The Epigenetic Transmission of Maternal Effects in Mammals: Evaluating and Extending Laboratory Knowledge to Natural Ecosystems

by

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ABSTRACT

THE EPIGENETIC TRANSMISSION OF MATERNAL EFFECTS IN MAMMALS: EVALUATING AND EXTENDING LABORATORY KNOWLEDGE TO NATURAL ECOSYSTEMS

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Epigenetic maternal effects are being studied in laboratories at an exponentially increasing rate and have routinely been found to have consequences on the physiological and behavioural function of mammalian offspring. Despite this wealth of knowledge, our understanding of how epigenetic maternal effects impact free-living animals within natural ecosystems is comparatively non-existent. Here, I examine laboratory epigenetic maternal effects literature from the last decade with a perspective informed by an eco-evolutionary framework and found that the type and timing of maternal effects influences the extent of offspring DNA methylation in mammals. Additionally, I use an innovative experimental field manipulation to assess the epigenetic maternal effects of stress in free-living North American red squirrels and found inherent sex-specific differences in *Nr3c1* and *Pomc* gene expression of red squirrel offspring despite no effect of maternal stress. Together, these studies highlight the importance of integrating evolutionary, ecophysiological, and molecular theory when studying epigenetic maternal effects.

DEDICATION

This thesis is dedicated to Chinook, Puck, Oberon, Mowat, and Thomson – an eclectic group of raptors who (unbeknownst to them) have taught me incredibly important lessons in vulnerability, mindfulness, focus, and succeeding against the odds while I worked on my thesis.

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Chapter 1: Prologue

1.1 Physiological Stress and the Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal (HPA) axis is an important mediator between how an organism interprets and responds to the stimuli that they encounter in their environment. A complex negative feedback loop that acts to both facilitate and regulate the physiological stress response (Finsterwald and Alberini, 2014), the HPA axis allows for an animal to resiliently navigate the wide variety of chaotic and unpredictable challenges they face in their daily lives. Physiological stress is a well-studied phenomenon within the field of human psychopathology, with physiological stress impacting a variety of biological functions such as long term memory formation, synaptic plasticity, metabolism, the autoimmune system, and behaviour, among others (Finsterwald and Alberini, 2014). Additionally, the study of physiological stress in mammals has predominantly occurred through the study of model organisms in laboratory settings, with minimal attention given to studying physiological stress in natural systems (Boonstra, 2013). Some argue that these laboratory studies cannot be used to sufficiently explain or interpret stress in natural systems because the stressors laboratory animals are exposed to bear minimal resemblance, if any, to those experienced in the wild (Boonstra, 2013). To confidently make inferences about how mammals function physiologically in their natural environments, and extending this to make appropriate behavioural hypotheses, it is crucial that an ecophysiological approach to studying stress and the incorporation of ecologically relevant experimental design becomes more prominent in the literature.

1.2 Stress Epigenetics and Maternal Effects

With the balance of glucocorticoid (GC) production and activation of glucocorticoid receptors (GRs) perpetually in flux (De Kloet et al., 2005), the precise regulation of this dynamic system is critical for maintaining the balance between eustress and distress at the physiological level. In the laboratory literature, the regulation of the dynamic relationship between GCs and GRs has been linked strongly to epigenetic mechanisms, specifically to the methylation of DNA that encodes for GRs (McGowan and Matthews, 2018). Not only does the regulation of DNA methylation allow for the modulation of physiological stress in response to the environment, but methylation of GRs allows for transgenerational parental influence to modify the stress response as well. Akin to the coverage of physiological stress in the literature, however, little is currently understood about how these epigenetic maternal effects are imparted on free-living organisms, the extent to which they do or do not influence an organism's physiology or fitness, or whether they occur outside of the laboratory at all (Bossdorf et al., 2008; Feil and Fraga, 2012; McGowan and Matthews, 2018). With an extensive laboratory literature but a comparatively non-existent set of experimental field validations, caution is required to avoid overstating the importance these effects play in natural systems – an oversight that has occurred a number of times with laboratory rodent research in the past (Wolff, 2003). There exists exciting potential in understanding the interplay between DNA methylation, parental effects, and the regulation of the HPA axis in free-living mammals however further work is needed to validate this potential outside of the laboratory.

1.3 Challenges in Understanding Maternal Effects Epigenetics in Free-Living Populations

A number of challenges currently constrain our ability to extend our understanding of the epigenetic maternal effects of stress to free-living populations of mammals. The study of transgenerational parental effects often demands a thorough understanding of a population's pedigree to accurately follow and measure the transmission of these effects. This poses a staggering barrier of entry for molecular biologists to extend their laboratory research into real world environments as the generation of a highly detailed pedigree for a free-living population costs a significant amount of time, effort, expertise, and money. In contrast, researchers like ecophysiologists have access to long-term ecological data sets, often tracking detailed pedigrees across several generations depending on the species, but they oftentimes lack the training, resources, and expertise required to independently conduct and analyze the technical and sensitive molecular biology experiments needed to validate laboratory trends with their study populations (Bossdorf et al., 2008; Ledón-Rettig et al., 2013). Individually, ecophysiologists, and molecular biologists possess the knowledge, technical skills, and resources to study epigenetic maternal effects (and parental effects in general) in free-living populations (Sheriff et al., 2017) but the collaboration of researchers in these disciplines is paramount in developing a robust understanding of these effects outside of the laboratory.

1.4 Overall Objective

This thesis addresses the previously described knowledge gaps and research challenges within the field of epigenetic maternal effects through the following:

1. A meta-analysis of current literature on the epigenetic maternal effects of stress, which explores to what extent the methylation of DNA is influenced by the type and perinatal timing of maternal effects. This analysis is then used to identify key trends in the literature and key gaps in our current knowledge on the role epigenetic maternal effects of stress play in populations of free-living mammals.
2. An empirical evaluation of the maternal effects of stress in the wild using red squirrels as a model system. I explored how the maternal effects of stress influence the expression of glucocorticoid receptors (*Nr3c1)* and pro-opiomelanocortin (*Pomc)* mRNA and DNA methylation of *Nr3c1* in the HPA axis of yearling red squirrels born to stressed mothers. These data reveal exciting inherent sex-specific regulations of the HPA axis in yearling red squirrels but found no effect of maternal stress exposure on the expression of *Nr3c1* and *Pomc* mRNA. Additionally, this analysis is used to explore similarities and differences between the transmission of these effects in an ecologically-relevant experimental system and the effects which have traditionally been observed in laboratory rodent model systems.

Chapter 2: Type and timing of maternal effects interact to influence transmission, strength and direction of epigenetic outcomes in mammals.

2.1 Abstract

The relationships between maternal effects and epigenetic changes like DNA methylation are becoming increasingly well understood. Maternal effects can alter DNA methylation in offspring via several mechanisms, including maternal diet or behaviour. What remains unclear, however, is whether the type of maternal effect and the timing with respect to developmental stage of the offspring (pre- or post-natal) may differentially influence DNA methylation in offspring. Thus, a meta-analysis was conducted to understand how the type of maternal of effect and pre- or post-natal timing influence the transmission, strength, and direction of offspring DNA methylation. Forty studies met the criteria for inclusion and included 264 individual instances of recorded changes in neonatal DNA methylation due to a maternal effect. We found a significant interaction between method of inheritance (maternal care or maternal diet) and timing (prenatal or postnatal) of maternal effects, with post-natal behavioural effects having the strongest influence on offspring DNA methylation. Notably, there was a dearth of studies in free-ranging non-model organisms, and a pronounced publication bias where there is a skew to published studies reporting large and precise effect sizes relative to sample sizes. With these findings, we discuss the implications of maternal effects being influenced by type and timing of effect and our current limitations in extending knowledge from the laboratory to free-living animals.

2.2 Introduction

Maternal effects are a broadly categorized phenomenon in which the phenotype of offspring is influenced in part by the genotype, phenotype and environment of their mother (Mousseau and Fox, 1998). Originally viewed as a troublesome source of inexplicable but confounding environmental resemblance within offspring (Marshall et al., 2008), maternal effects are now understood as a means by which a mother could shape the phenotype of her offspring. The environmental matching hypothesis highlights the potential for adaptive maternal effects whereby mothers alter the phenotypes of their young to tune their young to thrive in the anticipated perinatal or adult environments (Monaghan, 2008; Sheriff and Love, 2013). Diet and behaviour are two well-studied maternal factors known to influence offspring, especially in the perinatal period (Mousseau and Fox, 1998). Prenatal folic acid consumption in pregnant mice has been shown to influence the expression of multiple traits in offspring including obesity, coat colour, hyperinsulinemia, and life span (Wolff et al., 1998). Postnatal maternal care behaviour, such as licking and grooming of mouse pups, has been well established to have organizational effects on the hypothalamic pituitary adrenal axis in rodents (Cameron et al., 2005; Kaffman and Meaney, 2007; Liberman et al., 2012; Pan et al., 2014a). Additionally, in free-living red squirrels, increased levels of prenatal glucocorticoids in mothers were related to the post-natal growth rate of offspring (Dantzer et al., 2013). Multiple types of maternal effects (diet, behaviour, stress) across timescales during sensitive periods of development (gamete and zygote formation, gestation, parturition, post-parturition) yield potent opportunities to influence offspring phenotype and fitness.

Despite the well-established importance of maternal effects in the development of offspring, the molecular mechanisms linking an offspring's perinatal experience with downstream physiological and behavioural outcomes are only beginning to come into focus. With the advancement of molecular genetic tools, maternal effects have become closely associated with epigenetics; the study of the heritable alteration of gene expression patterns which do not involve the alteration of the genetic code (Champagne, 2008; McGowan and Matthews, 2018; van Otterdijk and Michels, 2016; Wang et al., 2017). While there are a number of different epigenetic processes, DNA methylation, where methyl groups are added or removed to a molecule of DNA to change the three-dimensional conformation of the molecule between heterochromatin and euchromatin (Feil and Fraga, 2012), is the process frequently associated with the study of maternal effects. This change in conformation alters the ability of DNA polymerases to bind to and transcribe the DNA at a gene-specific level (Feil and Fraga, 2012). Whether an increase or decrease in DNA methylation, and thus the promotion or inhibition of gene expression, is beneficial is entirely context dependent (Bossdorf et al., 2008; Finsterwald and Alberini, 2014). In addition to the direction of change in DNA methylation, the extent to which a region is methylated or unmethylated in a tissue is important in determining gene expression activity, making quantitative assessments of overall change in methylation levels a useful tool for understanding the strength of its effects.

