplification for each sample was performed in duplicate.

Table 2.3 Pyrosequencing-PCR Assay Validation Pre-PCR Reference Mixes

Percentage Mix	Volume	Volume of 0 %
100%	27.0µl of 100%	0.00µl of 0%
95%	22.8µl of 100%	1.20µl of 0%
90%	17.05µl of 95%	0.95µl of 0%
75%	11.67µl of 90%	2.33µl of 0%
50%	7.33µl of 75%	3.67µl of 0%
25%	5.00µl of 50%	5.00µl of 0%
10%	4.00µl of 25%	6.00µl of 0%
5%	3.50µl of 10%	3.50µl of 0%
0%	0.00µl of 5%	3.00µl of 0%

Table 2.4 CTCF1 and CTCF2 Reagents and Conditions used for Assay Validation and Pyrosequencing PCR

Primers CTCF1	**5' ⟶ 3'**		**Amplicon Length**
Forward	GAATTTTGTAAGGAGATTATG		132bp
Reverse	Bio-ACCAAAAATAAAAAAACTCT		
Reagents CTCF1	**Volume (µl)**		
HotStar® Taq Master Mix (QIAGEN)	12.5		
Forward Primer (10µM)	1.0		
Reverse Primer (10µM)	1.0		
dH_2O	9.5		
Bisulfite Modified DNA	1.0		
Total Volume	**25.0**		
Primers CTCF2	**5' ⟶ 3'**		**Amplicon Length**
Forward	GGTTTATTTTTTGTAAAGAA		147bp
Reverse	Bio-CCCATAACTATAAAATCAT		
Reagents CTCF2	**Volume (µl)**		
HotStar® Taq Master Mix (QIAGEN)	12.5		
Forward Primer (10µM)	1.0		
Reverse Primer (10µM)	1.0		
$MgCl_2$ (2.0mM) (QIAGEN)	2.0		
dH_2O	7.5		
Bisulfite Modified DNA	1.0		
Total Volume	**25.0**		
PCR Conditions CTCF1 and CTCF2	**Temperature (°C)**	**Time (min:sec)**	**Cycles**
Initiation	95	15:00	
Denaturation	95	00:15	50
Annealing	45	00:30	
Extension	72	00:15	
Final Extension	72	05:00	
Hold	4	∞	

Note: Bio –Biotin labelled

Following PCR, standard electrophoretic techniques were used for the visualisation of PCR products and contamination assessment. The amplified 0% and 100% CTCF1 and CTCF2 products were then mixed to generate post-PCR methylation ranges according to the dilutions in **Table 2.5.** All mixes, both pre- and post-PCR for CTCF1 and CTCF2 underwent Pyrosequencing analysis (**See 2.6** and **2.7**) with the use of the sequencing primers described in **Table 2.6**.

Table 2.5 Post-PCR Assay Validation Mixes

Percentage Mix	**Volume**	**Volume of 0 %**
100%	95.00µl of 100%	0.00µl of 0%
95%	87.40µl of 100%	4.60µl of 0%
90%	67.00µl of 95%	3.75µl of 0%
75%	45.75µl of 90%	9.25µl of 0%
50%	30.00µl of 75%	15.00µl of 0%
25%	20.00µl of 50%	20.00µl of 0%
10%	15.00µl of 25%	22.50µl of 0%
5%	12.50µl of 10%	12.50µl of 0%
0%	00.00µl of 5%	10.00µl of 0%

Table 2.6 Sequencing Primers for Pyrosequencing Reaction

Sequencing Primers	**5' ⟶ 3'**	**Number of CpGs to Analyse**
CTCF1	AATTAGTTGTGGGGTTTATA	6
CTCF2	TTGTGTGTAAAGATTAGGGT	5

2.6.2.3 Validation Analyses

Calibration curves were constructed for each primer pair and assessed at each CpG site within CTCF1 and CTCF2 ***(See Appendix H).*** Analysis of the pre-PCR R^2 values (**Table 2.7**) between expected and observed methylation levels showed that there was no preferential amplification of methylated DNA by the CTCF1 primer. Furthermore, the post-PCR R^2 values indicated that the assay was reproducible (**Table 2.7**).

Table 2.7 Validation Assay R^2 Values for the Six CpG sites of CTCF1

CpG Site	Pre-PCR Mix R^2	Post-PCR Mix R^2
1	0.9964	0.9984
2	0.9971	0.9993
3	0.9967	0.9990
4	0.9972	0.9995
5	0.9986	0.9999
6	0.9984	0.9991

Analysis of the CTCF2 pre-PCR R^2 values (**Table 2.7**) shows slight preferential amplification of the methylated DNA with the calibration curves **(*See Appendix H*)** showing departures from the expected at the extreme low percentages between 5% – 25%. However, the R^2 values are still above 0.9 suggesting a relatively robust assay (**Table 2.8**).

Table 2.8 Validation Assay R^2 Values for the Five CpG sites of CTCF2

CpG Site	Pre-PCR Mix R^2	Post-PCR Mix R^2
1	0.9495	0.9978
2	0.9662	0.9987
3	0.9463	0.9995
4	0.9527	0.9990
5	0.9580	0.9992

2.6.3 Pyrosequencing PCR

Amplicons for the CTCF1 and CTCF2 binding sites of the *H19* ICR were performed as per the conditions in **Table 2.4**. The PCR amplification for each sample was performed in duplicate**.**

Standard electrophoretic techniques were used to resolve the PCR amplified products. Visualisation of amplified products took place on a 1% (w/v) agarose gel containing SafeView® (NBS Biologicals, Huntingdon, UK) at 80 volts in 1 x TBE buffer. A 100bp DNA molecular size marker at 0.1µg/µl (Gene Ruler™ 100bp Plus, Thermo Scientific, MA, USA) was used as a size standard.

2.6.4 Pyrosequencing Preparation and Run

Pyrosequencing was performed using the PyroMark MD System (Biotage) (***full protocol in Appendix I***). Before Pyrosequencing commenced, 2µl of Streptavidin Sepharose™ HP beads, 30µl ddH_2O and 38µl of binding buffer was added to each well of a 96-well PCR plate that contained 10µl of PCR product. The plate was left on a shaker for 10 min at 300 rpm. This was done to facilitate binding of the biotin labelled PCR products to the sepharose beads.

The vacuum probe block was then used to capture the PCR product attached to the sepharose beads. The vacuum probe block, which contained bound PCR product, was placed sequentially into four different troughs containing 70% ethanol, denaturation (NaOH) solution, washing buffer and ddH_20 for 5 seconds each. This process allowed for the preparation of single-stranded PCR products as it separates the DNA strands, leaving only the biotin-labelled strand attached to the sepharose bead captured by the vacuum probe. The sepharose beads, with the single-stranded PCR products attached, were released into a PSQ 96 plate. Each well of the plate was pre-filled with 0.5µl of 10µM sequencing primer and 11.5µl annealing buffer. The PSQ 96 plate was then heated to 80˚C for 3 minutes and allowed to cool, facilitating the annealing of the sequencing primer to the single stranded PCR products.

A reagent cartridge (**Figure 2.7**) was prepared with the required amounts of enzyme, substrate and dNTPs using the PyroMark® Gold Q96 Kit (QIAGEN, CA, USA) as assigned by the PyroMark MD software. The PSQ 96 plate was run on a PyroMark MD instrument with each sample run in duplicate for each assay.

Figure 2.7: PyromarkMD Cartridge. Arrangement of tips in the Pyrosequencing MD Cartridge. S - Substrate; E- Enzyme; A,C,G,T- 4 nucleotides.

When analysing methylation data, the ratio of C (methylated): T (unmethylated) at a given CpG site within a specific DNA sample may vary, and is therefore unlike a traditional SNP where a sample is denoted as either heterozygous or homozygous. In this case, the C and T alleles are quantified and expressed as a percentage. Pyrosequencing data was analysed using the Pyro Q-CpG Software (Biotage, Uppsala, Sweden).

2.7 Hypothesis Testing

In this study it was hypothesised that chronic alcohol exposure *in utero* during the period of embryo sex determination would result in perturbations in a number of domains, these include: (1) pregnancy outcome; (2) parturition; (3) pup growth rate; (4) locomotor activity; (5) anxiety levels; (6) DNA methylation at the *H19* ICR. Furthermore, it was hypothesised that there would be (7) transgenerational inheritance of these phenotypic effects and (8) transgenerational inheritance of the *H19* epimutation and potentially (9) a lineage specific inheritance of the phenotypic effects and the *H19* epimutation.

2.8 Statistical Analysis

All descriptive statistical analyses were performed using STATA 11 (StataCorp LP). Associations between treatment and pregnancy outcome, offspring phenotype and

methylation levels were assessed using Independent Two-sample T-tests and Wilcoxon Mann-Whitney tests in STATA 11.

2.8.1 Descriptive Statistics

Data analysed by descriptive statistics are described in terms of central tendency, specifically the mean levels, median and mode. The measure of dispersion has been described by use of the standard deviation and where appropriate the minimum and maximum have been described. The discrete numerical outcome variables include gestational length, litter size, faecal boli count and amount of urine. The continuous outcome variables include survival rate, percentage of male offspring, pup weight (analysed as growth rates), DNA methylation level, total distance travelled and time spent in the inner zone. The categorical outcome variables are pregnancy outcome and parental lineage. The independent variables in this study are the treatment group and generation. The DNA methylation levels per CpG site within the CTCF1 and CTCF2 binding sites of the *H19* ICR are graphically represented as bar graphs constructed by use of mean ± SD values.

2.8.2 Association between Treatment, Pregnancy Outcome, Phenotype & Methylation

In this experiment, offspring in each treatment or control group were considered as independent samples, so that all offspring in each treatment group were compared with all offspring in the corresponding control group, without reference to their respective dams. This was due to each fertilisation event involving sperm and egg that may have been variably affected by treatment and subsequently causing each offspring to display individual and varying effects of exposure. Furthermore, during pregnancy each embryo has its own placenta and therefore the environment and interactions with the mother are unique to that experienced by its siblings.

To determine whether differences in the discrete and continuous data variables existed between the two treatment groups, EtOH and control, analysis was initiated by assessing the normality of the underlying distribution by the use of histograms and the Shapiro-Wilk

goodness of fit test. Where a normal distribution was seen an independent two-sample T-test was conducted in order to detect differences between treatment groups. Importantly before performing the T-test the variance between groups was investigated by use of the F-test. If the F-statistic was significant (< 0.05) equal variance between the two groups could not be assumed and therefore a T-test accounting for the unequal variance was performed. Where non-parametric distributions were found, a two-sample Wilcoxon Rank Sum test (Wilcoxon Mann-Whitney) was performed.

In the instance of DNA methylation data, the data were analysed using non-parametric tests, more specifically the two-sample Wilcoxon Mann-Whitney, due to the small sample size. These analyses use the median values rather than the mean values. In this test analyses are based on the ranks of observations rather than the raw data values themselves. All samples were run in duplicate and the mean percentage methylation values were calculated. Note that from this point onwards this mean methylation level will be referred to as the methylation level and the mean methylation level will refer to the average methylation level across all CpG sites within a binding site, i.e. CTCF1 or CTCF2. Analyses, stratified by treatment and generation, were performed for each individual CpG site, as well as the average methylation level across binding sites. Correlations between CpG sites in each CTCF binding factor were assessed using the Spearman's rank correlation test.

For categorical variables (i.e. pregnancy outcome) the Chi– squared (X^2) test would normally be performed, however, as several data points within this study had counts of less than five the Fisher's Exact test was used.

All tests underwent a two-tailed test as the direction of change in the variables was not presumed. Any findings with a P value (α) less than 0.05 were considered to be statistically significant, whilst *P* values significant at the 10% level ($p < 0.1$) were regarded as showing a trend.

2.8.3 Correlations between Pregnancy Outcome, Phenotype & Methylation

The linear relationships between variables were assessed by the use of the Pearson's or Spearman's Correlation. Pearson's correlation was used when data followed normal distributions, whilst and for non-parametric distributions the Spearman's correlation was used. The correlations determine whether one variable is associated with another and if that relationship is either negative (i.e. as one variable increases the other decreases) or positive (i.e. both variables increase or decrease together). Furthermore the strength of the correlation is assessed with weaker correlations closer to 0 and stronger associations tending closer to +/- one.

Following correlation assessments between growth rate data and littersize data the effect of litter size was assessed by use of the ANOVA. The ANOVA compared the mean differences in growth rate across treatment groups while adjusting for the effects of litter size.

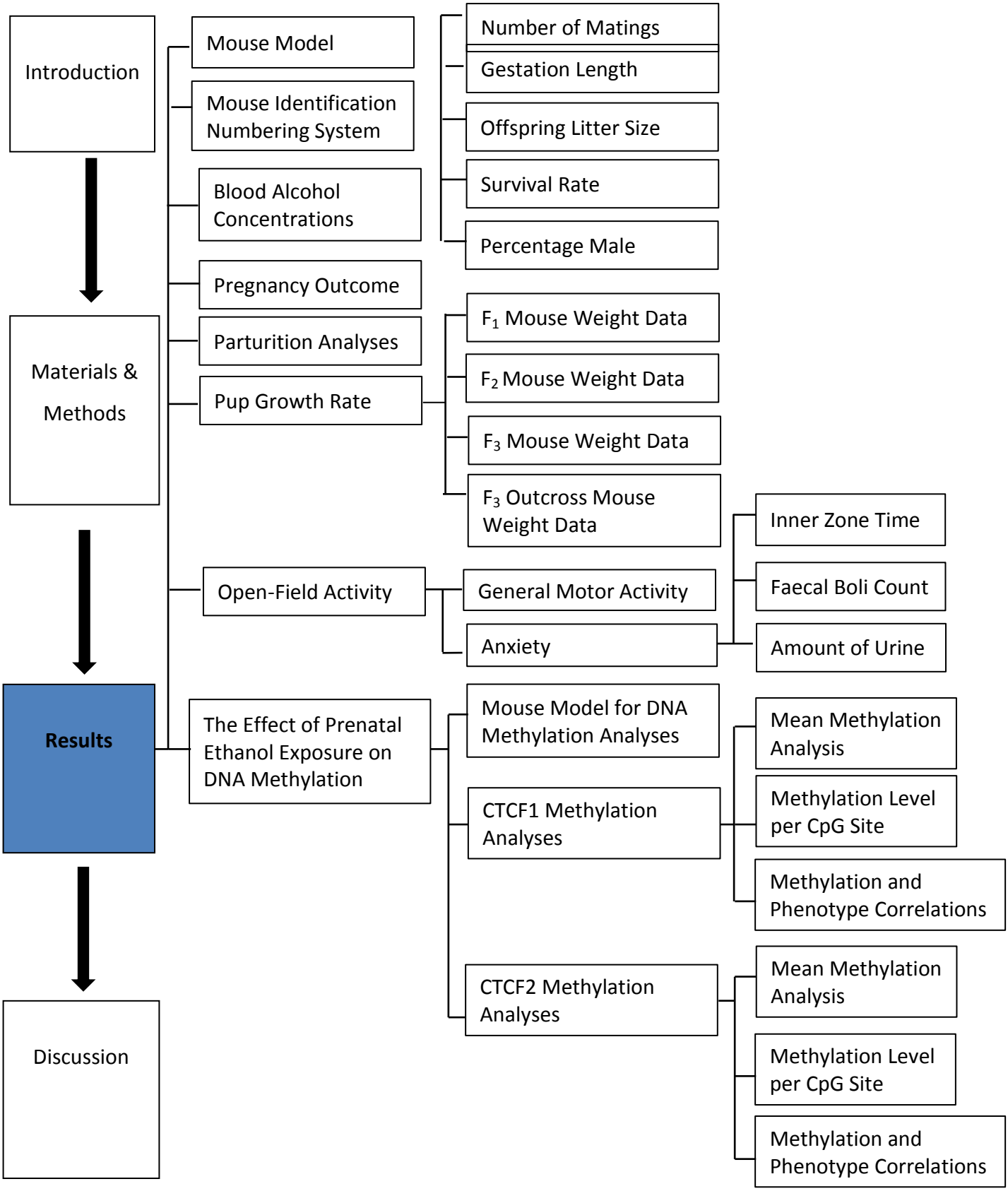
Spearman's correlations were performed on the combined treatment groups to determine if DNA methylation or mean methylation level was influenced by gestational alcohol exposure at the F_0 generation.

Correlation analyses were performed using STATA 11 (StataCorp LP).

Chapter 3

Results

3.0 Results


Introduction
Materials & Methods
Results
Discussion
Mouse Model
Mouse Identification Numbering System
Blood Alcohol Concentrations
Pregnancy Outcome
Parturition Analyses
Pup Growth Rate
Open-Field Activity
The Effect of Prenatal Ethanol Exposure on DNA Methylation
Number of Matings
Gestation Length
Offspring Litter Size
Survival Rate
Percentage Male
F_1 Mouse Weight Data
F_2 Mouse Weight Data
F_3 Mouse Weight Data
F_3 Outcross Mouse Weight Data
General Motor Activity
Anxiety
Inner Zone Time
Faecal Boli Count
Amount of Urine
Mouse Model for DNA Methylation Analyses
CTCF1 Methylation Analyses
CTCF2 Methylation Analyses
Mean Methylation Analysis
Methylation Level per CpG Site
Methylation and Phenotype Correlations
Mean Methylation Analysis
Methylation Level per CpG Site
Methylation and Phenotype Correlations

3.1 Chapter Summary

The primary objective of this study was to determine whether transgenerational inheritance of DNA methylation alterations at the *H19* imprinting control region occurred following chronic maternal ethanol exposure during embryonic development in mice. It was predicted that (1) DNA methylation alterations would occur in the germ line; and result in subsequent offspring (F_1-F_3) displaying (2) altered DNA methylation profiles; (3) growth fluctuations; (4) altered locomotor activity and anxiety levels; and (5) perturbations in parturition parameters in the F_0-F_2 generations. To test these predictions, phenotypic classification of the offspring occurred from PN7 to PN80 and the DNA methylation status of the *H19* ICR was analysed from the tail DNA of ethanol-exposed and control individuals across the four generations (F_0-F_3).

3.2 Mouse Model for Phenotypic Analyses

A total of 173 C57BL/6J mice were used for analysis in this study. The F_0 generation consisted of 11 males that underwent no treatment and 19 treated dams - 10 ethanol-exposed (EtOH) and 9 sucrose-exposed (control). From F1 to F3 EtOH refers to those mice directly descended from the F0 female exposed to EtOH and likewise for sucrose. The F_1 control group consisted of 26 individuals, equally divided by sex (n = 13). Within the F_1 EtOH group there were 10 males and 9 females. The F_2 generation (inclusive of those used in outcross experiments) totalled 63; 20 female and 17 male controls. In the EtOH group there were 13 individuals of each sex. The F_3 EtOH offspring consisted of 6 males and 6 females. In contrast, the F_3 control offspring contained 6 males and 4 females **(Table 3.1)**.

The F_2 outcross offspring, which no longer consisted of EtOH and control groups, contained 5 male and 10 female individuals. Crosses between 3 EtOH dams and 3 control sires resulted in 3 male and 5 female offspring. The reciprocal cross of 3 control dams mated with 3 EtOH sires resulted in 2 males and 3 females (**Table 3.1**).

Despite the disparity in offspring numbers from F_1 – F_3 the proportion of males to females in the two treatment groups across generations did not differ **(Table 3.1)**.

Table 3.1: Mice Frequencies according to Treatment and Sex at Generations F_0-F_3 Outcross

Generation	Treatment	Sex	Frequency (n)	*P*-value[a]
F_0	NT	M	11	-
	EtOH	F	10	
	S	F	9	
F_1	EtOH	M	10	1.000
	EtOH	F	9	
	S	M	13	
	S	F	13	
F_2	EtOH	M	13	0.802
	EtOH	F	13	
	S	M	17	
	S	F	20	
F_3	EtOH	M	6	0.691
	EtOH	F	6	
	S	M	6	
	S	F	4	
F_2 Outcross Offspring	FEtOH x MS	M	3	1.000
	FEtOH x MS	F	5	
	FS x MEtOH	M	2	
	FS x MEtOH	F	3	
Total			**173**	

NT – No Treatment, EtOH – Ethanol-treated group, S – Sucrose-control group,
FEtOH x MS – Ethanol-treated Female mated with Sucrose-treated Male,
SF x MEtOH – Sucrose-treated Female mated with Ethanol-treated Male
[a] *P*-value generated from Fisher's-Exact Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

3.3 Mouse Identification Numbering System

Unique identification numbers were assigned to each mouse used in the study by use of steel ear tags. ***Appendix A and Appendix B*** shows the treatment group, sex and parents of each individual, where appropriate.

3.4 Blood Alcohol Concentrations

Mean (± S.D) maternal blood alcohol levels 30 minutes post-gavage on GD6 were 1.15 ± 0.45mg/ml and 0.48 ± 0.17mg/ml for the EtOH (n = 5) and control (n = 6) F_0 females, respectively ***(See Appendix E)***. A statistically significant difference in the blood alcohol levels between the two treatment groups was seen (Mann Whitney, one-tailed test; *P = 0.0022*). The blood alcohol levels obtained in the EtOH exposed micereached physiologically significant levels of >1.0mg/ml (Rhodes *et al.*, 2005).

3.5 Pregnancy Outcome

Upon mating a resulting pregnancy is not always guaranteed and as such the outcome of each mating event was monitored and recorded as 'not pregnant' or 'successfully pregnant'. 'Not pregnant' was indicated by the lack of parturition, whilst 'successfully pregnant' was indicated by the birth of offspring. Across the F_0, F_1 and F_2 generations there were a total of 17 'not pregnant' and 41 'successfully pregnant' recordings. F_2 outcross experiments resulted in 5 'successfully pregnant' and 1 'not pregnant' dam.

Table 3.2: Pregnancy Outcome at Generations F_0-F_2 Outcross between Treatment Groups

Generation	Treatment	N	Successfully Pregnant	Not Pregnant	*P*-value[a]
F_0	EtOH	10	5	5	0.141
	S	9	8	1	
F_1	EtOH	9	9	0	-
	S	10	10	0	
F_2	EtOH	10	6	4	0.370
	S	10	3	7	
F_2 Outcross	EtOH	3	3	0	1.000
	S	3	2	1	

EtOH -treated, S – Sucrose-treated control
[a] *P*-value generated from Fisher's-Exact test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

Within the F_0 generation the EtOH females (n = 10) had an impregnation success rate of 50%, or five pregnancies out of a possible 10. Control dams (n = 9) showed an overall success rate of 89%, or 8 out of a potential 9 pregnancies. F_1 generation pregnancy success rates did not vary

between treatment groups with all dams mated in both the EtOH (n = 9) and control (n = 9) groups resulting in successful pregnancies **(Table 3.2)**.

In contrast, the dams of the F_2 generation differed in pregnancy success rates and together resulted in a number of 'not pregnant' recordings. The EtOH (n = 10) group had a 40% pregnancy failure rate, where 4 out of a possible 10 pregnancies resulted in no parturition. The control dams (n = 10) had almost double this with 70% of the dams failing to reach a successful delivery. In F_2 outcross experiments, where F_2 generation EtOH females (n = 3) were crossed with control males (n = 3), there was a 100% pregnancy success rate. In contrast, reverse crosses involving control females (n = 3) and EtOH males (n = 3) there was a 67% success rate with 2 out of a possible 3 pregnancies resulting in parturition **(Table 3.2)**.

Upon assessment there was no difference in pregnancy outcome between treatment groups at the three generations (F_0: *P = 0.141*, F_2: *P = 0.370,* F_2 Outcross: *P = 1.000*) **(Table 3.2)**.

Importantly it was noted that within this study some mice within the F_0 generation experienced the pregnancy complication of a miscarriage (CAS Veterinary Staff, Personal communication). There were 2 miscarriages in the F_0 EtOH (n = 10) group and 0 in the control (n = 9) group. Although tempting to speculate that EtOH treated pregnant mice are more likely to miscarry, there was no significant difference between the treatment groups, given the small sample size (Fisher's Exact Test; *P = 0.474*).

3.6 Parturition analyses

To determine whether the treatment each dam was exposed to at the F_0 generation had an impact on the parturition of successive generations several variables were measured on successfully pregnant dams from F_0 to F_2 outcross. The parameters assessed included the number of matings required for a successful pregnancy (**Table 3.3**), gestation length (**Table 3.3**), litter size (**Table 3.3**), survival rate per litter (**Table 3.3),** and percentage of male offspring (**Table 3.3).**

Table 3.3: Parturition Variables at Generations F_0-F_2 Outcross between Treatment Groups

Variable	Generation	Treatment	N	Mean ± SD	Mode (min, max)	*P*-value[a]
Number of Matings	F_1	EtOH	9	3.56 ± 1.24	4 (2,5)	0.003**[b]
		S	10	1.90 ± 0.88	1 (1,3)	
	F_2	EtOH	6	1.50 ± 0.84	1 (1,3)	0.877
		S	3	1.33 ± 0.58	1 (1,2)	
	F_2 Outcross	EtOH	3	1.67 ± 0.58	2 (1, 2)	0.182
		S	2	1.00 ± 0.00	1 (1, 1)	
Gestation Length (days)	F_0	EtOH	5	20.20 ± 0.84	20 (19, 21)	0.090^[b]
		S	8	19.50 ± 0.53	19 (19, 20)	
	F_1	EtOH	9	19.44 ± 0.88	19 (18, 21)	0.115
		S	10	19.90 ± 0.32	20 (19, 20)	
	F_2	EtOH	6	19.67 ± 0.52	20 (19, 20)	1.000
		S	3	19.67 ± 0.58	20 (19, 20)	
	F_2 Outcross	EtOH	3	19.33 ± 0.58	19 (19, 20)	0.182
		S	2	20.00 ± 0.00	20 (20, 20)	
Litter Size (pups/litter)	F_0	EtOH	5	5.40 ± 0.89	6 (4, 6)	0.263
		S	8	6.38 ± 1.69	7 (3, 8)	
	F_1	EtOH	9	6.00 ± 1.66	6 (3, 9)	0.439
		S	10	6.30 ± 0.95	7 (4, 7)	
	F_2	EtOH	6	4.80 ± 1.47	6 (3, 6)	0.890
		S	3	5.30 ± 0.58	5 (5, 6)	
	F_2 Outcross	EtOH	3	5.00 ± 1.00	- (4, 6)	0.083^[b]
		S	2	7.50 ± 0.71	- (7, 8)	
Survival Rate/litter (%)	F_0	EtOH	5	93.33 ± 9.13	100.0 (83.33, 100.0)	0.676
		S	8	77.80 ± 36.00	100.0 (0.0, 100.0)	
	F_1	EtOH	9	76.20 ± 43.40	100.0 (0.0, 100.0)	0.429
		S	10	95.24 ± 11.00	100.0 (66.67, 100.0)	
	F_2	EtOH	6	66.70 ± 51.60	100.0 (0.0, 100.00)	0.758
		S	3	94.44 ± 9.62	100.0 (83.33, 100.0)	
	F_2 Outcross	EtOH	3	100.00 ± 0.00	100.0 (100.0, 100.0)	-
		S	2	100.00 ± 0.00	100.0 (100.0,100.0)	
Percentage Male Offspring (%)	F_0	EtOH	5	63.33 ± 11.06	60.0 (50.0, 80.0)	0.079^[b]
		S	7	39.10 ± 25.78	- (0.0, 71.43)	
	F_1	EtOH	7	57.51 ± 23.81	33.33 (20.0, 83.33)	0.100[b]
		S	10	38.22 ± 0.95	16.67 (16.67, 71.43)	
	F_2	EtOH	4	57.50 ± 43.49	- (0.0, 100.0)	0.714
		S	3	73.30 ± 11.55	80.0 (60.0, 80.0)	
	F_2 Outcross	EtOH	3	33.33 ± 30.55	- (0.0, 60.0)	0.767
		S	2	37.50 ± 53.03	- (0.0, 75.0)	

EtOH – Ethanol-treated, S – Sucrose-treated control; [a] *P*-value generated from Wilcoxon Mann-Whitney Test, [b] *P*-value generated from Independent T-test; Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$)

3.6.1 Number of Mating Attempts

As no treatment regimen was being delivered to subsequent generations following the F_0 generation a number of mating events could take place to generate a pregnancy. In the case of a failed mating, a maximum of five mating attempts took place, after which no further attempt was made.

Within the F_1 generation the number of matings required to generate a successful pregnancy differed greatly between the two treatment groups. EtOH dams (n = 9) required, on average, double (1.87 times) the number of attempts than the control (n = 10). In contrast, the EtOH (n = 6) and control (n = 3) groups within the F_2 generation showed a similar mean number of attempts. The EtOH females (n = 3) of the F_2 Outcross group had a slightly higher mean number of attempts in comparison to the control females (n = 3) **(Table 3.3)**.

Upon statistical analysis a significant difference in mating attempts between treatment groups at the F_1 generation was found. However, no difference was observed in successive generations (F_1: *P = 0.003,* F_2: *P = 0.877,* F_2 Outcross *P = 0.182*) **(Table 3.3)**.

3.6.2 Gestation Length

The time taken (in days) from GD1 up to day of parturition was recorded and termed gestational length. Inter-treatment group comparisons at the F_0 generation showed EtOH females (n = 5) had a longer mean gestation length by 0.7 days, compared to the control females (n = 8). In contrast, at the F_1 and F_2 Outcross generation the control females showed a longer mean gestational period than the EtOH group. The mean gestation length between treatment groups at the F_2 generation did not vary **(Table 3.3)**.

Statistical analysis showed no significant difference in gestational length between treatment groups, however, F_0 gestational length trended towards significance (F_0: *P = 0.090,* F_1: *P = 0.115*, F_2: *P = 1.000,* F_2 Outcross: *P = 0.182*) **(Table 3.3)**.

3.6.3 Offspring Litter Size

The litter size in each treatment group was recorded for each successful pregnancy, and included offspring cannibalised after birth.

For all generations, the average litter size for the EtOH group was lower than that of the control group's mean littersize. Within the F_0 generation the EtOH (n = 5) group differed from the control (n = 9) group by one pup (0.98pups/litter). The F_1 generation produced the smallest litter size difference between treatment groups with EtOH dams (n = 9) and control dams (n = 10) producing average litters of 6.00 ± 1.66pups/litter and 6.30 ± 0.95pups/litter, respectively. The largest difference in litter size was seen within the F_2 outcross group, where EtOH dams (n = 3) produced litters with an average of 2 pups less than the control dams (n = 2) **(Table 3.3)**.

There was no difference in litter size at generations F_0-F_2. Although, whilst not significant, the difference between the two groups at the F_2 Outcross pregnancies did appear to be trending towards significance (F_0: *P = 0.263,* F_1: *P = 0.439,* F_2: *P = 0.890* and F_2 Outcross: *P = 0.083*) **(Table 3.3)**.

3.6.4 Survival Rate per Litter

To determine whether the treatment to the F_0 gestating females would have an effect on the future offspring's survival the percentage of offspring within a litter that survived the first week post parturition was recorded.

Survival rate at the F_0 generation was high amongst the EtOH (n = 5) group with 93 % ± 9 % of the pups surviving the first week of life. In contrast, the control (n = 8) group had a 15% lower mean survival rate of 78% ± 36%. The F_1 generation displayed an inverse relationship to the F_0 generation with an average of 95% ± 11% survival rate in the control (n = 10) group and a lower rate of 76% ± 43% pups surviving in the EtOH (n = 9) group. The largest survival rate difference (28%) was seen in the F_2 generation where the control (n = 3) group was the higher of the two groups. The survival rate between the two treatment groups within the F_2 Outcross dams did not vary as all offspring survived **(Table 3.3)**.

Despite the large differences in survival rates at generations F_0 – F_2 there was no significant difference in survival rate between treatment groups (F_0 *P = 0.676,* F_1 *P = 0.429,* F_2 *P= 0.890)* **(Table 3.3)**.

3.6.5 Percentage of Male Offspring

The percentage of male offspring in each litter was analysed to assess the effect of treatment exposure during gestation in the F_0 on sex outcome in future offspring. These data excluded cannibalised pups as their sex could not be determined.

Within the F_0 generation the average percentage of male offspring between treatment groups differed by 24% with EtOH (n = 5) litters having 63% ± 11% males and the control (n = 7) group 39% ± 10%. At the F_1 generation this trend continued, where the EtOH (n = 7) male percentage reached 58% ± 24% and the control (n = 10) reached 38% ± 1%. In contrast to F_0 and F_1, in the F_2 generation the control (n = 3) dams appeared to have the larger proportion of male offspring compared to the EtOH (n = 4) dams. The percentage of male offspring within the F_2 Outcross dams did not differ greatly between the EtOH (n = 3) and control (n = 2) groups **(Table 3.3)**.

Analysis revealed that the litters of the F_0 exposed trended towards significance (F_0: *P = 0.079,* F_1: *P = 0.100,* F_2: *P = 0.714,* F_2 Outcross: *P = 0.767*) **(Table 3.3)**.

3.6.6 Parturition Analysis Summary

The number of matings required to result in a successful delivery differed significantly (*P = 0.003*) amongst the F_1 treatment groups with the prenatally EtOH exposed dams requiring more attempts than the controls. This effect was not perpetuated in subsequent generations. Gestational length and percentage male trended towards significance within the F_0 EtOH-exposed dams (*P = 0.090; P = 0.083*). F_0-EtOH dams had an increased gestational length as well as a larger proportion of male offspring when compared to controls. Litter size was not significantly different between treatment groups at the F_0 to F_2 generations, however, at the F_2 outcross generation a difference trending towards significance was found (*P = 0.083*). Survival rate per litter was not different between treatment groups at any generation.

3.7 Mouse Weight Data

To determine the impact of F_0 chronic maternal ethanol exposure during embryonic development on the growth of offspring in successive generations (F_1- F_3) pup weight was measured and growth rates analysed. Weight was measured daily from PN7 to PN30 and reassessed every 5 days from then on to PN80. For each generation the overall growth rate (PN7-PN80) was assessed, as well as at three additional distinct growth periods that corresponded to a change in the mouse's environment and/or feeding habits.

Overall growth rate, time period 0, corresponded to PN7-PN80 and encompassed all time points at which the mice were assessed. Suckling, referred to as time period 1, spanned PN7 to PN21, and included the period during which pups relied solely on milk from their mother as a food source. Weaning (time period 2) was from PN22 to PN30. Over this time mice began self-feeding. In addition, at PN25 dams were removed from cages and mice remained housed with littermates to PN30. At time period three, PN35 to PN80, mice were same-sex group housed up until adulthood. The time periods are summarised in **Table 3.4**.

Table 3.4: Time Periods analysed for Growth Rates and their corresponding Time Frames

Time Period	Growth Period	Encompassed Days
0	Overall	7 - 80
1	Suckling	7 - 21
2	Weaning	22 - 30
3	Same-Sex Group Housed / Adult	35 - 80

As male mice tend to grow more rapidly than female mice and subsequently tend to weigh more; mice growth rates were separated according to sex at all generations so as not to skew the data due to sex ratio for all time points (F_1-F_2 Outcross Offspring).