Although the link between maternal effects and DNA methylation is clearly emerging, whether the magnitude of change in DNA methylation differs based on the type of maternal effect or the perinatal timing of the maternal effect is not well-known. It is not currently clear whether diet- or behaviour-derived maternal effects, or whether the pre- or post-natal timing of a

maternal effect, have differing impacts on the epigenomic and associated phenotypic outcome in offspring. These factors are important to consider when applying current epigenetic maternal effects literature to the study of ecological epigenetics and behavioural ecology as the relationship between the diversity of types and timings of maternal effects (which are inextricably linked to a diversity in life history strategies across taxa) and the relevance of DNA methylation on ecologically- and evolutionarily-relevant behavioural variation is poorly understood (Ledón-Rettig et al., 2013). Additionally, it is possible that maternal diet and maternal behaviour have interactive effects in influencing the extent of offspring DNA methylation and thus it is important to consider maternal effects and epigenetics from an interdisciplinary perspective.

In this meta-analysis, we explored how the type and timing of maternal effects influences the degree and direction of DNA methylation in offspring. While this is a first step in a more comprehensive understanding of the epigenetics of maternal effects, the findings of this study reveal distinct interactions for the pre- or post-natal timing of exposure in the strength and direction of maternal diet and maternal behaviour on DNA methylation in offspring. We also highlight evidence of strong publication bias in the current literature with respect to the over-reporting of strong study effect sizes with small sample study populations that may be masking some subtle yet important insights into the mechanisms that link a mother to its offspring. Lastly, this meta-analysis identifies a literature gap between eco-evolutionary theory and current knowledge of epigenetic mechanisms and underscores the importance of approaching the study of maternal epigenetics with both a molecular biology and eco-evolutionary toolset.

2.3 Methods

2.3.1 Literature Search

An electronic literature search was conducted using the Web of Science search engine on August 22nd, 2018. Articles were discovered using the search terms "Maternal Effect AND DNA methyl*" and "Maternal Effect AND DNA methyl* AND mamm*" and searches were restricted between 2008 and 2018 to assess maternal effects literature over the last decade. These terms were selected to retrieve papers focusing on mammalian maternal effects which were being measured through changes in DNA methylation.

2.3.2 Inclusion Criteria

Included studies focused on maternal diet or maternal behaviour. Studies were considered if they were a primary research article that involved the experimental treatment of a mother and measured a resulting change in DNA methylation in her offspring. Next, a study was further considered if a control group of untreated mothers, and their offspring, were also used in comparison to the primary treatment group. Last, a study was included if a mean percentage of methylation, standard error or standard deviation, and a sample size for each discrete treatment group were indicated within the methods, results, tables, or figures. For studies that did not explicitly report mean, standard error, or standard deviation but had them reported visually in figures, ImageJ was used to extract values (Schneider et al., 2012). These values were mandatory for inclusion as they are required to calculate effect sizes for individual data points.

2.3.3 Meta-Analysis

For included studies, additional information was recorded that could reasonably be expected to affect dynamics of DNA methylation. When available, the following covariates were included for the mother (species, type of maternal behaviour or diet modification, timing of maternal effect) and for the offspring (sex, gene target, and tissue sampled). However, offspring sex, gene target, and tissue sampled were excluded from the analyses due to insufficient depth of coverage.

All meta-analyses were conducted using the OpenMEE software (Build date July 26th, 2017), which relies heavily on the package “metafor” in R (Wallace et al., 2016). A Hedge’s d effect size was calculated for each recovered treatment and control group pairings from the literature using means and standard deviations. Analysis of subgroups was conducted for type of maternal effect, timing of maternal effect, behaviour type, dietary modification type, and species using the “subgroup” function in OpenMEE, under the same analysis settings described above for standard meta-analysis. Metaregression was used to investigate the effects of type of maternal effect, timing, and species using the “metaregression” function in OpenMEE. Species was included as subgroup instead of correcting for phylogenetic independence due to issues with a non-random polyphyletic bias inhibiting my ability to detect a true evolutionary signal with low sample sizes (Lajeunesse, 2009). Additionally, an absolute Hedge’s d effect size was calculated to remove any directional differences in effect sizes, and in doing so evaluate absolute change in DNA methylation percentage, on the maternal effects within the data (see Supplement 1) and absolute effect sizes were calculated for individual subgroup and metaregression data. This was conducted to determine the intercept for all combinations of type of maternal effect, timing, and

species present within the dataset. All analyses were conducted using a continuous random-effects model with a maximum likelihood random-effects method. All data presented graphically shows mean ± 95% confidence intervals, unless otherwise described.

2.3.4 Publication Bias

Publication bias was assessed using the "funnel plot" function in OpenMEE. A funnel plot was generated by comparing Hedge's d effect sizes generated using means and standard deviations against the standard error within each study using a continuous random-effects model with a maximum likelihood random-effects method. Presence of publication bias was assessed qualitatively based on the symmetry of data distribution as well as the distribution of data within a 95% confidence interval, with the assumption that studies with higher sample sizes would have higher precision (lower standard error) and lower overall effect sizes than those with lower sample sizes (Sterne et al., 2011).

2.4 Results

2.4.1 Literature Search

The two literature searches conducted using the Web of Science resulted in the discovery of 1298 and 155 articles, respectively, for a total of 1453 articles evaluated. After the exclusion of papers based on evaluation of titles, abstracts, keywords, and methods, 40 articles met inclusion criteria. Individual data points were recorded when a quantified percentage of DNA methylation was presented at a whole-gene level or for individual CpG sites for a treatment and control group. This resulted in a total of 264 data points across the 40 studies (Range: 1-27 entries per study, Mean: 6.47 entries per study) (see Supplement 2).

Studies were generally grouped into two primary types of maternal effects, maternal behaviour (n = 71 data points) and maternal diet (n = 193), and two primary timings of perinatal influence, pre-natal (n = 223) and post-natal (n = 41). Each of these mechanisms was found to have several different sub-components (see *Meta-Analysis*). Pre- and post-natal timing were limited to strict binary categorization for analysis. Additionally, there were five species used in the studies included in this meta-analysis: *Mus musculus* (n = 96), *Rattus norvegicus* (n = 93), *Macaca mulatta* (n = 20), *Homo sapiens* (n = 10), and *Sus scrofa domesticus* (n = 45). Lastly, studies included analysis of percentage of DNA methylation across 54 different genes and 23 different tissues.

2.4.2 Meta-Analysis

Analysis of all 264 data points found that there is a significant positive standardized mean difference in effect size as a result of maternal effects. Metaregression analysis revealed significant effects of type, timing, and species on the change in DNA methylation as a result of maternal effects (Figure 2.1). The post-natal behavioural modifications of *R. norvegicus* (mean ± SEM, p-value; 2.430 ± 0.256, p = <0.001) and *M. mulatta* (0.637 ± 0.299, p = 0.033) significantly modified DNA methylation in offspring and no other interactions of maternal effect type, timing, and species were found to have a significant influence on the magnitude of change of DNA methylation.

Several covariates were analyzed separately to determine their specific relevance to DNA methylation. Behavioural maternal effects significantly increased DNA methylation overall, with maternal separation being the only behaviour type to significantly modify DNA methylation (Table 2.1). Diet did not significantly influence DNA methylation overall, however vitamin D

restriction and supplementation with additional calories, betaine, and nicotinamide resulted in significant modifications of DNA methylation (Table 2.2).When comparing absolute effect sizes agnostic of directional biases in the data, behavioural maternal effects had effect sizes 3.2-fold larger than those of dietary maternal effects. Pre-natal timing of maternal effects did not significantly modify the extent of DNA methylation, however post-natal timing of maternal effects was found to significantly modify DNA methylation (Table 2.3). When comparing absolute effect sizes, maternal effects occurring post-natally had effect sizes 4-fold larger than those of maternal effects occurring pre-natally. Lastly, with respect to species specificity, maternal effects significantly altered DNA methylation only in *M. musculus* (decrease) and *R. norvegicus* (increase) (Table 2.4). However, there are minimal studies on other species, and *M. musculus* and *R. norvegicus* contained predominantly dietary and behaviourally focused studies, respectively, which also trended strongly in the same direction and magnitude across all data.

2.4.3 Publication Bias

Funnel plot asymmetry was found when including all 264 data points (Figure 2.2). Asymmetry was found across both axes, with a skew towards low standard error studies as well as a skew towards greater observed effect sizes than would be predicted by the 95% confidence intervals. Additionally, when analyzing funnel plot asymmetry for the behavioural and dietary data separately, both exhibit evidence of publication with a more prominent occurrence of skew towards greater observed effect sizes than would be predicted within the behavioural data.

2.5 Discussion

A meta-analysis conducted using literature on maternal effects and DNA methylation revealed that the type and timing of a maternal effect do influence the extent of change in

offspring DNA methylation in mammals. Differences were detected in the up- and down-regulation of DNA methylation with respect to these effects, with post-natal behavioural effects generally found to increase DNA methylation levels. Additionally, the extent of DNA methylation changes as a result of maternal effects appears to follow species-specific trends, however limitations in the current literature, and bias towards traditional lab rodent models, prevent the conductance of more robust phylogenetic analysis. Lastly, an analysis of effect sizes presented in literature over the last decade suggests the maternal effects DNA methylation literature suffers from publication biases in the form of stronger than expected effect sizes for small sample size studies. Overall, my analysis represents a novel approach to analyzing the current maternal effects literature informed by an underrepresented evolutionary perspective.

2.5.1 Type and Timing of Maternal Effects

Post-natal maternal behaviour was associated with the largest change in offspring DNA methylation (Figure 2.1). Post-natal behavioural modifications (such as maternal separation or licking/grooming behaviours) can facilitate immediate modifications in response to the environment surrounding a mother and her offspring. This would be especially important in altricial species (as are those included in this study), where newborns are incapable of reacting to the environment around them (Scheiber et al., 2017) and are entirely dependent on parental care for survival. Additionally, maternal behaviour could be much faster acting or more direct than dietary effects due to limiting factors such as digestion time and milk production and thus could allow for more precise tuning of offspring phenotype to the local environment. Surprisingly, perinatal diet did not have an overall impact on offspring DNA methylation in the subset of studies evaluated here. The biochemical nature of dietary effects could result in a highly specific

modulation of the offspring epigenome and may require a less exaggerated overall response in methylation changes making them far more subtle and difficult to detect. One alternate possibility is that selection has shaped an epigenome resilient to unpredictable resource availability, and thus the direct impact of dietary effects on overall methylation is reduced, though there are clear impacts of perinatal nutrition on offspring physiology and behaviour (Altmann et al., 2012; Painter et al., 2008; Schaible et al., 2011; Xu et al., 2016). More likely the limited scope of environmental conditions and study species in the current literature does not allow us to detect these effects. Regardless, framing the interpretation of epigenetic maternal effects data in a way that prioritizes the consequences of the costs and benefits to a free-living animal increases is critical for exploring the evolutionary importance of epigenetic maternal effects.

The significant effects of species, type, and timing additionally suggests that there are species-specific differences in the modification of offspring DNA methylation as a result of maternal effects, however phylogenetic analysis was constrained due to the small number of laboratory model species and humans. Post-natal behaviour in rats and macaques was found to have effect sizes significantly different from zero (Figure 2.1). However, the prevalence of species-specific trends may be an artefact of the dataset as no species could be compared to all four possible combinations of types of maternal effects and timing, and there was notable inequality regarding the frequency each were studied. For example, no studies covering post-natal maternal diet treatments met the criteria for inclusion across all species. The evaluation of species-specific trends is additionally hindered by the lack of standardization of maternal treatments across species within the dataset, making it difficult to parse out differences due to

evolutionary signals (as opposed to experimental variation). The post-natal effect of diet may be more common and influential in other taxa, such as in species of birds which regurgitate undigested or partially digested food for their young. Additionally, it appears from the available literature that there is a preference for studying behavioural maternal effects in rats and dietary maternal effects in mice, suggesting biases or technical limitations in the selection of a model organism may be interfering with even study coverage across species (Hafner and Hafner, 1984). However, qualitative studies were discovered in the literature search that did not meet the criteria for inclusion in this analysis but which suggest behavioural and dietary effects in non-rodent species may also have a meaningful role in the transmission of maternal effects (Paternain et al., 2016; Sasagawa et al., 2017). While we cannot conclusively determine that there are species-specific differences in DNA methylation patterns as a result of maternal effects, the qualitative indicators within the broader literature support this as a valuable area of further study.

2.5.2 Magnitude of DNA Methylation Changes with Type of Maternal Effect and Timing

In addition to having differing effect strengths between type and timing within the data set, directional effects of offspring DNA methylation strongly impacted the magnitude of effect sizes observed within the dataset. An analysis agnostic of the direction of effects is important as the outcome of DNA methylation is context dependent in the genome. For example, methylation of a promoter and demethylation of an inhibitor would result in the same impact on gene expression but would together result in a net effect size of zero, or no methylation change, in the analysis. In the overall analysis, dietary maternal effects overall did not tend to result in positive or negative changes to offspring DNA methylation (Table 2.2), however we see a substantial increase in overall effect size when looking at the absolute effects on DNA methylation

compared to the untransformed data. This suggests these effects occur with equal magnitude but in additive yet opposite directions in the dataset. Similarly, behavioural modifications that are known to either increase (maternal care) or decrease (maternal stress) DNA methylation were covered in the studies, however there was an overall increase in DNA methylation (Table 2.1). In contrast to the dietary effects data, comparing the untransformed data to absolute data with directionality removed did not result in a substantial increase in effect size like with the dietary data. This analysis additionally extends within the timing data (Table 2.3), with pre-natal effects substantially increasing in effect size when analyzing absolute data. Despite these changes to effect sizes with the absolute transformation, the behavioural effects and the post-natally timed maternal effects continue to exhibit the largest overall effect on offspring DNA methylation levels and may be occurring more strongly regardless of the consequence on gene expression in response to maternal treatments.

2.5.3 Publication Bias

Funnel plot analysis revealed a significant skew in the literature towards highly precise studies with larger than expected effect sizes than would be predicted based on sample size (Figure 2.2). Studies with smaller sample sizes are expected to have less precision but are likely to show greater effect sizes due to their variability (Sterne et al., 2011). In contrast, studies with larger sample sizes are expected to have much greater precision but smaller effect sizes due to a reduction in variability (Sterne et al., 2011). The 40 studies included in this meta-analysis represent only a small fraction of the published literature and many studies were excluded due to issues of experimental design (e.g. not using controls, chronologically overlapping treatments) or data reporting (e.g. not specifying error type, ambiguous sample sizes). However, within this

dataset the majority of studies (regardless of sample size) trended toward having low experimental error and large effect sizes, suggesting that studies are either: 1) not reporting findings with low effect sizes or 2) not reporting findings which show greater variability despite having smaller sample sizes, characteristics which are especially prevalent within the behavioural maternal effects literature (Figure 2.2). This could also suggest that there is a skew in the types of maternal effects studied in the literature or a bias in experimental conditions used in maternal effects studies which skew towards an over reporting of studies that find larger than predicted (based on sample size) increases or decreases in DNA methylation. These findings are not surprising, as much of the literature recovered comes from biomedical fields where there is rampant and recognized incidence of publication bias (Duval and Tweedie, 2000). This is symptomatic of a prevalent and important issue in academia where the pressure to publish is combined with the fact that manuscripts reporting large, significant results tend to be more favourably reviewed than studies with null findings, regardless of their importance, and funding decisions are often tightly linked with factors such as publication frequency or citation impact (Duval and Tweedie, 2000). Thus, this meta-analysis and commentary highlight the importance of supporting the publication of negative, variable, and small effect size results to ensure an accurate representation of the natural variation in the transmission of maternal effects (Dirnagl and Lauritzen, 2010; Fanelli, 2012). Further, attempts to extend the study of epigenetic maternal effects to free-living populations of animals have been minimal and are currently being informed and designed based on an insufficiently representative literature base. Publication bias within this dataset and the greater literature is important to consider when interpreting the strength of effects

in downstream analysis and, additionally, our ability to extrapolate laboratory data to natural systems with free-living mammals or when extrapolating to a greater diversity of taxa.

2.6 Conclusion

The relationship between maternal effects and epigenetic mechanisms such as DNA methylation is slowly coming into focus, however the important nuances about the relationship between various types of maternal effects and the extent to which they modify offspring DNA methylation has remained unclear. For the first time, this research revealed that the type and timing of maternal effects affects the transmission, strength, and direction of these epigenetic changes, and can be best understood through the combination of ecological- and evolutionary-driven questions and molecular analyses. While publication bias in the maternal effects epigenetics literature may sway the interpretation of experimental outcomes, influencing the ability to broadly apply knowledge established in the laboratory to free-living animals in wild systems, this analysis identified several important gaps in the current literature. Future research could fill these gaps by examining different types of maternal effects across species in the lab, and crucially, in the wild. Our literature search returned zero studies examining epigenetic maternal effects by changes in offspring DNA methylation in free-living mammal populations, and an extremely limited number of studies which occurred on non-traditional study organisms. The absence of important ecological contexts on our current understanding of maternal effects weakens our ability to understand how these effects will influence the early-life environment of free-living animals in dynamic and chaotic ecosystems.

2.7 Tables

Table 2.1. Behavioural maternal effects on offspring DNA methylation percentage in traditional laboratory model mammals.

	Subgroup	Mean Effect Size ± SEM	Confidence Interval	Sample Size†	p-value
Overall	**Separation**	**1.89 ± 0.390**	**(1.125, 2.655)**	**39**	**< 0.001**
	Maternal Care	1.119 ± 1.572	(-1.961, 4.199)	2	0.476
	Anxiety	0.473 ± 0.653	(-0.807, 1.752)	2	0.469
	Depression	2.821 ± 0.152	(2.523, 3.119)	1	NA
	Stress	-0.253 ± 0.204	(-0.652, 0.146)	27	0.214
	Total	*0.948 ± 0.265*	*(0.428, 1.467)*	*71*	*< 0.001*
Absolute	**Separation**	**2.542 ± 0.238**	**(2.057, 2.991)**	**39**	**< 0.001**
	Maternal Care	**2.340 ± 0.744**	**(0.882, 3.797)**	**2**	**0.002**
	Anxiety	**0.916 ± 0.337**	**(0.256, 1.576)**	**2**	**0.007**
	Depression	2.821 ± 0.152	(2.523, 3.119)	1	NA
	Stress	**0.664 ± 0.122**	**(0.425, 0.904)**	**27**	**< 0.001**
	Total	*1.774 ± 0.176*	*(1.429, 2.119)*	*71*	*< 0.001*

† Sample sizes are for number of treatment-control pairings retrieved from studies included in the meta-analysis.

Table 2.2. Dietary maternal effects on offspring DNA methylation percentage in traditional laboratory model mammals.

	Subgroup	Mean Effect Size ± SEM	Confidence Interval	Sample Size†	p-value
Overall	Methyl Donor +	-3.231 ± 1.759	(-6.679, 0.216)	4	0.066
	Ethanol +	0.111 ± 0.122	(-0.128, 0.350)	42	0.361
	Folic Acid +	-0.078 ± 0.108	(-0.134, 0.290)	17	0.47
	Protein -	-0.188 ± 0.176	(-0.533, 0.158)	33	0.287
	Sugar +	6.770 ± 2.118	(2.619, 10.922)	1	NA
	Nicotine +	0.273 ± 0.171	(-0.063, 0.608)	13	0.111
	Docosahexaenoic acid +	0.051 ± 0.049	(-0.046, 0.0147)	3	0.302
	Cadmium +	0.201 ± 0.105	(-0.005, 0.407)	14	0.056
	Vitamin D -	**-0.356 ± 0.165**	**(-0.679, -0.032)**	**14**	**0.031**
	Calorie -	-0.050 ± 0.376	(-0.787, 0.686)	2	0.893
	Calorie +	**0.436 ± 0.178**	**(0.086, 0.785)**	**4**	**0.015**
	Lipotropes +	0.109 ± 0.5	(-1.090, 0.871)	2	0.827
	Cocaine +	-0.223 ± 0.962	(-2.109, 1.663)	2	0.817
	Protein +	-0.161 ± 0.127	(-0.411, 0.088)	19	0.205
	Fat +	-0.248 ± 0.449	(-1.127, 0.632)	1	NA
	Betaine +	**0.637 0.258**	**(0.131, 1.143)**	**7**	**0.014**
	Vapour +	4.163 ± 1.453	(1.316, 7.011)	1	NA
	Bisphenol A +	0.046 ± 0.303	(-0.548, 0.639)	2	0.88
	Folic Acid -	-0.238 ± 0.2	(-0.630, 0.154)	4	0.234
	Nicotinamide +	**-1.576 ± 0.277**	**(-2.119, -1.034)**	**2**	**< 0.001**
	Nitrogen Dioxide +	-0.635 ± 0.405	(-1.429, 0.159)	6	0.117
	Total	*-0.047 ± 0.057*	*(-0.159, 0.064)*	*193*	*0.403*
Absolute	Methyl Donor +	3.231 ± 1.759	(-6.679, 0.216)	4	0.066
	Ethanol +	**0.619 ± 0.095**	**(0.432, 0.805)**	**42**	**< 0.001**
	Folic Acid +	**0.231 ± 0.108**	**(0.019, 0.443)**	**17**	**0.032**
	Protein -	**0.792 ± 0.076**	**(0.643, 0.941)**	**33**	**< 0.001**
	Sugar +	6.770 ± 2.118	(2.619, 10.922)	1	NA
	Nicotine +	**0.282 ± 0.102**	**(0.081, 0.482)**	**13**	**0.006**
	Docosahexaenoic acid +	0.051 ± 0.049	(-0.046, 0.0147)	3	0.302
	Cadmium +	**0.381 ± 0.104**	**(0.178, 0.585)**	**14**	**< 0.001**
	Vitamin D -	**0.531 ± 0.121**	**(0.295, 0.768)**	**14**	**< 0.001**

Calorie -	0.445 ± 0.376	(-0.291, -1.181)	2	0.236
Calorie +	**0.436 ± 0.178**	**(0.086, 0.785)**	**4**	**0.015**
Lipotropes +	0.109 ± 0.5	(-1.090, 0.871)	2	0.827
Cocaine +	**1.346 ± 0.351**	**(0.658, 2.034)**	**2**	**< 0.001**
Protein +	**0.480 ± 0.088**	**(0.308, 0.651)**	**19**	**< 0.001**
Fat +	-0.248 ± 0.449	(-1.127, 0.632)	1	NA
Betaine +	**0.702 ± 0.219**	**(0.273, 1.132)**	**7**	**0.001**
Vapour +	4.163 ± 1.453	(1.316, 7.011)	1	NA
Bisphenol A +	0.248 ± 0.303	(-0.345, 0.842)	2	0.412
Folic Acid -	-0.238 ± 0.2	(-0.630, 0.154)	4	0.234
Nicotinamide +	**-1.576 ± 0.277**	**(-2.119, -1.034)**	**2**	**< 0.001**
Nitrogen Dioxide +	**0.848 ± 0.302**	**(0.256, 1.439)**	**6**	**0.005**
Total	*0.554 ± 0.039*	*(0.478, 0.630)*	*193*	*< 0.001*

+/- denote supplementation and restriction within the subgroups, respectively.