3.7.1 F_1 Mouse Weight Data

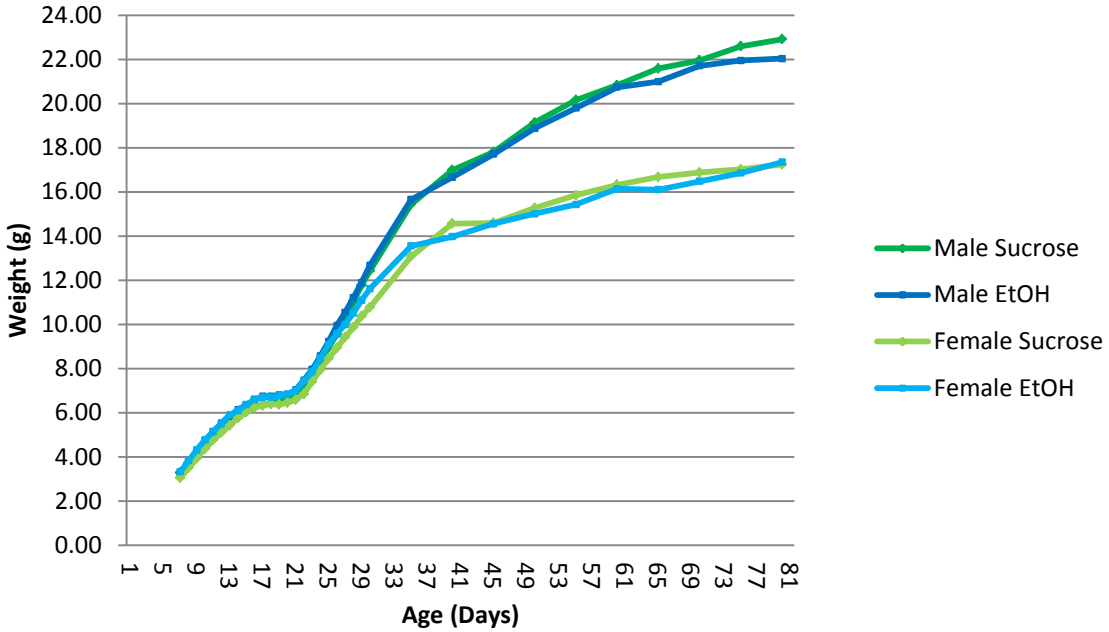


Figure 3.1: Overall Growth Rates of F_1 Individuals. Growth rates from day 7 to day 80 of EtOH and S - Control (sucrose – treated control) F_1 individuals stratified by sex.

Overall growth rates depicted in Figure 3.1 indicate that during time period 1 (PD 7 - PD21) there was little difference in growth rates between individuals. Following a plateau (PD13 - PD21), growth rate began to increase rapidly amongst all individuals albeit at varying rates. At approximately PD30 the difference in growth rates across sexes was evident and continued throughout analysis. At time period 3 (PD35-PD80) growth rates continued to increase although at a much slower rate than at previous time periods. Treatment appeared to have some effect in EtOH males and females as they exhibited lower growth rates than their control counterparts. More thorough analysis of the distinct growth periods is displayed in **Table 3.5**.

Table 3.5: F_1 Generation Growth Rates stratified by Sex and Treatment across Time Periods 0, 1, 2 and 3

Period	Treatment	Sex	N	Mean Growth Rate ± SD	Min, Max	*P*-value[a] sex	*P*-value[b] treatment
0	EtOH	M	10	0.257 ± 0.012	0.243, 0.285	6.21×10^{-9}***	0.121
	S	M	13	0.269 ± 0.020	0.234, 0.313		
	EtOH	F	9	0.192 ± 0.008	0.184, 0.212		0.571[a]
	S	F	14	0.194 ± 0.013	0.175, 0.225		
1	EtOH	M	10	0.271 ± 0.018	0.242, 0.294	0.590[b]	0.050*
	S	M	13	0.252 ± 0.030	0.196, 0.299		
	EtOH	F	9	0.262 ± 0.021	0.226, 0.287		
	S	F	14	0.252 ± 0.024	0.216, 0.297		
2	EtOH	M	10	0.649 ± 0.097	0.470, 0.755	5.78×10^{-7}***	0.853
	S	M	13	0.656 ± 0.064	0.543, 0.784		
	EtOH	F	9	0.522 ± 0.044	0.451, 0.608		0.250
	S	F	14	0.489 ± 0.072	0.330, 0.568		
3	EtOH	M	10	0.142 ± 0.022	0.098, 0.170	4.78×10^{-8}***	0.064^
	S	M	13	0.166 ± 0.035	0.095, 0.220		
	EtOH	F	9	0.084 ± 0.009	0.069, 0.095		0.377
	S	F	14	0.092 ± 0.019	0.071, 0.140		

EtOH – Ethanol-treated, S – Sucrose-treated control
[a] *P*-value generated from Wilcoxon Mann Whitney [b] *P*-value generated from Independent T-test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

At time period 0, growth rates between EtOH females (n = 9) and control females (n = 14) were similar, however, the EtOH males (n = 10) had a lower growth rate than the control males (n = 13). There was an effect of sex but none of treatment (Sex: $P = 6.21 \times 10^{-9}$, Treatment: Males: $P = 0.121$, Females: $P = 0.571$) **(Table 3.5).**

In contrast, EtOH male and EtOH female growth rates at time period 1 appeared higher than their control group counterparts. The intersex difference between EtOH-exposed individuals was relatively large whereas the control females did not differ from the control males. There was no effect of sex but a significant effect of treatment (Sex: $P = 0.590$, Treatment: $P = 0.050$) **(Table 3.5).**

At time period 2 the highest growth rates appeared for all groups. The difference between treatment groups amongst females was relatively large and, similar to time period 1, the EtOH group was heavier. The difference between treatment groups within males was relatively small and the control group was heavier. There was a significant effect of sex but none of treatment (Sex: $P = 5.78 \times 10^{-7}$, Treatment: Males: $P = 0.853$, Females: $P = 0.250$) **(Table 3.5).**

The growth rate of EtOH males and females at time period 3 was lower than that of the control males and females. Female growth rates were slower than the male growth rates, with the growth rate of EtOH females equating to almost 60% of the EtOH male growth rate and the control female growth rate equating to 55% of the respective male average growth rate. There was an effect of sex and no effect of treatment in females; however, a value trending towards significance was noted in males (Sex: $P = 4.78 \times 10^{-8}$, Treatment: Males: $P = 0.064$, Females: $P = 0.377$) **(Table 3.5).**

3.7.1.1. Effects of Litter Size on F_1 Growth Rates

The effect of litter size was assessed on all time period growth rates. At time period 0, 2 and 3 there was no effect of litter size when stratified by sex. However, at time period 1 (suckling) there was a significant negative correlation associated with litter size, where increased litter size resulted in decreased pup weight. The effect was most severe in females and although not as severe in males the negative correlation was still apparent in males and did trend towards significance (Males: $P = 0.061$, Females: $P = 0.0002$) **(Table 3.6)**.

Table 3.6: Correlation rho and *P* values obtained from assessments of Litter Size on Growth Rate in the F_1 Generation

Period	Sex	Rho	*P*-value
0	M	-0.103	0.640[a]
	F	0.551	0.131[a]
1	M	-0.397	0.061^[b]
	F	-0.697	0.0002***[b]
2	M	-0.068	0.759[b]
	F	0.282	0.193[b]
3	M	0.125	0.570[a]
	F	-0.242	0.266[a]

Correlation assessed with [a] Spearman's Correlation Test, [b] Pearson's Correlation Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

To account for the significant effect of litter size at weaning an ANOVA was performed adjusting for both treatment and litter size. An association between litter size and growth was still present ($P = 0.016$), however, the effect of treatment had been lost ($P = 0.604$).

3.7.2 F_2 Mouse Weight Data

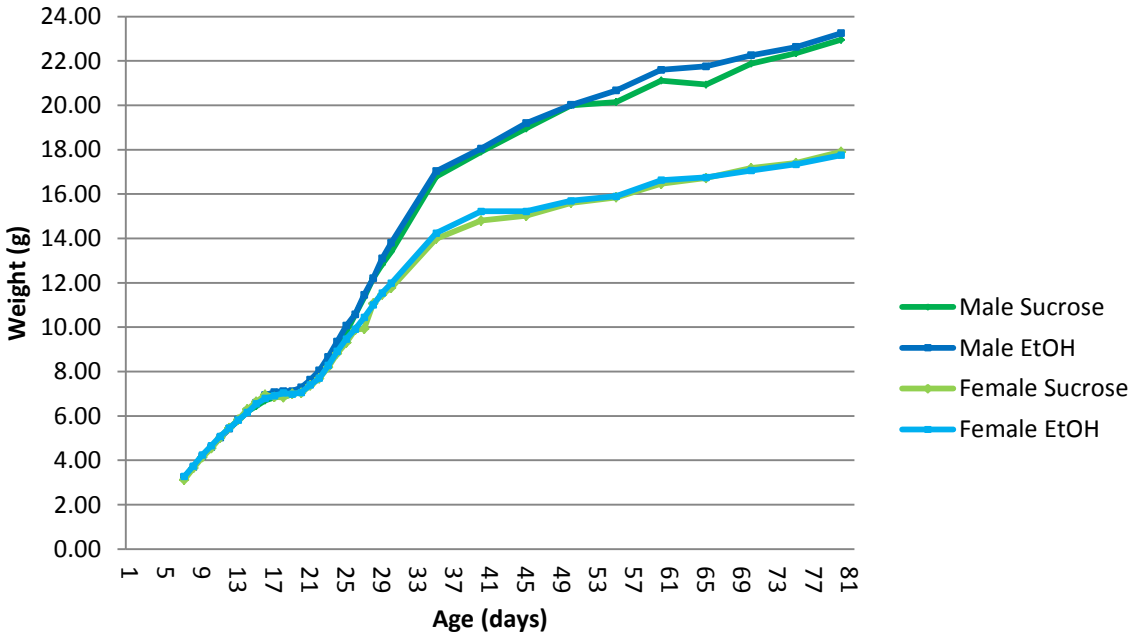


Figure 3.2: Overall Growth Rates of F_2 Individuals. Growth Rate of EtOH and S - Control (sucrose-treated control) F_2 individuals stratified by sex over the 73 day period.

Figure 3.2 indicates that there was little difference between all individuals at period 1 (PD7 – PD21). At PD22-30 a steep rise in growth rate was apparent and showed the most accelerated growth in all mice. From PD30 the sex divergence was evident and remained distinct throughout weight assessments. At all time periods little to no variability in growth rates between treatment groups was noted, however, at time period 3 some variability was apparent between treatment groups within males. More thorough analysis of the growth rate periods between treatment groups stratified by sex is displayed in **Table 3.7**.

Table 3.7: F_2 Generation Growth Rates stratified by Sex and Treatment across Time Periods 0, 1, 2 and 3

Period	Treatment	Sex	N	Mean Growth Rate ± SD	Min, Max	*P*-value[a] sex	*P*-value[a] treatment
0	EtOH	M	13	0.274 ± 0.016	0.248, 0.302	2.30×10^{-23}***	0.619
	S	M	17	0.271 ± 0.018	0.241, 0.303		
	EtOH	F	13	0.198 ± 0.007	0.186, 0.212		0.174
	S	F	20	0.203 ± 0.009	0.189, 0.220		
1	EtOH	M	13	0.313 ± 0.035	0.257, 0.367	0.376	0.931
	S	M	17	0.305 ± 0.049	0.187, 0.379		
	EtOH	F	13	0.262 ± 0.021	0.226, 0.287		
	S	F	20	0.304 ± 0.023	0.265, 0.349		
2	EtOH	M	13	0.721 ± 0.040	0.660, 0.778	7.52×10^{-11}***[b]	0.414[b]
	S	M	17	0.695 ± 0.070	0.510, 0.778		
	EtOH	F	13	0.535 ± 0.037	0.488, 0.594		0.159
	S	F	20	0.511 ± 0.053	0.354, 0.594		
3	EtOH	M	13	0.138 ± 0.036	0.092, 0.208	4.20×10^{-11}***	0.922
	S	M	17	0.137 ± 0.026	0.084, 0.182		
	EtOH	F	13	0.078 ± 0.013	0.058, 0.100		0.108
	S	F	20	0.087 ± 0.016	0.058, 0.114		

EtOH – Ethanol-treated, S – Sucrose-treated control
[a] *P*-value generated from Independent T-test [b] *P*-value generated from Wilcoxon Mann-Whitney Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

The overall growth rate of EtOH males (n = 13) was marginally higher than that of the control males (n = 17); whereas within females, the control females (n = 20) had a slightly higher growth rate than EtOH females (n = 13). However, these growth rate margins were small; males differed by 0.003g/day and females by 0.005g/day. The difference between sexes within treatment

groups was over ten times larger than the inter-treatment difference; EtOH males and females differed by 0.076g/day and control males and females differed by 0.067g/day. There was a significant effect of sex but no effect of treatment (Sex: $P = 2.30 \times 10^{-23}$, Treatment: Males: $P = 0.619$, Females: $P = 0.174$) **(Table 3.7).**

Within time period 1, EtOH females gained on average 0.262 ± 0.021g/day, whilst control females grew at a faster rate gaining 0.304 ± 0.023g/day. Within males, it appeared that the EtOH group grew at a slightly faster rate than the control group; 0.313 ± 0.035g/day and 0.305 ± 0.049g/day, respectively. There was little to no difference between males and females in the control group, however, there was a difference of 0.05g/day between EtOH males and females. There was no effect of sex or treatment (Sex: $P = 0.376$, Treatment: $P = 0.930$) **(Table 3.7).**

At time period 2 EtOH males and females grew at a faster rate than the controls. EtOH males grew 0.026g more per day than the controls and EtOH females grew an additional 0.024g/day. There was an effect of sex but none for treatment (Sex: $p = 7.52 \times 10^{-11}$, Treatment: Males: $P = 0.414$, Females: $P = 0.159$) **(Table 3.7).**

Time period 3 yielded little to no difference (0.001g/day) between the males of both treatment groups. Control females had a larger growth rate than that of EtOH females, 0.087 ± 0.016 and 0.078 ± 0.013, respectively. There was a significant effect of sex but none of treatment (Sex: $P = 4.20 \times 10^{-11}$, Treatment: Males: $P = 0.922$, Females: $P = 0.108$) **(Table 3.7).**

3.7.2.1 Effects of Litter Size on F_2 Growth Rates

The effect of litter size was assessed on all period growth rates and found to have no effect at time period 0 and 2. In contrast, there was a significant effect of litter size within females at time period 1 and 3, where a modest to strong negative correlation between litter size and pup weight was noted **(Table 3.8).**

Table 3.8: Correlation rho and *P* values obtained from Assessments of Litter Size on Growth rate in the F_2 Generation

Period	Sex	Rho	*P*-value[a]
0	M	-0.303	0.103
	F	-0.226	0.206
1	M	-0.087	0.648[b]
	F	-0.581	0.0004***[b]
2	M	-0.047	0.806
	F	0.164	0.361
3	M	-0.217	0.249
	F	-0.478	0.005**

Correlation assessed with [a] Spearman's Correlation Test, [b] Pearson's Correlation Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

As there was no effect of treatment litter size effect analysis was not necessary. However correlation assessments did show an effect of litter size on growth in females at time periods 1 and 3. Therefore, to ensure there was no effect of treatment ANOVAs were performed adjusting for both treatment and litter size at these periods. Associations between litter size and growth were still present (Time period 1: *P = 0.002;* Time period 3: *P = 0.023*), however, there were no effects of treatment (Time period 1: *P = 0.780;* Time period 3: *P = 0.555*).

3.7.3 F_3 Mouse Weight Data

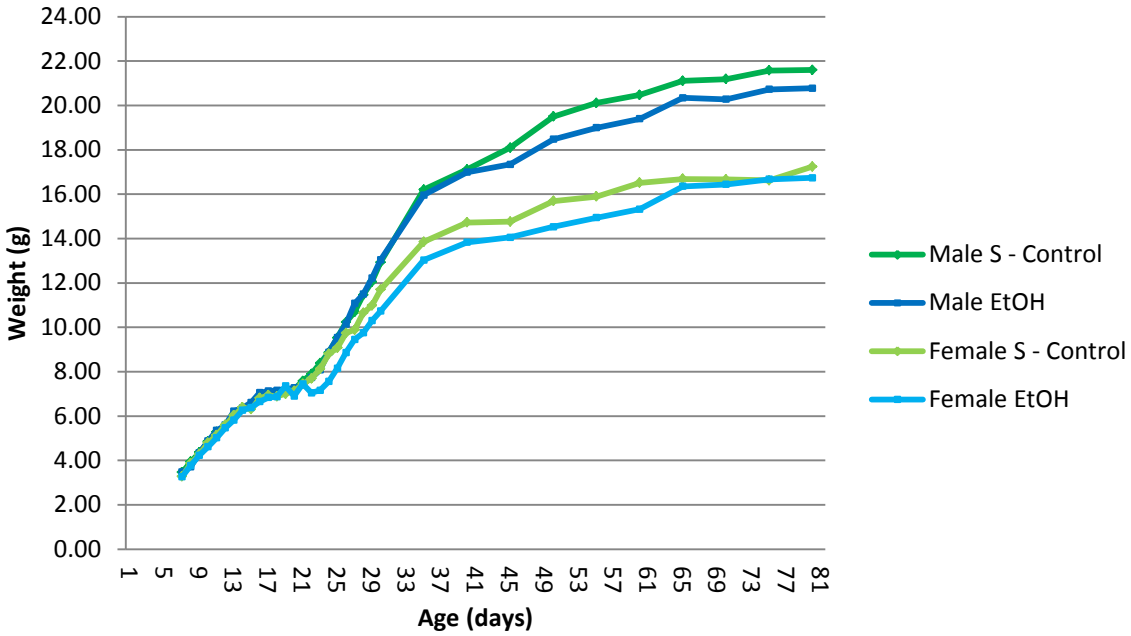


Figure 3.3: Overall Growth Rates of F_3 Individuals. Growth Rate of EtOH and S - Control (sucrose – treated control) F_3 individuals stratified by sex over the 73 day period.

Figure 3.3 shows little difference between pups at PD7 to PD21. Variance between female individuals appeared from about PD22 and continued to PD80, except where they coincided at 69 days to 77 days. Males appeared to have similar growth rates until age 35 days. Following this point their growth rates diverged and continued to do so throughout analysis. Similar to previous generations at age 22-35 days F_3 undergoes the most rapid growth. More detailed analysis of growth rates between treatment groups amongst the different time periods is depicted in **Table 3.9**.

Table 3.9: F_3 Generation Growth Rates stratified by Sex and Treatment across Time Periods 0, 1, 2 and 3

Period	Treatment	Sex	N	Mean Growth Rate ± SD	Min, Max	*P*-value[a] sex	*P*-value[a] treatment
0	EtOH	M	6	0.237 ± 0.014	0.221, 0.260	0.0001***	0.208
	S	M	6	0.248 ± 0.015	0.223, 0.265		
	EtOH	F	6	0.185 ± 0.004	0.180, 0.189		0.446
	S	F	4	0.191 ± 0.015	0.178, 0.213		
1	EtOH	M	6	0.283 ± 0.019	0.269, 0.320	0.553[b]	0.187[b]
	S	M	6	0.294 ± 0.023	0.274, 0.330		
	EtOH	F	6	0.299 ± 0.082	0.249, 0.459		
	S	F	4	0.296 ± 0.037	0.254, 0.339		
2	EtOH	M	6	0.663 ± 0.049	0.616, 0.750	1.06×10^{-5}***	0.426
	S	M	6	0.629 ± 0.089	0.488, 0.724		
	EtOH	F	6	0.461 ± 0.076	0.351, 0.558		0.372
	S	F	4	0.500 ± 0.041	0.459, 0.544		
3	EtOH	M	6	0.107 ± 0.026	0.079, 0.134	0.0004***	0.317
	S	M	6	0.120 ± 0.014	0.098, 0.139		
	EtOH	F	6	0.082 ± 0.017	0.054, 0.107		0.501
	S	F	4	0.075 ± 0.013	0.060, 0.091		

EtOH – Ethanol-treated, S – Sucrose-treated control
[a] *P*-value generated from Independent T-Test, [b] *P*-value generated from Wilcoxon Mann-Whitney Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

The overall growth rate of EtOH males (n = 6) was lower than that of control males (n = 6). This trend between treatment groups was also present within females, where EtOH females (n = 6) showed a growth rate of 0.185 ± 0.004g/day and control females (n = 4) 0.191 ± 0.015g/day. Males of both groups tended to grow at around 0.05g more per day than their respective females. Overall growth rate did not differ based on treatment group (Males: *P = 0.208*, Females: *P = 0.446*). However, there was a significant effect of sex (*P = 0.0001*) **(Table 3.9).**

During suckling, time period 1, control males had a growth rate that was slightly greater than that of the EtOH males; however, within females the EtOH group had a marginally higher growth rate. The variation between sexes and treatment groups was low with the biggest difference of 0.016g/day being seen between EtOH males and females, with the latter having the greater

growth rate. There was no effect of sex or treatment (Sex: *P = 0.553*, Treatment: *P = 0.187*) **(Table 3.9).**

In contrast to time period 1, at weaning (time period 2) the EtOH males grew at a much higher rate than the control males. However, amongst females the control group grew at a higher rate when compared to the EtOH group. A difference of 0.034g/day between males of the two treatment groups and a difference of 0.039g/day between females was noted. There was an effect of sex ($P = 1.06 \times 10^{-5}$), however there was no significant difference based on treatment (Males *P = 0.426*, Females *P = 0.372*) **(Table 3.9).**

Growth rates of mice in time period 3 followed a similar pattern to time period 1 where control males exhibited the highest growth rate. Within females it was the control females with the higher growth rate. Within the EtOH group females grew at a rate equivalent to 76% of the male growth rate. In the control group females grew at a rate equivalent to 62.5% of that of males. There was no difference based on treatment group (Males *p = 0.317*, Females *p = 0.501*). However, there was a significant effect of sex (*p = 0.0004*) **(Table 3.9).**

3.7.3.1 Effects of Litter Size on F_3 Growth Rates

Litter size had no effect on growth rates at any time period when stratified by sex **(Table 3.10)**.

Table 3.10: Correlation rho and p values obtained Assessments of Litter Size on Growth Rate in the F_3 Generation

Period	Sex	Rho	*P*-value[a]
0	M	-0.048	0.882[a]
	F	-0.348	0.324[a]
1	M	-0.290	0.361.[a]
	F	-0.348	0.324[a]
2	M	-0.202	0.576[b]
	F	0.112	0.728[b]
3	M	0.056	0.863[b]
	F	0.189	0.602[b]

Correlation assessed with [a] Spearman's Correlation Test, [b] Pearson's Correlation Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending Significance^ ($0.1 \leq P < 0.05$)

3.7.4 F_3 Outcross Mouse Weight Data

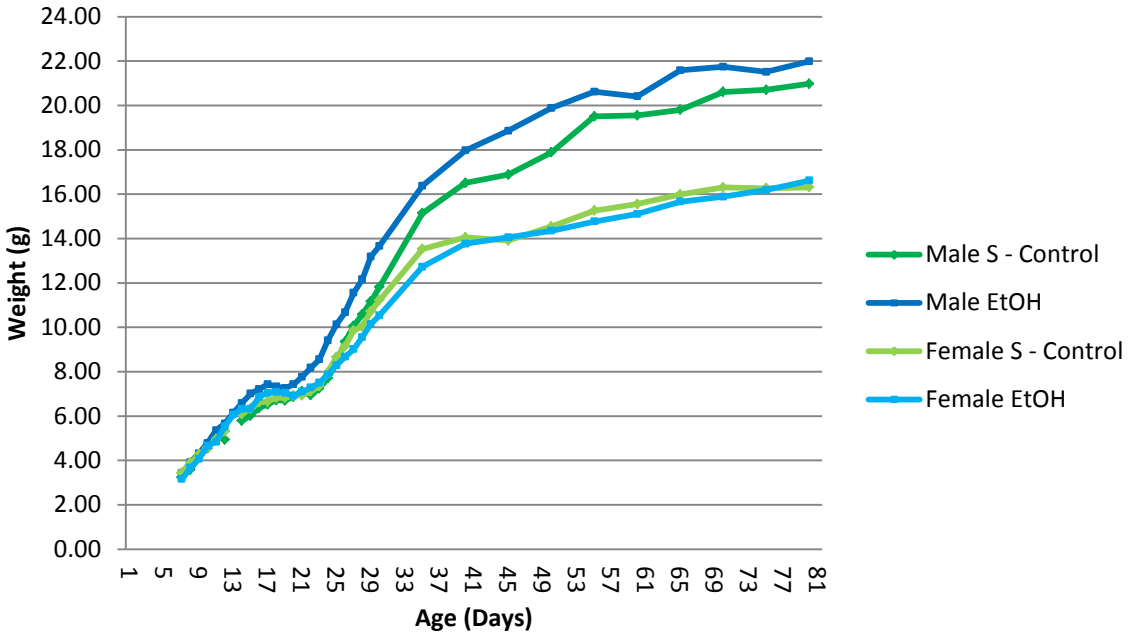


Figure 3.4: Overall Growth Rates of F_3 Outcross Individuals. Growth Rate of EtOH and S - Control (sucrose – treated control) F_3 Outcross individuals stratified by sex over the 73 day period.

Figure 3.4 shows that from PD13 there was variability across treatment groups, with the male EtOH group having the highest growth rate. Between male treatment groups variability continued throughout analysis; however amongst female treatment groups a difference was noted, although variability was to a much lower extent. From age 22 days to age 35 days the fastest growth rate for all individuals occurred. More detailed analysis of growth rates at the different time periods between treatment groups is depicted in **Table 3.11**.

Table 3.11: F_3 Outcross Generation Growth Rates stratified by Sex and Treatment across Time Periods 0, 1, 2 and 3

Period	Treatment	Sex	N	Mean Growth Rate ± SD	Min, Max	*P*-value[a] sex	*P*-value[a] treatment
0	EtOH	M	3	0.254 ± 0.013	0.240, 0.265	0.0034**	0.564
	S	M	2	0.243 ± 0.023	0.227, 0.259		
	EtOH	F	5	0.184 ± 0.016	0.165, 0.202		0.655
	S	F	3	0.176 ± 0.003	0.173, 0.180		
1	EtOH	M	3	0.310 ± 0.019	0.294, 0.332	0.188	0.107
	S	M	2	0.276 ± 0.053	0.240, 0.315		
	EtOH	F	5	0.281 ± 0.028	0.256, 0.313		
	S	F	3	0.249 ± 0.015	0.234, 0.263		
2	EtOH	M	3	0.686 ± 0.024	0.664, 0.711	0.0084**	0.564
	S	M	2	0.609 ± 0.112	0.530, 0.689		
	EtOH	F	5	0.406 ± 0.143	0.188, 0.530		0.297
	S	F	3	0.513 ± 0.033	0.475, 0.538		
3	EtOH	M	3	0.125 ± 0.028	0.092, 0.141	0.0128*	0.564
	S	M	2	0.130 ± 0.022	0.114, 0.145		
	EtOH	F	5	0.086 ± 0.031	0.059, 0.133		0.297
	S	F	3	0.062 ± 0.004	0.058, 0.064		

EtOH – Ethanol-treated, S – Sucrose-treated control
[a] *P*-value generated from Wilcoxon Mann-Whitney Test
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

At time period 0, the overall growth rate of F_3 Outcross EtOH males (n = 3) and females (n = 5) was higher than that of that of the control males (n = 2) and females (n = 3). The trend, whereby the EtOH group displayed a higher growth rate, continued into time period 1 and 2. Unlike previous time periods at time period 2 control females had a much higher growth rate than that of EtOH females. At time period 3 control males displayed the highest growth rate between males, however, EtOH females returned to having the largest growth rate between the two treatment groups **(Table 3.11)**.

3.7.4.1 Effects of Litter Size on F_3 Outcross Growth Rates

Litter size had no effect on growth rates at any time period when stratified by sex **(Table 3.12)**. However, females did demonstrate modest negative correlations between litter size and growth

at overall growth rate, time period 1 and 3. Time period 3 trended towards significance but the remaining periods did not. The small number of males within this group reduced effective correlation analysis between litter size and growth.

Table 3.12: Correlation rho and p values obtained Assessments of Litter Size on Growth Rate in the F_3 Outcross Generation

Period	Sex	Rho	*P*-value[a]
0	M	-0.474	0.420
	F	-0.582	0.130
1	M	0.000	1.000
	F	-0.449	0.265
2	M	0.000	1.000
	F	0.461	0.251
3	M	0.000	1.000
	F	-0.679	0.064^

Correlation assessed with [a] Spearman's Correlation Test,
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending Significance^ ($0.1 \leq P < 0.05$)

3.7.5 Growth Rate Analysis Summary

Overall growth rate displayed a significant sex effect at all generations. This sex effect was also apparent during weaning and adulthood but not at suckling. At the F_1 generation the overall growth rate between EtOH and control groups did not differ significantly. However, the EtOH males did display a lower growth rate than the control males. A treatment effect was noted at the F_1 generation during suckling but this was attributed to litter size. At the F_2 generation the overall growth rate of the EtOH group (males and females) was marginally higher than that of the control (males and females). Within the F_3 generation there was no significant difference between the growth rates of EtOH and control mice. However, the overall growth rate of EtOH males and females was lower than that of control males and females. At the F_3 Outcross generation the overall growth rate of the individuals arising from crosses between EtOH-treated dams and control sires was higher than that of those arising from control dam and EtOH-treated sires.

3.8 Open-Field Activity Analysis

The open-field test was used to determine whether ethanol exposure at the F_0 generation had an effect on general motor activity and anxiety on successive generations.

3.8.1 General Motor Activity

Total distance travelled in the open-field testing area served as the performance measure for general motor activity and exploratory behaviour. Increased total distance travelled indicated a measure of hyperactivity yielding from increased locomotor activity and increased exploratory behaviour with low levels of anxiety. The parametric Two-sample Independent T-test was used to assess whether a significant difference between treatment groups existed following chronic maternal ethanol consumption at the F_0 generation. Results are tabulated in **Appendix J** and presented graphically below in **Figure 3.5**.

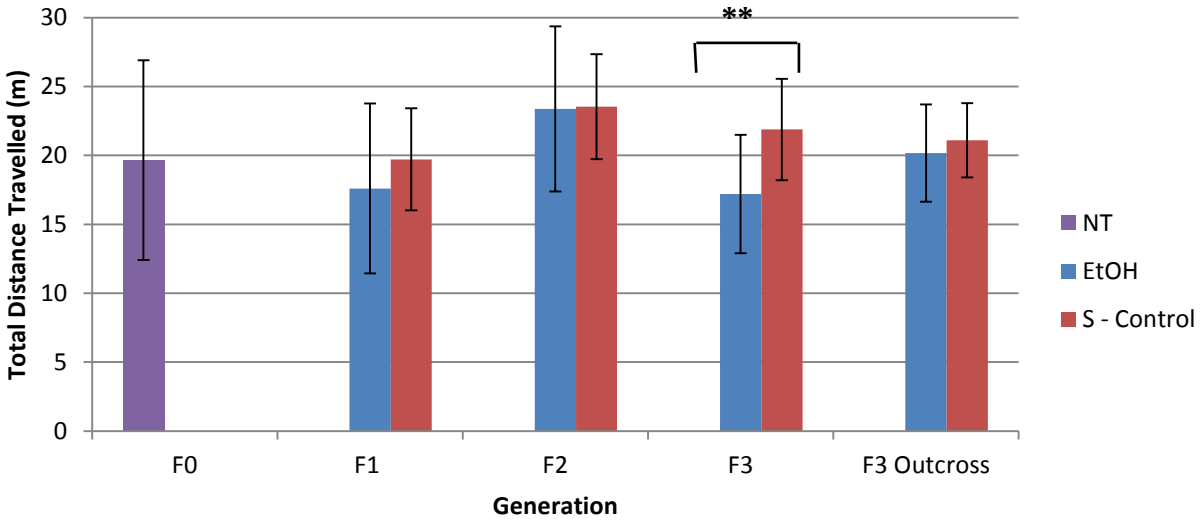


Figure 3.5: Total Distance Travelled between Treatment Groups at Generations F_0-F_3 Outcross. The total distance travelled in meters between treatment groups; NT – No treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1, F_2, F_3 and F_3 Outcross generation. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$).

The F_0 generation contained no treatment groups as the test was performed prior to the initiation of treatment in females. The mean distance travelled amongst the F_0 individuals (n = 30) was 19.66m ± 7.24m. At subsequent generations the EtOH-exposed mice appeared to travel

less than the sucrose-exposed (control) mice. At the F_1 generation the EtOH (n = 19) group travelled 17.60m ± 6.16m and the control (n = 24) group travelled 19.71m ± 3.7m. The difference between treatment groups at the F_2 generation was marginal; control (n = 37) mice traversed on average 23.53m ± 3.81m and the EtOH (n = 26) group 23.37m ± 5.99m. The F_3 generation showed the greatest difference between treatment groups. The F_3 EtOH (n = 12) group travelled 17.20m ± 4.30m, whilst the F_3 control (n = 10) group travelled 21.87m ± 3.68m. The F_3 Outcross group continued the trend where EtOH individuals travelled less than their control counterparts; however, the difference in distance was relatively small. The EtOH (n = 8) group moved on average 20.17m ± 3.53m and the control (n = 5) group 21.10m ± 2.70m. Upon assessment there was no effect of sex (F_0: *P = 0.875*, F_1: *P = 0.432* F_2**:** *P = 0.128,* F_3: *P = 0.954,* F_3 Outcross: *P = 0.954*). However, there was an effect of treatment at the F_3 generation (F_1: *P = 0.205*, F_2: *P = 0.917*, F_3: *P = 0.009,* F_3 Outcross: *P = 0.625*) **(Appendix and Figure 3.5)**. In addition to differences in average distances travelled, differences in standard deviation were observed with mice in the EtOH group showing greater variability in distance travelled compared to those of the control group. This was most apparent at the F_1 generation (F-test *P = 0.0441*), whereas F_2 and F_3 generations did not reach statistical significance.

3.8.2 Anxiety

Anxiety was measured by assessing the time spent in the inner zone, faecal boli count, and amount or urine produced while in the open-field during the 300 second testing period. An increase in the time spent in the inner zone indicated an increase in exploratory behaviour and lower levels of anxiety. Increased faecal boli count and amount of urine are also associated with the increased levels of anxiety. Hall (1934) indicates that the reduced amount of locomotor activity in the novel environment will activate the rodents autonomic system increasing the amount of faeces and urine produced. To assess if there was a significant difference between treatment groups in these measures the parametric Two–sample Independent T-test or non-parametric Wilcoxon Mann-Whitney Test was used depending on the distribution of the data.

3.8.2.1 Inner Zone Time

As mice tend to seek protection from the relatively more vulnerable centre of the open field area they spend more time in the peripheral regions. Mice that are less anxious will spend more time in the centre. Time spent in the inner zone was measured as the amount of time (in seconds) the mouse spent in the demarcated inner zone of the open-field testing area. Results are tabulated in **Appendix K** and presented graphically below in **Figure 3.6**.

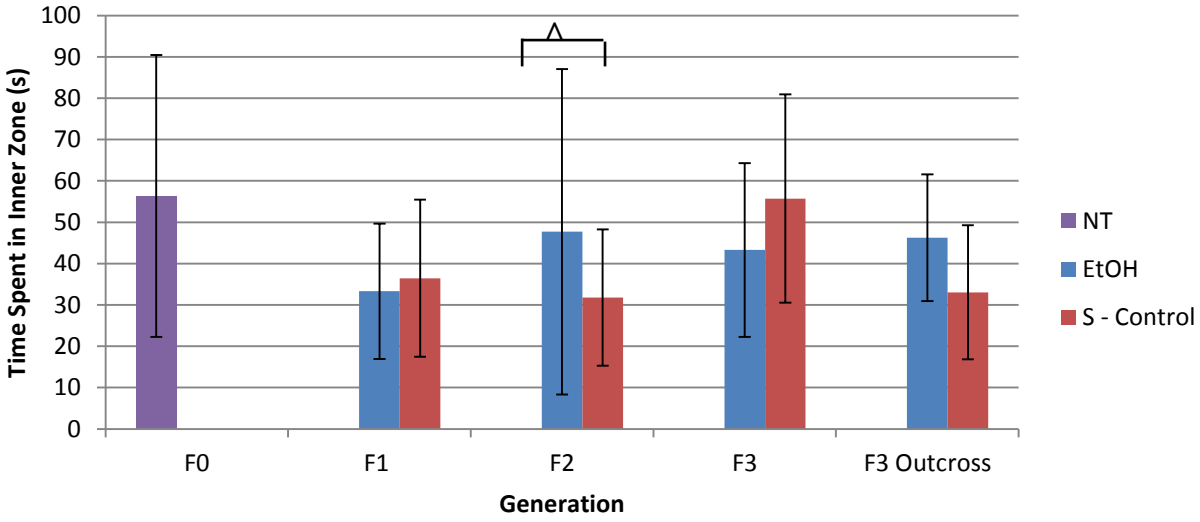


Figure 3.6: Time Spent in the Inner Zone between Treatment Groups at Generations F_0-F_3 Outcross. The time spent in the inner zone, in seconds, between treatment groups; NT – No treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1, F_2, F_3 and F_3 Outcross generations. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$).