† Sample sizes are for number of treatment-control pairings retrieved from studies included in the meta-analysis.

Table 2.3. Perinatal timing of maternal effects on offspring DNA methylation percentage in traditional laboratory model mammals.

	Subgroup	Mean Effect Size ± SEM	Confidence Interval	Sample Size†	p-value
Overall	Pre-natal	-0.035 ± 0.059	(-0.150, 0.079)	223	0.546
	Post-natal	**1.847 ± 0.380**	**(1.103, 2.591)**	**41**	**< 0.001**
	Total	*0.176 ± 0.075*	*(0.030, 0.323)*	*264*	*0.018*
Absolute	**Pre-natal**	**0.620 ± 0.042**	**(-0.537, 0.703)**	**223**	**< 0.001**
	Post-natal	**2.505 ± 0.227**	**(2.059, 2.950)**	**41**	**< 0.001**
	Total	*0.796 ± 0.049*	*(0.701, 0.891)*	*264*	*< 0.001*

† Sample sizes are for number of treatment-control pairings retrieved from studies included in the meta-analysis.

Table 2.4. Species-specific trends of maternal effects on offspring DNA methylation percentage in traditional laboratory model mammals.

	Subgroup	Mean Effect Size ± SEM	Confidence Interval	Sample Size†	p-value
Overall	***Mus musculus***	**-0.182 ± 0.075**	**(-0.329, -0.035)**	**96**	**0.015**
	Rattus norvegicus	**0.545 ± 0.167**	**(0.219, 0.872)**	**93**	**0.001**
	Macaca mulatta	0.891 ± 0.599	(-0.283, 2.065)	20	0.137
	Homo sapiens	0.361 ± 0.298	(-0.224, 0.945)	10	0.227
	Sus scrofa domesticus	0.102 ± 0.128	(-0.149, 0.354)	45	0.426
	Total	*0.176 ± 0.075*	*(0.030, 0.323)*	*264*	*0.018*
Absolute	***Mus musculus***	**0.553 ± 0.051**	**(0.454, 0.652)**	**96**	**< 0.001**
	Rattus norvegicus	**1.113 ± 0.115**	**(0.886, 1.339)**	**93**	**< 0.001**
	Macaca mulatta	**2.278 ± 0.306**	**(-1.679, 2.877)**	**20**	**< 0.001**
	Homo sapiens	**0.537 ± 0.270**	**(0.007, 1.067)**	**10**	**0.047**
	Sus scrofa domesticus	**0.629 ± 0.064**	**(0.503, 0.754)**	**45**	**< 0.001**
	Total	*0.796 ± 0.049*	*(0.701, 0.891)*	*264*	*< 0.001*

† Sample sizes are for number of treatment-control pairings retrieved from studies included in the meta-analysis.

2.8 Figures

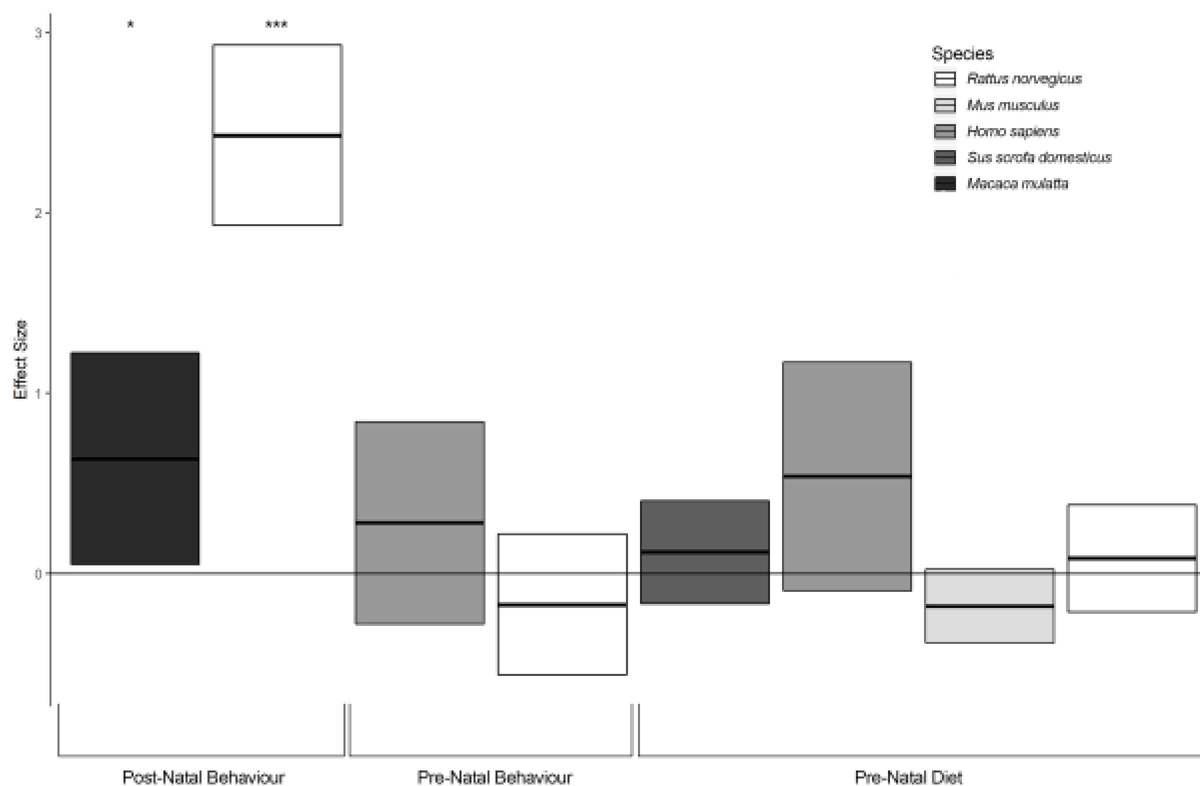

Figure 2.1. Interaction of type of maternal effect, timing, and species on effect size of offspring DNA methylation percentage in traditional mammalian laboratory model organisms. Boxplots of mean effect size for all combinations of interactions between type of maternal effect, timing, and species on offspring DNA methylation percentage available within the dataset. Post-natal behavioural effects in rats and macaques were found to have significant effect on effect size. Boxes represent mean (center line) and 95% confidence intervals. * $0.01 < p < 0.05$, *** $p < 0.001$.

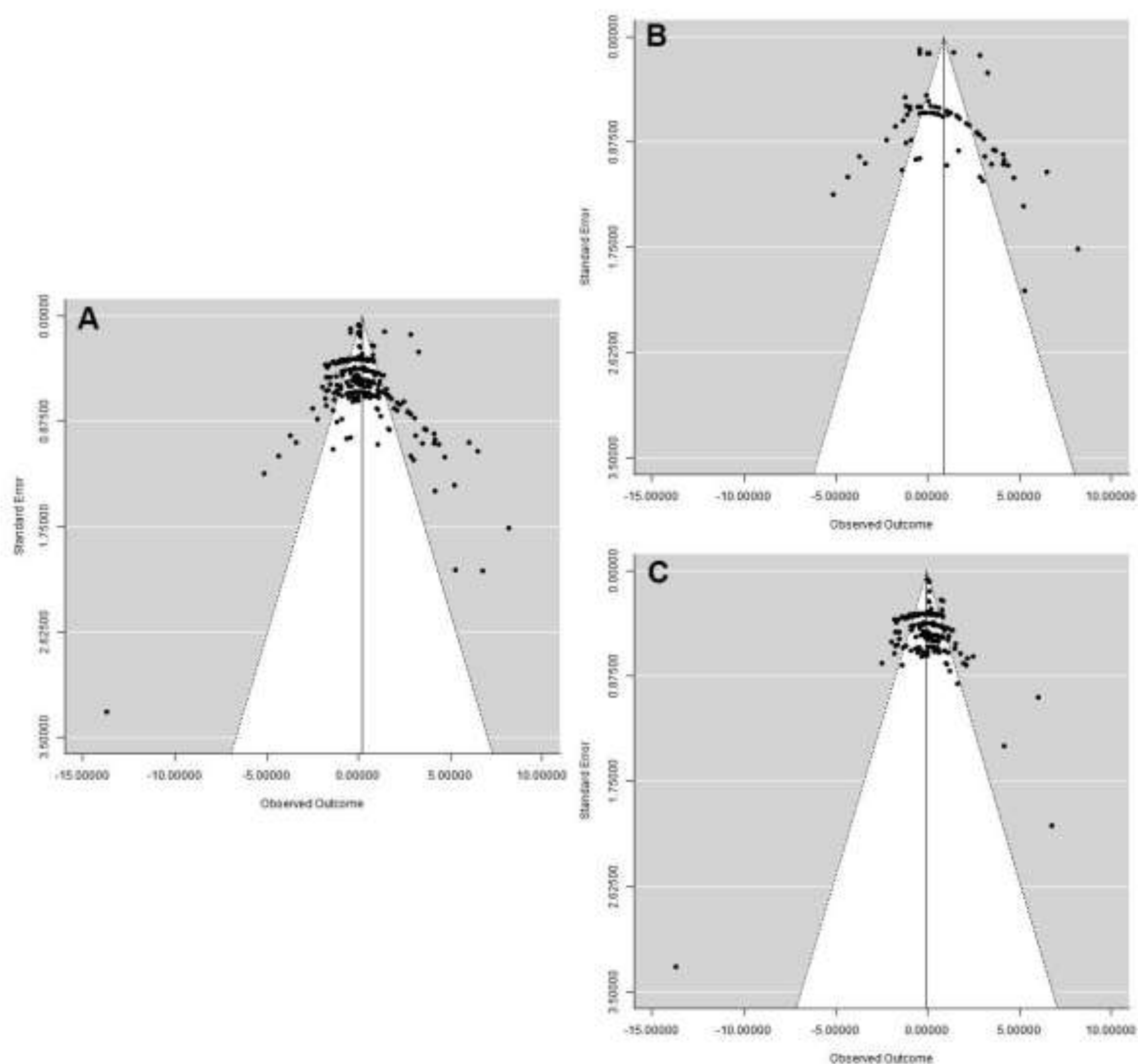

Figure 2.2. Funnel Plots of Publication Bias in Maternal Effects Epigenetics Literature from 2008-2018. Scatterplot graphing observed outcome of effect size against standard error for 264 data points in the dataset (A) overall, and additionally separated based on the (B) behavioural and (C) dietary maternal effects data. Distribution of data within the funnel plot indicates a greater recovery of studies with larger observed outcomes than would be predicted, as well as a skew towards low error regardless of sample size. Skew towards high effect sizes with low error is more prevalent within the behavioural data. White spacing represents distribution expected within a 95% confidence interval.