The F_0 generation contained no treatment groups. On average the F_0 (n = 30) mice spent 56.34s ± 34.07s in the inner zone. At the F_1 generation there was a small difference between treatment groups, the EtOH (n = 19) group spent 33.30s ± 16.39s in the inner zone and the control (n = 24) group spent 36.43s ± 19.00s in the inner zone. At the F_3 generation the difference between treatment groups was larger, the EtOH (n = 12) group frequented the inner zone for 43.28s ± 21.03s and the control (n = 10) for 55.72m ± 25.18m. In contrast, at the F_2 and F_3 Outcross generations the control group spent more time in the inner zone. The F_2 EtOH (n = 26) mice spent 47.70s ± 39.36s in the inner zone, whereas the F_2 control (n = 37) mice were in the inner zone for 31.80s ± 16.47s. This was the largest difference between treatment groups amongst all generations. The F_3 Outcross EtOH (n = 8) group were in the inner zone for 46.24s ± 15.32s and

the control (n = 5) for 33.04s ± 16.23s. Analysis revealed no effect of sex (F_0: *P = 0.378,* F_1: *P = 0.651;* F_2: *P = 0.625,* F_3: *P = 0.847,* F_3 Outcross*: P = 0.242*). There was a trending effect of treatment at the F_2 generation (F_1: *P = 0.680,* F_2*: P = 0.079,* F_3*: P = 0.221*, F_3 Outcross: *P = 0.242*) **(Appendix K and Figure 3.6)**.

3.8.2.2 Faecal Boli Count

Faecal boli count was measured as the number of stools produced by the mouse during the 300 second testing period. Male mice of subsequent generations produced higher faecal boli counts than females irrespective of treatment group. Data were stratified by sex due to significant sex differences (F_1: EtOH *P = 0.009,* S-Control: *P = 0.020;* F_2: EtOH *P = 0.934,* S - Control *P = 0.002;* F_3: EtOH *P = 0.022, S-Control: P = 0.073;* F_3 Outcross: EtOH *P = 0.010, S - Control P = 1.00*). Results are tabulated in **Appendix L** and displayed in **Figure 3.7** and **Figure 3.8**.

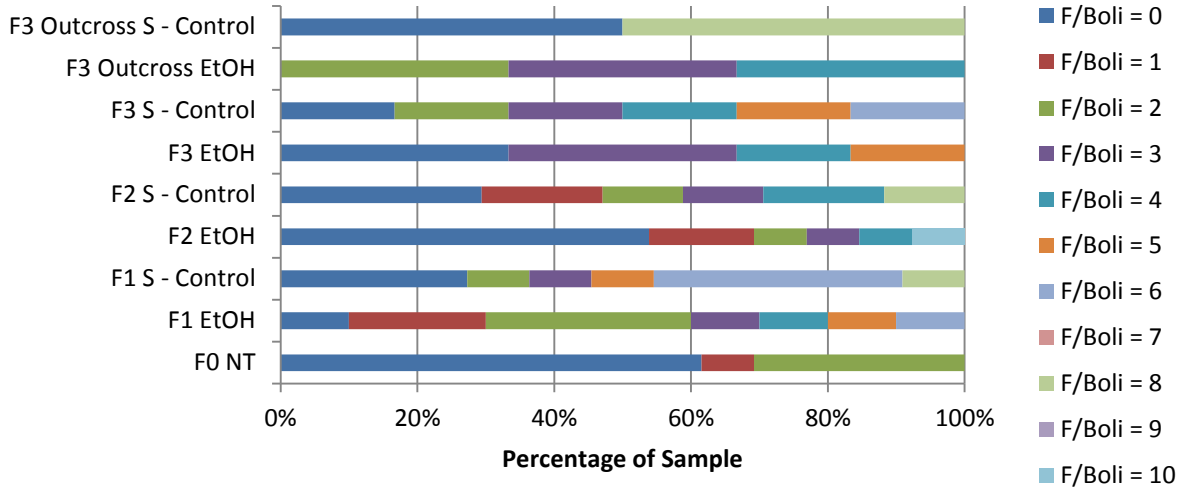


Figure 3.7: Number of Faecal Boli produced by Male Mice between Treatment Groups at Generations F_0-F_3 Outcross. The number of faecal boli (F/Boli) produced by male mice between treatment groups; NT – No treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1, F_2, F_3 and F_3 Outcross generations. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$).

The F_0 males (n = 11) showed some variability in defecation with sires producing 0, 1 or 2 boli. However, the majority of untreated sires produced no boli. At all subsequent generations the number of boli produced varied greatly despite treatment group. However, control males deposited more faecal boli than their EtOH counterparts at all generations **(Appendix L)**. The F_1

control (n = 11) group showed the greatest variability with faecal boli counts ranging from 0 to 8. The largest proportion (36%) of the F_1 control sires produced 6 boli. In the F_1 EtOH (n = 10) group the boli produced ranged from 0 to 6, with the majority producing 2 boli. At the F_2 generation the EtOH (n = 13) males 54% of the sires produced 0 boli, whilst the F_2 control (n = 17) sires showed greater variability with 18% producing 1 bolus, 185 producing 4 and 29% of sires producing no boli. The F_3 controls (n = 6) showed the greatest variability with the number of boli produced ranging from 0 to 6 and each number of boli being produced at an equal proportion (17%). The F_3 EtOH (n = 6) group showed less variability with an equal proportion of mice (33%) producing 0 and 3 boli. produced no boli, whilst the controls of the F_3 Outcross (n = 3) mainly produced 1 bolus (66%). At the F_3 Outcross EtOH (n = 3) group each sire produced a different number of boli, 2, 3 and 4. The control (n = 2) group consisted of one individual producing 1 bolus another producing 8 boli. There was no effect of treatment at all generations (F_1: *P = 0.256,* F_2: *P = 0.200,* F_3: *P = 0.511,* F_3 Outcross: *P = 1.000*) **(Appendix L and Figure 3.7)**.

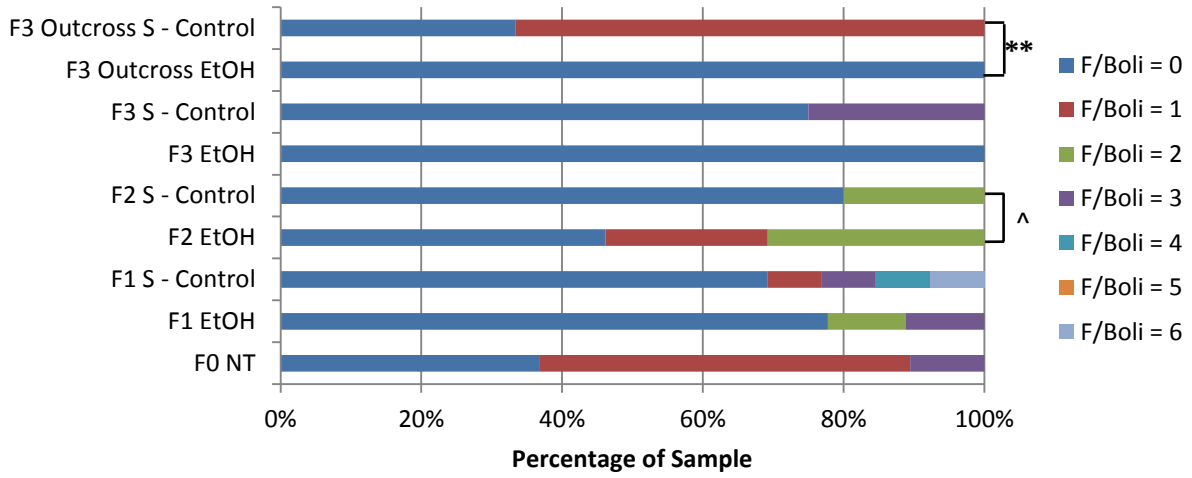


Figure 3.8: Number of Faecal Boli produced by Female Mice between Treatment Groups at Generations F_0-F_3 Outcross. The number of faecal boli (F/Boli) produced by female mice between treatment groups; NT – No treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1, F_2, F_3 and F_3 Outcross generations. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$).

The F_0 females (n = 19) showed some variability in defecation with dams producing 0, 1 or 3 boli. However, 52% of the dams produced 1 bolus. At the F_1, F_3 and F_3 Outcross generations the control group produced more boli than their EtOH counterparts. However, the majority of all

groups produced no boli. The F_1 control (n = 13) group showed the greatest variability with faecal boli counts ranging from 0 to 6; however, 69% of dams produced no boli. Although the degree of variability was slightly less in the F_1 EtOH (n = 9) group, as with the control the majority (78%) had a boli count of zero. At the F_2 generation the EtOH (n = 13) females 46% of the dams produced 0 boli, whilst 80% of the F_2 controls (n = 20) produced no boli. The F_3 and F_3 Outcross EtOH (n = 6; n = 5) groups showed no variability with all dams producing no boli. The majority of the F_3 controls (n = 4) showed produced no boli, whilst the controls of the F_3 Outcross (n = 3) mainly produced 1 bolus (66%). A significant effect of treatment was noted at the F_3 Outcross generation and an effect trending towards significance was noted in the F_2 females (F_1: *P = 0.654,* F_2: *P = 0.092,* F_3: *P = 0.221,* F_3 Outcross: *P = 0.008*) **(Appendix L and Figure 3.8)**.

3.8.2.3 Amount of Urine

Amount of urine was measured as the number of urine pools or urine streaks produced by a mouse during the 300 second testing period. In all generations and treatment groups male mice produced more urine than females during testing **(Appendix L).** Generations F_1, F_2 and F_3 displayed significant sex differences in at least one treatment group (F_0: *P = 0.536*, F_1: EtOH *P = 0.018,* S – Control *P = 0.019,* F_2: EtOH *P = 0.605,* S - Control *P = 0.002,* F_3: EtOH *P = 0.058,* S - Control *P = 0.027,* F_3 Outcross: EtOH *P = 0.124,* S - Control *P = 0.739*). Consequently, data were analysed stratified for sex.

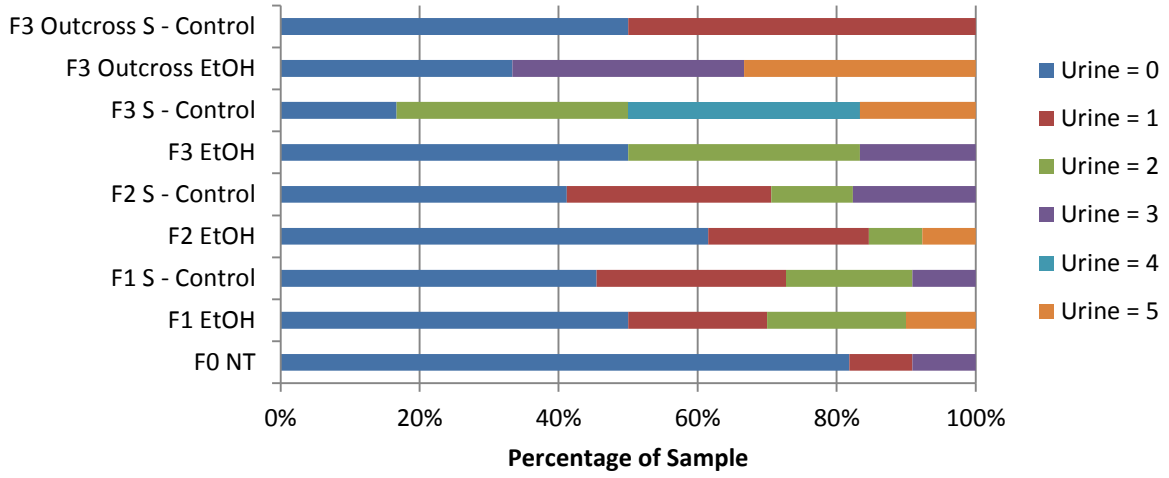


Figure 3.9: Amount of Urine produced by Male Mice between Treatment Groups at Generations F_0-F_3 Outcross. The amount of urine (number of pools or streaks) produced by male mice between treatment groups; NT – No treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1, F_2, F_3 and F_3 Outcross generations. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$).

Within the F_1 and F_3 Outcross generations the EtOH males produced more urine than the control males. The males show great variability within the amount of urine produced. The F_1 control and EtOH groups show similar levels of variability. 50% of F_1 EtOH (n = 10) male mice produced 0 pools/streaks, whereas 38% controls (n = 11) produced 0 pools/streaks. The largest difference between treatment groups was seen at the F_3 Outcross generation; the EtOH (n = 3) group deposited 33% of 1.3 and 5 pools/streaks, respectively. 50% of the control (n = 2) group produced no pools/streaks the other 1. At the F_2 and F_3 generations the inverse relationship between groups was apparent as control males produced more urine. 69% of F_2 EtOH (n = 13) males produced no pools/streaks of urine, whereas 59% of the control (n = 20) produced 1 or above pools/streaks of urine. The EtOH (n = 6) group of the F_3 generation produced 50% no pools/streaks of urine, whereas 83% of the control (n = 6) male mice produced more than 2 boli. There was no effect of treatment at all generations assessed (F_1: $P = 0.970$, F_2: $P = 0.277$, F_3: $P = 0.114$, F_3 Outcross: $P = 0.374$) **(Appendix L and Figure 3.9)**.

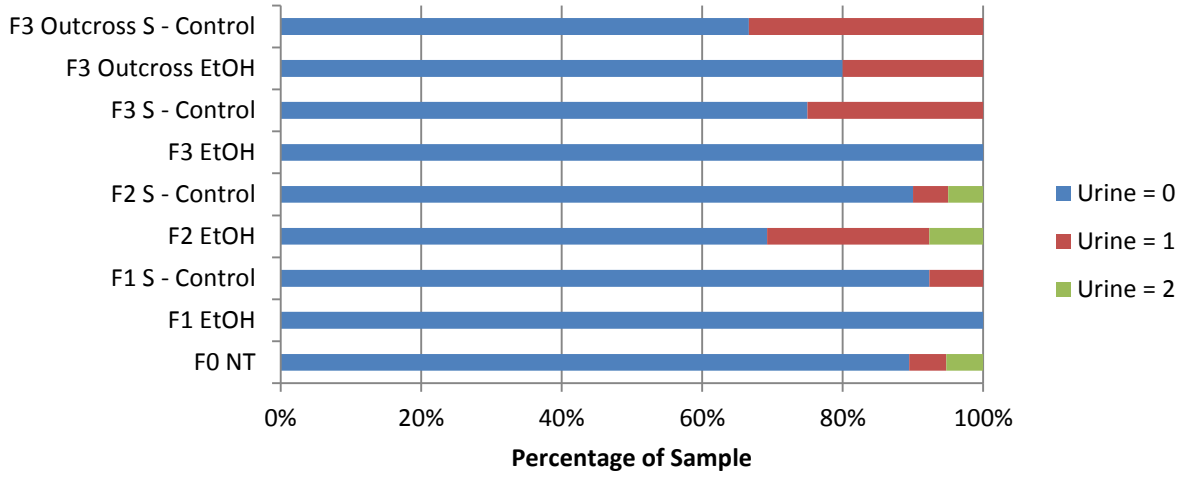


Figure 3.10: Amount of Urine produced by Female Mice between Treatment Groups at Generations F_0-F_3 Outcross. The amount of urine (number of pools or streaks) produced by female mice between treatment groups; NT – No treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1, F_2, F_3 and F_3 Outcross generations. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$), Trending significance ^ ($0.1 \leq P < 0.05$).

Within the females, the control group produced more urine in all generations apart from in F_2 where the EtOH females produce a higher amount of urine. The F_1 and F_3 EtOH (F_1: n = 9; F_3: n = 6) group produced no pools/streaks of urine. The majority of the F_1 (92%) and F_3 (75%) control (F_1: n = 13; F_3: n = 4) groups produced no pools/streaks of urine. In contrast to the other generations at the F_2 generation the EtOH group produced more pools/streaks of urine than the control females. However, the majority (89%) of control (n = 20) females produced no pools/streaks of urine. 69% of EtOH (n = 13) females produced no pools/streaks of urine, whereas 29% produced 1 pools/streaks of urine. 80% of the F_3 Outcross EtOH group produced no pools/streaks of urine, whilst the 67% of F_3 Outcross control group produced no pools/streaks of urine. There was no effect of treatment at all generations when accounting for sex (F_1: *P = 0.405,* F_2: *P = 0.154,* F_3: *P = 0.221,* F_3 Outcross: *P = 0.693*) **(Appendix L and 3.10)**.

3.8.3 Open-Field Analysis Summary

There was no significant effect of sex on locomotor activity and time spent in the inner zone. EtOH treatment appeared to have an effect on locomotor activity with decreased total distance travelled being found in all generations (F_1 – F_3). However, the F_3 generation was the only

generation to display a significant effect of treatment (*P = 0.009*). The time spent in the inner zone was decreased in the EtOH groups of the F_1 and F_3 generations and increased at the F_2 generation. The F_2 generation displayed a treatment effect trending towards significance (*P = 0.079*). Faecal boli and the amount of urine produced displayed significant sex effects. Males showed great variability in the number of faecal boli and amount of pools/streaks of urine produced. Control males produced more boli than their EtOH counterparts, whereas EtOH males produced more urine than the controls. In contrast, females showed little variability in number of faecal boli or amount of urine produced with the majority of females producing no boli and no urine despite treatment group or generation. There was no significant effect of treatment amongst males. Females displayed a significant and trending towards significant treatment effect at the F_2 and F_3 Outcross generation, respectively (F_3 Outcross: *P = 0.008*; F_2: *P = 0.092*).

3.9 The Effect of Prenatal Ethanol Exposure on DNA Methylation

The methodology and quality control measures used in methylation assessment included samples being PCR amplified in duplicate, as well as samples being amplified within the same PCR machine so as to reduce variation associated with different PCR machine use. Each PCR sample was assayed on the Pyrosequencer. Assay validation ensured minimum PCR amplification bias (**See 2.6.2**). Following PCR and the assessment of products for amplification and contamination, samples underwent Pyrosequencing. Assay reproducibility was assessed with assay validation confirming that the Pyrosequencer was calling true and correct methylation percentages (**See 2.6.2**). The average methylation percentage for each CpG site was calculated by averaging duplicate values for each CpG site for each sample. Inter-sample variation greater than 5% was excluded from the study. The average methylation percentage per CpG site was then averaged across the six CpG sites of CTCF1 to give CTCF1 mean methylation percentage per sample, the same was done for the five CpG sites of CTCF2 to obtain the mean methylation of CTCF2.

Batch effects were kept to a minimum in both the Pyrosequencing PCR and Pyrosequencing run by forming 96 well plates that contained samples ranging in treatment group, sex, and generation. Each PCR and Pyrosequencing plate contained a 0% and a 100% methylated control, as well as a common sample to allow for inter-plate comparisons. In addition to the above controls the Pyrosequencing plate contained a bisulfite modified (BM) water control and a PCR control. The BM control was the water control from the bisulfite modification step prior to Pyrosequencing PCR. The BM and PCR controls were carried through all subsequent methodology steps. Several attempts yielded results with clear PCR controls and the correct 0% and 100% methylation control readings, however, data were obtained at the BM control level, subsequently leading to the results being discarded and the assays repeated. Pyrosequencing data for the CTCF1 binding site is displayed in **Appendix M** and Pyrosequencing data for the CTCF2 binding site is displayed in **Appendix N**.

3.9.1 Mouse Model for DNA Methylation Analyses

Data were obtained for 30 (17%) of the original 173 samples. Tail biopsies were not collected from all individuals due to difficulties with surgery (9%), as well as samples being removed from the study due to time constraints (74%). The samples assessed represented generations F_0 to F_3. The females at the F_0 generation represented 2 mothers destined to become EtOH-exposed and 2 mothers destined to become sucrose-exposed. Generations F_1 to F_3 were equally divided by sex and treatment **(Table 3.13)**.

Table 3.13: Mice Frequencies for DNA Methylation Analyses according to Treatment and Sex at Generations F_0-F_3

Generation	Treatment	Sex	N
F_0	NT	M	2
	NT	F	4
F_1	EtOH	M	2
	EtOH	F	2
	S	M	3
	S	F	1
F_2	EtOH	M	2
	EtOH	F	2
	S	M	2
	S	F	2
F_3	EtOH	M	2
	EtOH	F	2
	S	M	1
	S	F	3
Total			30

NT – No Treatment, EtOH – Ethanol-treated group, S – Sucrose-control group

3.9.2 CTCF1 Methylation Analysis

Due to the small sample size of methylation data non-parametric tests were used for statistical analysis. In addition, in order to increase power the effect of sex upon both the mean methylation level and individual CpG sites was initially assessed across all generations together. It was assumed that if there was an effect of sex at the generation level it would be perpetuated across all generations and therefore better observed when looking at the group as a whole. Analyses were subsequently performed stratified by generation for confirmation. No significant

effect of sex was seen on mean methylation level or per CpG site when looking at all samples together or when stratified per generation **(Appendix O).** Furthermore, strong pairwise correlations were observed between methylation levels at the 6 CpG sites spanning the CTCF1 region **(Figure 3.11)**.

Figure: 3.11 CTCF1 CpG site Correlation Matrix. Correlation between Methylation levels at CpG sites 1 to 6 in binding site CTCF1 (n = 30).

3.9.2.1 Mean Methylation Analysis

The mean methylation levels at CTCF1 between treatment groups and at generations F_0 to F_3 are depicted in **Table 3.14** and are displayed graphically in **Figure 3.12**. The non-parametric Wilcoxon Mann-Whitney test was used to assess if there was a significant difference between treatment groups following chronic maternal ethanol consumption at the F_0 generation.

Table 3.14: Mean Methylation Level across CTCF1 CpG sites 1 to 6 between Treatment Groups at Generations $F_0 – F_3$

Generation	Treatment	N	Mean Methylation Level ± SD (%)	Median (Range)	*P*-value[a]
0	NT	6	56.50 ± 1.69	(55.03, 59.79)	-
1	EtOH	4	54.88 ± 0.83	(54.17, 56.07)	0.021*
	S	4	57.74 ± 1.36	(56.23, 59.03)	
2	EtOH	4	60.71 ± 2.63	(58.20, 64.36)	0.083^
	S	4	54.31 ± 6.90	(44.37, 60.07)	
3	EtOH	4	54.62 ± 1.74	(52.65, 56.53)	0.083^
	S	4	56.77 ± 1.79	(54.16, 58.04)	

NT–No treatment, EtOH–Ethanol-treated, S–Sucrose-treated control
[a] *P*-values generated from Wilcoxon Mann-Whitney
Statistically significant ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$).

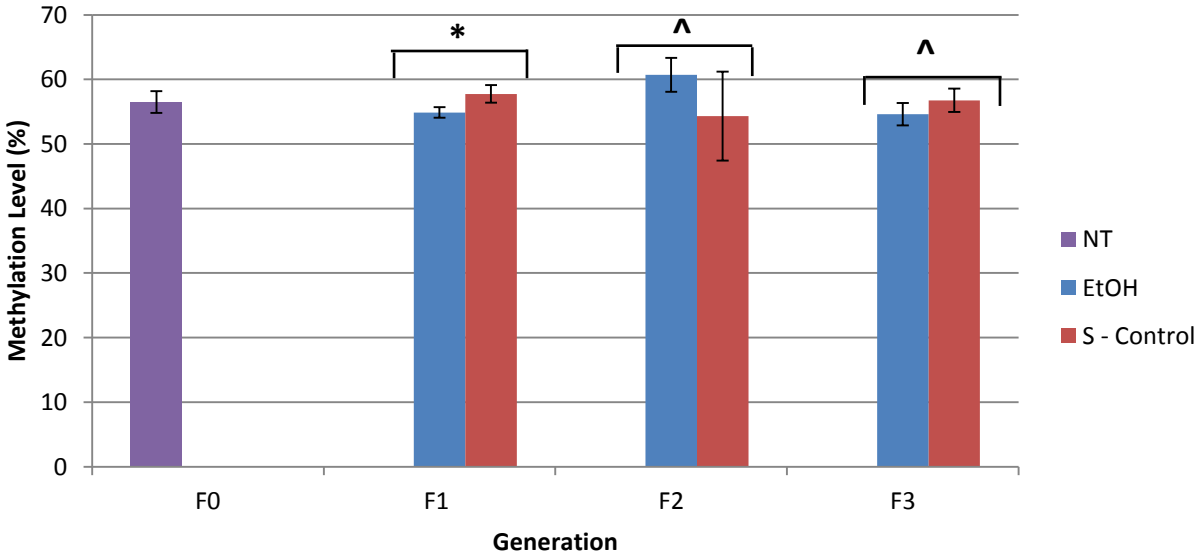


Figure 3.12: Mean Methylation Levels across CTCF1. Mean Methylation levels across CTCF1 between Treatment Groups, NT- No Treatment, EtOH – Ethanol-treated, Sucrose – Sucrose-treated control, across Generations F_0, F_1, F_2 and F_3. Statistically significant ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

The F_1 EtOH (n = 4) and F_3 EtOH (n = 4) groups showed lower mean methylation levels than their respective control (n = 4) groups. In contrast, at the F_2 generation the EtOH (n = 4) group appeared to have a higher mean methylation than the control group (n = 4). Analysis revealed a significant difference in mean methylation between EtOH and control groups at the F_1

generation (*P = 0.021*). At the F_2 and F_3 generations the difference in methylation appeared to be trending towards significance (*P = 0.083*) **(Figure 3.12 and Table 3.14)**.

3.9.2.2 Methylation Level per CpG site

The methylation levels for each of the six CpG sites within CTCF1 between treatment groups and at generations F_0 to F_3 are depicted in **Table 3.15** and are displayed graphically in **Figures 3.13, 3.14, 3.15 and 3.16**. The non-parametric Wilcoxon Mann-Whitney test was used to assess if there was a significant difference between treatment groups.

Table 3.15: CpG site Methylation Levels across CTCF1 between Treatment Groups at Generations F_0 – F_3

Generation	CpG	Treatment	N	Methylation Level ± SD (%)	Median (Range)	*P*-value[a]
0	1	NT	6	56.12 ± 1.72	55.96 (54.72, 59.41)	-
	2	NT	6	57.48 ± 1.04	57.50 (55.90, 59.10)	-
	3	NT	6	59.75 ± 3.48	59.03 (56.53, 66.38)	-
	4	NT	6	54.93 ± 1.65	55.02 (53.04, 57.22)	-
	5	NT	6	57.30 ± 2.41	56.70 (55.00, 61.97)	-
	6	NT	6	53.41 ± 0.93	53.40 (51.90, 54.70)	-
1	1	EtOH	4	54.63 ± 0.95	54.48 (53.29, 55.42)	0.021**
		S	4	57.76 ± 1.21	57.65 (56.63, 59.40)	
	2	EtOH	4	56.37 ± 1.10	59.96 (54.98, 57.67)	0.021**
		S	4	59.83 ± 1.38	56.42 (58.07, 61.01)	
	3	EtOH	4	57.22 ± 0.85	57.06 (56.38, 58.34)	0.149
		S	4	59.37 ± 2.13	59.34 (57.33, 61.63)	
	4	EtOH	4	53.51 ± 0.93	53.41 (52.85, 54.89)	0.021**
		S	4	57.01 ± 1.12	56.92 (55.52, 58.21)	
	5	EtOH	4	55.75 ± 1.09	55.74 (55.13, 57.37)	0.081^
		S	4	57.48 ± 1.51	57.73 (55.73, 59.01)	
	6	EtOH	4	51.80 ± 0.74	51.52 (51.08, 52.75)	0.021**
		S	4	55.00 ± 1.55	55.17 (53.12, 56.52)	
2	1	EtOH	4	60.43 ± 1.95	60.04 (58.55, 63.10)	0.083^
		S	4	54.43 ± 6.22	55.76 (45.85, 60.38)	
	2	EtOH	4	62.44 ± 2.46	61.78 (60.24, 65.97)	0.043**
		S	4	55.29 ± 7.06	57.74 (45.02, 61.06)	
	3	EtOH	4	64.66 ± 2.81	64.80 (61.13, 67.92)	0.043**
		S	4	56.66 ± 6.88	58.50 (46.99, 62.65)	
	4	EtOH	4	59.36 ± 2.66	58.93 (56.97,62.63)	0.149
		S	4	52.73 ± 8.33	55.95 (40.46, 58.56)	
	5	EtOH	4	61.17 ± 3.22	59.98 (57.86, 65.20)	0.083^
		S	4	55.30 ± 6.46	57.03 (46.08, 61.07)	

	6	EtOH	4	56.19 ± 3.55	54.97 (53.45, 61.36)	0.248
		S	4	51.42 ± 6.59	53.57 (41.84, 56.71)	
3	1	EtOH	4	54.37 ± 1.99	54.47 (52.06, 56.50)	0.248
		S	4	56.71 ± 2.46	57.21 (53.47, 58.94)	
	2	EtOH	4	55.89 ± 1.49	55.81 (54.18, 57.77)	0.083^
		S	4	58.06 ± 1.50	58.39 (55.96, 59.48)	
	3	EtOH	4	56.86 ± 2.38	57.06 (54.06, 59.28)	0.149
		S	4	58.78 ± 2.56	59.76 (54.99, 60.61)	
	4	EtOH	4	53.28 ± 1.86	52.66 (51.86, 55.95)	0.083^
		S	4	55.90 ± 1.49	55.97 (54.07, 57.59)	
	5	EtOH	4	55.59 ± 2.38	55.82 (52.78,57.94)	0.149
		S	4	57.17 ± 1.93	58.09 (54.28, 58.23)	
	6	EtOH	4	51.73 ± 1.36	51.87 (50.19, 52.99)	0.083^
		S	4	53.99 ± 1.63	54.09 (52.19, 55.62)	

NT–No treatment, EtOH–Ethanol-treated, S–Sucrose-treated control
[a] *P*-values generated from Wilcoxon Mann-Whitney
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

F_0 Methylation Level per CpG Site

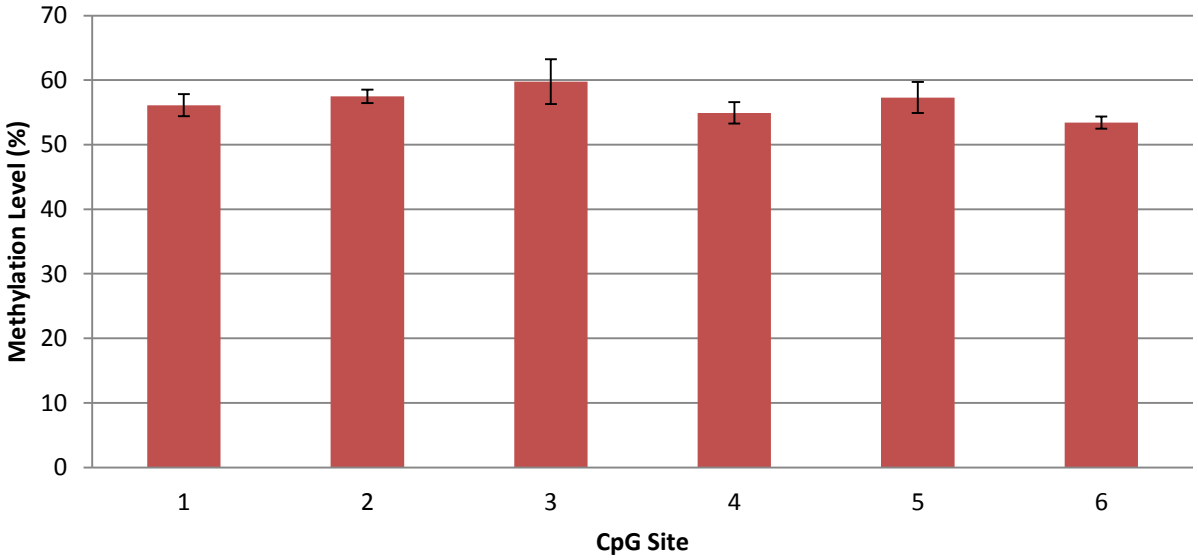


Figure 3.13: Methylation Level per CpG site within CTCF1 at the F_0 Generation. Methylation levels at CpG site 1 to 6 of the CTCF1 binding site amongst F_0 mice prior to exposure.

Within the F_0 generation CpG site 3 had the highest level of methylation ranging between 66.38% and 56.53%; however this site displayed the largest standard deviation of all sites

assessed with ± 3.48%. In contrast, CpG site 6 had the lowest methylation with a range of 51.90% to 54.70%. CpG sites 2 and 5 had similar methylation levels, equating to 57.48% and 57.30%, respectively. The CpG sites with the lowest standard deviations were site 2 and 6 with ± 1.04% and ± 0.93%, respectively (**Figure 3.13 and Table 3.15)**.

F_1 Methylation Level per CpG Site

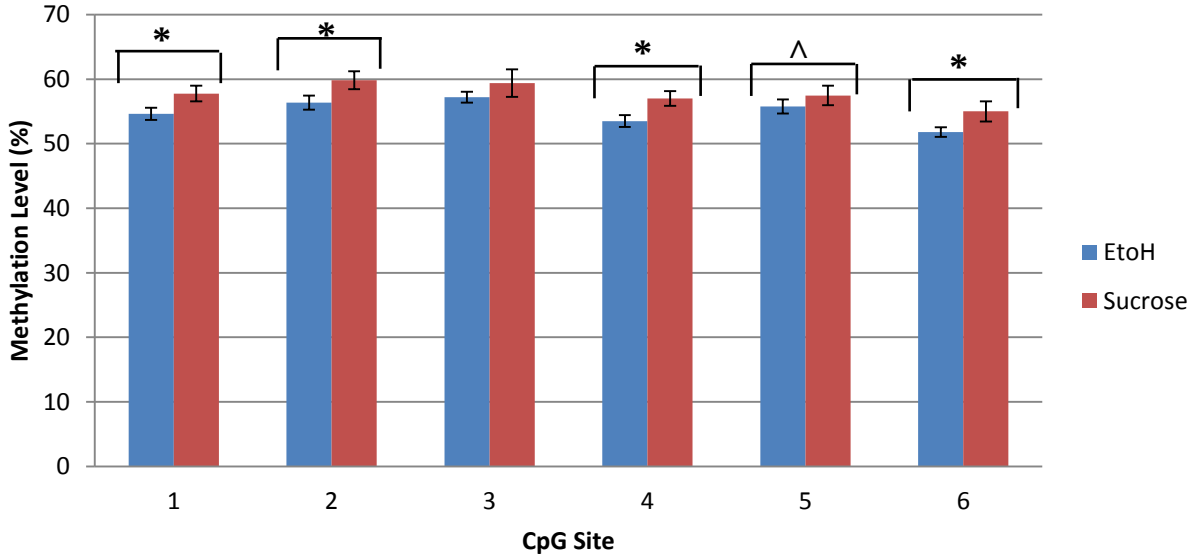


Figure 3.14: Methylation Level per CpG site within CTCF1 at the F_1 Generation. Methylation levels at CpG site 1 to 6 within CTCF1 between Treatment Groups, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, in the F_1 generation. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

At the F_1 generation the effect of treatment on the methylation level at each CpG site was assessed. The control group had a higher methylation level than the EtOH group at each of the six CpG sites. The greatest methylation level difference between treatment groups occurred at CpG site 4. At this site the control group's methylation level was 57.01% ± 1.12% and the EtOH group's was 53.51% ± 0.93%, resulting in a 3.5% difference. CpG sites 1, 2 and 6 showed differences similar to this with differences of 3.13%, 3.2% and 3.46%, respectively. In contrast, the lowest methylation difference of 1.73% occurred at CpG site 5. A significant effect of treatment occurred at CpG sites 1, 2, 4 and 6 ($P = 0.021$). Furthermore, a trend towards significance was seen at CpG site 5 ($P = 0.081$), however, there was no significance at site 3 ($P = 0.149$) **(Figure 3.14 and Table 3.15)**.