Chapter 3: Free-living red squirrels exhibit sex-specific Nr3c1 mRNA expression patterns with no change in mRNA expression or DNA methylation in response to maternal stress.

3.1 Abstract

Physiological stress influences an organism's perception and response to their environment. Maternal stress extends this influence further, from mother to offspring, and is known to be regulated epigenetically through DNA methylation. In wild, free-living, red squirrels I explored the epigenetic effects of maternal stress on offspring neurophysiology using qRT-PCR and sodium-bisulfite conversion next-generation sequencing to measure changes in mRNA expression of *Nr3c1* and *Pomc* and DNA methylation of *Nr3c1* within key brain regions of the hypothalamic-pituitary-adrenal axis. Yearling red squirrels born to mothers with experimentally induced elevations in glucocorticoid levels did not significantly modify mRNA expression in response to maternal stress, however *Nr3c1* mRNA expression was significantly higher in male yearling red squirrels than in females. However, DNA methylation did not differ significantly across 17 CpG sites in a CpG island adjacent to the *Nr3c1* locus in response to maternal stress. Combining cutting edge molecular techniques with ecologically relevant field manipulations represents a crucial advance in understanding persistent and adaptive effects of maternal stress in mammals.

3.2 Introduction

Physiological stress, traditionally measured through the production and activity of glucocorticoids (GCs), can shape the development and function of the mammalian nervous system via the hypothalamic-pituitary-adrenal (HPA) axis and has been well studied under laboratory conditions (de Kloet et al., 2008; Finsterwald and Alberini, 2014). The HPA axis is a primary mediator of how an organism interprets and responds to their environment (Flinn et al., 2011). GCs, once produced, interact with glucocorticoid receptors (GRs) (encoded for by the nuclear receptor subfamily 3 group C member 1 [*Nr3c1*] gene) (Liberman et al., 2012)) throughout the body to facilitate the physiological stress response. Activated GRs in the hypothalamus stimulate the secretion of corticotropin-releasing hormone (CRH) into the hypothalamo-hypophyseal portal system (Gunnar and Quevedo, 2007). Once released CRH can stimulate corticotropes within the pituitary to release adrenocorticotropic hormone (ACTH) (produced by the cleavage of the pro-opiomelanocortin [*Pomc*] precursor peptide (Aguilera, 1994)); the hormone which stimulates the production of GCs in the adrenal glands and completes the HPA axis feedback loop (Gunnar and Quevedo, 2007). In human psychopathology, chronic physiological stress due to prolonged exposure to GCs is well-known to impact a variety of biological functions such as long term memory formation, synaptic plasticity, metabolism, the autoimmune system, and behaviour, among others (Finsterwald and Alberini, 2014). In traditional lab model systems, such as mice, maternal stress or GC exposure during pregnancy is known to impact HPA axis physiology and behaviour in offspring.

Recently, it has been suggested that wild populations may serve as more accurate model systems for understanding biologically-relevant consequences of physiological stress, in part

because the stressors laboratory animals are exposed to bear minimal resemblance, if any, to those experienced in free-living animals (Boonstra, 2013; Newman et al., 2015). The inbred nature of laboratory animals has erased much genetic variation among individuals, an asset in understanding preliminary experimental effects, however, this barren genetic landscape is not representative of the variation among conspecific individuals in the natural world. Without field-tested studies in natural systems, there are questions of whether rodent based laboratory research represent "facts or artifacts" (Wolff, 2003). Further, lab studies are typically of short duration, thus not only is the role that high amplitude or long duration stresses play in free-living populations poorly understood and highly debated (Dantzer et al., 2014), but the long-term impacts across life-history phases is also not known. It has been reported that roughly 1% of studies on stress make explicit reference to free-living animals (Boonstra, 2013), despite the prevalent implicit common goals of explaining biological function or examining pathology in organisms in natural environments (humans included). This stunning underrepresentation of quantitative studies on stress that embrace ecologically relevant contexts makes it difficult to interpret results and draw conclusions regarding physiological stress.

The importance of ecological context when studying stress is exemplified by the "environmental matching hypothesis" which proposes a link between HPA axis dynamics and the maternal effects of stress in natural, free-living, populations (Gluckman et al., 2005; Sheriff et al., 2017). Maternal effects are a subset of parental effects in which the mother's genotype and environment influence the phenotype of offspring, in addition to the genotype and environment of the offspring themselves (Mousseau and Fox, 1998), and the environmental/maternal matching hypotheses suggest these maternal effects occur to improve maternal and offspring fitness (Gluckman et al., 2005; Sheriff et al., 2017). In free-living snowshoe hares (*Lepus*

americanus), fecal glucocorticoid metabolite (FGM) levels reflect population densities of their primary predator, the Canadian lynx (*Lynx canadensis*), where FGM are elevated when predator pressure is also high (Sheriff et al., 2009). Additionally, newborn hares exhibit patterns of FGM levels that closely reflect those of their mother, despite having minimal-to-no exposure to lynx predation, suggesting that the predation stress experienced by their mothers has shaped the offspring HPA axis (Sheriff et al., 2010). In the laboratory, the maternal effects of stress are thought to be transmitted to offspring via the prenatal or postnatal modification of epigenetic markers, such as DNA methylation (Pan et al., 2014a; Schaible et al., 2011; Thanos et al., 2016). Methylation of DNA induces a three-dimensional conformation change in chromosomal state from euchromatin to heterochromatin, causing the DNA to change shape and prevent the binding of RNA polymerase for transcription (Hunter, 2012). In laboratory rodents, the extent to which a mother physically grooms and licks her offspring is known to directly modify DNA methylation levels for *Nr3c1* within the hippocampus of her offspring where maternal care is associated with a reduction in DNA methylation and a consequent lower level of HPA axis activity in offspring. (Feder et al., 2009). Whether in the lab, or in the natural world, the connections between maternal stress and offspring physiology are evident. Nonetheless, it is not known if the mechanisms by which the intrinsic and extrinsic maternal environment influence offspring apply to natural, free-living, organisms (Bossdorf et al., 2008). Understanding the mechanisms by which maternal stress affects offspring HPA axis physiology is critical as more and more wild populations are exposed to changing or challenging environments that may affect HPA axis function and influence fitness (Sheriff et al., 2017).

Here, using a wild population of red squirrels in the Yukon, Canada, I quantify mRNA expression in discrete brain regions for two key genes in the HPA axis, *Nr3c1* and *Pomc,* of

young adult offspring born to stressed mothers. For the first time in a natural system, I examine the hypothesis that maternal stress modifies the HPA axis in offspring by altering levels of GR expression through the methylation of DNA. Based on literature from captive studies (Feder et al., 2009), this hypothesis predicts reduced GR expression and increased DNA methylation of *Nr3c1* in tissue collected from offspring of stressed mothers, and thus there will be decreased mRNA expression of *Pomc* in the pituitary gland downstream in the HPA axis. By combining sophisticated laboratory techniques with an innovative ecological manipulation of maternal stress (Dantzer et al., 2013), I harness the power of a long-term population study to examine the persistent neurological effects of maternal stress on offspring in the wild and set the stage for understanding the downstream implications for fitness.

3.3 Methods

3.3.1 Study System

Maternal stress treatments were conducted on free-living North American red squirrels (*Tamiasciurus hudsonicus*) during 2010 outside of the Kluane National Park and Reserve in the southwest Yukon, Canada. Maternal stress was induced for treatment squirrels using audio playback of red squirrel calls (treatment) to simulate increased population density, an ecologically relevant stimulus known to increase maternal glucocorticoids and influence neonatal offspring physiology (Dantzer et al., 2013). Control mothers were exposed to boreal chickadee (*Poecile hudsonicus)* calls (control), a non-predatory native species to the area, as described by (Dantzer et al., 2013). Previous research within this experimental system has shown that manipulations of perceived social density increase maternal glucocorticoid levels to the same magnitude as manipulations of actual density, and offspring born from these mothers have an increased growth rate (Dantzer et al., 2013). Offspring were tagged in the nest in 2010 and

eighteen 10-12 month old offspring (n = 9 treatment; n = 9 control) were re-captured and sacrificed for tissue collection in the spring of 2011 to allow for a sufficient amount of growth prior to tissue collection. Whole brains were excised immediately after cervical dislocation, and pituitary tissues, left and right brain hemispheres, and cerebellums were separated with a sterile scalpel blade, frozen immediately on dry ice and stored at -80°C until analysis.

North American red squirrels have a relatively recent evolutionary divergence (approximately 71 MYA (Hedges et al., 2015)) from traditionally studied laboratory rodent models (compared to other commonly used laboratory models such as fruit flies, flatworms, and yeast) resulting in a conserved genetic architecture, morphological structure, HPA axis physiology (Boonstra et al., 2008), and maternal care behaviours (Larsen and Boutin, 1994). However, red squirrels have vastly different life histories than laboratory rodents which are strongly influenced by their ecological environments, including differences in age of sexual maturity and critical territory acquisition and food caching demands required for overwintering survival in red squirrels (Larsen and Boutin, 1994). Because of these characteristics, red squirrels are an excellent experimental organism for testing the applicability of knowledge from laboratory rodents to free-living animals.

3.3.2 Brain microdissection

One brain hemisphere per individual (randomly selected left or right) was dissected under sterile conditions. Dissections were made in reference to the day 56 mouse (*Mus musculus*) brain maps to ensure accurate dissection of the hippocampus and hypothalamus in the absence of a detailed red squirrel brain map. Reference material was gathered from the Allen Mouse Brain Atlas (Sunkin et al., 2013) and the C57BL/6J Mouse Brain Library (Rosen et al., 2000) for use in

anatomical precision and Bregma coordinate determination. All further references to Bregma location are with respect to the day 56 adult mouse brain. The day 56 mouse brain was selected as a reference due to the large level of anatomical detail available, the timing being ontogenetically similar to yearling red squirrels (Layne, 1954), and their relatively close phylogenetic relationship (Order: Rodentia).

Microdissection of red squirrel brain regions was conducted using a Leica CM3050 S Research Cryostat in combination with a modified Palkovitz punch technique. One hemisphere from each individual was mounted in the cryostat at -20°C for 30 minutes prior to dissection to prevent physical damage to the tissues during sectioning. Brains were sectioned coronally in a rostral-to-caudal direction at a thickness of 100 μm until the first appearance of the hippocampus at approximately -1.06 mm Bregma (Figure 3.1). Hypothalamic tissue was microdissected using 1.00 mm diameter brain punch cores from -1.06 mm to -2.75 mm Bregma, while hippocampal tissue was microdissected using 1.25 mm diameter brain punch cores from -1.06 mm to -3.75 mm Bregma. Additionally, after target sections were removed, the brain surrounding the cores was sectioned to quantify average whole brain expression (referred to as whole brain slice homogenates) in the absence of the target regions (hippocampus, hypothalamus, pituitary). Tissues were maintained at -20°C for the duration of the dissection process, then stored at -80°C until the extraction of genomic materials was conducted. Brain punches (Leica Biosystems), punch handles, forceps, paintbrushes, and cryostat blades were sterilized in an Eliminase (Decon Labs, Inc., PA, USA) bath then rinsed in 3 consecutive de-ionized water washes prior to use. Interior surfaces of the cryostat were sterilized using 100% ethanol while external surfaces were sterilized using Eliminase and 100% ethanol. Microdissection punches and forceps were stored

in previously sealed sterile, RNAase free 1.5-mL microcentrifuge tubes and stored on dry ice or at -20°C, respectively, to minimize tissue thaw during dissection.

3.3.3 Tissue Extraction

Extraction of RNA and DNA from tissues was conducted using the TRIzol reagent method. Order of tissue preparation was randomly determined to minimize biases in extraction protocol for treatment and control groups. Extractions were conducted as recommended by the manufacturer, with the following exceptions: tissue samples were homogenized in 1 mL TRIzol reagent using a BeadBlaster 24 bead homogenizer (Benchmark), run at a speed of 6.00 m/s, for three 30 seconds on/30 seconds off cycles using five beads per sample. All beads were sterilized immediately prior to homogenization using Eliminase (Decon Labs, Inc, PA, USA) then rinsed three times with RNA-free molecular grade water. Prior to centrifugation during the initial phase separation, the TRIzol-chloroform-homogenate mixture was transferred via pipette to a new 1.5-mL microcentrifuge tube, to prevent the formation of pellets on the beads during downstream processing steps. RNA-free glycogen was added to samples of RNA isolates which had less than 10 µg starting tissue, as suggested by the manufacturer during RNA precipitation. RNA was eluted to a final volume of 50 µL of RNA-free molecular grade water and DNA was eluted to a final volume of 300 µL of 8 mM NaOH. All RNA samples were treated with DNase in order to remove any contaminating DNA resulting from the extraction process using the TURBO DNA-*free* kit (Invitrogen). All RNA and DNA extracts were assessed for quality of genetic materials immediately after extractions occurred using 2% agarose gel electrophoresis and spectrophotometry with a NanoDrop™-8000 (Thermo Scientific™).

3.3.4 RT-PCR

Reverse transcription PCR (RT-PCR) was conducted using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Reactions were prepared as described by the manufacturer, with each reaction containing a final RNA quantity of 1 µg in 20 µl. Reactions were conducted and a subset of samples were randomly evaluated using gel electrophoresis to determine reaction success. For each cDNA conversion, a 2-fold (*Nr3c1, Pomc, Fau*) and 1000-fold *(Rn18s)* dilution was created using molecular grade H_2O to minimize concentration-related template inhibition. *Fau* (40S ribosomal protein S30) and *Rn18s* (18S ribosomal RNA) were selected as reference genes due to previous studies suggesting their expression is not influenced by exposure to stress (Mitter et al., 2009) (for additional information, refer to Supplement 3).

3.3.5 Nr3c1 *and* Pomc *mRNA Expression*

Expression of mRNA was quantified via qRT-PCR. All qRT-PCR reactions were conducted using a StepOnePlus™ Real-Time PCR system (Applied Biosystems) at a final reaction volume of 20 µL and contained 10 µL 2x TaqMan Fast Advanced Master Mix (Applied Biosystems), 2 µL of variably concentrated forward primers, 2 µL of variably concentrated reverse primers, 4 µL of nuclease-free H_2O, and 200 ng of cDNA template. All qPCR reactions described henceforth were conducted under the following run conditions: one 2 minute 50˚C hold followed by a 2 minute 95˚C hold for polymerase activation, then 40 cycles consisting of a 1 second denaturation at 95˚C followed by a 20 second combined annealing and extension at 60˚C. A standard curve was conducted for each assay to assess their utility for quantification and assays with an amplification efficiency between 90%-100% and an R^2 greater than 0.99 were used for further analysis (Figure 3.2). Standard curves were run as a 5-fold dilution series across 6 concentrations, starting at 200 ng/µL of gDNA. Standards were run in triplicate and all assay

standards were run simultaneously. For further description of the design and optimization for qPCR protocols, refer to Supplement 3.

Gene expression assays were conducted using the optimized reaction conditions described above. One volume of reaction master mix was prepared and used for all PCR reactions to ensure consistency in reaction conditions across plates. Samples were organized across 96 well plates to include all gene assays for a tissue on the same reaction plate, with all genes for a specific tissue extract or RT-NTC (reverse transcription no template control) run in triplicate. Additionally, each plate included a no-template control for each assay master mix in singlet and one interplate control (IPC) sample run in triplicate using the *Rn18s* assay and a 200 ng/µL red squirrel whole brain homogenate gDNA extract. Gene expression data was transformed using a modified version of the Hellemann (2007) $\Delta\Delta C_T$ relative expression comparison method, correcting for interplate variance using the *Rn18S* IPC for all genes. Data were processed as described by the formulas in Hellemann (2007) for the generation of assay-specific normalized relative quantities (NRQ) of gene expression using these calibrated C_T values, with the geometric mean of *Rn18s* and *Fau* being used as a reference comparison for *Nr3c1* and *Pomc* expression.

3.3.6 Sodium Bisulfite Treatment

Genomic DNA samples from red squirrel hypothalamus, hippocampus, and pituitary were treated with sodium bisulfite using the MethylEdge™ Bisulfite Conversion System (Promega). Sodium bisulfite treatments were conducted using 20 µL of variably concentrated genomic DNA following the manufacturers protocols and eluted to a final volume of 20 µL. Concentration of treated DNA samples was determined using the "RNA-40" protocol on a

NanoDrop™-8000 (Thermo Scientific™). Sodium bisulfite treated DNA was stored without exposure to light at -20°C until analysis.

3.3.7 Nr3c1 *DNA Methylation*

Sodium-bisulfite treatment compatible primers were designed to target the nearest CpG island to the *Nr3c1* locus used for the qPCR assays described above. A 153 bp CpG-rich fragment of the CpG island was designed and amplified with the forward primer 5' – TTCAAAAGGCTCTGTAAGCCATGC – 3' and reverse primer 5' – TCCCCAGAGGAAAGGCTGAT – 3'. For further description of the design and validation of the sodium bisulfite DNA methylation sequencing assay, refer to Supplement 4. Library preparation was conducted on sodium bisulfite converted red squirrel DNA normalized to 5 ng/µL with an Illumina MiSeq system using a modified version of the Illumina 16S metagenomic sequencing library preparation protocol (Illumina part # 15044223 revision B). Amplicon and indexing PCR was conducted using 12.5 µL and 25 µL of 2x KAPA HiFi Uracil+ master mix per reaction, respectively. Amplicon PCR was conducted using 5 µL of 1 µM forward and reverse primers per reaction. Index PCR was conducted using custom primers designed by the Advanced Analysis Centre at the University of Guelph. Additionally, PCR clean-up steps were conducted as prescribed using NucleoMag ® NGS Clean-up and Size Select magnetic beads (Macherey-Nagel). Individual libraries were quantified using a 2100-Bioanalyzer (Agilent Technologies), normalized, pooled, and paired-end sequenced on a single flow cell.

CLC Genomics Workbench 10.0.1 (https://www.qiagenbioinformatics.com/) was used for sequence bioinformatics. Sequences were pair merged with a maximum of 2 ambiguous bases and trimming of ambiguous sequence ends. Sequences were further quality filtered with no

masking and a cumulative mismatch score threshold of 10, with individual mismatched base pairs costing a score of 1 mismatch and with insertions and deletions costing 3 mismatches. Additionally, length fractions and general sequence similarity fractions were valued at 0.5 and 0.8, respectively. Red squirrel sequences which successfully met these quality standards were mapped back to the original thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) amplicon fragment used in the primer design process with an in-silico sodium-bisulfite conversion with 100% efficiency. DNA methylation levels were assessed by determining conversion efficiency at 17 individual CpG sites, with the number of sequences containing cytosines compared against the total number of sequences containing a cytosine or thymine at that base location.

3.3.8 Statistical Analysis

Statistical analysis was conducted using R v3.4.2 (R Core Team, 2017). Log-transformed NRQs for *Nr3c1* and *Pomc* gene expression and sodium-bisulfite conversion percentages for *Nr3c1* were analyzed using a general linear mixed effects model with the lme function in the R package lme4 (Bates et al., 2014), including tissue type when more than one tissue was analyzed (hypothalamus, hippocampus, pituitary, whole brain slice homogenate), sex (male, female), and treatment (playback, control) as fixed effects and squirrel identity as a random effect (for sample sizes, refer to Table 3.1). Additionally, for the analysis of DNA methylation, CpG site identity was included as a fixed effect. Fixed effects were tested for significance by comparing likelihoods of each fitted model using the function “anova.lme”. Specific fixed effects determined to be statistically significant were further explored using a Tukey’s HSD post-hoc test. Outliers greater than 2 standard deviations from the mean were excluded from gene expression analyses (n = 1). All results are presented as a mean of relative mRNA expression or percentage DNA methylation ± SEM, where applicable.