F_2 Methylation Level per CpG Site

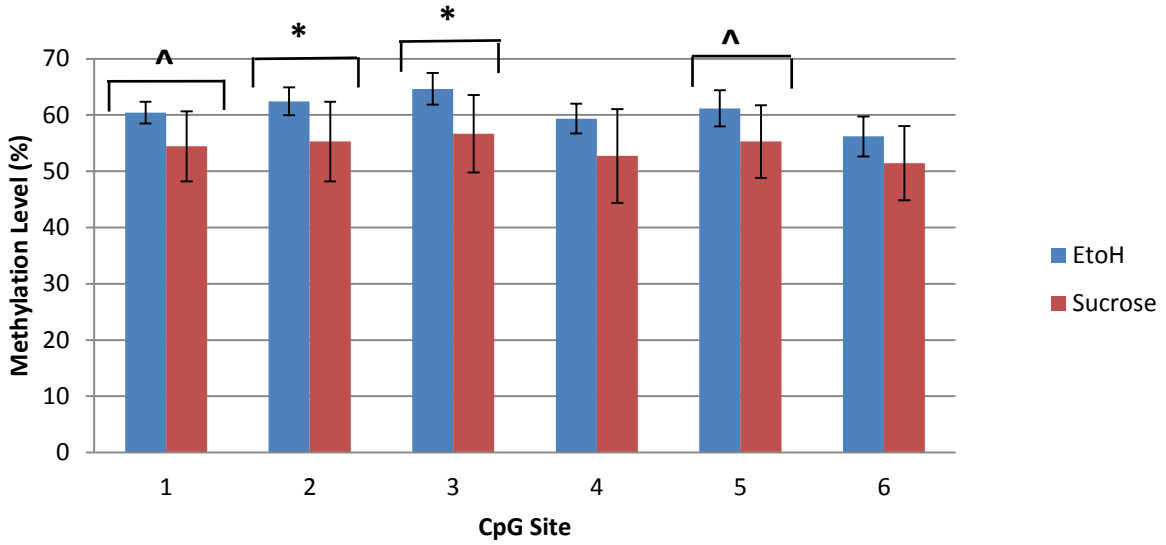


Figure 3.15: Methylation Level per CpG site within CTCF1 at the F_2 Generation. Methylation levels at CpG site 1 to 6 within CTCF1 between Treatment Groups, EtOH – Ethanol-treated, S - Control – Sucrose-treated control, in the F_2 generation. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

In contrast to the F_1 generation, the EtOH group within the F_2 generation appeared to have a higher average methylation than the control group at each CpG site. CpG 4 and 6 displayed the lowest methylation levels amongst all CpG sites for both treatment groups. CpG 3 displayed the highest methylation level for EtOH (64.66% ± 2.81%) and the control (56.66% ± 6.88%). Furthermore, this CpG site had a methylation difference of 8%, the greatest difference between treatment groups. The control samples showed large standard deviations at all CpG sites ranging from ± 6.22% to ± 8.33%, with CpG site 4 possessing the largest standard deviation. Analysis revealed an effect of treatment at CpG site 2 and 3 ($P = 0.043$) and a trend towards significance at CpG site 1 and 5 ($P = 0.083$) **(Figure 3.15 and Table 3.14)**.

F_3 Methylation Level per CpG Site

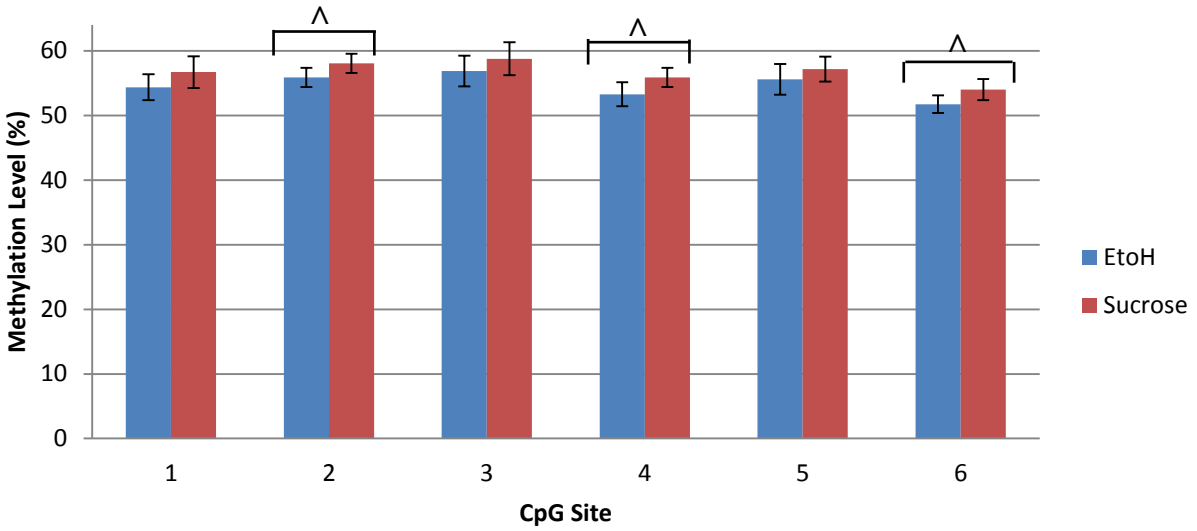


Figure 3.16: Methylation Level per CpG site within CTCF1 at the F_3 Generation. Methylation levels at CpG site 1 to 6 within CTCF1 between Treatment Groups, EtOH – Ethanol-treated, S - Control – Sucrose-treated control, in Generation F_3. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$)

At the F_3 generation the relationship between treatment groups was contradictory to the F_2 generation, however it showed similarities to that of the F_1 generation. At all CpG sites the EtOH group had lower methylation levels than the controls. The greatest difference in methylation levels between groups was at CpG site 4, the difference was 2.62%. The lowest difference of 1.58% was seen at CpG site 5. In this generation there was no significant effect of treatment at any CpG site, however, at positions 2, 4 and 6 a difference trending towards significance was noted ($P = 0.083$) **(Figure 3.16 and Table 3.15)**.

3.9.3 Correlations between CTCF1 Methylation and Mouse Phenotype

The non-parametric Spearman's Correlation was used to assess if there was an association between methylation and mouse phenotype. Correlations were performed between methylation and growth (**Appendix Q**) and open-field behaviour (**Appendix R**). Due to small sample sizes data was not stratified by sex and treatment.

At the F_1 generation there was a strong positive correlation (*rho = 0.5238*) between overall growth rate and mean methylation. In contrast to the F_1 generation there was weak to little correlation at the F_2 and F_3 generation (F_2: *rho = -0.0476*; F_3: *rho = 0.1429*) between mean methylation and overall growth rate. At suckling, the F_1 generation showed a strong negative correlation (*rho = -0.619*) was present with CpG site 3 (*P = 0.028*) being significantly correlated to growth at this time period. The F_2 generation showed a similar pattern of negative correlation between mean methylation and growth at suckling (*rho = -0.4524*). Within the F_3 generation CpG site 1 (*P = 0.0065*) and CpG site 5 (*P = 0.0366*) was significantly correlated to adult growth rate. Despite these moderate to strong correlations between growth and mean methylation at the CTCF1 binding site mean methylation was significantly correlated to growth only in the F_3 generation during adulthood growth (**Appendix Q**).

For open field measures the F_1 generation revealed no significant correlations for the total distance travelled. However, for the anxiety measures (time spent in the inner zone/ faecal boli/amount of urine) CpG site 5 displayed a strong negative correlation (> - 0.5). However, these were not significant. At the F_2 and F_3 generation there were no significant correlations between methylation, at the mean methylation level or per CpG site, and behaviour. However at the F_2 generation a positive correlation between methylation and time spent in the inner zone was noted. Furthermore, a negative correlation between amount of urine and methylation was found. Neither relationship was found to be significant (**Appendix R).**

3.9.4 CTCF2 Methylation Analysis

Using the same analysis procedure as in CTCF1 no significant effect of sex on methylation level per CpG site or mean methylation was found when looking at all samples or when stratified per generation (***See Appendix P***). Strong pairwise correlations were observed between methylation levels at the 5 CpG sites spanning the CTCF2 region **(Figure 3.17)**.

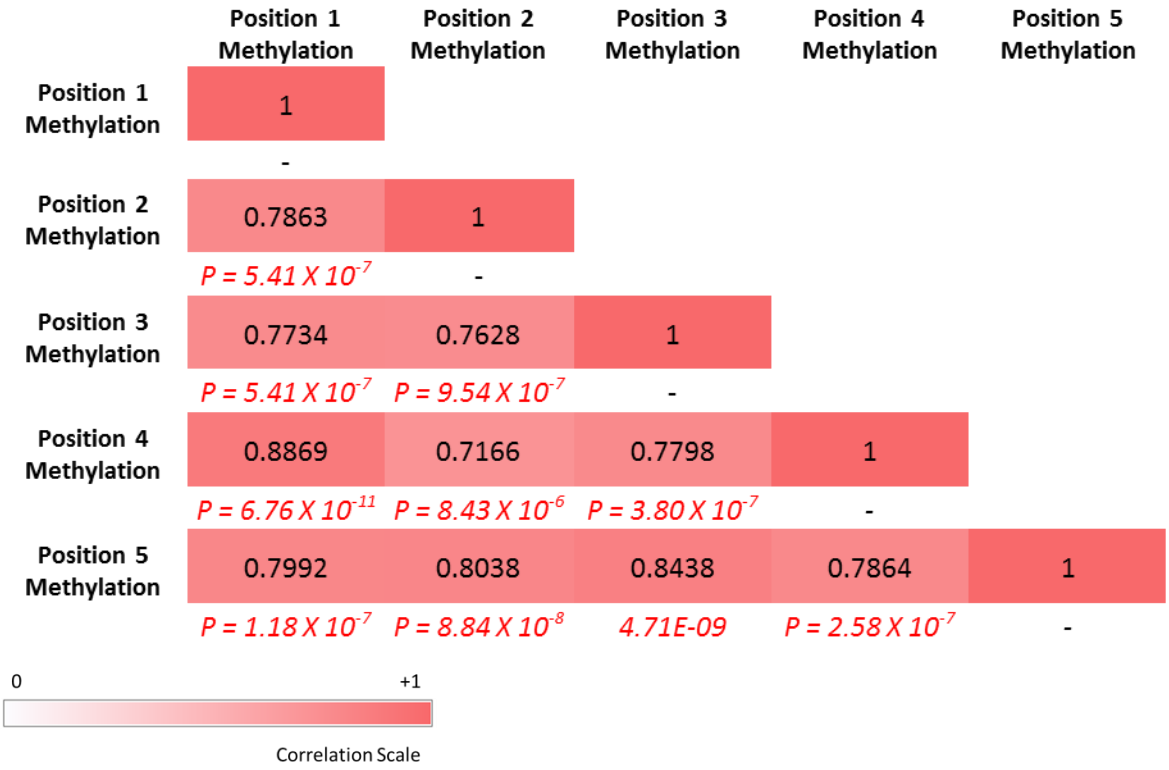


Figure 3.17: CTCF2 CpG site Correlation Matrix. Correlation between Methylation levels at CpG sites 1 to 5 in binding site CTCF2 (n = 30).

3.9.4.1 Mean Methylation Levels

The mean methylation levels at CTCF2 between treatment groups and at generations F_0 to F_3 are depicted in **Table 3.16** and are displayed graphically in **Figure 3.18**. The non-parametric Wilcoxon Mann-Whitney test was used to assess if there was a significant difference between treatment groups following chronic maternal ethanol consumption at the F_0 generation.

Table 3.16: Mean Methylation Level across CTCF2 between Treatment Groups at Generations F_0 – F_3

Generation	Treatment	N	Mean Methylation Level ± SD (%)	Median (Range)	*P*-value[a]
0	NT	6	55.66 ± 2.65	54.79 (53.70, 60.61)	-
1	EtOH	4	53.95 ± 0.81	53.69 (53.32, 55.11)	0.021**
	S	4	56.85 ± 1.08	57.05 (55.38, 57.94)	
2	EtOH	4	57.64 ± 0.56	57.69 (57.00, 58.18)	0.248
	S	4	54.31 ± 4.45	55.30 (48.12, 58.50)	
3	EtOH	4	54.89 ± 0.43	55.03 (54.27, 55.24)	0.248
	S	4	55.09 ± 0.72	55.36 (54.02, 55.60)	

NT–No treatment, EtOH–Ethanol-treated, S–Sucrose-treated control
[a] *P*-values generated from Wilcoxon Mann-Whitney
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

The mean methylation level at the F_0 generation (n = 6) was 55.65% ± 2.64%; analysis within the F_0 generation revealed no significant difference in mean methylation levels within females destined to become EtOH and sucrose-exposed (control) (Kruskal-Wallis; *P= 0.439*). Furthermore, no difference in mean methylation was noted between these females and the males of the F_0 generation (Kruskal-Wallis; *P = 0.156*).

The EtOH group (n = 4) of the F_1 generation displayed the lowest mean methylation level of all groups analysed. The mean methylation ranged between 53.32% and 55.11% in the F_1 EtOH-exposed and between 55.38% and 57.94% in the F_1 control. In contrast to the F_1 generation, the EtOH group (n = 4) of the F_2 generation displayed a higher level of mean methylation than the control (n = 4). The standard deviation, which provides an indication of the spread of the data, at the F_2 control was the highest of all generations analysed with a deviation of 4.45%. At the F_3 generation the ranges covered by the EtOH (54.27% to 55.24%) and control (54.02% to 55.60%) groups were similar, however, the control group (n = 4) displayed a marginally higher level of mean methylation than that of the EtOH group (n = 4). Analysis revealed a significant effect of treatment on the mean methylation level at the F_1 generation but no effect was seen in the F_2 and F_3 generation (F_1: *P = 0.021*, F_2: *P = 0.248*, F_3: *P = 0.248*) **(Figure 3.18 and Table 3.16)**.

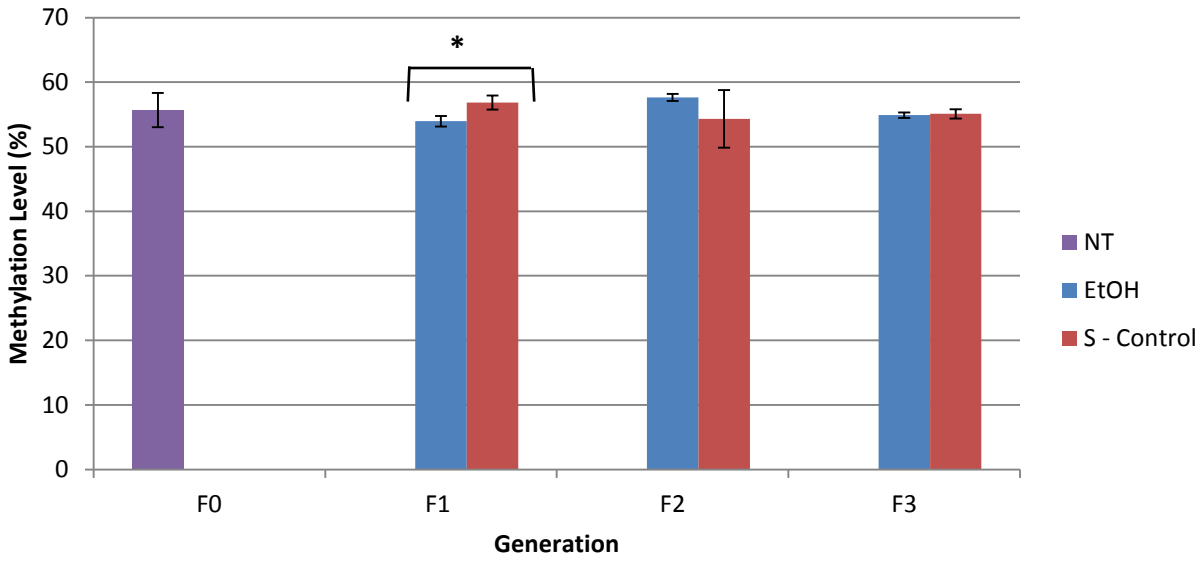


Figure 3.18: Mean Methylation Levels across CTCF2. Mean Methylation levels across CTCF2 between Treatment Groups; NT- No Treatment, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, across Generations F_0, F_1, F_2 and F_3. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

3.9.4.2 Methylation Levels per CpG Site

The methylation levels of the individual CpG sites within CTCF2 across generations F_0 to F_3 are depicted in **Table 3.17**. The non-parametric Wilcoxon Mann-Whitney test was used to assess whether there was a significant difference between treatment groups. For each generation bar graphs were constructed around the means with error bars representing the standard deviation (**Figures 3.19, 3.20, 3.21 and 3.22**).

Table 3.17 CpG site Methylation Levels across CTCF2 between Treatment Groups at Generations F_0-F_3

Generation	CpG	Treatment	N	Methylation Level ± SD (%)	Median (Range)	*P*-value[a]
0	1	NT	6	58.85 ± 4.18	57.48 (55.38, 66.94)	-
	2	NT	6	55.33 ± 1.35	54.94 (54.23, 57.90)	-
	3	NT	6	53.61 ± 1.01	53.27 (52.51, 55.07)	-
	4	NT	6	55.15 ± 4.23	54.10 (51.26, 62.81)	-
	5	NT	6	55.35 ± 2.76	54.39 (52.95, 60.32)	-
1	1	EtOH	4	55.31 ± 1.39	55.58 (53.95, 56.68)	0.021*
		S	4	58.17 ± 0.99	58.24 (56.99, 59.20)	
	2	EtOH	4	54.16 ± 0.89	54.20 (53.08, 55.17)	0.021*
		S	4	57.81 ± 0.50	57.79 (57.21, 58.45)	
	3	EtOH	4	53.18 ± 1.19	52.70 (52.38, 53.95)	0.043*
		S	4	55.71 ± 1.65	55.70 (53.80, 57.66)	
	4	EtOH	4	53.33 ± 0.80	53.38 (52.39, 54,19)	0.083^
		S	4	55.62 ± 1.98	55.95 (53.10, 57.48)	
	5	EtOH	4	53.78 ± 0.99	53.44 (53.00, 55.23)	0.021*
		S	4	56.95 ± 0.93	57.09 (55.80, 57.82)	
2	1	EtOH	4	59.33 ± 0.16	59.30 (59.21, 59.54)	0.245
		S	4	56.51 ± 4.74	57.43 (49.94, 61.23)	
	2	EtOH	4	57.88 ± 0.71	57.97 (57.04, 58.54)	0.149
		S	4	54.50 ± 3.61	55.23 (49.89, 57.66)	
	3	EtOH	4	56.38 ± 0.52	56.46 (55.67, 56.94)	0.248
		S	4	53.01 ± 4.48	54.22 (46.22, 56.97)	
	4	EtOH	4	57.23 ± 1.66	57.02 (55.46, 59.46)	0.149
		S	4	53.66 ± 5.11	54.40 (46.83, 59.02)	
	5	EtOH	4	57.35 ± 1.29	57.17 (56.12, 58.98)	0.083^
		S	4	53.85 ± 4.52	55.22 (47.32, 57.64)	
3	1	EtOH	4	57.39 ± 0.43	57.36 (56.94, 57.92)	0.564
		S	4	56.63 ± 1.63	56.99 (54.36, 58.16)	
	2	EtOH	4	55.20 ± 0.89	55.46 (53.93, 55.94)	0.100
		S	4	55.22 ± 1.05	55.14 (54.10, 56.50)	
	3	EtOH	4	53.90 ± 0.55	53.90 (53.28, 54.53)	0.387
		S	4	53.70 ± 0.39	54.70 (53.20, 54.05)	
	4	EtOH	4	53.74 ± 0.62	53.47 (53.35, 54.67)	0.387
		S	4	54.85 ± 1.96	54.85 (52.46, 56.62)	
	5	EtOH	4	54.23 ± 1.12	53.76 (53.51, 55.91)	0.248
		S	4	55.04 ± 0.62	54.94 (54.44, 55.83)	

NT–No treatment, EtOH–Ethanol-treated, S–Sucrose-treated control

[a] *P*-values generated from Wilcoxon Mann-Whitney

Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)

Trending significance ^ ($0.1 \leq P < 0.05$)

F_0 Methylation Level per CpG Site

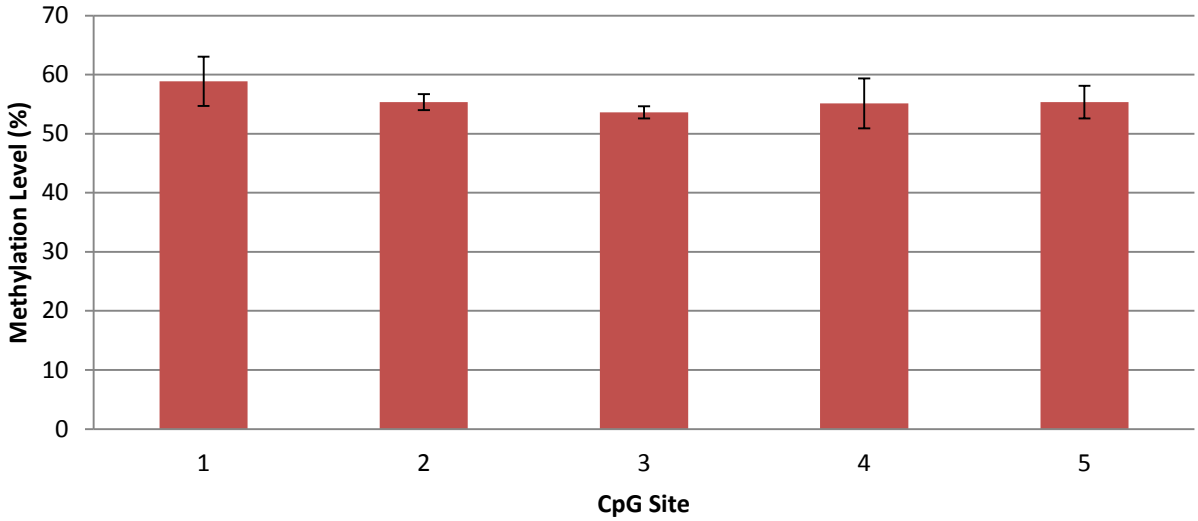


Figure 3.19: Methylation Level per CpG site within CTCF2 at the F_0 Generation. Methylation levels at CpG site 1 to 5 of the CTCF2 binding site amongst F_0 mice prior to exposure.
Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$)
Trending significance ^ ($0.1 \leq P < 0.05$)

Within the F_0 generation CpG site 1 had the highest level of methylation ranging between 55.38% and 66.94%, whilst CpG site 3 had the lowest methylation with a range of 52.51% to 55.07%. CpG sites 2, 4 and 5 had similar methylation levels, ranging between 55.15% and 55.35% methylation. The standard deviations amongst CpG sites 2 and 3 (1.35% and 1.03%) were quite low in comparison to the larger standard deviations of CpG sites 1 and 4 (4.18% and 4.23%) **(Figure 3.19 and Table 3.17)**.

F_1 Methylation Level per CpG Site

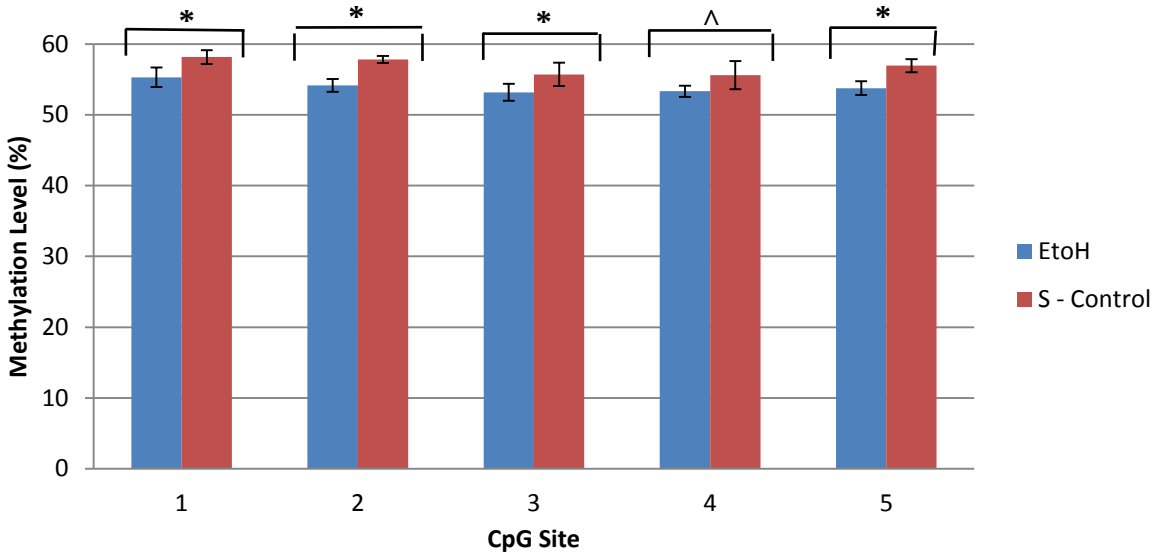


Figure 3.20: Methylation Level per CpG site within CTCF2 at the F_1 Generation. Methylation levels at CpG site 1 to 5 within CTCF2 between Treatment Groups, EtOH – Ethanol-treated, S – Control – Sucrose-treated control, at the F_1 Generation. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

For all sites the EtOH group (n = 4) displayed lower levels of methylation than the control (n = 4). At CpG sites 1, 2, 3 and 5 there was a significant effect of treatment on methylation level (CpG1: $P = 0.021$, CpG2: $P = 0.021$, CpG3: $P = 0.043$ and CpG5: $P = 0.021$). At CpG4 although not significant the effect was trending ($P = 0.083$) **(Figure 3.20** and **Table 3.17)**.

F_2 Methylation Level per CpG Site

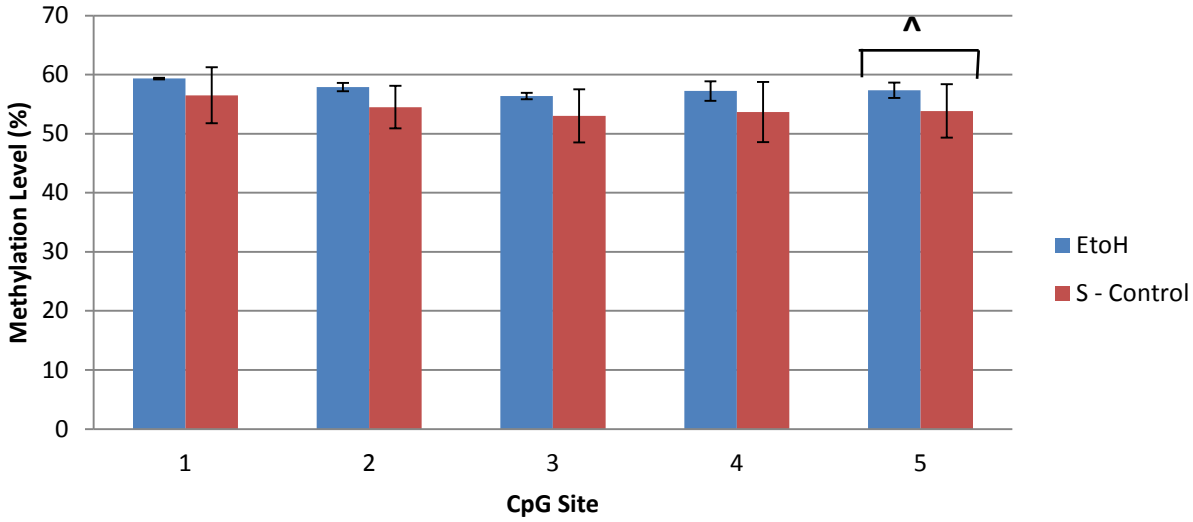


Figure 3.21: Methylation Level per CpG site within CTCF2 at the F_2 Generation. Methylation levels at CpG sites 1 to 5 within CTCF2 between Treatment Groups, EtOH – Ethanol-treated, S - Control – Sucrose-treated control, in Generations F_2. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

In contrast to the F_1 generation, the EtOH group (n = 4) at the F_2 generation displayed higher methylation levels than the control group at all CpG sites. Analysis revealed no effect of treatment on methylation level at CpG sites 1 to 4, however, CpG site 5 showed an effect trending towards significance (CpG1: $P = 0.245$, CpG2: $P = 0.149$, CpG3: $P = 0.248$, CpG4: $P = 0.149$ and CpG5: $P = 0.083$) **(Figure 3.21** and **Table 3.17)**.

F_3 Methylation Level per CpG Site

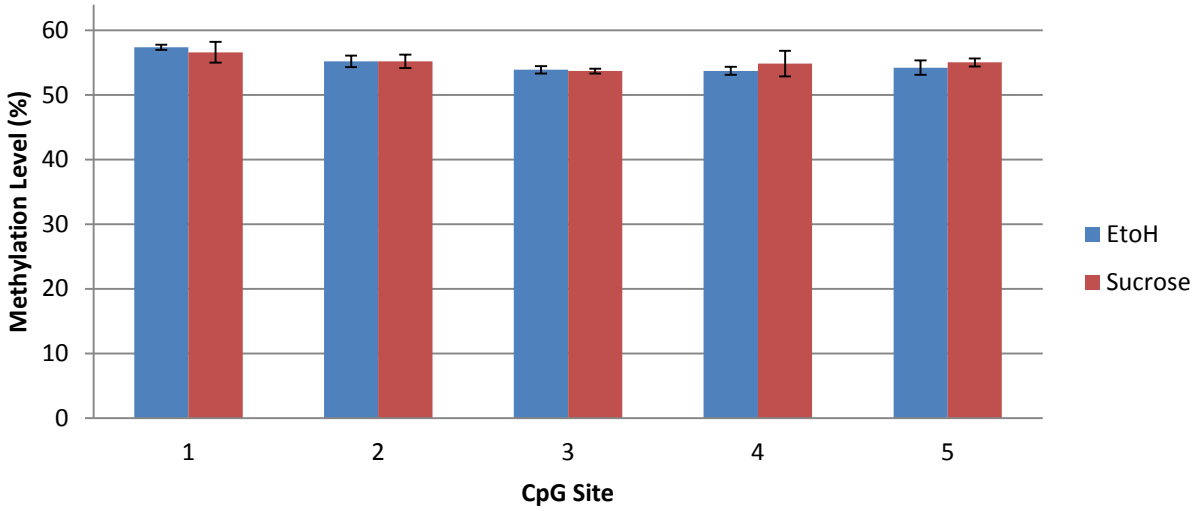


Figure 3.22: Methylation Level per CpG site within CTCF2 at the F_3 Generation. Methylation levels at CpG sites 1 to 5 within CTCF2 between Treatment Groups, EtOH – Ethanol-treated, S - Control – Sucrose-treated control, in Generation F_3. Statistically significant *** ($P \leq 0.001$) ** ($0.001 < P \leq 0.01$) * ($0.05 \leq P < 0.01$); Trending significance ^ ($0.1 \leq P < 0.05$).

At the F_3 generation the relationships between treatment groups did not follow a consistent trend throughout all CpG sites. At CpG site 1 the EtOH group (n = 4) displayed a higher level of methylation than the control group (n = 4), whereas at CpG sites 2 and 3 the two treatment groups displayed similar methylation levels. At CpG sites 4 and 5 the control group displayed higher levels of methylation than the EtOH group. Analysis of all CpG sites revealed no effect of treatment on methylation level (CpG1: $P = 0.564$, CpG2: $P = 0.100$, CpG3: $P = 0.387$, CpG4: $P = 0.387$ and CpG5: $P = 0.248$) **(Figure 3.22** and **Table 3.17)**.

3.9.5 Correlations between CTCF2 Methylation and Mouse Phenotype

The non-parametric Spearman's Correlation was used to assess if there was an association between methylation and mouse phenotype. Correlations were performed between methylation and growth (**Appendix S**) and open-field behaviour (**Appendix T**). Due to small sample sizes data was not stratified by sex and treatment.

At the F_1 generation there were no significant correlations present between growth and mean methylation level or per methylation level per CpG site. The F_2 generation showed significant (*P = 0.0465*) strong negative correlation (*rho = -0.7143*) between suckling growth rate and mean methylation. Furthermore, these significant strong negative correlations were present at CpG site 1 (*rho =-0.6347*; *P = 0.0909*) and CpG site 3 (*rho = -0.785, P= 0.0208*). At the F_3 generation there was no significant correlation at all time periods, except for at CpG site 3 where a positive correlation between methylation and suckling growth rate was found (*rho = 0.833; P= 0.102*) (**Appendix S**).

For open field measures the F_1 generation revealed no significant correlations for the total distance travelled. However, there was a strong negative correlation between methylation and the time spent in the inner zone and amount of urine. At the F_2 a positive correlation between methylation and time spent in the inner zone was noted yet no correlations were of significance. At the F_3 generation a phenotypic measure revealed positive and negative correlations at different CpG sites. Significant correlations included the strong positive correlation (*rho = 0.7559*) between faecal boli and CpG site 1 (*P = 0.03*) and the strong negative correlation (*rho = -0.7638*) between amount of urine and CpG site 1 (P = 0. 0274) (**Appendix T).**

3.9.6 Methylation Analysis Summary

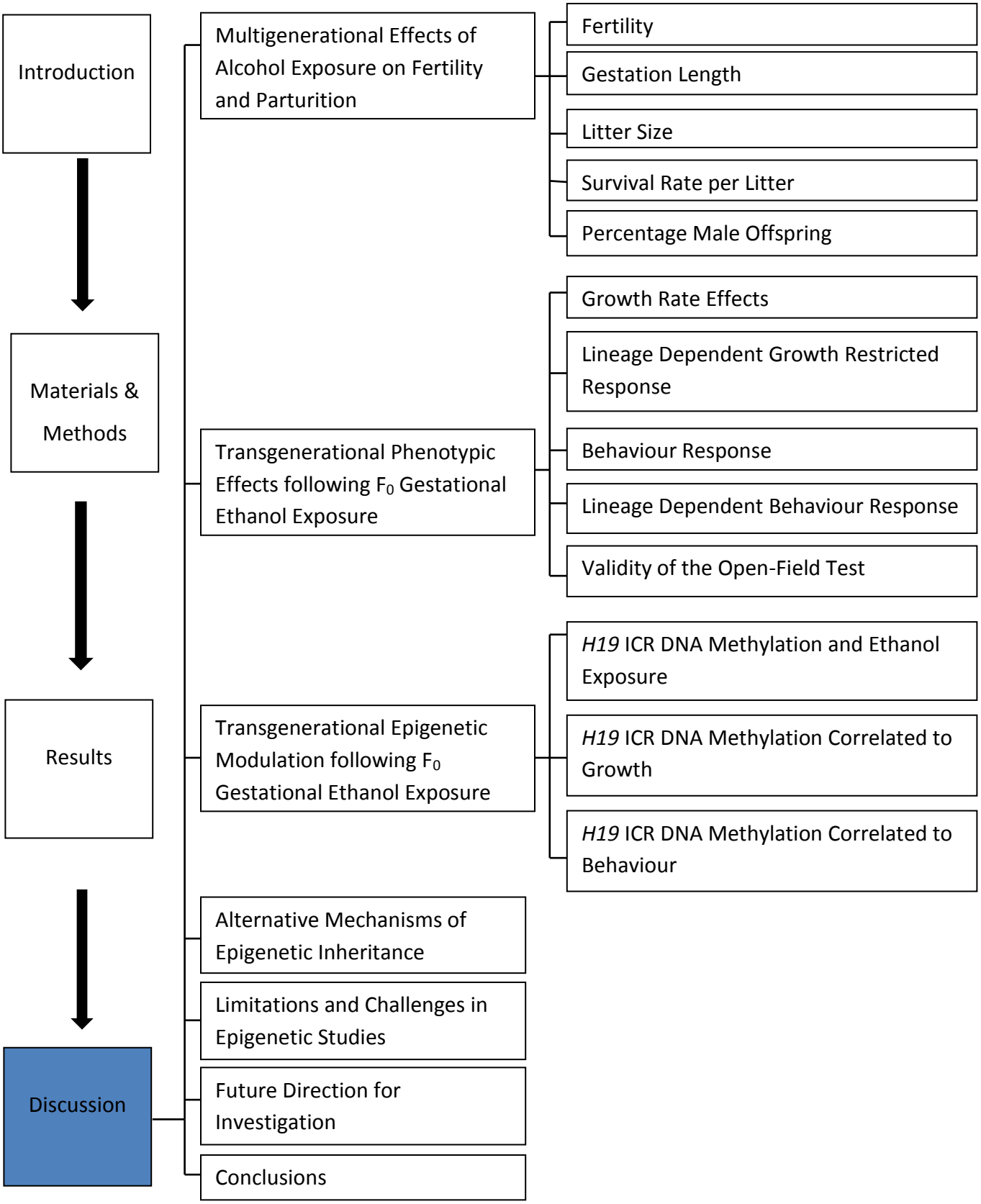
At generations F_1 and F_3 the control group displayed on average higher mean methylation levels than that of the EtOH group in both CTCF1 and CTCF2 binding sites. Furthermore, both regions displayed the inverse trend at the F_2 generation, where the EtOH group had on average higher mean methylation levels than that of the control. Despite the similar trends in mean methylation levels between treatment groups at each generation significant results only occurred at the CTCF1 binding site. The F_1 generation displayed a statistically significant difference *(P = 0.021)* between treatment groups whereas the F_2 and F_3 generations trended towards significance *(P = 0.083).*

For the methylation level per CpG site the F_1 generation provided statistically significant differences at both the CTCF1 and CTCF2 binding sites. At CTCF1 CpG sites 1, 2, 4 and 6 displayed statistically significant differences *(P = 0.021)* and at CTCF2 CpG sites 1, 2, 3 *(P = 0.021)* and 5 *(P = 0.043)* showed significant differences. Subsequent generations, F_2 and F_3, yielded no significant differences at each of the 5 CpG sites within the CTCF2 binding site. However, at CTCF1 the F_2 generation showed significant differences *(P = 0.021)* at 2 CpG sites (2 and 3) and the F_3 generation had 3 CpG sites (2, 4 and 6) that trended towards significance *(P = 0.083).*

Chapter 4

Discussion & Conclusion

4.0 Discussion



The current study was designed to investigate potential transgenerational effects of prenatal EtOH exposure. The objectives were to search for morphological, behavioural and epigenetic evidence for these transgenerational effects. A C57BL/6J mouse model was utilised as the strain readily consumes high doses of alcohol and is sensitive to the teratogenic effects of alcohol displaying both the physiological and neurobehavioral traits associated with FAS/FASD (Kleiber *et al.*, 2011; Parnell *et al.*, 2009; Sulik, 1984). Furthermore, effective transgenerational studies require the use of a model that displays early sexual maturity, has a short gestational period and produces large numbers of offspring, otherwise statistically sound studies cannot be performed in a reasonable timeframe (Rosenfeld, 2010). The *H19* ICR was the target gene of this study due to the important role this gene plays in foetal growth and development. Furthermore several studies have highlighted the effects of prenatal EtOH exposure on *H19* methylation with Stouder *et al.* (2011) showing its susceptibility and potential as a target across generations.

The discussion that follows explores the implications of each of these findings. Firstly, evidence of reduced fertility in those prenatally exposed is discussed followed by the lack of other multigenerational parturition effects. The transgenerational effects (or lack thereof) of F_0 maternal ethanol exposure on F_1 – F_3 growth rates and behaviour measures are discussed with potential lineage dependency of these phenotypes assessed. The methylation levels at the *H19* ICR are assessed and the relationships between treatment, DNA methylation and phenotypes are investigated. Lastly, possible mechanisms accounting for the altered epigenome evident in the EtOH group are discussed and their implications will be explored. It must be noted that a vast number of studies have assessed the effects of prenatal EtOH exposure on a single generation. However, to date studies assessing the transgenerational effects of EtOH on behaviour are somewhat lacking and only a single study has assessed the transgenerational effects of EtOH on methylation, making comparisons within the context of other literature difficult.

4.1 The Multigenerational effect of Alcohol Exposure on Fertility and Parturition

4.1.1 Fertility

Intuitively one might expect reduced fertility to be associated with EtOH exposure, yet published studies provide no conclusive evidence to support this. In this study a successful pregnancy was indicated by the birth of offspring. At the F_0 generation the EtOH-treated and sucrose-treated females had impregnation success rates of 50% and 89%, respectively, however, this was not statistically significant (*P = 0.141*) as the sample size was small (EtOH n = 5; control n = 9). The directional trend runs counter to other studies (Grinfeld *et al.*, 1999). Grinfeld *et al.* (1999) describes an equal pregnancy success rate of 75% (EtOH n = 16; control n = 16) between two similar treatment groups. However, the route of administration and dosage differed to my study, Grinfeld and co-workers exposed mice to low doses of EtOH from GD5 to GD19 through an *ad lib* liquid diet.

The difference in pregnancy success rate within my study may be due to the occurrence of miscarriages and/or the resorption of foetuses. Two miscarriages occurred in the F_0 EtOH group *(P = 0.474).* Several other studies have noted the occurrence of miscarriages in the F_0 EtOH-exposed group [as reviewed (Becker *et al.*, 1996)]. However, within guinea pigs the number of miscarriages appears to increase with increased EtOH dose (Wang *et al.*, 2009). The lack of miscarriages in subsequent generations within my study suggests that direct EtOH exposure on the developing foetus is required for this outcome to occur. Embryo resorption involves the death and resorption of the embryo between implantation and the completion of organogenesis. Gilliam *et al.* (1989) noted that EtOH exposure over the period of organogenesis and sex determination induced a ten times greater number of reabsorbed foetuses at implantation sites when compared to sucrose controls within pregnant C57BL/6J mice *(P < 0.001)* (Gilliam *et al.*, 1989). In contrast, Haycock and Ramsay (2009) noted no effect on the number of resorptions when exposure occurred during the preimplantation period. These studies indicate that the timing of EtOH exposure is critical in the occurrence of resorptions.

Therefore, as my study exposed C57BL/6J mice over a similar period to Gilliam *et al*. (1989) a plausible explanation for the reduced number of successful pregnancies within the EtOH group may be an increase in the number of resorptions.

Within the F_1 generation the pregnancy success rate was not different between treatment groups indicating that prenatally exposed offspring are capable of becoming pregnant and maintaining the pregnancy to term. Despite all mice obtaining a successful pregnancy, the number of attempts required to achieve this was significantly different between treatment groups *(P = 0.003)*. Potential mechanisms for decreased fertility within the EtOH group include total embryo resorption, delayed sexual maturity and altered development in both male and female offspring, delaying successful reproduction. Studies on rodent models have noted delayed vaginal opening and decreased plasma levels of luteinising hormone (LH) in prenatally EtOH exposed female offspring (Boggan *et al.*, 1979; Esquifino *et al.*, 1986). McGivern (1989) noted a lack of the normal testosterone surge expected on GD18 and GD19 in prenatally exposed rat foetuses. These decreased testosterone levels appear to persist throughout development, with exposed male rats showing reduced testes weights and reduced testosterone levels during both puberty and adulthood (Udani *et al.*, 1985).

The F_2 generation showed a similar number of matings required between treatment groups *(P = 0.877)*, for those dams that became pregnant and had offspring. The pregnancy failure rates of the EtOH and control dams were 40% and 70%, respectively. Contrary to expectation the failure rate was higher in the control group. Within the F_2 Outcross matings EtOH females had a slightly higher mean number of attempts in comparison to the control females but there was no statistical difference (*P = 0.182*). Given the small sample size (EtOH n = 3; control n = 2), even moderate effects would not be statistically significant. During the time of F_2 matings there were air flow problems within the animal unit that may have created an environmental stress reducing effective fertilization, but this would have affected both groups in a similar way.

Unfortunately the reduced fertility could not be correlated to methylation data at the two binding sites of *H19* due to the lack methylation data for F_1 dams (EtOH n = 2; control = 1).

However, the role of aberrant DNA methylation in the aetiology of infertility cannot be ruled out as other research groups have found associations between environmental exposures, DNA methylation and infertility. Following vinclozolin exposure in gestating rats over the period of embryo sex determination offspring displayed increased spermatogenic cell apoptosis, reduced sperm count, decreased sperm motility and increased incidences of infertility. Altered DNA methylation profiles at several imprinted loci (including *H19*) within the male germline were correlated with the transgenerational (F_1-F_4) male-specific effect (Anway *et al.*, 2005).

4.1.2 Gestation Length

The average gestation length for the C57BL/6J mouse is 19 - 21 days. Within our study the F_0 EtOH dams gestated for an average of 20.20 days and the F_0 control dams for 19.50 days. The difference in gestational length at the F_0 generation trended towards significance *(P = 0.090*). The pattern of increased gestational length within an EtOH exposed group was also observed in rats and guinea pigs (Bond, 1988; Hayward *et al.*, 2004). Another study within mice found no significant difference in gestational length between treatment groups (Grinfeld *et al.*, 1999). Within human studies gestation length information is provided in only about half of published reports on FAS with the majority of studies finding no difference in gestational length (Abel, 1990). Some human studies show EtOH exposure leads to preterm delivery [as reviewed by (Hannigan & Armant, 2000)].

Within my study subsequent generations displayed no difference in gestational length between treatment groups suggesting no perpetuation or exacerbation of prenatal EtOH exposure effects and that the change in gestational period, if any, is due to direct alcohol exposure during pregnancy.

4.1.3 Litter Size

The average litter size of female C57BL/6J mice is around 6.2 pups, however, first time mothers often produce litter sizes less than this (MGI: Jackson Lab). Within our study the average litter size was relatively high for nulliparous females. The F_0 EtOH-exposed females generated litter

sizes with an average of 5.40 pups and the F_0 sucrose-exposed generated litters of on average of 6.38 pups. Our study revealed no effect on litter size following F_0 chronic maternal EtOH exposure *(P = 0.263)*. Despite differences in the dosage and timing of prenatal EtOH exposure several mouse models support our finding (Gilliam *et al.*, 1989; Middaugh *et al.*, 1988; Mothes *et al.*, 1996; Stouder *et al.*, 2011). Studies involving rats have also revealed no effect on litter size (Abel, 1979; Bond, 1988; Caul *et al.*, 1979; Dursun *et al.*, 2006; Osborne *et al.*, 1980; Vorhees, 1989). However, some studies have noted significant differences in litter size (Wainwright & Gagnon, 1985; Wang *et al.*, 2009). In a study by Wainwright and Gangnon (1985) C57BL/6J mice were fed 10% (v/v) EtOH in their drinking bottles from GD5-19. Exposure in this study was much greater than that of my study. Wainwright and Gagnon (1985) obtained dosages of 18.6g/kg/day and 16.8g/kg/day between GD5-12 and GD12-19, respectively, whilst in my study the dosage was 2.9g/kg/day from GD6 -16.

The F_1 generation within our study did not display a significant difference in litter size between treatment groups which is in line with previous reports (Becker & Randall, 1987). This suggests no exacerbation of the effect of alcohol on littersize and, as there was no effect in the F_2 generation, there was no effect on the germline of the F_1 generation. However, the F_2 generation females involved in outcross experiments showed differences trending towards significance *(P = 0.083).* The EtOH exposed dams produced litter sizes smaller than the control dams. However, this trending effect may not be a 'real' effect due to the small sample size of the control group (n = 2).

4.1.4 Survival Rate per Litter

The survival rate per litter, measured as the number of pups who survived past the first week of parturition, was not significantly different between treatment groups at each generation (F_0 to F_2 Outcross). Furthermore, at all generations each treatment group had a modal number of 100% survival rate per litter suggesting that the majority of pups survived despite treatment. The effect is in accordance with other rodent models (Abel & Dintcheff, 1986; Caul *et al.*, 1979) including guinea pigs (Wang *et al.*, 2009), which showed no difference in the survival rate

between offspring of the F_0 exposed groups. In contrast, trends towards decreased pup survival and reduced viable litter numbers following EtOH exposure have been found, although these have not been significant (Kleiber *et al.*, 2011; Wainwright *et al.*, 1996). The lack of significant post natal mortality within my study indicates that a dosage of 2.9g/kg of EtOH over GD6-16 is not sufficient to induce significant risk to pup post-natal survival.

4.1.5 Percentage Male Offspring per Litter

Within my study, percentage male offspring, the measurement used as an indicator of sex difference, trended towards significance within the F_0 generation *(P = 0.079).* EtOH litters comprised of 63% males, whereas control litters contained 39% males. Subsequent generations showed no statistical difference between treatment groups. However, a trend similar to F_0 was seen in the F_1 generation, whilst the F_2 generation showed the reverse relationship. Several rodent (mouse and rat) models have noted no difference in sex proportion amongst the offspring of the F_0 treated dams (Caul *et al.*, 1979; Osborne *et al.*, 1980; Stouder *et al.*, 2011; Vorhees, 1989). Furthermore, a study in guinea pigs with chronic maternal EtOH exposure at the same dose found no difference in the number of male to female offspring (Hayward *et al.*, 2004). In stark contrast to the above studies Grinfeld *et al.* (1999) noted a significant difference in the number of males to females between treatment groups, however, the EtOH exposed group had fewer males than the control group.

4.2 Transgenerational Phenotypic Effects following F_0 Gestational Ethanol Exposure

4.2.1 Growth Rate Effects

Growth is dependent on adequate nutrition, oxygen, growth factors and hormones. Prenatal EtOH exposure may have altered the intrauterine environment and subsequently resulted in the perturbation of the balance of these factors, possibly leading to altered growth patterns. Postnatal growth retardation is a clinical feature of those diagnosed with FAS but is not required for the diagnosis of FASD. Within our study F_0 EtOH exposure during the period of organogenesis and gonadal sex determination resulted in no significant difference in overall

growth rate between treatment groups at generations F_1 to F_3. At all generations there was a significant sex effect on pup growth after weaning, in accordance with other studies (Caul *et al.*, 1979).

At the F_1 generation there was no significant effect on overall growth between the two treatment groups. However, the EtOH pups, whilst appearing to grow at a faster rate than the control pups during suckling (due to a litter size effect), had a slower overall growth rate than the controls. Several discrepancies occur when it comes to growth data in the literature. Some studies have noted a growth deficit in the prenatally exposed offspring (Haycock & Ramsay, 2009; Padmanabhan & Hameed, 1988), whilst others have not (Caul *et al.*, 1979; Stouder *et al.*, 2011). The variability in outcome may be partly due to the timing and dosage of exposure. The period during which exposure takes place will influence the measure of growth and development that is affected. For example within mice, exposure between GD7-GD14 would affect organogenesis, whilst exposure from GD15–GD19 would affect somatic growth and central nervous system development [as reviewed by (Becker *et al.*, 1996)]. Studies that exposed mice to a similar dose to that of my study, but during the preimplantation period, found a significant effect on embryonic weight and noted the EtOH exposed mice to be significantly smaller than that of the control group (Haycock & Ramsay, 2009). The genetic background of the mice used within the Haycock and Ramsay (2009) study were different to that of my own. Both studies utilised C57BL/6J dams, however, Haycock and Ramsay (2009) used Castaneus sires for matings, generating hybrid embryos. An additional study utilising the MF1 strain exposed their mice to a higher dose of 5.8g/kg ethanol over GD1 - GD6. Exposure over the preimplantation period was associated with decreased embryonic weight at GD15 (Padmanabhan & Hameed, 1988). The difference in significance of foetal growth and its dependency on the timing of exposure suggests that the preimplantation period may be more sensitive in determining a growth restricted phenotype. Furthermore, genetic background may contribute to the susceptibility of this phenotype.

Other possible factors contributing to a growth restricted phenotype within prenatally exposed offspring include caloric deficiency, deficient maternal care, impaired pup suckling and a delay in establishing independent feeding. The use of lab chow controls in conjunction with isocaloric sucrose pair-fed controls in studies by Middaugh and Boggan (1991) resulted in no growth differences between the two controls, however, a significant effect between the isocaloric sucrose control and EtOH exposed group was seen and suggested that caloric deficiency was not responsible for growth reduction. Furthermore, several cross-fostering experiments have shown that maternal factors, including, deficient maternal care, are not responsible for the growth restricted phenotype (Middaugh & Boggan, 1991). However, several studies have found morphological abnormalities in both the foetus and neonate following *in utero* exposure (Kaminen-Ahola *et al.*, 2010; Sulik, 1984). The possibility of these morphological changes impairing suckling in neonates and feeding in adults cannot be ruled out as a mechanism responsible for the growth restricted phenotype observed in rodent models (Kaminen-Ahola *et al.*, 2010).

Middaugh and Boggan (1991) utilised a similar strain and exposure period to my study and found that mice had comparable birth weights between treatment groups. However, between 19 and 28 days of age the growth restricted phenotype became evident in male and female offspring and persisted for at least 35 days. Middaugh and Boggan (1991) suggested that as exposure was halted before the latter portion of pregnancy *in utero* nutrition improved and the dehydrating effects of alcohol decreased, thus accounting for the lack of a significant treatment effect between birth weights. The absence of a difference at the F_1 generation within my study may be due to Middaugh and Boggan's suggestions, which would allow for pups to recover from *in utero* weight deficits. The trend toward lower birth weight in the EtOH exposed group, but lack of significant effect, may be due to the variability of *in utero* environment each pup experiences. Individual placentae are produced for each pup and subsequently the effects of alcohol, including the EtOH levels, are unique and may influence the effects that each pup experiences.

At the F_2 generation no difference in growth was noted between the treatment groups. However, the EtOH males displayed higher growth rates at all time periods, although these were not significant. The lack of a difference between treatment groups runs counter to a study by Becker and Randall (1987). Becker and Randall (1987) utilised a mouse model that included two generations and investigated birth weight effects on those prenatally exposed to EtOH (F_1) and their offspring (F_2). It was found that those prenatally exposed had reduced birth weights. Furthermore, when prenatally exposed offspring did not consume EtOH during their own pregnancy their offspring were still at risk for low birth weights. However, if these females did consume alcohol during pregnancy the low birth weight effect was greater than that caused by *in utero* exposure alone (Becker & Randall, 1987). Importantly it must be noted that this group assessed birth weight and not growth rate. Birth weight was not assessed in my study due to the risk of cannibalisation following handling of the pups. The F_3 generation showed no significant difference between treatment groups, however, the EtOH groups had slower growth rates overall.

4.2.1.1 Lineage Dependent Growth Restricted Response

Importantly at the F_3 Outcross generation, the male and female offspring arising from the cross involving an EtOH exposed dam and control sire displayed higher overall growth rates than those arising from the unexposed dam and exposed sire cross. Although not statistically significant, potentially due to small sample size, the trend is evident during suckling and within the males at weaning. The apparent trend on those arising from exposed sires suggests a paternal lineage of the phenotype. Several transgenerational studies have highlighted paternal lineage in the effects of alcohol and other molecules such as endocrine disruptors (Anway *et al.*, 2005; Stouder & Paoloni-Giacobino, 2010; Stouder *et al.*, 2011).

4.2.2 Behaviour Responses

The open field test was designed for the simultaneous measurement of locomotor activity and anxiety. Within this study each young adult mouse (PN 63) was placed in the open field testing area for 300 seconds and the total distance travelled, time spent in the inner zone, faecal boli

count and amount of urine streaks and/or pools were assessed. It is hypothesised that increased total distance travelled reflects an indication of locomotor hyperactivity. A decrease in the time spent in the inner zone is suggested to indicate an increase in anxiety levels. Increased faecal boli count and amount of urine were also proposed to be associated with increased levels of anxiety.

In contrast to other studies, my study showed no difference in locomotor activity between males and females (Caul *et al.*, 1979; Kleiber *et al.*, 2011; Osborne *et al.*, 1980). Although not significant at all generations, EtOH-exposed mice appeared to travel less than the sucrose-exposed (control) mice indicating decreased activity. The F_2 generation showed a marginal difference, whilst, the F_1 and F_3 generations showed greater differences with the latter being significant. The decreased activity in the EtOH groups suggested that those groups were 'hypoactive' when compared to the control group. The literature on the effect of prenatal EtOH exposure on the activity of the F_1 generation differs greatly. Some studies suggest increased activity, others decreased activity and some no change in activity (Abel & Dintcheff, 1986; Bond & Di Giusto, 1977; Dursun *et al.*, 2006; Kleiber *et al.*, 2011; Osborne *et al.*, 1980; Westergren *et al.*, 1996). In the above mentioned studies activity outcome following exposure appears to be age dependent. The majority of studies indicated increased activity when rodents were assessed at pre-weaning and juvenile ages, whereas those claiming no change in activity only assessed rodents as adults (Bond & Di Giusto, 1977; Dursun *et al.*, 2006; Osborne *et al.*, 1980; Westergren *et al.*, 1996). Bond and Di Giusto highlighted this age dependency in activity when they assessed prenatally EtOH exposed rats at various time points and found the rats were more active than their control counterparts at age 28 and 56 days, but not at 112 days. This age dependency in testing suggests that the hyperactivity experienced in prenatally exposed individuals is exclusive to younger offspring and dissipates in adulthood. Subsequently, the timing of testing is critical and as we tested young adult mice the period of most sensitivity for this measure may have been missed. Furthermore, as some behaviours, such as head dipping, occurred at each assessment age up until adulthood it suggests that prenatal EtOH exposure

may cause permanent effects on some measures and not on others (Dursun *et al.*, 2006; Plonsky & Riley, 1983).

As mentioned, locomotor activity was lower in the EtOH groups of subsequent generations. Unfortunately activity levels following ancestral exposure to EtOH have not been assessed in other studies and cannot be compared. The perpetuation of the treatment effect trend within locomotor activity suggests the transgenerational inheritance of an altered behaviour in response to F_0 maternal EtOH exposure. However, as transgenerational inheritance requires the persistence of a significant effect through generations F_1, F_2 and F_3 a significant effect observed only at the F_3 generation is not substantial evidence to declare transgenerational inheritance (Skinner, 2008). The significant effect present at only the F_3 generation in my study may be an artefact from altered experimental procedures beyond my control. Deviations from protocol at this generation included the shorter habituation before testing, as well as rats being present during the testing period.

In addition to locomotor activity, the time spent in the inner zone was assessed for all generations. There was no effect of sex at the F_0 generation or at subsequent generations. A marginal difference was observed between treatment groups at the F_1 generation indicating little to no difference in anxiety levels. This is in contrast to other studies where prenatal alcohol exposure has been seen to result in elevated anxiety in the F_1 offspring as measured using the open field test and elevated plus maze (Kleiber *et al.*, 2011; Osborn *et al.*, 1998). Kleiber and co-workers (2011) found females spending significantly less time in the centre zone in comparison to males but both male and female EtOH-treated mice spent less time in the centre in comparison to their control counterparts (Kleiber *et al.*, 2011).

Within my study, the EtOH group of the second generation spent more time in the centre when compared to the controls. This difference trended towards significance and indicated a decrease in anxiety levels in those with grand-maternal EtOH exposure. Due to the lack of similar studies this decrease in anxiety within the F_2 EtOH mice cannot be compared to other

literature. However, the large spread of the data in this group does suggest that the effect is artefactual. The effect resolved by the third generation with no significant difference being apparent, yet the relationship between groups altered with those of the EtOH group spending less time in the centre. The decreased anxiety experienced by the EtOH group of the F_2 generation indicates that EtOH exposure during F_0 gestation directly altered the F_1 germ line (Skinner, 2008). The lack of propagation of the effect to the F_3 generation may be due to the erasure and reestablishment of methylation marks during meiosis and offspring development (Reik *et al.*, 2001b). This germline epigenetic reprogramming allows not only for the resetting of parental imprints in successive generations but also prevents the propagation of epimutations (Reik *et al.*, 2001b).

Additional measures assessed in the open-field that provide an indication of anxiety include defecation and the amount of urine produced. Similar to previous studies, within my study a significant sex effect was present in the defecation and urination response (Caul *et al.*, 1979). Within males the amount of boli and urine produced was highly variable despite treatment. The EtOH group produced on average less boli than the controls at all generations. However, this was not significantly different. Within females, the number of boli trended towards significance at the F_2 generation but as the majority of female mice produced no boli it appears this effect is not a true effect and is rather due to outliers skewing the data. Most females produced little to no urine with no difference being found between treatment groups and/or generations. The lack of a significant effect on these measures runs similar to other findings (Abel, 1979; Caul *et al.*, 1979). However, unlike other studies there was no negative correlation between the number of boli and activity (Hall, 1934; Milner & Crabbe, 2008).

In my study, the lack of significant effects found within the F_1 prenatally exposed offspring may be due to the BACs reached. Although significant they are relatively low compared to those reached by others (Kelly *et al.*, 1987). Alcohol-induced alterations to the CNS and behaviour are shown to be more highly correlated with the BACs reached rather than the dose or amount of alcohol administered (Kelly *et al.*, 1987; Pierce & West, 1986a, 1986b). Kelly *et al.* (1987)

investigated the effects of BACs during the neonatal growth spurt from PD 4-10 in rats. An EtOH dose of 6.6 g/kg/day was administered over this period either as 'condensed' or 'uniform'. The condensed dose was administered 8 hours apart and resulted in high cyclic BACs, whilst the uniform dose was administered every 24 hours and resulted in stable, but low BACs. Both the condensed and uniform EtOH exposures resulted in microencephaly, with significant growth restrictions in specific regions of the brain, such as the cerebellum. However, hyperactivity measured in 90 day old rats, was only present in those who experienced the condensed treatment paradigm and subsequently were exposed to higher BACs (Kelly *et al.*, 1987). Importantly, Kelly *et al.* (1987) administered alcohol during the neonatal period. Unlike humans, where brain structures develop *in utero*, the murine brain growth spurt occurs postnatally and subsequently EtOH exposure during the postnatal period might provide a more accurate measure of EtOH's effect on the developing brain and subsequent behaviour alterations providing a more comparable measure between human and rodent FAS/FASD phenotype (Cudd, 2005).

4.2.2.1 Lineage Dependent Behavioural Response

The F_3 Outcross offspring derived from F_2 Outcross mating experiments indicated no lineage specific inheritance for locomotor activity. However, although not significant, a relationship for the time spent in the inner zone was present. Offspring of control females and EtOH-exposed males spent less time in the inner zone when compared with the offspring of those derived from EtOH females and control males. The increased anxiety in those with fathers derived from the EtOH exposed lineage suggests that the increased anxiety phenotype is transmitted through the paternal line. This is in accordance with the findings of Stouder *et al.* (2011) who found the effects of prenatal EtOH exposure to be inherited through the male line. More specifically, it was shown that the effects were correlated to methylation alterations in the sperm (Stouder *et al.*, 2011).

The lineage of reduced growth and altered behaviour through the male line suggests the transmission of altered epigenetic states through the male epigenome. Sperm cells are capable

of transmitting these altered methylation profiles from generation to generation. However, as DNA methylation is reported to be erased during gametogenesis and in the early embryo other epigenetic signals, such as histone modifications and ncRNAs, may be the epigenetic marks that are responsible for the altered epigenetic states present in the offspring upon fertilization.

Upon the completion of spermatogenesis in rodents and humans, the haploid genome in the sperm head undergoes compaction by removing and replacing the majority of histones with protamines. Despite this repackaging of the sperm genome with protamines, there is increasing evidence that some of the genome remains histone-bound and that these regions are at loci important for development, including promoters of miRNAs, imprinted genes, transcription and signalling factors (Hammoud *et al.*, 2009).

4.2.2.2 Validity of the Open field Test

As hyperactivity is a classic symptom of FAS, increased activity in the EtOH group would be expected. Mothes *et al.* (1996) showed no difference in activity within the open-field between treatment groups. It was suggested that neophobia (anxiety due to a novel environment) in the mice was responsible for the lack of activity difference, rather than a lack of treatment effect. Assessment of home cage activity showed a significant difference and indicated hyperactivity amongst the prenatally exposed mice. The familiar environment provided a more sensitive test with the confounding variables of a novel environment not interfering with assessment. In conclusion they suggested that locomotor activity may be masked by the fear experienced by mice within the novel environment (Mothes *et al.,* 1996). Kleiber *et al.* (2011) noted a significant decrease in activity levels within those prenatally exposed to EtOH, however, in addition to decreased activity they noted an increase in anxiety (measured as the time spent in the inner zone) when using the open-field. They suggested that the decreased activity levels are not a true effect of treatment but rather that of the neophobia experienced. This anxiety associated behaviour was validated when assessing home cage activity. Unlike Mothes *et al*. (1996) Kleiber and co-workers found no significant difference amongst treatment groups. Caul *et al.* (1979) noted no difference in activity on the first day of open-field testing. However, the

second day of testing revealed a significant effect of maternal treatment where EtOH exposed mice were more active than their control counterparts (Caul *et al.,* 1979). As a result of the neophobia experienced by mice the application of the open-field, as used in this study, may not provide a sufficiently sensitive measure to test locomotor activity. As per Caul *et al.* (1979), providing additional time to allow mice to acclimatise to the surroundings may be more effective. However, this acclimatisation may confound the additional application of testing anxiety in mice. Furthermore, the validity of using defecation as a measure of anxiety has been questioned (Lister, 1990). Defecation and urination have been described as an indicator of emotionality within a novel environment, yet they are not to be confused with nervousness or anxiousness (Bindra & Thompson, 1953).

Furthermore, comparisons amongst various open-field data can be misleading as, although individual laboratories may have developed standard practices, there is great variability in the equipment and techniques used (Walsh & Cummins, 1976). With the use of automated systems the deviations between the observations of researchers may have been reduced. However, differences persist in the shape, size, colour, light position and intensity, subdivisions and timings of the test. The variations in the above mentioned dimensions and parameters result in significant changes to the test result [as reviewed by (Walsh & Cummins, 1976)].

4.3 Transgenerational Epigenetic Modulation following F_0 Maternal Ethanol Exposure

4.3.1 H19 ICR DNA Methylation and Ethanol Exposure

Despite the phenotypic effects on the offspring being variable and modest within my study numerous other studies examining the promoter regions of specific genes have noted altered DNA methylation levels in response to prenatal EtOH exposure (Downing *et al.*, 2010). Within my study, analysis of the CTCF1 and CTCF2 binding sites of the *H19* ICR showed significant hypomethylation following prenatal EtOH exposure. Furthermore, within the CTCF1 and CTCF2 binding sites CpG sites 1, 2, 4 and 6 and CpG sites 1, 2, 3 and 5, respectively, showed specific differences between treatment groups. Site-specific demethylation at the *H19* ICR following

alcohol exposure has been previously reported (Haycock & Ramsay, 2009; Stouder *et al.*, 2011). Alcohol is known to disrupt the folate-dependent pathway and subsequent activity of methionine synthase. The altered activity of methionine synthase results in an accumulation of DNMT inhibitors causing disruption to the establishment and maintenance of normal methylation patterns (Halsted *et al.*, 2002). This altered one-carbon metabolism may be responsible for both the decreased mean methylation and methylation levels per CpG site observed at the F_1 generation.

The F_2 EtOH group displayed increased mean methylation at both binding sites when compared to the control. The CTCF1 binding site showed a significant effect of treatment, whereas CTCF2 did not, despite displaying a similar trend. The lack of effect may be due to the large variability within the F_2 control group. CTCF1 site-specific hypermethylation occurred at CpG sites 2 and 3. Stouder *et al.* (2011) did find site-specific methylation changes within the F_2 EtOH generation. However, these changes involved decreases in methylation and were confined to brain tissue (Stouder *et al.*, 2011). Following hypermethylation at the F_2 generation, at the F_3 generation CTCF1 mean methylation levels of the EtOH group had returned to slightly lower than that of the control group, whilst at the CTCF2 binding site the methylation levels were equal between the treatment groups.

It may be hypothesised that the increased methylation at the F_2 generation is the result of a 'recovery' mechanism in which the DNA methylation errors (due to EtOH exposure) incurred in the F_1 generation were 'rectified', albeit slightly overcompensated. Within my study EtOH exposure occurred during the period of embryo sex determination and resulted in both the F_1 embryo and F_1 germ cells being directly exposed. Due to the effect alcohol has on one-carbon metabolism it was hypothesised that the F_2 EtOH group would display similar hypomethylation to that observed in the F_1 generation. F_0 gestating dams were exposed from GD6 – GD16, during this period F_1 germ cells undergo epigenetic reprogramming. Reprogramming is initiated with a wave of demethylation where methylation marks, including imprints, are removed. Following this methylation marks are re-established. However, re-establishment is only initiated at the

prospermatogonia stage (GD15-16) within males and postnatally within females concurrently with oocyte growth (Reik *et al.*, 2001b). This would allow for the epigenetic machinery to re-establish imprints within the F_1 germ cells without continued exposure to EtOH. Haycock and Ramsay discuss the possibility of recovery mechanisms within the gastrulating embryo, but not the placenta. The confinement of methylation alterations within the placenta and not the developing embryo led the researchers to believe that epigenetic control was more stringent within the embryo. The more serious epigenetic control within the embryo was suggested to be due to the embryo having to maintain imprinted expression for a longer period of time to ensure successful development, whereas the placenta is discarded at birth (Haycock & Ramsay, 2009).

Remethylation following induced demethylation has also been found in plants and appears to be a protective function against long term epigenetic defects, such as deleterious transposable element activity (Teixeira *et al*., 2009). *Arabidopsis* mutants deficient for the adenosine triphosphatase chromatin remodeler gene *ddm1* display up to 70% losses in DNA methylation. When crossed with wild-type plants, DNA methylation losses persist into the F_1 and subsequent generations, despite *ddm1* mutations behaving as recessive traits. The persistent DNA methylation losses led several scientists to believe that DNA methylation could not be restored. However, Teixeira *et al.* (2009) investigated the stability of DNA methylation across repeat elements in several generations following these *Arabidopsis* crosses. Methylation analysis was investigated following the F_5 generation and of the 47 lines investigated 21 remained hypomethylated and 22 showed wild-type methylation levels. Teixeira *et al.* (2009) claim that the return of some lines' methylation levels to wild-type levels are due to a remethylation process. Furthermore, they note that the remethylation occurred at specified previously hypomethylated regions throughout the genome suggesting a targeted but robust approach. Further investigation into the mechanisms responsible for the remethylation process involved crosses involving *rdr2* (*rdr2* = *RNA dependent RNA Polymerase 2*) *Arabidopsis* mutants. Progeny displayed impaired RNA interference (RNAi), the process by which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules (Castel and

Martienssen, 2013). However, the progeny displayed restored *ddm1* functions with similar methylation profiles as those homozygous for the null allele of the maintenance methyltransferase enzyme *MET1.* The aberrant methylation profiles despite restored *ddm1* function highlighted the essential need for RNAi in remethylation (Teixeira *et al.*, 2009). In plants, the remethylation of repeat elements by RNAi provides a protective effect against transgenerational epigenetic effects, but the role of the RNAi machinery in mammals as a measure to protect against deviations in methylation at other genomic locations, such as, imprinted genes, needs further investigation.

At the F_3 generation the mean methylation levels of the EtOH group decrease at the CTCF1 binding site but do not significantly differ between treatment groups at the CTCF2 binding site. The change in methylation level may be attributed to the recovery mechanism postulated above adjusting for the increased methylation levels at the F_2 generation. As there is no change in the CTCF2 binding factor it appears once the methylation levels are at the 'correct' level no adjustments are necessary in subsequent generations. The significant change in CTCF1 methylation at the F_1 generation and trending towards significance changes in the F_2 and F_3 generation, despite being in different directions, indicate potential transgenerational inheritance of altered H19 ICR DNA methylation.

4.3.2 H19 ICR Correlated to Growth

In my study the overall growth rates within the F_1, F_2 and F_3 generations were not significantly different between treatment groups. However, at the F_1 and F_3 generations the EtOH group had a slower overall growth rate than the control, whereas, at the F_2 generation the control group had a slower overall growth rate than the EtOH group. The mean methylation levels at the CTCF1 binding site followed a similar pattern between treatment groups with the EtOH groups of the F_1 and F_3 generations decreasing and the F_2 generation increasing in relation to their control counterparts. These altered DNA methylation profiles at the CTCF1 binding site of the *H19* ICR may have had profound effects on CTCF binding and subsequent *Igf2/H19* gene regulation. A decrease in methylation level could potentially result in the decreased binding of

the methylation-sensitive CTCF binding factor to the paternal *H19* ICR. Thus, activating the paternal *H19* allele and subsequently reducing the expression of paternally expressed *Igf2*. In addition to the paternal *H19* expression, the maternal *H19* allele still has normal gene regulation and expression resulting in double the amount of *H19.* This overexpression would be expected to result in a growth restricted phenotype. In the case of increased methylation, increased CTCF binding and tighter regulation of the *H19* allele would occur, potentially increasing *Igf2* expression and subsequently resulting in an increased growth phenotype [as highlighted by (Knezovich & Ramsay, 2012)].