3.4 Results

3.4.1 Nr3c1 *and* Pomc *mRNA Expression*

Nr3c1 expression significantly differed among tissue types ($F_{3,44} = 11.46$, $p < 0.0001$, Figure 3.3), and between sexes where males generally had higher expression than females ($F_{1,14} = 5.018$, $p = 0.0418$, Figure 3.3), however was not significantly affected by the maternal playback treatment ($F_{1,14} = 0.1534$, $p = 0.7012$). Squirrel identity was responsible for 41.09% of the variation ($\sigma^2 = 0.0177$) observed within the data ($\sigma^2_{residual} = 0.0430$). Tukey's HSD post-hoc analysis revealed that levels of expression in the pituitary (0.2393 ± 0.0701) were significantly higher than all other tissues, that expression levels in the hippocampus (-0.0822 ± 0.0639) and whole brain slice homogenate (-0.0684 ± 0.0639) were similar, and that expression in hypothalamus was lower than all other tissues (-0.3859 ± 0.0654). Further, there were no significant interactions between tissue and treatment ($F_{3,44} = 0.1820$, $p = 0.9081$), or sex and treatment ($F_{1,14} = 0.4873$, $p = 0.4966$).

Expression of *Pomc* significantly differed among tissues ($F_{3,38} = 218.1$, $p < 0.0001$), however did not significantly differ between the sexes ($F_{1,14} = 0.8618$, $p = 0.3690$) or between treatment groups ($F_{1,14} = 0.3168$, $p = 0.5824$, Figure 3.4). Additionally, the level of expression of *Pomc* was not found to significantly differ as a result of the interaction of tissue and treatment ($F_{3,38} = 0.3688$, $p = 0.7759$), or sex and treatment ($F_{1,14} = 0.00241$, $p = 0.9615$). Squirrel identity was responsible for 70.47% of the variation ($\sigma^2 = 0.1190$) observed within the data ($\sigma^2_{residual} = 0.1689$). Tukey's HSD post-hoc analysis revealed that levels of expression in the pituitary (3.308 ± 0.147) were significantly higher than all other tissues and that expression levels in the hippocampus (-1.755 ± 0.1481), whole brain slice homogenate (-1.337 ± 0.1467), and hypothalamus (-0.9750 ± 0.1567) were low, with pairwise comparison of hypothalamus and

whole brain slice homogenate ($p = 0.1625$) and the comparison of hippocampus and whole brain slice homogenate ($p = 0.0627$) showing no statistically significant differences in expression.

3.4.2 Nr3c1 *DNA Methylation Analysis by Sodium-Bisulfite Next Generation Sequencing*

Next generation sequencing generated 16 436 544 total sequences. Additionally, sequences were successfully pair merged and mapped to the in-silico sodium-bisulfite sequence generated from the thirteen-lined ground squirrel reference genome (see Supplement 5 for a summary of sequence generation and mapping by sample).

Analysis of the sodium bisulfite conversion treatment found that DNA methylation levels differed significantly among individual CpG sites within free-living red squirrels ($F_{1,861} = 6.184$, $p = 0.0131$) (Figure 3.5). DNA methylation was not found to be significantly affected by treatment ($F_{1,14} = 0.1517$, $p = 0.7028$), sex ($F_{1,14} = 1.501$, $p = 0.2408$), or tissue ($F_{2,861} = 1.329$, $p = 0.2652$). Additionally, for DNA methylation levels, there was no significant interaction between treatment and tissue ($F_{2,861} = 0.5452$, $p = 0.5799$) or between treatment and sex ($F_{1,14} = 0.4188$, $p = 0.5280$).

3.5 Discussion

3.5.1 Sex-specificity of Nr3c1 *mRNA expression*

Yearling red squirrels exhibited sex-specific patterns of *Nr3c1* mRNA expression in key neural tissues of the HPA axis, including the hippocampus, hypothalamus, and pituitary (Figure 3.3). In male offspring, *Nr3c1* expression was significantly higher than in females, and maternal stress was consistently associated with non-significant decreases in *Nr3c1* expression in males but did not alter expression in females. There were no overall differences between the sexes in the expression of *Pomc* (Figure 3.4), and when especially considering the pituitary, suggests that

inherent sex-specific differences may be restricted to the glucocorticoid receptors. While I was hampered by limited statistical power due to low sample sizes, one of the common challenges when working with free-living animals in natural populations, the consistency in the responses generates an exciting hypothesis that the long-term effects of maternal stress may be influenced by sex, with differing effects on gene expression in the HPA axis of males versus females.

Sex differences, while not particularly surprising, are generally understudied and underappreciated in both laboratory and field studies (Caro, 2012). The protein families for glucocorticoid receptors are closely related to those for the gonadal steroid hormones, (e.g. estrogen receptor protein family: NR3A; testosterone and progesterone receptor protein family: NR3C) (Handa and Weiser, 2014) and sex-specific interactions between the HPA and hypothalamic-pituitary-gonadal (HPG) axes are well described in the literature (Handa and Weiser, 2014; Toufexis et al., 2014). In laboratory rodents, androgens have been shown to play a role in the reactivity of the HPA axis to stress (Groeneweg et al., 2011) and other non-reproductive behaviours (Bender et al., 2017; Kuo et al., 2013). Similarly, estrogen receptors are located in and affect a number of brain nuclei within the HPA axis (Simerly and Young, 1991), with ER-β receptors having been shown to specifically play a role in the dampening of anxiety- and stress-related behaviours in rodents (Krężel et al., 2001). Interestingly, female wild red squirrels were found to have lower levels of GR expression in the brain relative to males in this study. While I cannot yet assign a value to these relative differences, if female GR mRNA expression is relatively lower, we might predict associated behavioural profiles in line with those described in laboratory studies where GR expression is reduced relative to control: increased anxiety and increased stress sensitivity (Grundwald and Brunton, 2015; Iqbal et al., 2012; Pan et al., 2014b). Alternatively, if males have relatively high GR expression in the brain, this too may

be associated with certain behavioural profiles. Regardless, the tendency for maternal stress to decrease GR expression in the male brain as described in the literature suggests that male offspring could be more sensitive to early life stress. Future work in the laboratory and field should further explore proximate and ultimate phenomena associated with sex-specific responses to maternal stress.

3.5.2 DNA Methylation as a Possible Regulatory Mechanism

DNA methylation within the CpG island upstream of the *Nr3c1* gene locus was generally low, though similar to levels reported in previous studies on lab mammals (Kalpachidou et al., 2016; Londono Tobon et al., 2016; Lupien et al., 2009; Pan et al., 2014b). Contrary to our prediction, DNA methylation did not differ based on treatment, sex, or brain region (Figure 3.5). While I did not find evidence for maternal stress effects on DNA methylation in young adults, it is possible that a less conservative approach, using a larger fragment of the predicted CpG island, could reveal differences if structural conformation of chromatin is dependent in other regions of the CpG island. An alternative ecological explanation is that patterns of DNA methylation in these individuals have been modified due to the powerful selective filter of first winter survival, one of the most intensive agents of natural selection in this population of red squirrels (Kemp and Keith, 1970; Larsen and Boutin, 1994), and as a result I can only make inferences on adult individuals who successfully pass through this ecological filter. Indeed, juvenile mortality has immense inter-annual variation the proportion of weaned juveniles that survive the first winter and recruit into the population ranges from 0.10 – 0.88 (Studd et al., 2015). Lastly, despite the extensive support for the role of DNA methylation in the laboratory maternal effects literature, it is also possible that the maternal effects of stress are not regulated epigenetically via DNA methylation in free-living red squirrels. The absence of a significant effect of treatment

interestingly suggests that the epigenetic maternal effects of stress are less important in free-living populations of mammals than are observed under laboratory conditions. One potential hypothesis to explain this is that the physiological stress experienced in response to an ecologically-relevant stressor is insufficient to trigger the epigenetic regulation of the stress axis, and that levels of physiological stress required to provoke this response in the lab are unnatural or only occur when faced with exceptionally traumatic events. Alternatively, effect sizes similar to those observed in this study may occur in the laboratory, but due to the over-representation of large effect size findings in the maternal epigenetics literature (as described in Chapter 2) our expectations for offspring responses to maternal stress are biased towards the regular occurrence of large magnitudes of change in DNA methylation or gene expression. The inclusion of important life history characteristics and relevant ecological information is integral when interpreting and understanding the relationship between DNA methylation and maternal effects in complex and variable natural systems.

Alternative mechanisms associated with the transmission of maternal effects of stress in laboratory rodents may also be at work. Alternative epigenetic regulation of GRs such as microRNA that specifically target GR mRNA, which are known to be differentially expressed in mice exposed to maternal stress (Schouten et al., 2013), could cause sex- or tissue-specific modifications of mRNA levels regardless of methylation level. Additionally, it may be possible that there are non-epigenetic maternal effects which are responsible for the differential expression of *Nr3c1* expression, such as a possible differential distribution of resources to offspring by the mother during perinatal development (Johnson et al., 2013; Mousseau and Fox, 1998). Further exploration into the mechanistic role DNA methylation may play in regulating the transmission of the maternal effects of stress in free-living mammals is required.

3.5.3 Comparability of Lab and Field Studies

This study represents the first to explore and quantify the complex relationship between DNA methylation and the maternal effects of stress in free-living mammals, to the best of our knowledge. The study of maternal effects epigenetics has historically taken place within laboratory settings, however there is an important need for field-tested studies in the wild to understand these processes under natural, real-world conditions. Laboratory conditions often supply simple and sterile environments to study animals in order to minimize environmental variation on stress variables (Calisi and Bentley, 2009). However in so doing, experimental conditions often invoke irrelevant levels of stress (be it more or less than in natural systems) and thus it is difficult to interpret and transfer laboratory-based knowledge (Newman et al., 2015). In laboratory rodents, offspring born to stressed mothers exhibit increased anxiety- and depressive-like behaviours such as reduced willingness to explore novel environments (Pan et al., 2014b; Sasaki et al., 2013), modified social behaviours (Cinquea et al., 2012), and increased apathy in stressful situations (Borsini and Meli, 1988; Mizoguchi et al., 2008). Though traditionally viewed as factors associated with increased pathophysiological responses to stress (Champagne and Curley, 2009; Turecki and Meaney, 2016), I hypothesize that similar behavioural responses to physiological stress could pose beneficial to a free-living North American red squirrel when matched with an appropriate environment. Within the ecological context of our study population, the reduced exploratory and social behaviours (that would be predicted by mRNA expression patterns observed in male red squirrels this study) could increase the likelihood that an individual squirrel successfully commits to and defends a territory from conspecifics in their first year of life, thus increasing the likelihood of first year overwintering survival. A greater attention to life

history and ecological environment when designing studies would enhance the accurate interpretation of results and advance the study of epigenetic maternal effects.

3.6 Conclusion

In this study, yearling North American red squirrels had sex-specific and tissue-specific differences in Nr3c1 mRNA expression within tissues in the HPA axis but showed no difference in expression in response to an ecologically-relevant maternal stress treatment in the wild. This novel experiment highlights the potential for quantifying epigenetic regulation of the HPA axis in wild, free-living mammals, and both expands and informs laboratory-based studies with the clear importance of considering sex-specific regulation of the HPA axis and how the epigenetic regulation of maternal stress may differ between laboratory and field experiments. This exploration of maternal stress epigenetics is also important in understanding the role epigenetics plays in ecological systems and highlights the value of both extending complex molecular genetic methodologies into natural systems and introducing ecological context into the design and interpretation of laboratory research. Future research is needed to understand the role epigenetics plays in fitness outcomes related to maternal effects across a broad scope of taxa both inside and outside of the lab.