The link between decreased/increased methylation and decreased/increased growth rate suggested a link between the two variables. Correlation analysis revealed moderate to strong correlations between methylation levels of CTCF1/CTCF2 and growth at suckling and adulthood. The spurious occurrence of significant effects suggested that they may have occurred by chance. Due to the small sample size there may have been inadequate power to detect a significant difference. In order to determine if there is a true effect of methylation on growth, sample size would need to be increased.

4.3.3 H19 ICR Correlated to Behaviour

In my study both the anxiety and activity levels of the EtOH-exposed groups at all generations appear to follow the same pattern of mean methylation at the CTCF1 binding site across generations. That is the EtOH groups of the F_1 and F_3 generation had decreased activity/ time spent in the inner zone and decreased mean methylation at the CTCF1 binding site, whilst, at the F_2 generation the activity/time spent in the inner zone and methylation levels are all increased. This similar relationship between increased methylation and increased activity and time spent in the inner zone suggests a potential link between the two open-field measures and methylation. However, upon correlation analysis, no significant associations were evident between the mean methylation at the CTCF1 binding site of the *H19* ICR and locomotor activity/time spent in the inner zone. Similar relationships are seen at the CTCF2 binding site, however, the F_3 generation does not follow the same pattern as within this binding site there is

no difference between treatment groups. However, the link between DNA methylation and behaviour is not unlikely due to the effects alcohol has on neuronal gene expression.

Ethanol is known to disrupt neurodevelopmental gene expression, which is likely to lead to abnormal brain development (Hard *et al.*, 2005; Kleiber *et al.*, 2012; Liu *et al.*, 2009). Kleiber *et al.* (2012) analysed the transcriptome of the brain following prenatal EtOH exposure and noted subtle changes to global gene expression. The genes identified, such as *Ache* and *Bcl2,* are associated with neurodevelopmental pathways and relate to ADHD and anxiety, classic features of the FASD phenotype. Liu *et al.* (2009) exposed mouse embryos and showed both increases and decreases in methylation at 1028 and 1136 genes, respectively. Gene expression analysis revealed that the altered methylation was associated with significant expression changes at 84 genes. These genes were associated with multiple functions including chromatin remodelling, neuronal morphogenesis, synaptic plasticity and neuronal developmental genes (Liu *et al.*, 2009). The locus specific methylation alterations that occur throughout the genome highlight that although the methylation alteration of the *H19* ICR was the focus of this study there may be alterations at other loci, including those involved in neurodevelopment, and these may be responsible for the behavioural phenotypic effects in subsequent generations (Kleiber *et al.*, 2012; Liu *et al.*, 2009).

4.4. Alternative Mechanisms of Epigenetic Inheritance

Alcohol exposure is known to not only affect DNA methylation, but has also been shown to affect non-coding RNAs (ncRNAs) and histone modifications. Evidence for histone marks being a causal variant in EtOH's negative effects have recently been assessed (Guo *et al.*, 2011; Zhong *et al.*, 2010). Prenatal EtOH exposure may result in congenital heart disease (CHD), but the underlying mechanisms are not clear. Zhong and co-workers investigated the effects of high and low levels of EtOH exposure on H3 acetylation and subsequent gene expression of heart-development related genes (*GATA4, Mef2c*, and *Tbx5*) in cardiac progenitor cells. Results indicated low levels of EtOH increased the H3 acetylation but did not significantly change the expression of the heart development-related genes, whilst high levels of EtOH induced both H3

acetylation and significant gene expression changes, which was suggested to be a potential mechanism underlying alcohol-induced CHD (Zhong *et al.*, 2010). An additional study by Guo *et al.* (2011) assessed the effect of alcohol on histone modifications in the cerebellum. It was found that perinatal EtOH exposure decreased the expression and function of the histone acetyl transferase, CREB binding protein (CBP). Altered CBP function resulted in decreased lysine H3 and H4 acetylation within the cerebellum which they suggest may be responsible for the motor activity deficits associated with FAS/FASD patients (Guo *et al.*, 2011).

In addition to histone modifications ncRNAs, such as microRNAS (miRNAs), have also seen to be affected by EtOH exposure and may subsequently contribute to disease aetiology. A bioinformatic analysis of global gene expression data obtained from EtOH exposed embryos was used to detect potential *cis*-acting elements that may underlie the biological effects of alcohol (Wang *et al.*, 2008). Analyses revealed that alcohol treatment stimulated expression of miRNAs, as detected by increased levels of miRNA binding sites (3'-UTRs).

It is therefore important to study the different epigenetic mechanisms; DNA methylation, histone modifications and ncRNAs, to get a more complete understanding of the role of epigenetic modulation in alcohol teratogenesis.

4.5 Limitations and Challenges in Epigenetic Studies

Within this study sample size was a major limitation. It occurred in the transgenerational mouse model due to the limited numbers of EtOH-exposed dams who became pregnant and/or maintained their pregnancy. This was beyond my control and resulted in a small sample size in not only the F_1 generation but at subsequent generations throughout the study. The time constraints in terms of allowing for repeated matings further reduced the number of samples used in methylation analyses and further exacerbated small sample size effects. Methylation analysis underwent a further bottleneck due to challenges with equipment and time constraints. These effects were most apparent when assessing the correlations between mouse phenotype and methylation.

Additional limitations included that the most severe cases of imprinting errors and/or developmental malformations may not have been seen due to alcohol exposure inducing miscarriages and/or embryo resorptions; subsequently some of the true effects of alcohol on DNA methylation could not be investigated. The foetuses that died within the first week of life were often eaten by their mothers before tissue could have been collected. These pups may have displayed the most severe imprinting errors and methylation analysis could not be carried out. Furthermore, they did not reproduce, so the potentially most severe effects were not propagated through generations.

A further limitation of my study includes assessing the ICR region of a single locus, *H19*. Although Stouder *et al.* (2011) found *H19* to be a specific target for maternal EtOH consumption several studies have found alcohol to have site-specific effects, inducing both hypermethylation and hypomethylation, at various loci throughout the entire genome (Hard *et al.*, 2005; Liu *et al.*, 2009). In this study, the assessment of the paternally imprinted *H19* ICR does not provide insight into the effects on maternally imprinted loci. Furthermore, most epigenetic studies investigate the methylation status of the promoter regions or regions surrounding the transcription start sites, due to their CpG rich-density, however, recent research has shown that other regions, such as inter-genic CpG island shores and intra-genic CpG islands, may be more important in controlling phenotype outcome (Heijmans & Mill, 2012).

Analysis of a single cell type, namely the somatic cells of the tail, was an additional limitation to my study. Although methylation analysis occurred on an imprinted gene ICR several studies have shown a tissue-specific effect in DNA methylation at these regions (Schneider *et al.*, 2010). Furthermore, in response to an environmental exposure DNA methylation changes appear to persist in certain tissues and not in others (Anway *et al.*, 2005; Stouder & Paoloni-Giacobino, 2010; Stouder *et al.*, 2011). Analysis of both behavioural and methylation changes at a single time point in adulthood was also a weakness of this research as the changes that may occur during development could not be examined (Dursun *et al.*, 2006; Schneider *et al.*, 2010).

Within my study Pyrosequencing technology was used to analyse methylation levels. Pyrosequencing allows for CpG site-specific quantitative analysis of DNA methylation based on sequencing-by-synthesis technology. However, validation of these results still needs to be performed. Within the context of global epigenetic research there are a variety of techniques used to quantify DNA methylation level with some researchers using antibody based methods and others next generation sequencing. The different platforms differentiate in their ability to perform genome wide or site-specific analysis. The accuracy and resolution across these varying platforms also differ and subsequently with the variability in technology and approach, making inter-study comparisons difficult, which highlight the importance of validation using independent methods (Heijmans & Mill, 2012). More recently the discovery of hydroxymethylation (hmCpG), an intermediary of CpG methylation, and the inability of certain techniques, such as Pyrosequencing, to detect it has led to an additional layer of complexity when analysing DNA methylation signatures (Kobayashi *et al.*, 2012).

The relatively small difference in methylation between groups (~2.5%) at specific CpG sites within my study did not have its biological relevance determined. I did not assess the functionality of altered methylation levels within the tail tissue as it was beyond the scope of this study. However, whole brains were collected from all mice and stored to allow for the assessment of mRNA transcript levels and DNA methylation at a later date. The biological implications of such small alterations in DNA methylation are not fully understood but the accumulation of several deviations and the effect they have as a group on the methylome and subsequent biological processes may be far greater (Stoger, 2008). Several international initiatives are underway that aim to generate reference epigenomes and transcriptomes for different cell types. The National Institutes of Health (NIH) Roadmap and International Human Epigenome Consortium will provide improved reference material for future epigenetic studies (Heijmans & Mill, 2012).

4.6 Future Directions for Investigation

Future directions stemming from my project should involve the analysis of the collected mouse brain tissue. DNA methylation and gene expression analysis (real-time PCR or gene expression

arrays) would provide an indication of the tissue-specific effects of EtOH exposure and the functional consequences of these methylation changes. Analysis of additional genomic regions to include other paternally imprinted loci, such as *Rasgrf1* and *IG-DMR*, and maternally imprinted loci, such as *Snrpn* and *Peg3*, would allow for a more extended view of the effects of EtOH exposure on imprinted loci. Furthermore, the inheritance pattern, multigenerational or transgenerational, could be determined to provide evidence (or lack thereof) for the long lasting effects of prenatal EtOH exposure on brain development.

Within the global context of epigenetic epidemiology, researchers investigating the aetiology of FAS/FASD should be cognisant of the importance of taking an integrated approach to FAS/FASD research. Understanding the genomic, epigenomic, gene expression and phenotypic outcomes and the interdependent nature of these outcomes following maternal EtOH exposure will provide invaluable insight into determining the aetiology of the disease.

Previous research has highlighted the importance of timing and dosage in generating EtOH's negative impact on the phenotypic measures of offspring, such as pup weight and growth. Future studies need to investigate the effects that various EtOH concentrations given at different periods of pregnancy (i.e. preconception, preimplantation and organogenesis) may have on DNA methylation levels. The regions under investigation should include multiple loci as the effects of EtOH are not restricted to imprinted genes (Liu *et al.*, 2009). More recently, methylation at CpG island shores and intragenic CpG islands have been highlighted as regions of interest in the regulation of phenotypic variation (Deaton *et al.*, 2011; Irizarry *et al.*, 2009). Furthermore, the effect EtOH exposure has on all three levels of epigenetic regulation, including DNA methylation, RNA species and histone modifications need to be investigated. Histone modifications are closely related to chromatin conformation and should be correlated with DNA methylation levels and gene expression of associated regions. In addition to tissue-specific methylation profiles EtOH exposure induces tissue-specific effects and some studies have highlighted epigenetic inheritance of altered methylation patterns within certain tissues (Schneider *et al.*, 2010; Stouder *et al.*, 2011). Future studies will need to account for this tissue-specificity while being aware at which developmental stage sampling and/or analysis occurs, as

age is known to influence the epigenome (Schneider *et al.*, 2010). Following the above considerations the transgenerational inheritance of these changes should be investigated.

4.7 Conclusions

The findings of this study provide some evidence of transgenerational epigenetic alterations following F_0 chronic maternal EtOH exposure. Although multigenerational effects on parturition could not be declared there was a significant effect on F_1 generation fertility. In addition, although not significant at all generations, EtOH's effects on phenotypic measures and methylation were apparent at all generations. The increased methylation observed at the F_2 EtOH generation may suggest a recovery mechanism within the epigenetic machinery. This recovery mechanism would ameliorate the negative effects associated with altered DNA methylation levels and may have important implications for the inheritance of FAS/FASD traits. Correlation analysis between DNA methylation at the *H19* CTCF1 and CTCF2 binding sites with growth rate and behaviour measures revealed no significant relationships.

Despite the lack of conclusive evidence for transgenerational effects this dissertation provides additional epigenetic evidence for the effects of prenatal EtOH exposure. The effect of chronic maternal EtOH treatment on growth and behaviour were not significant in this study. However, further studies assessing a range of behavioural measures throughout postnatal development following exposure at a number of *in utero* developmental stages are required to determine the full effect of prenatal EtOH exposure on behaviour. Although much further research is necessary, this project supports the involvement of epigenetic mechanisms in alcohol teratogenesis.

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Web Resources

- G*Power 3.1.3®.
- Ensembl (release 65, December 2011) - http://www.ensembl.org/index.html
- Lars Lewejohann (2004). Software for your behavioural data and analysis purposes. Retrieved 18 October 2012 from http://www.phenotyping.com/mdigital.html. Last modified 7 April 2004.
- MGI Jackson Lab: Festing, M.F.W. (1998). Inbred strains of Mice. Retrieved 18 November 2013 from http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml
- Schumacher, A. (2009). Bisulfite conversion of DNA for methylation profiling. Retrieved 25 October 2012 from http://www.methylogix.com
- UCSC human genome browser (Feb. 2009, GRCh37/hg19 assembly) - http://genome.ucsc.edu/cgi-bin/hgGateway

Appendices

Appendices

Appendix A: Mating Schedules

Sucrose Mating Schedule

F_0 Mating	F_0 Offspring / F_1 Generation	F_1 Mating	F_1 Offspring / F_2 Generation	F_2 Mating	F_2 Offspring / F_3 Generation
43 X 76 ***F X M***	***133 (M)*** ***134 (M)*** 137 (M) 135 (F) ***136 (F)*** ***138 (F)***	136 X 152 F X M (43, 76 X 47, 71)	***319 (M)*** ***259 (F)*** ***324 (F)*** ***261 (F)***	259 X 243 F X M (136, 152, 43, 76, 47, 71 X 96, 168, 45, 73, 57, 77)	***401 (M)*** 402 (M) ***403 (M)*** 404 (M) ***405 (F)***
		138 X 159 F X M (43, 76 X 56, 72)	***320 (M)*** ***256 (F)*** 257 (F) ***258 (F)*** 322 (F) 1 (F)	258 X 242 F X M (138, 159, 43, 76, 56, 72 X 96, 168, 45, 73, 57, 77)	0
44 X 66 ***F X M***	***139 (M)*** 140 (M) ***141 (M)*** ***143 (F)*** ***144 (F)*** 145 (F) 146 (F)	143 X 149 F X M (44, 66 X 47, 71)	***374 (M)*** 375 (F) 378 (F) 379 (F) ***380 (F)*** ***381 (F)***	381 X 396 F X M (143, 149, 44, 66, 47, 71 X 170, 124, 57, 77, 45, 73)	0
45 X 74 ***F X M***	***98 (M)*** 123 (M) ***124 (M)*** ***96 (F)*** ***100 (F)***	96 X 168 F X M (45, 73 X 57, 77)	***242 (M)*** ***243 (M)*** 264 (F) ***265 (F)*** ***266 (F)*** 267 (F)	265 X 319 F X M (96, 168, 45, 73, 57, 77 X 136, 152, 43, 76, 47, 71)	0
		100 X 167 F X M (45, 73 X 57, 77)	***383 (M)*** ***385 (M)*** 386 (M) ***387 (M)*** 388 (M) 382 (F) 384 (F)	384 X 356 F X M (100, 167, 45, 73, 57, 77 X 157, 139, 56, 72, 44, 66)	***408 (M)*** ***409 (M)*** 410 (M) ***431 (F)*** ***407 (F)***

47 X 71 ***F X M***	148 (M) ***149 (M)*** 151 (M) ***152 (M)*** ***153 (M)*** ***147 (F)*** ***150 (F)***	147 X 141 F X M (47, 71 X 44, 66)	***323 (M)*** ***278 (M)*** ***253 (F)*** ***321 (F)***	253 X 385 F X M (147, 141, 47, 71, 44, 66 X 100, 167, 45, 73, 57, 77)	0
50 X 65 ***F X M***	1 1 1	-	-	-	-
51 X 62 ***F X M***	***154 (F)*** ***155 (F)*** 156 (F)	154 X 153 F X M (51, 62 X 47, 71)	***364 (M)*** ***365 (M)*** ***376 (M)*** 1 (M) **348 (F)** ***352 (F)*** 351 (F)	348 X 320 F X M (154, 153, 51, 62, 47, 71 X 138, 159, 43, 76, 56, 72)	0
52 X 64 ***F X M***	0	-	-	-	-
56 X 72 ***F X M***	***159 (M)*** ***157 (F)*** 158 (F) 160 (F) 161 (F) 162 (F) ***163 (F)***	157 X 139 F X M (56, 72 X 44, 66)	***353 (M)*** 354 (M) 355 (M) ***356 (M)*** ***293 (F)*** ***360 (F)*** 362 (F)	360 X 387 F X M (157, 139, 56, 72, 44, 66 X 100, 167, 45, 73, 57, 77)	427 (M) 421 (M) ***426 (M)*** ***424 (M)*** ***433 (F)***
		163 X 164 F X M (56, 72 X 57, 77)	***299 (M)*** ***358 (M)*** 357 (F) ***359 (F)*** 361 (F) ***363 (F)***	363 X 323 F X M (163, 164, 56, 72, 57, 77 X 147, 141, 47, 71, 44, 66)	0
57 X 77 ***F X M***	***164 (M)*** 166 (M) ***167 (M)*** ***168 (M)*** ***165 (F)*** 169 (F) 170 (F)	170 X 124 F X M (57, 77 X 45, 73)	***396 (M)*** ***394 (F)*** 395 (F) ***397 (F)*** ***398 (F)*** 399 (F)	398 X 374 F X M (170, 124, 57, 77, 45, 73 X 143, 149, 44, 66, 47, 71)	0

Note: All crosses are denoted by the female first followed by the male crossed with; numbers within brackets indicate parental lineage; numbers in bold indicate subjects used for study analyses; 1 = cannibalised pups; 0 = no pregnancy; M = Male; F = Female

Ethanol Mating Schedule

F_0 Mating	F_0 Offspring / F_1 Generation	F_1 Mating	F_1 Offspring / F_2 Generation	F_2 Mating	F_2 Offspring / F_3 Generation
39 X 74 ***F X M***	82 (M) ***83 (M)*** 113 (M) ***128 (M)*** ***81 (F)*** ***112 (F)***	112 X 115 F X M (39, 74 X 41, 76)	***330 (M)*** ***329 (M)*** ***326 (F)*** 377 (F) ***327 (F)*** 328 (F)	326 X 337 F X M (112, 115, 39, 74, 41, 76 X 104, 130, 53, 65, 58, 64)	0
				327 X 335 F X M (112, 115, 39, 74, 41, 76 X 110, 126, 58, 64, 53, 65)	440 (F) 474 (F) ***473 (F)*** 437 (F) ***472 (F)*** 1
		81 X 121 F X M (39, 74 X 42, 66)	1 1 1	-	-
40 X 59 ***F X M***	0	-	-	-	-
41 X 76 ***F X M***	***90 (M)*** ***115 (M)*** ***116 (F)*** ***117 (F)***	116 X 95 F X M (41, 76 X 42, 66)	230 (M) 231 (M) 232 (M) ***233 (M)*** ***234 (M)*** ***274 (F)*** ***275 (F)***	275 X 283 F X M (116, 95, 41, 76, 42, 66 X 118, 83, 53, 65, 39, 74)	1 1 1
		117 X 120 F X M (41, 76 X 53, 65)	0	-	-

42 X 66 ***F X M***	94 (M) ***95 (M)*** ***121 (M)*** 122 (M) ***91 (F)***	91 X 128 F X M (42, 66 X 39, 74)	347 (M) ***346 (M)*** 345 (M) ***342 (M)*** ***343 (M)*** ***344 (F)***	344 X 336 F X M (91, 128, 42, 66, 39, 74 X 110, 126, 58, 64, 53, 65)	***446 (M)*** 484 (M) ***487 (M)*** 488 (M) ***483 (F)*** ***481 (F)***
46 X 75 ***F X M***	0	-	-	-	-
48 X 72 ***F X M***	0	-	-	-	-
49 X 62 ***F X M***	0	-	-	-	-
53 X 65 ***F X M***	119 (M) ***120 (M)*** ***126 (M)*** ***104 (F)*** ***118 (F)***	104 X 130 F X M (53, 65 X 58, 64)	341 (M) ***338 (M)*** ***337 (M)*** ***340 (F)*** ***339 (F)*** 1 (F)	339 X 330 F X M (104, 130, 53, 65, 58, 64 X 112, 115, 39, 74, 41, 76)	***464 (M)*** ***461 (M)*** 463 (M) 462 (F) ***460 (F)*** ***459 (F)***
				340 X 346 F X M (104, 130, 53, 65, 58, 64 X 91, 128, 39, 74, 42, 66)	0
		118 X 83 F X M (53, 65 X 39, 74)	283 (M) 268 (F) 269 (F) ***270 (F)*** ***271 (F)***	270 X 239 F X M (118, 83, 53, 65, 39, 74 X 106, 91, 58, 64, 41, 76)	0
				271 X 233 F X M (118, 83, 53, 65, 39, 74 X 116, 95, 41, 76, 42, 66)	0
55 X 71 ***F X M***	0	-	-	-	-

***58* X 64** ***F* X M**	***127 (M)*** ***130 (M)*** 132 (M) ***106 (F)*** ***110 (F)***	106 X 90 F X M (58, 64 X 41, 76)	235 (M) ***236 (M)*** 238 (M) ***239 (M)*** 240 (M) 241 (M) 318 (M) ***272 (F)*** ***273 (F)***	273 X 331 F X M (106, 90, 58, 64, 41, 76 X 110, 126, 58, 64, 53, 65)	***411 (M)*** 412 (M) ***413 (M)*** 414 (M) 429 (M)
		110 X 126 F X M (58, 64 X 53, 65)	***336 (M)*** ***335 (M)*** 333 (M) 331 (M) ***334 (F)*** ***332 (F)***	332 X 342 F X M (110, 126, 58, 64, 53, 65 X 91, 128, 42, 66, 39, 74)	1 1 1

Note: All crosses are denoted by the female first followed by the male crossed with; numbers within brackets indicate parental lineage; numbers in bold indicate subjects used for study analyses; 1 = cannibalised pups; 0 = no pregnancy; M = Male; F = Female

Appendix B: Outcross Mating Schedule

F_2 Outcross Mating	F_2 Outcross Offspring / F_3 Outcross Generation
274 X 355 ***FE X MS***	***482 (F)*** ***434 (F)*** 480 (F) 1 (F)
334 X 383 ***FE X MS***	***489 (M)*** 486 (M) ***485 (M)*** ***442 (F)*** 443 (F)
268 X 364 ***FE X MS***	***428(M)*** 418 (M) 432 (F) ***417 (F)*** ***420 (F)***
261 X 230 ***FS X ME***	465 (M) ***468 (M)*** 466 (M) 475 (M) 471 (M) ***469 (M)*** ***467 (F)***
378 X 343 ***FS X ME***	476 (F) 457 (F) 479 (F) ***478 (F)*** 458 (F) ***456 (F)*** 477 (F)
394 X 330 ***FS X ME***	0

Note: All crosses are denoted by the female first followed by the male crossed with; numbers in bold indicate subjects used for study analyses; 1 = cannibalised pups; 0 = no pregnancy; M = Male; F = Female

Appendix C: Ethics Clearance Certificate

AESC3

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2011/02/04

APPLICANT: Ms M Ungerer

SCHOOL: Pathology
DEPARTMENT: Human Genetics
LOCATION: de Korte Street

PROJECT TITLE: Transgenerational inheritance of DNA methylation alteration at imprinted loci following maternal ethanol exposure in mice

Number and Species

158 male and 158 female mice (mus musculus)

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20110125. This approval remains valid until 20130124.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

1. **Applicant must explain/justify size of the tail slice - is 1 cm excessive?**
2. **Is the quantity of ethanol sufficient to produce the desired effect?**
3. **96% pharmaceutical ethanol to be used, whatever the quantity**
4. **Administration of analgesia to be discussed with Director of CAS**

Signed: NORTON (Chairperson, AESC) Date: 07/02/2011

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: (Registered Veterinarian) Date: 08/02/2011

cc: Supervisor: Professor M Ramsay
Director: CAS

Works 2000/Iain0015/AESCCert.wps

Appendix D: Preparation of Solutions

Isocaloric Sucrose Solution

Sucrose	704g

Make up to 1000 ml with ddH_2O

50% Ethanol Solution

96% Ethanol Solution	521ml

Make up to 1000 ml with ddH_2O

1% Agarose Gel

Agarose	0.5g
1 x TBE	50ml
Safe View ®	2µl

0.5M Ethylenediamine Tetra-acetic Acid (EDTA)

$Na_2EDTA.2H_2O$	93.05g
dH2O	300ml
Final volume (H_2O)	500ml

pH adjusted to 8.0 with 10M NaOH and autoclaved

10 x TBE

Tris	216g
Boric Acid	110g
EDTA	14.88g

Make up to 2000ml with dH_2O

TE Buffer (10mM Tris-HCl, pH7.4; 0.1mM EDTA)

2M Tris-HCL (pH 7.4)	0.5ml

EDTA (pH 8.0)	20µl
dH_2O	99.48ml
	100ml

Autoclave and store at room temperature

2M Tris-HCl pH 7.4

Tris base	242.2g
dH_20	700ml
Final volume (dH_20)	1L

pH was adjusted by adding ~140µl concentrated HCl and autoclaved

10M NaOH

NaOH pellets	4g
dH_2O	10ml

Appendix E: Blood Ethanol Concentration Protocol

The following protocol was taken from the BioVision Ethanol Assay Kit (BioVision Research Products, California, United States) instruction manual.

Reagent Preparation

Ethanol Probe

Ready to use as supplied. Warm to room temperature prior to use. Store at – 20˚C, avoid contamination with water, protect from light. Use within two months.

Ethanol Enzyme Mix

Add 220 µl Ethanol Assay Buffer to the ethanol Enzyme Mix and mix well. Store at 4˚C. Use within two months.

Ethanol Assay Protocol:

Note: Extreme care should be taken to ensure that no alcohol vapours (Ethanol, Methanol, and Propanol) are in the laboratory air where this assay is to be performed. Alcohol vapours in the air will be rapidly absorbed by kit components resulting in very high background making the kit unusable. Laboratories where HPLC (High Performance Liquid Chromatography) equipment and solvents are standing or where alcohol is used to wipe down laboratory benches or equipment are inappropriate locations to perform this assay.

Standard Curve Preparations

For the colourimetric assay, add 50 µl of pure ethanol to 808.7 µl Ethanol Assay Buffer, mix well. Then take 10 µl of the dilution into 990 µl assay buffer to generate 10 nmol/µl of ethanol standard. Take 100 µl of the dilution into 900 µl assay buffer to generate 1mM (1 nmol/µl). Add

0, 2, 4, 6, 8, 10 µl to a series of wells in a 96 well plate and adjust the volume of each to 50 µl with Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/ well ethanol Standard.

Sample Preparation

Samples can be diluted directly in Assay Buffer and tested. Biological samples such as serum (containing ~0.01-0.016% w/v) should be diluted 1:10-1:100 and volumes in the range of 10-30 µl used. For beverages which contain 100x more alcohol, correspondingly greater dilutions should be used. We suggest making several dilutions of your sample so that the sample reading is within the standard curve range.

Within our study dilutions of 1:200 were utilised and volumes of 20µl used

Reagent Mix Preparation

Mix enough reagents for the number of assays performed: For each well, prepare a total 50 µl reaction Mix containing:

46 µl Ethanol Assay Buffer
2 µl Ethanol Probe
2 µl Ethanol Enzyme Mix

Add 50 µl of the Reaction Mix to all wells

Incubate for 60 minutes at room temperature or 30 minutes at 37˚C protected from light. *In this experiment, plates were incubated at 37˚C for 30 minutes.*
Measure O.D 570 nm for colourimetric assay in a micro-plate reader.
Correct background by subtracting the background value derived from the 0 ethanol control from all samples (The background reading can be significant and must be subtracted from sample readings). Calculate ethanol concentrations of the test samples from the standard curve, multiplied by the dilution factor.

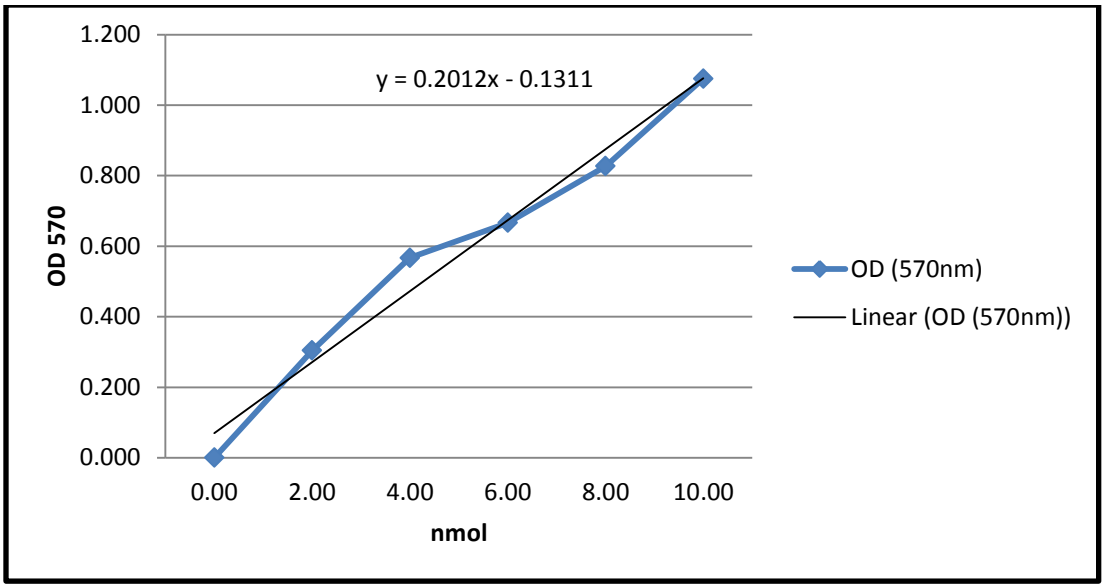


Figure E.1: Standard Curve obtained for BAC Analysis

Sample	EtOH 1	EtOH 2	EtOH 3	EtOH 4	EtOH 5	Con 1	Con 2	Con 3	Con 4	Con 5	Con 6	0	2	4	6	8	10
OD	1.899	2.037	1.884	2.314	1.814	1.358	1.329	1.317	1.221	1.298	1.444	1.512	1.816	2.079	2.178	2.339	2.587
Subtract Blank (ABS)	0.387	0.525	0.372	0.802	0.302	0.154	0.183	0.195	0.291	0.214	0.068	0	0.304	0.567	0.666	0.827	1.075
EtOH reading (x) from std curve (x=y/0.2012+ 0.1311)	2.055	2.740	1.980	4.117	1.632	0.897	1.041	1.100	1.577	1.195	0.469	0.131	1.642	2.949	3.441	4.241	5.474
Concentration = std curve value (x)/volume used (20µl)	0.103	0.137	0.099	0.206	0.082	0.045	0.052	0.055	0.079	0.060	0.023	0.007	0.082	0.147	0.172	0.212	0.274
Multiply by dilution factor (x 200) to obtain actual concentration (mM)	20.55	27.40	19.80	41.17	16.32	8.97	10.41	11.00	15.77	11.95	4.69	1.31	16.42	29.49	34.41	42.41	54.74
mg/ml	0.947	1.262	0.912	1.897	0.752	0.413	0.479	0.507	0.727	0.550	0.216	0.060	0.756	1.359	1.585	1.954	2.522

Appendix F: DNA Extraction Protocol

The protocol 'Purification of Total DNA from Animal Tissue' is the supplementary protocol to the DNeasy Blood and Tissue Preparation Kit (QIAGEN). This protocol is designed for the purification of Total (genomic and mitochondrial) DNA from animal tissues, including rodent tails.

Protocol:

Cut up to 25mg tissue (up to 10mg spleen) into small pieces, and place in a 1,5ml microcentrifuge tube. For rodent tails, place one (rat) or two (mouse).4-0.6cm lengths of tail into a 1.5ml microcentrifuge tube. Add 180µl Buffer ATL. ***In this case one 0.6cm adult mouse tail was used.***

Add 20µl proteinase K. Mix thoroughly by vortexing, and incubate at 56˚C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample. ***In this case the sample was left to lyse overnight as to ensure complete lysis. This does not affect the sample adversely.***

Vortex 15s. Add 200µl Buffer AL to the sample and mix thoroughly by vortexing. Then add 200µl ethanol (96-100%), and mix again thoroughly by vortexing.

Pipette the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x g (8000rpm) for 1min. Discard flow through and collection tube.

Place the DNeasy Mini spin column into a new collection tube, add 500µl AW1, and centrifuge for 1min at ≥6000 x g (8000rpm). Discard flow through and collection tube.

Place the DNeasy Mini spin column into a new collection tube, add 500 µl AW2, and centrifuge for 3min at 20 000 x g (14 000rpm) to dry the DNeasy membrane. Discard flow through and collection tube.

Place the DNeasy Mini spin column in a clean 1.5ml or 2ml microcentrifuge tube, and pipette 200µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1min,

and then centrifuge for 1min at ≥6000 x g (8000rpm) to elute. *In this case an additional elution step was performed to increase overall DNA yield.*

Appendix G: Bisulfite Modification Protocol

The following protocol was taken from the Zymo Research's EZ DNA Methylation-Gold™ Kit instruction manual.

Reagent Preparation:

Preparation of CT Conversion Reagent

The CT Conversion Reagent supplied with this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:

Add 900µl water, 300 µl of M-Dilution Buffer, and 50 µl M-Dissolving Buffer to a tube of CT Conversion Reagent.

Mix at room temperature with frequent vortexing or shaking for 10 minutes.

Preparation of M-Wash buffer

Add 96 ml of 100% ethanol to the 24 ml M-Wash Buffer concentrate before use

Protocol:

1. Add 130 µl of the CT Conversion Reagent to 20 µl of your DNA sample in a PCR tube. If the volume of the DNA sample is less than 20 µl, make up the difference with water.

Mix the sample by flicking the tube or pipetting the sample up and down, then centrifuge the liquid to the bottom of the tube.

2. Place the sample tube in a thermal cycler and perform the following steps:

98°C for 10 minutes

64°C for 2.5 hours

4°C storage up to 20 hours.

3. Add 600 µl of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided collection tube.

4. Load the sample (from Step 2) into the Zymo-Spin™ IC column containing the M-Binding buffer. Close the cap and mix by inverting the column several times.

5. Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.

6. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.

7. Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20°C – 30°C) for 15 - 20 minutes. After the incubation, centrifuge at full speed for 30 seconds.

8. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.

Add another 200 µl of M-Wash Buffer and centrifuge for an additional 30 seconds.

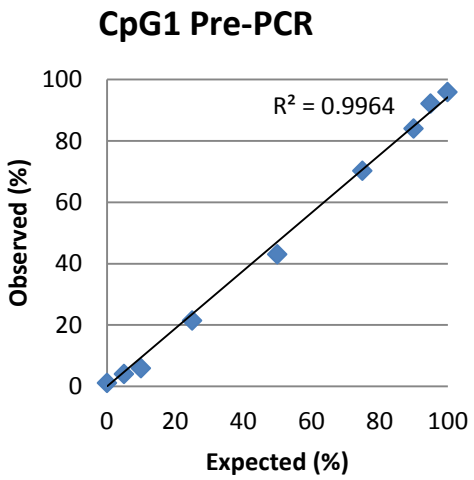
9. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

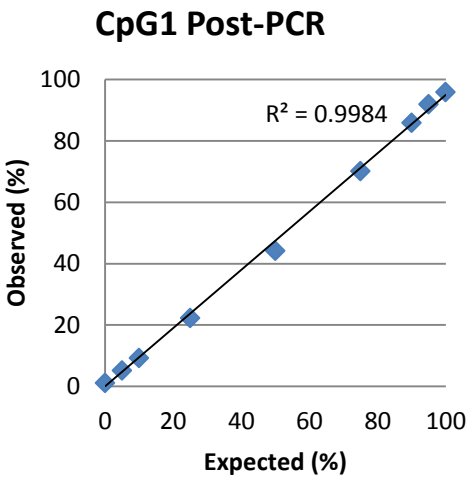
The elution volume can be >10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

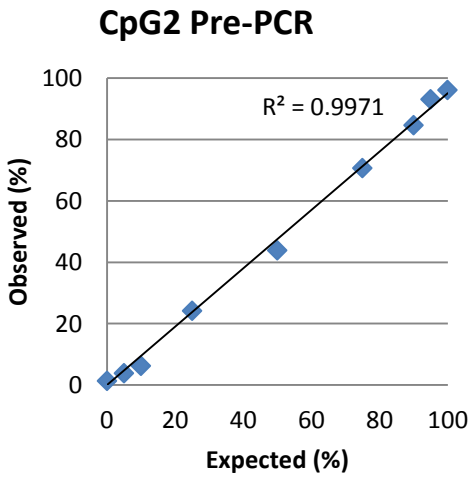
Appendix H: Assay Validation Calibration Curves

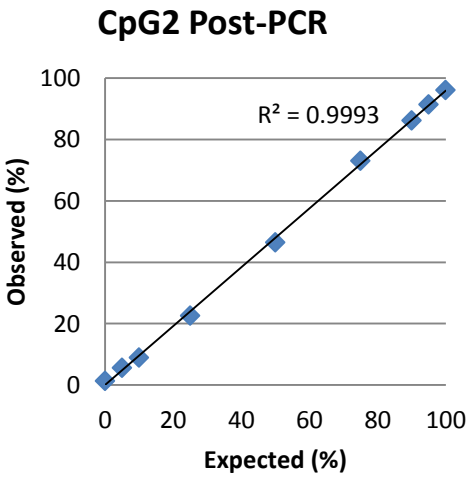
Validation of the CTCF1 and CTCF2 primer sets occurred to ensure that no preferential amplification towards methylated or unmethylated DNA occurred during the PCR reaction prior to Pyrosequencing. Following bisulfite modification, reference-level mixing, PCR and Pyrosequencing calibration curves were constructed for each primer pair and assessed at each CpG site. The calibration curves for the pre and post PCR mixes at the CTCF1 and CTCF2 binding site are shown below:

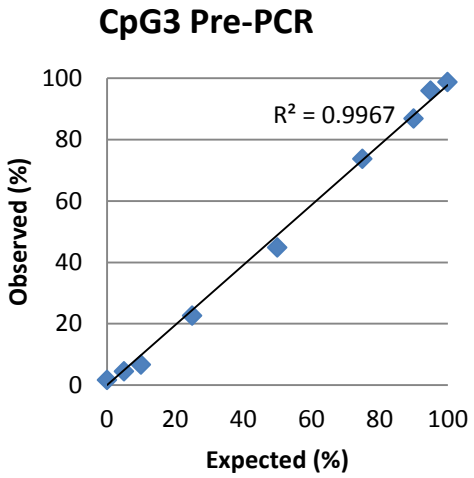
CTCF1:

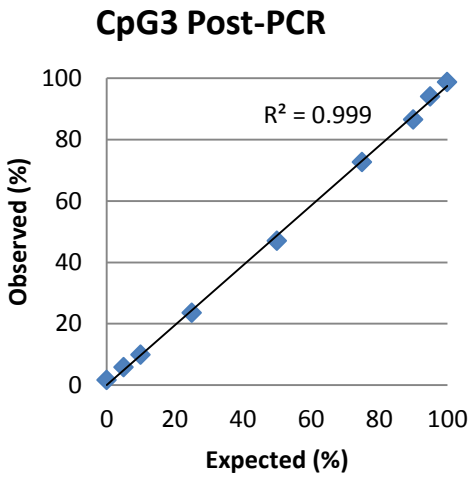


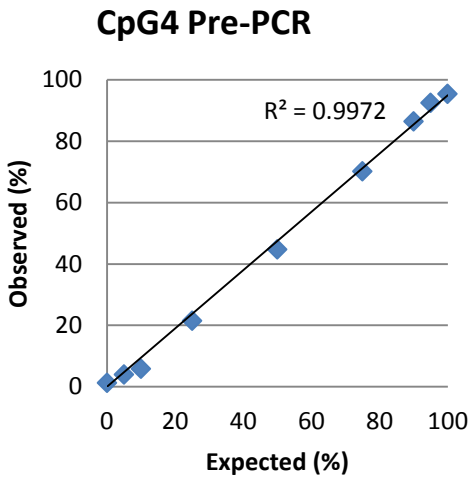


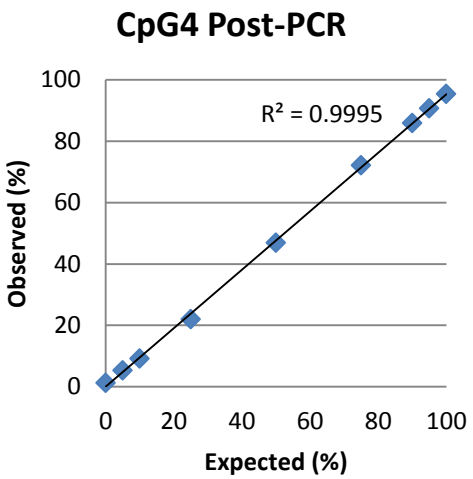


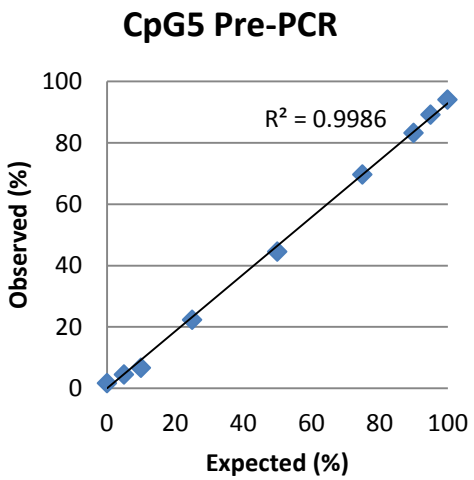


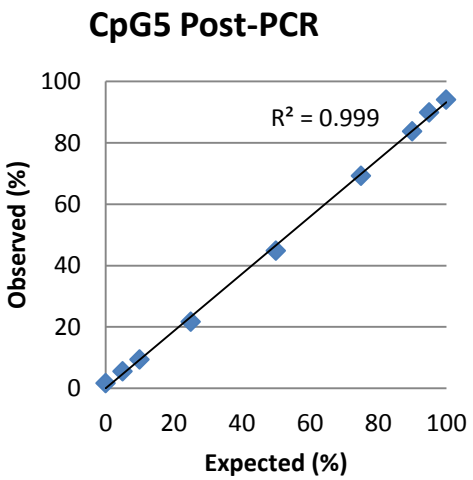

CpG3 Pre-PCR
R² = 0.9967
Observed (%)
Expected (%)
0
20
40
60
80
100

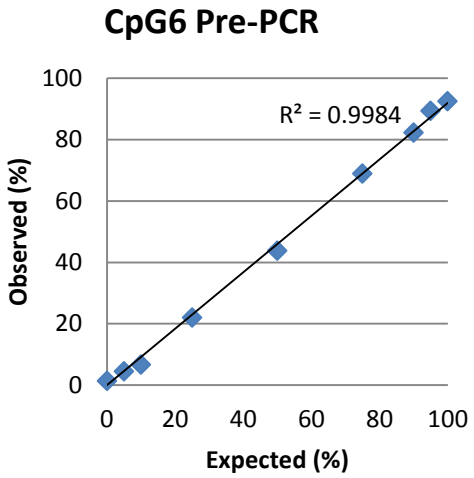

CpG3 Post-PCR
R² = 0.999
Observed (%)
Expected (%)
0
20
40
60
80
100

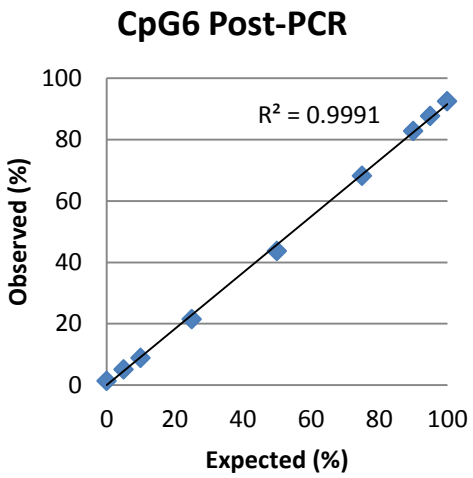

CpG4 Pre-PCR
R² = 0.9972
Observed (%)
Expected (%)
0
20
40
60
80
100


CpG4 Post-PCR
R² = 0.9995
Observed (%)
Expected (%)
0
20
40
60
80
100

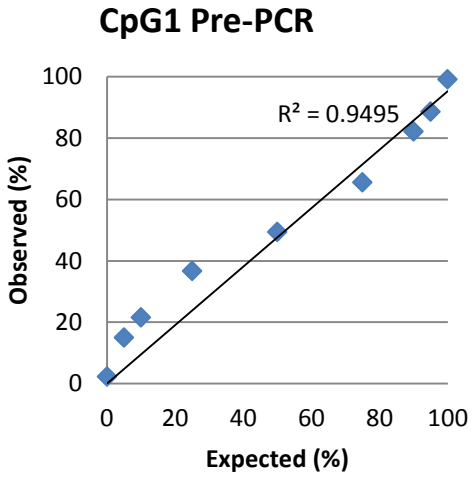

CpG5 Pre-PCR
R² = 0.9986
Observed (%)
Expected (%)
0
20
40
60
80
100

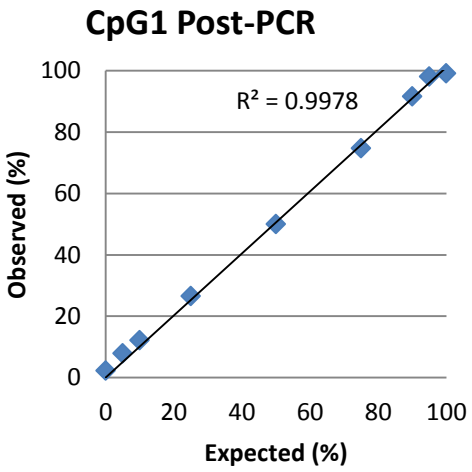

CpG5 Post-PCR
R² = 0.999
Observed (%)
Expected (%)
0
20
40
60
80
100

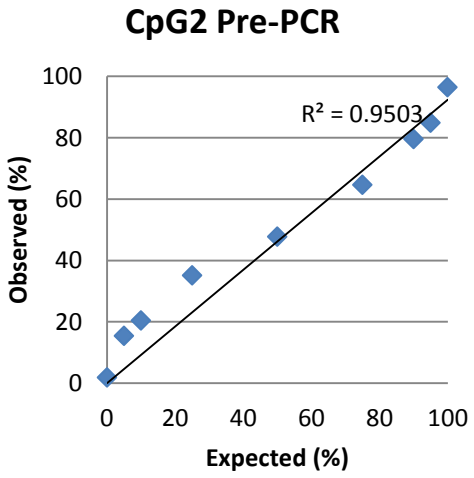

CpG6 Pre-PCR
R² = 0.9984
Observed (%)
Expected (%)
0
20
40
60
80
100

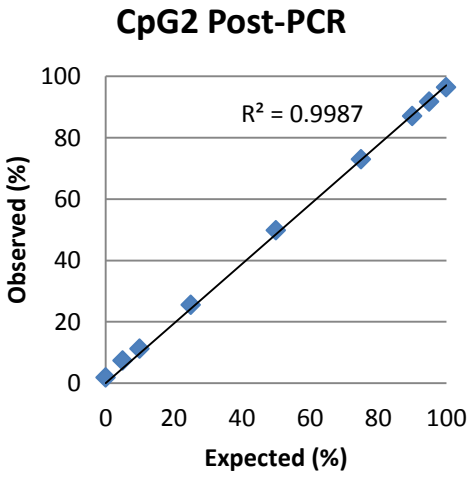

CpG6 Post-PCR
R² = 0.9991
Observed (%)
Expected (%)
0
20
40
60
80
100

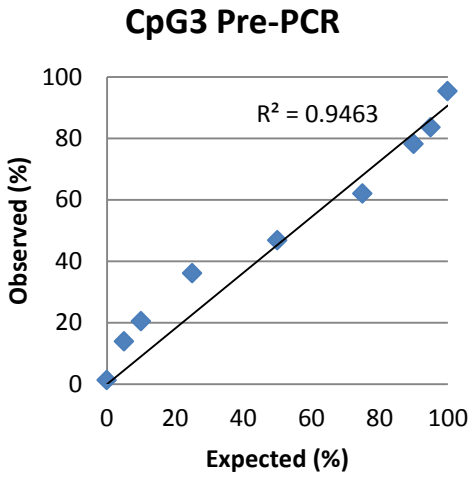
CTCF2

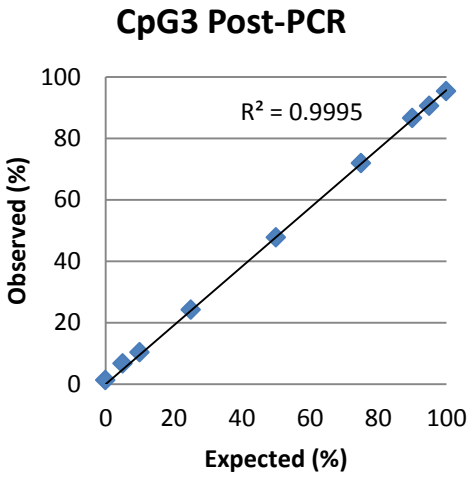

CpG1 Pre-PCR
R² = 0.9495
Observed (%)
Expected (%)
0
20
40
60
80
100

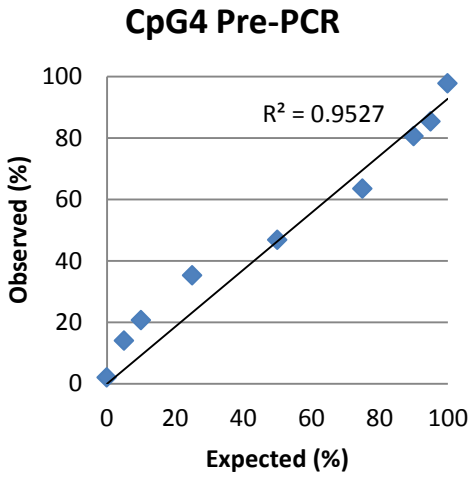

CpG1 Post-PCR
R² = 0.9978
Observed (%)
Expected (%)
0
20
40
60
80
100

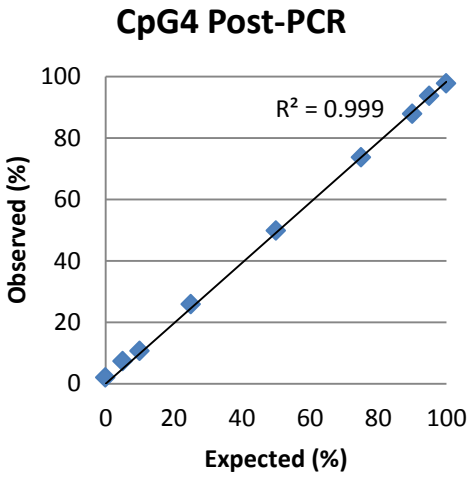

CpG2 Pre-PCR
R² = 0.9503
Observed (%)
Expected (%)
0
20
40
60
80
100

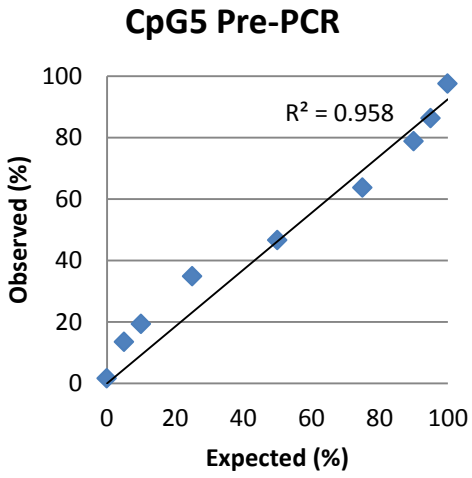

CpG2 Post-PCR
R² = 0.9987
Observed (%)
Expected (%)
0
20
40
60
80
100

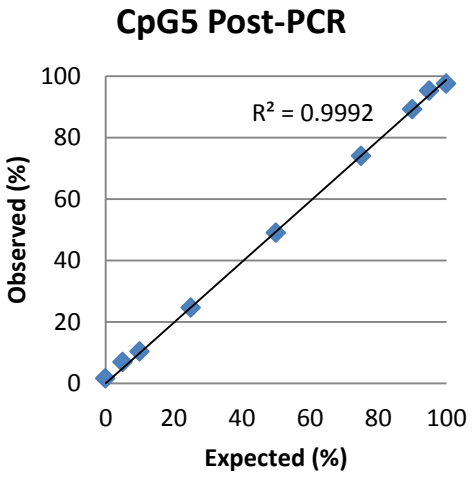

CpG3 Pre-PCR
R² = 0.9463
Observed (%)
Expected (%)
0
20
40
60
80
100


CpG3 Post-PCR
R² = 0.9995
Observed (%)
Expected (%)
0
20
40
60
80
100


CpG4 Pre-PCR
R² = 0.9527
Observed (%)
Expected (%)
0
20
40
60
80
100


CpG4 Post-PCR
R² = 0.999
Observed (%)
Expected (%)
0
20
40
60
80
100


CpG5 Pre-PCR
R² = 0.958
Observed (%)
Expected (%)
0
20
40
60
80
100


CpG5 Post-PCR
R² = 0.9992
Observed (%)
Expected (%)
0
20
40
60
80
100

Appendix I: Pyrosequencing Run Protocol

Once the PCR is complete, the products should be run on a 3% agarose gel to ensure PCR was successful.

Setting up PSQ run

1. Clean the needles of the vacuum prep:

Aliquot 80 µl of ddH_20 into all wells of a 96 well plate. Switch on the vacuum pump and lower the vacuum prep tool onto the plate for 20 sec so that all the ddH_2O is taken up by the vacuum prep tool (all wells should be empty after this).

2. Immobilization of PCR products to beads:

Use 5 µl or 10µl PCR products depending on the strength of the band.

Shake the bottle of streptavidin sepharose beads until a homogenous solution is obtained.

Make up a sepharose beads and binding buffer master mix where each reaction contains

2 µl of sepharose beads

38 µl of binding buffer

Make up to 70/75µl with ddH_2O – A total volume of 80µl must be obtained when placed with PCR product.

Aliquot 70/75 µl of the sepharose/binding buffer mix to each sample

Cover the 96 well plate with a plastic seal and place on a shaker for 10 min at 300 rpm so as to ensure that the beads remain in solution and do not settle.

Prepare the sequencing primer and annealing buffer master mix. Each reaction must contain

0.5 µl of 10 µM sequencing primer

11.5 µl of annealing buffer.

Aliquot 12 µl into each well of the PSQ plate.

3. Strand separation of PCR products:

Place the 96 well plate with PCR products and the PSQ plate on the Pyrosequencing workstation

Place four troughs on the Vacuum Prep work station in the following order and fill each trough with the following:

i.70% Ethanol

ii. Denaturation buffer

iii. Wash Buffer

iv. ddH_20

Turn the vacuum pump on and apply the vacuum to the 96 well plate containing the samples and sepharose/binding buffer mix. The beads with the immobilized templates will be captured on the on the filter probes of the vacuum probe block.

Make sure all the liquid has been captured onto the filter probes.

Move the vacuum probe block to the 70% ethanol trough for 5 sec

Move the vacuum probe block to the denaturation buffer trough for 5 sec, following this tilt the block at 90˚ to ensure all buffer has passed through the tube.

Place the vacuum probe block into the wash buffer trough for 5 sec, tilt the block at 90˚C to allow for all the liquid to completely drain from it for a few seconds and return to a horizontal position.

Turn the vacuum off to release the vacuum.

Place the vacuum probe block onto the PSQ 96 well plate and release the beads from the filter probes onto the plate by shaking the vacuum probe block while allowing the filter probes to rest on the bottom of the wells.

Place the vacuum in the ddH_2O trough.

4. Primer annealing:

Heat the PSQ plate with the sequencing primer and annealing buffer on a heating block set at 80˚C for 3 min.

Allow to cool to room temperature.

5. Cartridge preparation

Add the required amount of enzyme, substrate and dNTPs into the cartridge, as specified by the software once all the necessary information has been entered.

Place the PSQ plate and cartridge into the Pyrosequencer and start the run.

Analysis

Click on the run button once it is complete and press analyse to analyse all the samples.

Appendix J: Total distance travelled at Generations F_0-F_3

Generation	Treatment	Sex	N	Mean ± SD (m)	Median (m)	*P*-value sex[a]	*P*-value treatment[a]
F_0	NT	M	11	19.38 ± 7.55	18.70	0.875	-
	NT	F	19	19.82 ± 7.62	17.83		
	NT	M/F	30	19.66 ± 7.24			
F_1	EtOH	M	10	17.27 ± 6.13	17.81	0.812	0.205
	EtOH	F	9	17.97 ± 6.54	16.51		
	EtOH	M/F	19	17.60 ± 6.16			
	S	M	11	19.37 ± 4.64	20.19	0.713	
	S	F	13	19.99 ± 3.46	19.89		
	S	M/F	24	19.71 ±3.7			
F_2	EtOH	M	13	23.08 ± 6.17	22.54	0.808	0.917
	EtOH	F	13	23.66 ± 6.03	22.95		
	EtOH	M/F	26	23.37 ± 5.99			
	S	M	17	21.68 ± 4.31	22.26	0.074^	
	S	F	20	25.10 ± 6.52	25.88		
	S	M/F	37	23.53 ± 3.81			
F_3	EtOH	M	6	17.50 ± 2.96	18.63	0.651	0.009[+]
	EtOH	F	6	16.30 ± 5.53	16.26		
	EtOH	M/F	12	17.20 ± 4.3			
	S	M	6	22.40 ± 4.65	21.96	0.626	
	S	F	4	21.15 ± 1.79	21.42		
	S	M/F	10	21.87 ± 3.68			
F_3 Outcross	FEtOH x MS	M	3	22.60 ± 3.01	22.70	0.140	0.625
	FEtOH x MS	F	5	18.71 ± 3.19	19.40		
	FEtOH x MS	M/F	8	20.17 ± 3.53			
	FS x MEtOH	M	2	22.43 ± 3.02	22.43	0.564[b]	
	FS x MEtOH	F	3	20.21 ± 2.65	20.56		
	FS x MEtOH	M/F	5	21.10 ± 2.68			

NT- No treatment, EtOH – Ethanol-treated, S – Sucrose-treated control; [+]Statistically significant

[a] p-values generated from Independent T-test

Appendix K: Inner Zone Time at Generations F_0-F_3

Generation	Treatment	Sex	N	Mean ± SD (s)	Median (s)	p-value[a] sex	p-value[a] treatment
F_0	NT	M	11	66.53 ± 42.97	49.20	0.378[b]	-
	NT	F	19	50.44 ± 27.28	50.20		
	NT	M/F	30	56.34 ± 34.07			
F_1	EtOH	M	10	33.55 ± 18.84	30.35	0.806	0.578
	EtOH	F	9	33.11 ± 14.31	25.30		
	EtOH	M/F	19	33.30 ± 16.39			
	S	M	11	35.26 ± 18.27	31.40	0.789	
	S	F	13	37.42 ± 20.37	34.20		
	S	M/F	24	36.43 ± 19			
F_2	EtOH	M	13	37.85 ± 19.51	33.70	0.555	0.079^[b]
	EtOH	F	13	57.55 ± 51.36	34.10		
	EtOH	M/F	26	47.70 ± 39.36			
	S	M	17	31.58 ± 18.20	26.90	0.659	
	S	F	20	31.99 ± 15.32	28.85		
	S	M/F	37	31.80 ± 16.47			
F_3	EtOH	M	6	35.28 ± 16.19	33.25	0.202	0.221
	EtOH	F	6	51.27 ± 23.62	48.70		
	EtOH	M/F	12	43.28 ± 21.03			
	S	M	6	66.72 ± 21.59	67.30	0.090^	
	S	F	4	39.22 ± 22.82	31.45		
	S	M/F	10	55.72 ± 25.18			
F_3 Outcross	FEtOH x MS	M	3	39.10 ± 24.10	22.70	0.345	0.242[b]
	FEtOH x MS	F	5	50.52 ± 7.71	50.10		
	FEtOH x MS	M/F	8	46.24 ± 15.23			
	FS x MEtOH	M	2	26.20 ± 6.65	26.20	0.564[b]	
	FS x MEtOH	F	3	37.60 ± 20.66	28.10		
	FS x MEtOH	M/F	5	33.04 ± 16.23			

NT- No treatment, EtOH – Ethanol-treated, S – Sucrose-treated control; [+]Statistically significant

[a] p-values generated from Independent T-test [b] p-values generated from Wilcoxon Mann-Whitney Test

Appendix L: Faecal Boli and Amount of Urine at Generations F_0-F_3

Variable	Gen	Treatment	Sex	N	Mean ± SD (number of boli)	Mode	p-value-sex[a]	p value-treatment[a]
Faecal Boli	F_0	NT	M	11	0.45 ± 0.82	0	0.149	-
		NT	F	19	0.84 ± 0.89	1		
	F_1	EtOH	M	10	2.60 ± 1.90	2	0.009^{+}	M 0.317
		EtOH	F	9	0.56 ± 1.13	0		
		S	M	11	3.82 ± 2.92	6	0.020^{+}	F 0.580
		S	F	11	1.08 ± 1.98	0		
	F_2	EtOH	M	13	1.62 ± 2.84	0	0.934	M 0.200
		EtOH	F	13	0.85 ± 0.90	0		
		S	M	17	2.41 ± 2.58	0	0.002^{+}	F 0.092^
		S	F	20	0.40 ± 0.82	0		
	F_3	EtOH	M	6	2.50 ± 2.07	0	0.022^{+}	M 0.514
		EtOH	F	6	0.00 ± 0.00	0		
		S	M	6	3.33 ± 2.16	-	0.073^	F 0.221
		S	F	4	0.75 ± 1.50	0		
	F_3 Out-cross	FEtOH x MS	M	3	3.00 ± 1.00	-	0.010^{+}	M 1.000[b]
		FEtOH x MS	F	5	0.00 ± 0.00	0		
		FS x MEtOH	M	2	4.00 ± 5.66	-	0.100[b]	F 0.008^{+} [b]
		FS x MEtOH	F	3	1.00 ± 0.00	1		

Variable	Gen	Treatment	Sex	N	Mean ± SD (pools/streaks)	Mode	p-value-sex[a]	p value-treatment[a]
Amount of Urine	F_0	NT	M	11	0.36 ± 0.92	0	0.536	-
		NT	F	19	0.16 ± 0.50	0		
	F_1	EtOH	M	10	1.10 ± 1.60	0	0.018^{+}	M 0.970
		EtOH	F	9	0.00 ± 0.00	0		
		S	M	11	0.91 ± 1.04	0	0.019^{+}	F 0.405
		S	F	13	0.15 ± 0.55	0		
	F_2	EtOH	M	13	0.77 ± 1.42	0	0.605	M 0.277
		EtOH	F	13	0.38 ± 0.65	0		
		S	M	17	1.05 ± 1.14	0	0.002^{+}	F 0.154
		S	F	20	0.15 ± 0.49	0		
	F_3	EtOH	M	6	1.17 ± 1.33	0	0.058^	M 0.114
		EtOH	F	6	0.00 ± 0.00	0		
		S	M	6	2.83 ± 1.83	4	0.027^{+}	F 0.221
		S	F	4	0.25 ± 0.50	0		
	F_3 Out-cross	FEtOH x FS	M	3	2.67 ± 2.52	-	0.124[b]	M 0.374[b]
		FEtOH x FS	F	5	0.20 ± 0.45	0		
		FS x MEtOH	M	2	0.50 ± 0.71	-	0.739[b]	F 0.693[b]
		FS x MEtOH	F	3	0.33 ± 0.58	-		

Appendix M: Pyrosequencing Data at CTCF1 for Generations F_0-F_3

Sample	Generation	Sex	Treatment		CpG Site Analysed						CTCF1 Mean Methylation (%)/Sample
					1	2	3	4	5	6	
66	0	M	NT	PCR 1	56.6	57.20	65.64	56.19	60.51	51.81	
				PCR 2	62.21	60.99	67.12	58.25	63.42	57.58	
				AVERAGE Methylation (%)	**59.41**	**59.10**	**66.38**	**57.22**	**61.97**	**54.70**	**59.79**
76	0	M	NT	PCR 1	54.13	56.02	59.14	54.36	56.60	52.61	
				PCR 2	55.31	58.6	60.11	56.58	57.17	53.82	
				AVERAGE Methylation (%)	**54.72**	**57.31**	**59.63**	**55.47**	**56.89**	**53.22**	**56.20**
41	0	F	NT	PCR 1	55.06	56.71	60.27	52.56	56.91	53.17	
				PCR 2	56.24	58.68	59.54	53.52	57.47	53.99	
				AVERAGE Methylation (%)	**55.65**	**57.70**	**59.91**	**53.04**	**57.19**	**53.58**	**56.18**
43	0	F	NT	PCR 1	55.29	58.51	58.82	55.64	57.99	52.84	
				PCR 2	55.79	55.77	56.45	53.51	54.50	50.95	
				AVERAGE Methylation (%)	**55.54**	**57.14**	**57.64**	**54.58**	**56.25**	**51.90**	**55.51**
51	0	F	NT	PCR 1	55.81	56.83	57.46	54.27	56.41	53.79	
				PCR 2	57.05	54.96	55.58	52.08	53.58	52.53	
				AVERAGE Methylation (%)	**56.43**	**55.90**	**56.52**	**53.18**	**55.00**	**53.16**	**55.03**
58	0	F	NT	PCR 1	53.20	56.37	57.39	55.45	54.93	53.50	
				PCR 2	56.69	59.08	59.48	56.76	58.09	54.27	
				AVERAGE Methylation (%)	**54.95**	**57.73**	**58.44**	**56.11**	**56.51**	**53.89**	**56.27**

115	1	M	EtOH	PCR 1	56.16	56.83	57.91	53.48	56.64	52.81	
				PCR 2	54.16	56.22	55.67	52.85	53.62	51.13	
				AVERAGE Methylation (%)	**55.16**	**56.53**	**56.79**	**53.17**	**55.13**	**51.97**	**54.79**
130	1	M	EtOH	PCR 1	53.46	55.95	57.13	52.63	56.17	51.51	
				PCR 2	53.12	56.69	55.62	53.07	54.09	50.64	
				AVERAGE Methylation (%)	**53.29**	**56.32**	**56.38**	**52.85**	**55.13**	**51.08**	**54.17**
133	1	M	S - Control	PCR 1	57.58	59.44	58.56	55.95	57.73	54.22	
				PCR 2	55.67	56.70	56.10	55.08	55.73	52.02	
				AVERAGE Methylation (%)	**56.63**	**58.07**	**57.33**	**55.52**	**56.73**	**53.12**	**56.23**
153	1	M	S - Control	PCR 1	59.09	61.00	61.40	58.65	59.05	57.00	
				PCR 2	59.71	60.69	60.09	57.77	57.83	56.03	
				AVERAGE Methylation (%)	**59.40**	**60.85**	**60.75**	**58.21**	**58.44**	**56.52**	**59.03**
167	1	M	S - Control	PCR 1	56.99	60.08	58.26	56.87	55.16	54.31	
				PCR 2	57.23	58.70	57.31	57.61	56.30	54.45	
				AVERAGE Methylation (%)	**57.11**	**59.39**	**57.79**	**57.24**	**55.73**	**54.38**	**56.94**
91	1	F	EtOH	PCR 1	54.12	54.86	58.43	53.78	56.47	50.93	
				PCR 2	55.16	55.09	56.31	52.51	54.27	51.84	
				AVERAGE Methylation (%)	**54.64**	**54.98**	**57.37**	**53.15**	**55.37**	**51.39**	**54.48**
96	1	F	S - Control	PCR 1	57.84	60.96	62.31	56.43	59.38	55.45	
				PCR 2	57.93	61.06	60.95	57.69	58.63	56.55	
				AVERAGE Methylation (%)	**57.89**	**61.01**	**61.63**	**57.06**	**59.01**	**56.00**	**58.77**
81	1	F	EtOH	PCR 1	57.35	60.33	60.45	56.26	58.42	52.75	
				PCR 2	53.48	55.01	56.23	53.51	56.32	52.75	
				AVERAGE Methylation (%)	**55.42**	**57.67**	**58.34**	**54.89**	**57.37**	**52.75**	**56.07**

299	2	M	S - Control	PCR 1	55.55	56.89	60.58	56.42	58.08	53.79		
				PCR 2	58.53	59.47	59.85	57.94	57.35	54.93		
				AVERAGE Methylation (%)	**57.04**	**58.18**	**60.22**	**57.18**	**57.72**	**54.36**		**57.45**
319	2	M	S - Control	PCR 1	43.27	42.29	43.68	37.63	43.28	38.52		
				PCR 2	48.42	47.74	50.3	43.28	48.87	45.15		
				AVERAGE Methylation (%)	**45.85**	**45.02**	**46.99**	**40.46**	**46.08**	**41.84**		**44.37**
329	2	M	EtOH	PCR 1	62.12	61.76	64.98	59.5	61.06	56.9		
				PCR 2	56.96	61.79	63.54	55.37	57.83	54.09		
				AVERAGE Methylation (%)	**59.54**	**61.78**	**64.26**	**57.44**	**59.45**	**55.50**		**59.66**
337	2	M	EtOH	PCR 1	60.53	61.78	65.33	60.42	62.18	53.45		
				PCR 2	-	-	-	-	-	-		
				AVERAGE Methylation (%)	**60.53**	**61.78**	**65.33**	**60.42**	**62.18**	**53.45**		**60.62**
352	2	F	S - Control	PCR 1	60.89	61.93	64.35	58.94	62.47	56.88		
				PCR 2	59.87	60.18	60.96	58.17	59.67	56.54		
				AVERAGE Methylation (%)	**60.38**	**61.06**	**62.66**	**58.56**	**61.07**	**56.71**		**60.07**
253	2	F	S - Control	PCR 1	54.39	56.33	58.32	54.71	57.12	52.63		
				PCR 2	54.55	57.46	55.25	54.71	55.59	52.92		
				AVERAGE Methylation (%)	**54.47**	**56.90**	**56.79**	**54.71**	**56.36**	**52.78**		**55.33**
271	2	F	EtOH	PCR 1	63.1	65.97	67.92	62.63	65.20	61.36		
				PCR 2	-	-	-	-	-	-		
				AVERAGE Methylation (%)	**63.10**	**65.97**	**67.92**	**62.63**	**65.20**	**61.36**		**64.36**
272	2	F	EtOH	PCR 1	58.19	59.16	60.41	56.85	57.91	55.05		
				PCR 2	58.91	61.31	61.85	57.08	57.81	53.84		
				AVERAGE Methylation (%)	**58.55**	**60.24**	**61.13**	**56.97**	**57.86**	**54.45**		**58.20**

487	3	M	EtOH	PCR 1	57.25	57.28	58.41	57.02	58.86	53.54		
				PCR 2	55.74	58.26	58.21	54.87	57.02	51.94		
				AVERAGE Methylation (%)	**56.50**	**57.77**	**58.31**	**55.95**	**57.94**	**52.74**		**56.53**
413	3	M	EtOH	PCR 1	51.96	55.11	55.14	52.54	54.79	49.69		
				PCR 2	55.02	55.82	56.46	53.8	54.24	50.69		
				AVERAGE Methylation (%)	**53.49**	**55.47**	**55.80**	**53.17**	**54.52**	**50.19**		**53.77**
424	3	M	S - Control	PCR 1	58.02	59.82	61.44	59.01	58.88	55.89		
				PCR 2	58.5	56.54	58.1	56.17	57.57	54.31		
				AVERAGE Methylation (%)	**58.26**	**58.18**	**59.77**	**57.59**	**58.23**	**55.10**		**57.85**
459	3	F	EtOH	PCR 1	54.48	56.04	60.33	51.85	57.49	52.99		
				PCR 2	56.43	56.25	58.22	52.45	56.76	52.99		
				AVERAGE Methylation (%)	**55.46**	**56.15**	**59.28**	**52.15**	**57.13**	**52.99**		**55.52**
481	3	F	EtOH	PCR 1	51.36	52.32	54.18	50.24	51.93	49.78		
				PCR 2	52.75	56.04	53.93	53.47	53.62	52.21		
				AVERAGE Methylation (%)	**52.06**	**54.18**	**54.06**	**51.86**	**52.78**	**51.00**		**52.65**
407	3	F	EtOH	PCR 1	55.25	57.54	59.93	55.70	58.60	52.22		
				PCR 2	57.05	59.67	61.28	55.29	57.77	53.93		
				AVERAGE Methylation (%)	**56.15**	**58.61**	**60.61**	**55.50**	**58.19**	**53.08**		**57.02**
405	3	F	S - Control	PCR 1	53.16	55.21	56.07	53.5	54.63	51.39		
				PCR 2	53.78	56.71	53.9	54.64	53.92	52.98		
				AVERAGE Methylation (%)	**53.47**	**55.96**	**54.99**	**54.07**	**54.28**	**52.19**		**54.16**
433	3	F	S - Control	PCR 1	59.36	60.23	59.73	56.86	58.30	56.20		
				PCR 2	58.52	58.72	59.8	56.01	57.67	55.03		
				AVERAGE Methylation (%)	**58.94**	**59.48**	**59.77**	**56.44**	**57.99**	**55.62**		**58.04**

0%	-	-	-	PCR 1	0.81	0.91	1.92	0.86	1.53	1		
				PCR 2	1.2	1.18	1.99	1.31	1.87	1.2		
				AVERAGE Methylation (%)	**1.01**	**1.05**	**1.96**	**1.09**	**1.70**	**1.10**		**1.32**
0%	-	-	-	PCR 1	1.06	0	2.15	0	1.37	0		
				PCR 2	1.11	0.79	2.09	0	1.5	0		
				AVERAGE Methylation (%)	**1.09**	**0.40**	**2.12**	**0.00**	**1.44**	**0.00**		**0.84**
BM Water	-	-	-	PCR 1								
				PCR 2		Failed Run = No DNA						
				AVERAGE Methylation (%)								-
BM Water	-	-	-	PCR 1								
				PCR 2		Failed Run = No DNA						
				AVERAGE Methylation (%)								-
PCR Control	-	-	-	PCR 1								
				PCR 2		Failed Run = No DNA						
				AVERAGE Methylation (%)								-

Appendix N: Pyrosequencing Data at CTCF2 for Generations F_0-F_3

Sample	Generation	Sex	Treatment		CpG Site Analysed 1	2	3	4	5	CTCF2 Mean Methylation (%)/Sample
76	0	M	NT	PCR 1	59.93	54.26	54.23	57.30	56.97	
				PCR 2	58.68	56.98	52.86	55.64	56.36	
				AVERAGE Methylation (%)	**59.31**	**55.62**	**53.55**	**56.47**	**56.67**	**56.32**
66	0	M	NT	PCR 1	67.34	57.39	54.46	63.17	60.05	
				PCR 2	66.53	58.41	55.67	62.44	60.59	
				AVERAGE Methylation (%)	**66.94**	**57.90**	**55.07**	**62.81**	**60.32**	**60.61**
41	0	F	NT	PCR 1	57.08	54.08	54.01	51.57	52.69	
				PCR 2	56.02	55.63	51.99	54.18	53.20	
				AVERAGE Methylation (%)	**56.55**	**54.86**	**53.00**	**52.88**	**52.95**	**54.05**
43	0	F	NT	PCR 1	57.28	55.06	52.91	53.84	52.95	
				PCR 2	58.67	54.99	56.27	56.79	56.61	
				AVERAGE Methylation (%)	**57.98**	**55.03**	**54.59**	**55.32**	**54.78**	**55.54**
51	0	F	NT	PCR 1	55.83	54.84	52.91	54.12	52.66	
				PCR 2	54.93	53.61	52.94	50.22	55.35	
				AVERAGE Methylation (%)	**55.38**	**54.23**	**52.93**	**52.17**	**54.01**	**53.74**
58	0	F	NT	PCR 1	57.71	54.20	52.45	50.09	52.71	
				PCR 2	56.24	54.55	52.57	52.43	54.00	
				AVERAGE Methylation (%)	**56.98**	**54.38**	**52.51**	**51.26**	**53.36**	**53.70**

				PCR 1	60.92	57.80	57.76	57.03	57.57	
133	1	M	S - Control	PCR 2	56.51	57.80	54.84	56.73	55.66	
				AVERAGE Methylation (%)	**58.72**	**57.80**	**56.30**	**56.88**	**56.62**	**57.26**
				PCR 1	58.17	58.24	55.70	56.17	57.80	
153	1	M	S - Control	PCR 2	57.35	58.65	54.50	53.88	57.83	
				AVERAGE Methylation (%)	**57.76**	**58.45**	**55.10**	**55.03**	**57.82**	**56.83**
				PCR 1	55.08	56.97	53.71	51.97	54.11	
167	1	M	S - Control	PCR 2	58.90	57.45	53.88	54.22	57.48	
				AVERAGE Methylation (%)	**56.99**	**57.21**	**53.80**	**53.10**	**55.80**	**55.38**
				PCR1	56.17	52.38	54.35	53.97	55.20	
130	1	M	EtOH	PCR2	54.47	53.77	50.40	52.03	51.71	
				AVERAGE Methylation (%)	**55.32**	**53.08**	**52.38**	**53.00**	**53.46**	**53.45**
				PCR 1	54.15	52.59	52.90	54.52	53.39	
115	1	M	EtOH	PCR 2	52.64	55.19	52.22	53.01	52.61	
				AVERAGE Methylation (%)	**53.40**	**53.89**	**52.56**	**53.77**	**53.00**	**53.32**
				PCR 1	59.31	56.52	58.02	58.39	58.40	
96	1	F	S - Control	PCR 2	59.10	59.03	57.29	56.56	56.74	
				AVERAGE Methylation (%)	**59.21**	**57.78**	**57.66**	**57.48**	**57.57**	**57.94**
				PCR 1	55.35	55.68	54.20	56.42	55.57	
91	1	F	EtOH	PCR 2	58.01	53.32	55.69	51.95	54.89	
				AVERAGE Methylation (%)	**56.68**	**54.50**	**54.95**	**54.19**	**55.23**	**55.11**

81	1	F	EtOH	PCR 1	54.75	55.87	52.79	53.14	52.06	
				PCR 2	56.92	54.46	52.89	51.63	54.77	
				AVERAGE Methylation (%)	**55.84**	**55.17**	**52.84**	**52.39**	**53.42**	**53.93**
319	2	M	S - Control	PCR 1	49.94	49.89	46.62	46.83	47.32	
				PCR 2	-	-	-	-	-	
				AVERAGE Methylation (%)	**49.94**	**49.89**	**46.62**	**46.83**	**47.32**	**48.12**
299	2	M	S - Control	PCR1	59.02	55.79	54.75	55.82	54.81	
				PCR2	56.66	58.38	54.92	54.92	56.77	
				AVERAGE Methylation (%)	**57.84**	**57.09**	**54.84**	**55.37**	**55.79**	**56.18**
337	2	M	EtOH	PCR 1	61.92	58.57	58.62	58.95	59.71	
				PCR 2	57.16	58.20	55.25	55.19	58.25	
				AVERAGE Methylation (%)	**59.54**	**58.39**	**56.94**	**57.07**	**58.98**	**58.18**
329	2	M	EtOH	PCR 1	58.92	58.78	55.58	56.03	56.34	
				PCR 2	59.49	58.29	55.76	54.88	55.90	
				AVERAGE Methylation (%)	**59.21**	**58.54**	**55.67**	**55.46**	**56.12**	**57.00**
352	2	F	S - Control	PCR 1	61.21	58.36	57.05	59.40	58.30	
				PCR 2	61.25	56.96	56.88	58.64	56.97	
				AVERAGE Methylation (%)	**61.23**	**57.66**	**56.97**	**59.02**	**57.64**	**58.50**
253	2	F	S - Control	PCR 1	56.74	53.55	53.06	54.56	54.56	
				PCR 2	57.29	53.19	54.15	52.30	54.75	
				AVERAGE Methylation (%)	**57.02**	**53.37**	**53.61**	**53.43**	**54.66**	**54.42**
271	2	F	EtOH	PCR 1	-	-	-	-	-	
				PCR 2	59.40	57.04	56.45	59.46	57.78	
				AVERAGE Methylation (%)	**59.40**	**57.04**	**56.45**	**59.46**	**57.78**	**58.03**

272	2	F	EtOH	PCR 1	56.99	56.83	54.75	55.43	54.97	
				PCR 2	61.42	58.28	58.19	58.49	58.13	
				AVERAGE Methylation (%)	**59.21**	**57.56**	**56.47**	**56.96**	**56.55**	**57.35**
413	3	M	EtOH	PCR 1	57.00	54.15	53.75	51.90	52.53	
				PCR 2	56.87	53.70	53.51	54.80	54.48	
				AVERAGE Methylation (%)	**56.94**	**53.93**	**53.63**	**53.35**	**53.51**	**54.27**
424	3	M	S - Control	PCR 1	56.38	56.21	54.26	54.07	55.31	
				PCR 2	57.01	56.79	52.86	53.99	56.35	
				AVERAGE Methylation (%)	**56.70**	**56.50**	**53.56**	**54.03**	**55.83**	**55.32**
487	3	M	EtOH	PCR 1	58.52	54.96	55.54	52.30	53.57	
				PCR 2	57.31	56.36	53.51	54.80	53.96	
				AVERAGE Methylation (%)	**57.92**	**55.66**	**54.53**	**53.55**	**53.77**	**55.08**
459	3	F	EtOH	PCR 1	57.99	56.62	53.38	54.40	53.10	
				PCR 2	56.47	55.26	53.18	54.93	54.40	
				AVERAGE Methylation (%)	**57.23**	**55.94**	**53.28**	**54.67**	**53.75**	**54.97**
481	3	F	EtOH	PCR 1	54.40	53.72	51.84	54.29	53.12	
				PCR 2	60.56	56.79	56.51	52.49	58.69	
				AVERAGE Methylation (%)	**57.48**	**55.26**	**54.18**	**53.39**	**55.91**	**55.24**
433	3	F	S - Control	PCR 1	57.50	54.27	54.25	56.56	56.04	
				PCR 2	57.10	55.11	53.85	56.03	53.29	
				AVERAGE Methylation (%)	**57.30**	**54.69**	**54.05**	**56.30**	**54.67**	**55.40**
405	3	F	S - Control	PCR 1	55.51	52.95	54.78	52.99	55.54	
				PCR 2	53.21	55.25	53.17	51.92	54.89	
				AVERAGE Methylation (%)	**54.36**	**54.10**	**53.98**	**52.46**	**55.22**	**54.02**

407	3	F	EtOH	PCR1	57.20	55.99	52.15	56.44	54.39	
				PCR2	59.11	55.17	54.25	56.80	54.49	
				AVERAGE Methylation (%)	**58.16**	**55.58**	**53.20**	**56.62**	**54.44**	**55.60**
				PCR1	54.40	53.72	51.84	54.29	53.12	
481	3	F	EtOH	PCR2	60.56	56.79	56.51	52.49	58.69	
				AVERAGE Methylation (%)	**57.48**	**55.26**	**54.18**	**53.39**	**55.91**	**55.24**
				PCR 1	2.16	1.86	1.18	1.80	1.20	
0%	-	-		PCR 2	2.13	1.77	1.03	1.92	1.14	
				AVERAGE Methylation (%)	**2.15**	**1.82**	**1.11**	**1.86**	**1.17**	**1.62**
				PCR 1	2.75	2.61	0.91	2.23	1.68	
0%	-	-		PCR 2	3.03	2.72	1.21	2.33	1.40	
				AVERAGE Methylation (%)	**2.89**	**2.67**	**1.06**	**2.28**	**1.54**	**2.09**
				PCR 1						
BM Water	-	-		PCR 2		Failed Run = No DNA				
				AVERAGE Methylation (%)						-
				PCR 1						
BM Water	-	-		PCR 2		Failed Run = No DNA				
				AVERAGE Methylation (%)						-
				PCR 1						
PCR Control	-	-		PCR 2		Failed Run = No DNA				
				AVERAGE Methylation (%)						-

Appendix O: Effect of Sex on CTCF1 Methylation at Generations F_0-F_3

CpG Site	Generation	N	P- value[a]
Mean Methylation	All	30	0.645
	0	8	0.165
	1	8	0.882
	2	8	0.564
	3	8	0.882
1	All	30	0.589
	0	8	1.000
	1	8	0.882
	2	8	0.773
	3	8	0.882
2	All	30	0.618
	0	8	0.355
	1	8	0.882
	2	8	0.773
	3	8	0.297
3	All	30	0.967
	0	8	0.165
	1	8	0.882
	2	8	0.773
	3	8	0.882
4	All	30	0.280
	0	8	0.165
	1	8	0.655
	2	8	0.773
	3	8	0.297
5	All	30	0.835
	0	8	0.165
	1	8	0.294
	2	8	0.564
	3	8	0.456
6	All	30	0.803
	0	8	0.355
	1	8	0.882
	2	8	0.248
	3	8	0.655

Appendix P: Effect of Sex on CTCF2 Methylation Generations F_0-F_3

CpG Site	Generation	N	p- value[a]
Mean Methylation	All	30	0.708
	0	8	0.064
	1	8	0.655
	2	8	0.387
	3	8	0.655
1	All	30	0.835
	0	8	0.064
	1	8	0.655
	2	8	0.468
	3	8	0.655
2	All	30	0.212
	0	8	0.064
	1	8	0.882
	2	8	0.564
	3	8	0.655
3	All	30	0.934
	0	8	0.165
	1	8	0.456
	2	8	0.387
	3	8	0.655
4	All	30	0.835
	0	8	0.064
	1	8	0.882
	2	8	0.248
	3	8	0.456
5	All	30	0.454
	0	8	0.064
	1	8	0.882
	2	8	0.564
	3	8	0.456

Appendix Q: CTCF1 Methylation and Growth Rate Correlations

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Position 6 Methylation	Mean Methylation	Overall (TP0)	Suckling (TP1)	Weaning (TP2)	Adult (TP3)
Overall (TP0)	0.5238	0.4524	-0.0238	0.619	0.0359	0.5238	0.5238	1.00			
	P = 0.1827	*P = 0.2604*	*P = 0.9554*	*P = 0.1017*	*P = 0.9327*	*P = 0.1827*	*P = 0.1827*	-			
Suckling (TP1)	-0.619	-0.5238	-0.7619	-0.6429	-0.6108	-0.619	-0.619	-0.0238	1.00		
	P = 0.1017	*P = 0.1827*	*P = 0.028*	*P = 0.0856*	*P = 0.1077*	*P = 0.1017*	*P = 0.1017*	*P = 0.9554*	-		
Weaning (TP2)	-0.0714	-0.119	-0.5238	-0.0238	-0.4791	-0.0714	-0.0714	0.6905	0.3333	1.00	
	P = 0.8665	*P = 0.7789*	*P = 0.1827*	*P = 0.9554*	*P = 0.2297*	*P = 0.8665*	*P = 0.8665*	*P = 0.058*	*P = 0.4198*	-	
Adult (TP3)	0.2619	0.1905	-0.3095	0.381	-0.1796	0.2619	0.2619	0.881	0.2619	0.619	1.00
	P = 0.5309	*P = 0.6514*	*P = 0.4556*	*P = 0.3518*	*P = 0.6703*	*P = 0.5309*	*P = 0.5309*	*P = 0.0039*	*P = 0.5309*	*P = 0.1017*	-

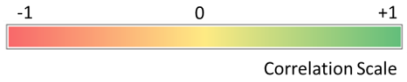


Figure Q.1: F_1 CTCF1 Methylation & Growth Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF1 and Growth Rates. F_1 (n = 8) (Spearmans Correlation)

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Position 6 Methylation	Mean Methylation	Overall (TP0)	Suckling (TP1)	Weaning (TP2)	Adult (TP3)
Overall (TP0)	-0.0476	0.0476	0.0476	0	-0.0476	-0.3333	-0.0476	1.00			
	P = 0.9108	*P = 0.9108*	*P = 0.9108*	*P = 1.000*	*P = 0.9108*	*P = 0.4198*	*P = 0.9108*	-			
Suckling (TP1)	-0.4524	-0.3095	-0.3095	-0.3571	-0.4524	-0.3571	-0.4524	0.4048	1.00		
	P = 0.2604	*P = 0.4556*	*P = 0.4556*	*P = 0.3851*	*P = 0.2604*	*P = 0.3851*	*P = 0.2604*	*P = 0.3199*	-		
Weaning (TP2)	-0.2857	-0.1667	-0.1667	-0.2381	-0.2857	-0.3095	-0.2857	0.9048	0.5	1.00	
	P = 0.4927	*P = 0.6932*	*P = 0.6932*	*P = 0.5702*	*P = 0.4927*	*P = 0.4556*	*P = 0.4927*	*P = 0.002*	*P = 0.207*		
Adult (TP3)	0.0238	0.0952	0.0952	0.0476	0.0238	-0.4524	0.0238	0.619	0.5238	0.3571	1.00
	P = 0.9554	*P = 0.8225*	*P = 0.8225*	*P = 0.9108*	*P = 0.9554*	*P = 0.2604*	*P = 0.9554*	*P = 0.1017*	*P = 0.1827*	*P = 0.3851*	

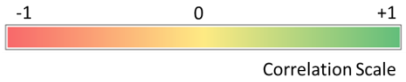


Figure Q.2: F_2 CTCF1 Methylation & Growth Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF1 and Growth Rates. F_2 (n = 8) correlated

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Position 6 Methylation	Mean Methylation	Overall (TP0)	Suckling (TP1)	Weaning (TP2)	Adult (TP3)
Overall (TP0)	0.381	0	-0.119	0.4762	0.1667	-0.0476	0.1429	1.00			
	P = 0.3518	*P = 1.000*	*P = 0.7789*	*P = 0.2329*	*P = 0.6932*	*P = 0.9108*	*P = 0.7358*	-			
Suckling (TP1)	0.2857	0.1905	-0.2619	0.4286	-0.0714	0.1429	0.2381	0.5238	1.00		
	P = 0.4927	*P = 0.6514*	*P = 0.5309*	*P = 0.2894*	*P = 0.8665*	*P = 0.7358*	*P = 0.5702*	*P = 0.1827*	-		
Weaning (TP2)	0.4286	0.0714	0.0476	0.5476	0.2857	0.0476	0.2381	0.8571	0.2857	1.00	
	P = 0.2894	*P = 0.8665*	*P = 0.9108*	*P = 0.16*	*P = 0.4927*	*P = 0.9108*	*P = 0.5702*	*P = 0.0065*	*P = 0.4927*	-	
Adult (TP3)	0.8571	0.6429	0.5952	0.6905	0.7381	0.619	0.7143	0.5714	0.1905	0.4762	1.00
	P = 0.0065	*P = 0.0856*	*P = 0.1195*	*P = 0.058*	*P = 0.0366*	*P = 0.1017*	*P = 0.0465*	*P = 0.139*	*P = 0.6514*	*P = 0.2329*	-

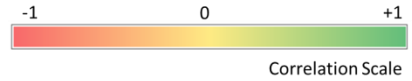


Figure Q.3: F_3 CTCF1 Methylation & Growth Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF1 and Growth Rates. F_3 (n = 8) correlated

Appendix R: CTCF1 Methylation and Open Field Measure Correlations

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Position 6 Methylation	Mean Methylation	Total Distance	Time in Inner Zone	Faecal Boli	Amount of Urine
Total Distance	-0.2381	-0.0952	-0.0952	-0.2143	0.0719	-0.2381	-0.2381	1.000			
	P = 0.5702	*P = 0.8225*	*P = 0.8225*	*P = 0.6103*	*P = 0.8657*	*P = 0.5702*	*P = 0.5702*	-			
Time in Inner Zone	-0.4524	-0.381	-0.3333	-0.3571	-0.5988	-0.4524	-0.4524	0.0000	1.000		
	P = 0.2604	*P = 0.3518*	*P = 0.4198*	*P = 0.3851*	*P = 0.1168*	*P = 0.2604*	*P = 0.2604*	*P = 1.000*	-		
Faecal Boli	-0.3448	-0.2171	-0.7534	-0.2554	-0.5588	-0.3448	-0.3448	0.332	0.2171	1.000	
	P = 0.4029	*P = 0.6056*	*P = 0.0309*	*P = 0.5415*	*p = 0.1499*	*P = 0.4029*	*P = 0.4029*	*P = 0.4217*	*P = 0.6056*	-	
Amount of Urine	-0.3712	-0.2474	-0.5774	-0.2474	-0.6845	-0.3712	-0.3712	0.0825	0.7835	0.6414	1.000
	P = 0.3654	*P = 0.5546*	*P = 0.134*	*P = 0.5546*	*P = 0.0611*	*P = 0.3654*	*P = 0.3654*	*P = 0.8461*	*P = 0.0214*	*P = 0.0865*	-

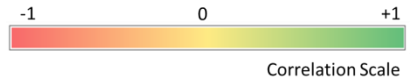


Figure R.1: F_1 CTCF1 Methylation & Open-Field Behaviour Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF1 and Open field Measurements. F_1 (n = 8) (Spearman's Correlation)

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Position 6 Methylation	Mean Methylation	Total Distance	Time in Inner Zone	Faecal Boli	Amount of Urine
Total Distance	0.2143	0.2381	0.2381	0.3333	0.2143	0.5952	0.2143	1.000			
	P = 0.6103	*P = 0.5702*	*P = 0.5702*	*P = 0.4198*	*P = 0.6103*	*P = 0.1195*	*P = 0.6103*	-			
Time in Inner Zone	0.619	0.5952	0.5952	0.6429	0.619	0.9524	0.619	0.7857	1.000		
	P = 0.1017	*P = 0.1195*	*P = 0.1195*	*P = 0.0856*	*P = 0.1017*	*P = 0.0003*	*P = 0.1017*	*P = 0.0208*	-		
Faecal Boli	0.000	0.0412	0.0412	0.1237	0.000	0.3299	0.000	0.5361	0.4536	1.000	
	P = 1.000	*P = 0.9228*	*P = 0.9228*	*P = 0.7704*	*P = 1.000*	*P = 0.4248*	*P = 1.000*	*P = 0.1708*	*P = 0.2589*	-	
Amount of Urine	-0.4949	-0.4949	-0.4949	-0.5361	-0.4949	-0.2887	-0.4949	-0.165	-0.2062	-0.1429	1.000
	P = 0.2125	*P = 0.2125*	*P = 0.2125*	*P = 0.1708*	*P = 0.2125*	*P = 0.4881*	*P = 0.2125*	*P = 0.6963*	*P = 0.6242*	*P = 0.7358*	-

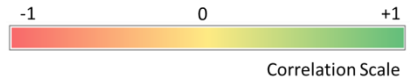


Figure R.2: F_2 CTCF1 Methylation & Open-Field Behaviour Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF1 and Open field Measurements. F_2 (n = 8) (Spearman's Correlation)

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Position 6 Methylation	Mean Methylation	Total Distance	Time in Inner Zone	Faecal Boli	Amount of Urine
Total Distance	0.4524	0.4762	0.5238	0.4762	0.5238	0.6429	0.5714	1.000			
	P = 0.2604	*P = 0.2329*	*P = 0.1827*	*P = 0.2329*	*P = 0.1827*	*P = 0.0856*	*P = 0.139*	-			
Time in Inner Zone	0.0476	-0.0952	-0.119	-0.2381	-0.1667	0.1905	0	0.2619	1.000		
	P = 0.9108	*P = 0.8225*	*P = 0.7789*	*P = 0.5702*	*P = 0.6932*	*P = 0.6514*	*P = 1.000*	*P = 0.5309*			
Faecal Boli	0.252	0.378	0.378	0.252	0.378	0.126	0.252	-0.252	-0.7559	1.000	
	P = 0.5472	*P = 0.3559*	*P = 0.3559*	*P = 0.5472*	*P = 0.3559*	*P = 0.7663*	*P = 0.5472*	*P = 0.5472*	*P = 0.03*		
Amount of Urine	-0.1364	-0.3	-0.1364	0.1364	-0.0273	-0.3273	-0.1909	0.1637	-0.1364	-0.433	1.000
	P = 0.7474	*P = 0.4703*	*P = 0.7474*	*P = 0.7474*	*P = 0.9489*	*P = 0.4287*	*P = 0.6506*	*P = 0.6986*	*P = 0.7474*	*P = 0.284*	

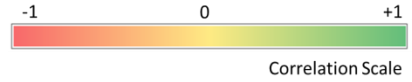


Figure R.3: F_3 CTCF1 Methylation & Open-Field Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF1 and Open field Measurements. F_3 (n = 8) (Spearman's Correlation)

Appendix S: CTCF2 Methylation and Growth Rate Correlation

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Mean Methylation	Overall (TP0)	Suckling (TP1)	Weaning (TP2)	Adult (TP3)
Overall (TP0)	0.2619	0.5	0.1667	0.2857	0.5	0.2619	1			
	P = 0.5309	*P = 0.207*	*P = 0.6932*	*P = 0.4927*	*P = 0.207*	*P = 0.5309*	-			
Suckling (TP1)	-0.1429	-0.4286	-0.1429	0.1667	-0.1429	-0.1429	-0.0238	1		
	P = 0.7358	*P = 0.2894*	*P = 0.7358*	*P = 0.6932*	*P = 0.7358*	*P = 0.7358*	*P = 0.9554*	-		
Weaning (TP2)	-0.3333	-0.0952	-0.3095	0.0714	-0.0476	-0.3333	0.6905	0.3333	1	
	P = 0.4198	*P = 0.8225*	*P = 0.4556*	*P = 0.8665*	*P = 0.9108*	*P = 0.4198*	*P = 0.058*	*P = 0.4198*	-	
Adult (TP3)	0.2143	0.381	0.1429	0.2619	0.3333	0.2143	0.881	0.2619	0.619	1
	P = 0.6103	*P = 0.3518*	*P = 0.7358*	*P = 0.5309*	*P = 0.4198*	*P = 0.6103*	*P = 0.0039*	*P = 0.5309*	*P = 0.1017*	-

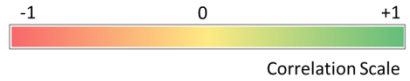


Figure S.1: F_1 CTCF2 Methylation & Growth Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF2 and Growth Rates. F_1 (n = 8) (Spearman's Correlation)

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Mean Methylation	Overall (TP0)	Suckling (TP1)	Weaning (TP2)	Adult (TP3)
Overall (TP0)	-0.1796	0.0952	-0.2381	-0.2381	-0.0476	-0.2143	1			
	P = 0.6703	*P = 0.8225*	*P = 0.5702*	*P = 0.5702*	*P = 0.9108*	*P = 0.6103*	-			
Suckling (TP1)	-0.6347	-0.2143	-0.7857	-0.6429	-0.6429	-0.7143	0.4048	1		
	P = 0.0909	*P = 0.6103*	*P = 0.0208*	*P = 0.0856*	*P = 0.0856*	*P = 0.0465*	*P = 0.3199*	-		
Weaning (TP2)	-0.3713	0.0952	-0.381	-0.4286	-0.3095	-0.4048	0.9048	0.5	1	
	P = 0.3652	*P = 0.8225*	*P = 0.3518*	*P = 0.2894*	*P = 0.4556*	*P = 0.3199*	*P = 0.002*	*P = 0.207*	-	
Adult (TP3)	-0.0838	0.0476	-0.2143	-0.2143	0	-0.1429	0.619	0.5238	0.3571	1
	P = 0.8435	*P = 0.9108*	*P = 0.6103*	*P = 0.6103*	*P = 1.000*	*P = 0.7358*	*P = 0.1017*	*P = 0.1827*	*P = 0.3851*	-

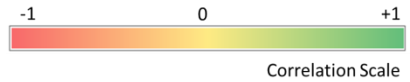


Figure S.2: F_2 CTCF2 Methylation & Growth Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF2 and Growth Rates. F_2 (n = 8) (Spearman's Correlation)

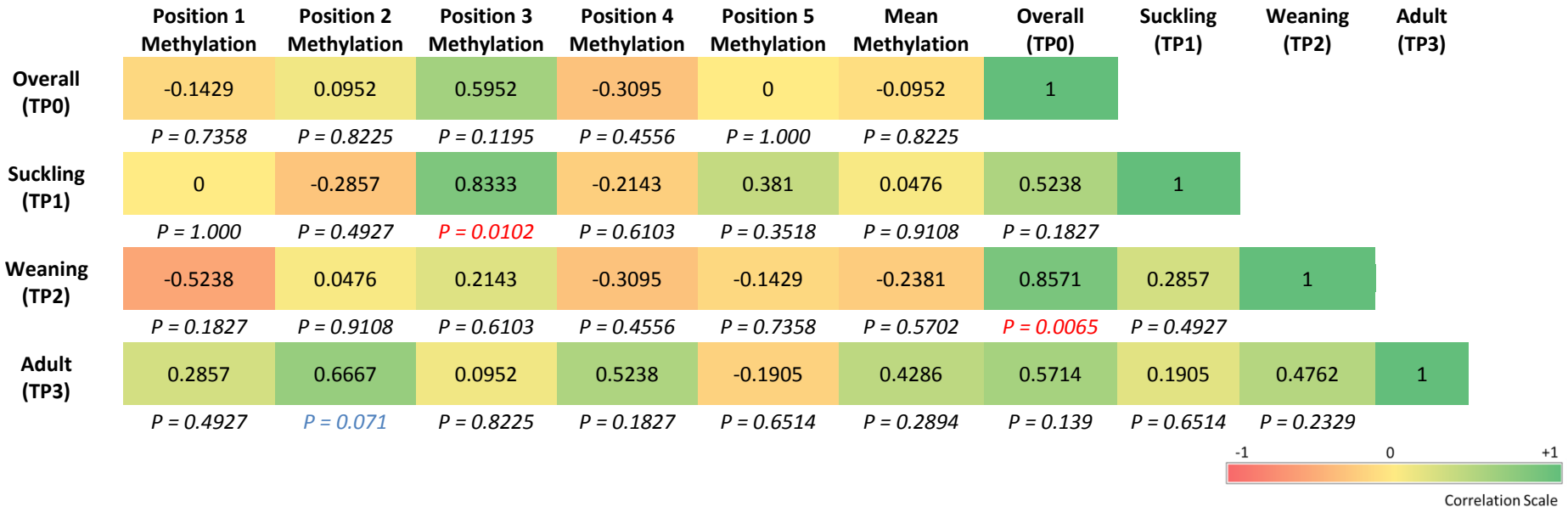


Figure S.3: F_3 CTCF2 Methylation & Growth Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF2 and Growth Rates. F_3 (n = 8) (Spearman's Correlation)

Appendix T: CTCF2 Methylation and Open Field Measure Correlations

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Mean Methylation	Total Distance	Time in Inner Zone	Faecal Boli	Amount of Urine
Total Distance	0	-0.119	-0.1905	-0.4762	-0.1667	0	1			
	P = 1.000	*P = 0.7789*	*P = 0.6514*	*P = 0.2329*	*P = 0.6932*	*P = 1*	-			
Time in Inner Zone	-0.4524	-0.6667	-0.5476	-0.4762	-0.381	-0.4524	0	1		
	P = 0.2604	*P = 0.071*	*P = 0.16*	*P = 0.2329*	*P = 0.3518*	*P = 0.2604*	*P = 1.000*	-		
Faecal Boli	-0.1788	-0.2426	-0.332	-0.166	-0.1788	-0.1788	0.332	0.2171	1	
	P = 0.6719	*P = 0.5626*	*P = 0.4217*	*P = 0.6944*	*P = 0.6719*	*P = 0.6719*	*P = 0.4217*	*P = 0.6056*	-	
Amount of Urine	-0.5361	-0.5774	-0.7011	-0.5361	-0.4124	-0.5361	0.0825	0.7835	0.6414	1
	P = 0.1708	*P = 0.134*	*P = 0.0527*	*P = 0.1708*	*P = 0.31*	*P = 0.1708*	*P = 0.8461*	*P = 0.0214*	*P = 0.0865*	-

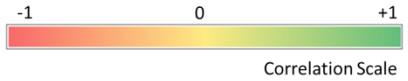


Figure T.1: F_1 CTCF2 Methylation & Open-Field Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF2 and Open Field Measures. F_1 (n = 8) (Spearman's Correlation)

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Mean Methylation	Total Distance	Time in Inner Zone	Faecal Boli	Amount of Urine
Total Distance	0.024	-0.0952	-0.1667	0.2143	-0.0476	-0.0238	1			
	P = 0.9551	*P = 0.8225*	*P = 0.6932*	*P = 0.6103*	*P = 0.9108*	*P = 0.9554*	-			
Time in Inner Zone	0.5509	0.3095	0.4286	0.6905	0.4286	0.5238	0.7857	1		
	P = 0.157	*P = 0.4556*	*P = 0.2894*	*P = 0.058*	*P = 0.2894*	*P = 0.1827*	*P = 0.0208*	-		
Faecal Boli	0.1037	0.4949	0.0412	-0.0412	-0.1237	0.0412	0.5361	0.4536	1	
	P = 0.8069	*P = 0.2125*	*P = 0.9228*	*P = 0.9228*	*P = 0.7704*	*P = 0.9228*	*P = 0.1708*	*P = 0.2589*	-	
Amount of Urine	-0.4356	-0.2887	-0.2474	-0.3712	-0.3712	-0.3712	-0.165	-0.2062	-0.1429	1
	P = 0.2807	*P = 0.4881*	*P = 0.5546*	*P = 0.3654*	*P = 0.3654*	*P = 0.3654*	*P = 0.6963*	*P = 0.6242*	*P = 0.7358*	-

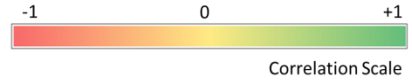


Figure T.2: F_2 CTCF2 Methylation & Open-Field Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF2 and Open Field Measures. F_2 (n = 8) (Spearman's Correlation)

	Position 1 Methylation	Position 2 Methylation	Position 3 Methylation	Position 4 Methylation	Position 5 Methylation	Mean Methylation	Total Distance	Time in Inner Zone	Faecal Boli	Amount of Urine
Total Distance	-0.5238	0.5238	-0.5	0.2857	0.0952	0.0238	1			
	P = 0.1827	*P = 0.1827*	*P = 0.207*	*P = 0.4927*	*P = 0.8225*	*P = 0.9554*	-			
Time in Inner Zone	-0.2857	0.1429	0.0238	0.119	0.0952	-0.0476	0.2619	1		
	P = 0.4927	*P = 0.7358*	*P = 0.9554*	*P = 0.7789*	*P = 0.8225*	*P = 0.9108*	*P = 0.5309*	-		
Faecal Boli	0.7559	0.252	0	0.378	-0.252	0.378	-0.252	-0.7559	1	
	P = 0.03	*P = 0.5472*	*P = 1.000*	*P = 0.3559*	*P = 0.5472*	*P = 0.3559*	*P = 0.5472*	*P = 0.03*	-	
Amount of Urine	-0.7638	-0.3	-0.1909	-0.5728	-0.0818	-0.4637	0.1637	-0.1364	-0.4637	1
	P = 0.0274	*P = 0.4703*	*P = 0.6506*	*P = 0.1378*	*P = 0.8472*	*P = 0.2471*	*P = 0.6986*	*P = 0.7474*	*P = 0.2471*	-

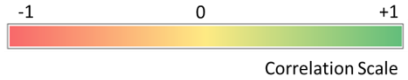


Figure T.3: F_3 CTCF2 Methylation & Open-Field Correlation Map. Correlations between Mean Methylation Level/Methylation Level per CpG site of CTCF2 and Open Field Measures. F_3 (n = 8) Spearman's Correlation