3.7 Tables

Table 3.1. Sample sizes for qRT-PCR and DNA methylation in North American Red Squirrels sorted by tissue, treatment, and sex subgroups.

Tissue	Treatment	Sex	*Nr3c1* mRNA Sample Count	*Pomc* mRNA Sample Count	*Nr3c1* DNA Methylation Sample Count
Hippocampus	Playback†	Male	6	6	6
		Female	3	2	3
	Control∆	Male	5	4	3
		Female	4	4	4
Hypothalamus	Playback	Male	6	6	6
		Female	3	2	3
	Control	Male	4	3	3
		Female	4	3	4
Pituitary	Playback	Male	6	6	6
		Female	3	3	3
	Control	Male	2	3	3
		Female	4	4	4
Brain Slice Homogenate	Playback	Male	6	5	-
		Female	3	3	-
	Control	Male	5	5	-
		Female	4	3	-

†Playback = simulated increased social density via playback of squirrel vocalizations
∆Control = simulated unchanged social density via playback of boreal chickadee vocalizations

3.8 Figures

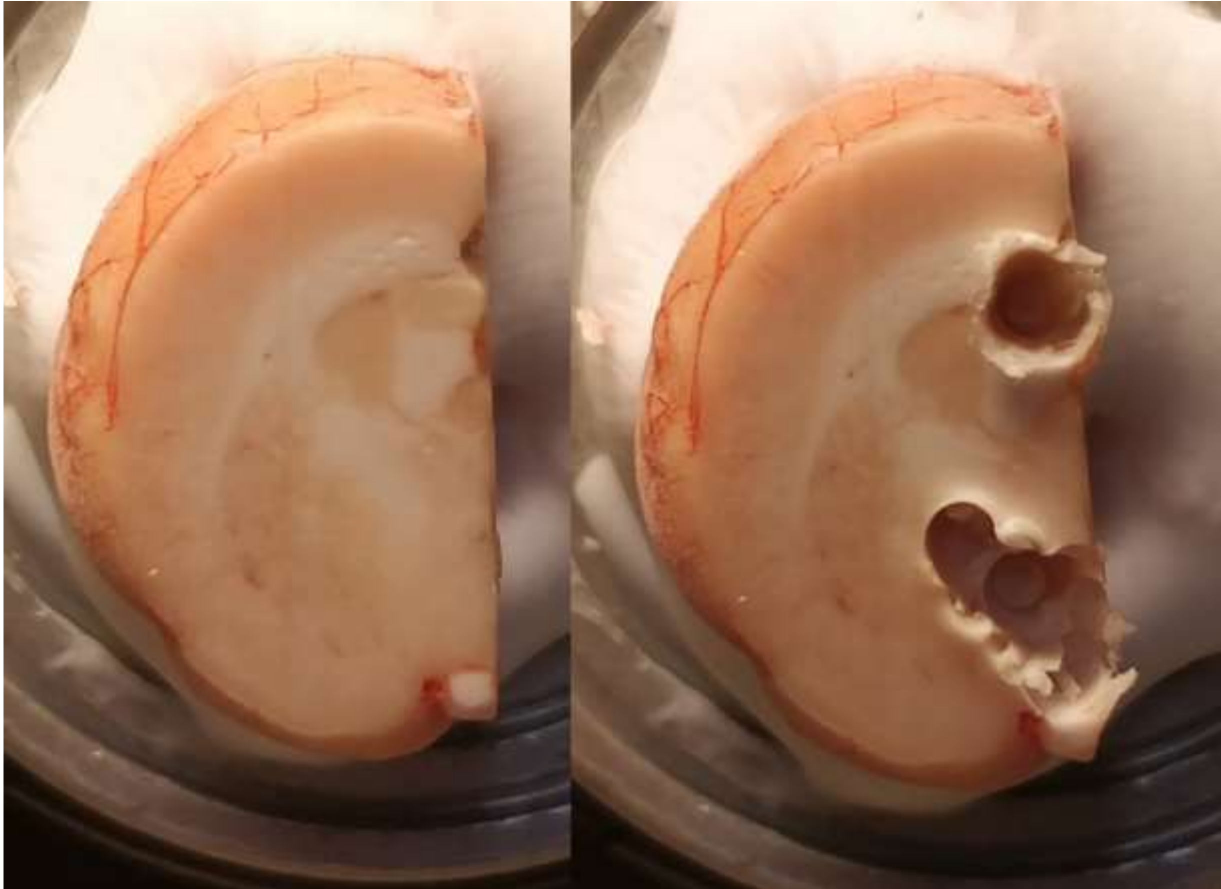

Figure 3.1. Microdissection of a red squirrel brain. Before (left) and after (right) the first removal of hippocampal and hypothalamic tissue from the right hemisphere of a yearling red squirrel brain at Bregma -1.06 (relative to the day 56 mouse brain) using a modified Palkovitz punch technique.

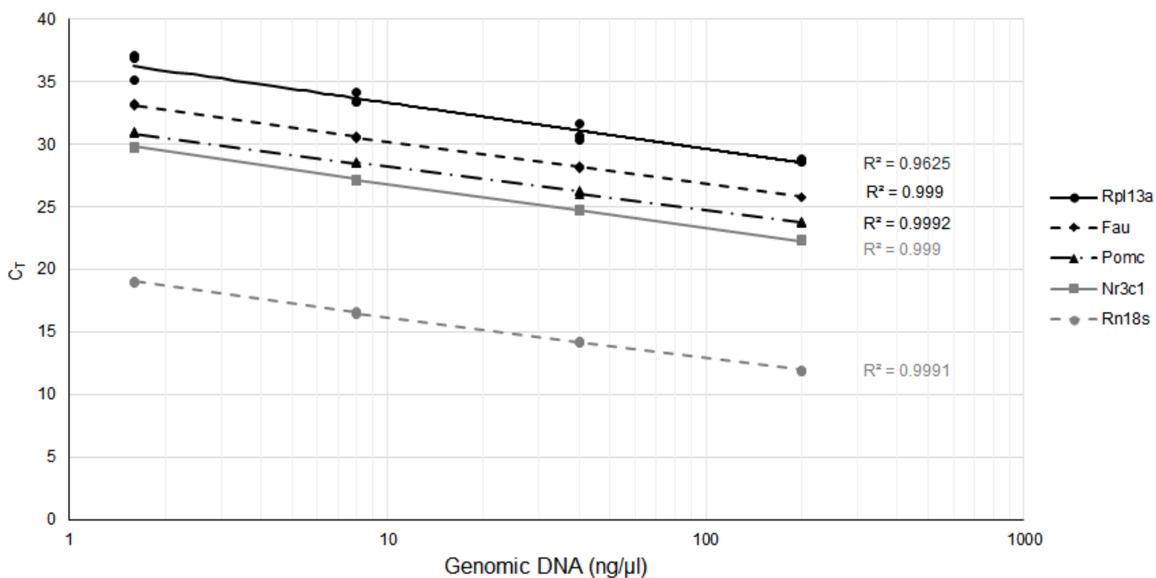

Figure 3.2. Standard curves for the quantification of gene expression in multiple target and reference genes in North American red squirrels. Analysis of cycle threshold (C_T) values over 4 concentrations spanning a biologically-relevant range was used to validate gene expression assay reliability and efficiency.

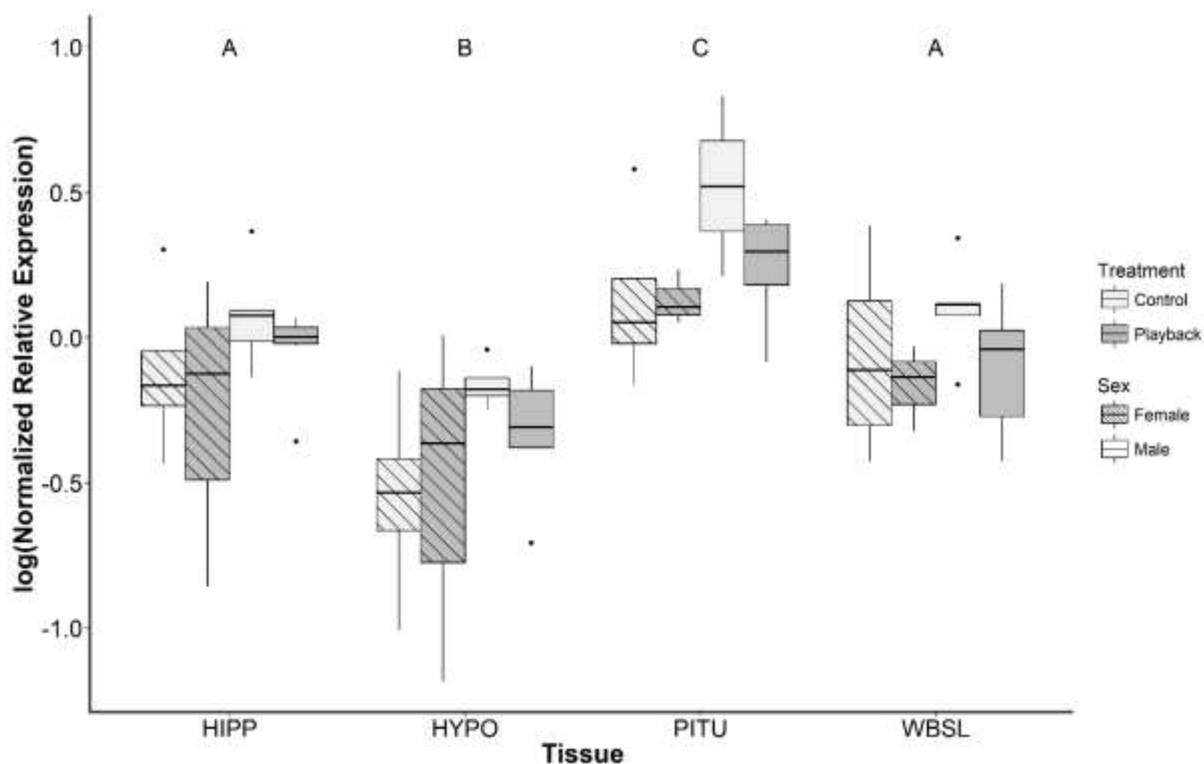

Figure 3.3. Normalized Relative Expression analysis for the expression of Nr3c1 in North American red squirrel yearlings. Gene expression significantly differed among tissues and between sexes. Dashed boxes represent females and open boxes represent males. HIPP = hippocampus, HYPO = hypothalamus, PITU = pituitary gland, WBSL = whole brain slice homogenate. Letters above groups indicate significant differences among tissue types.

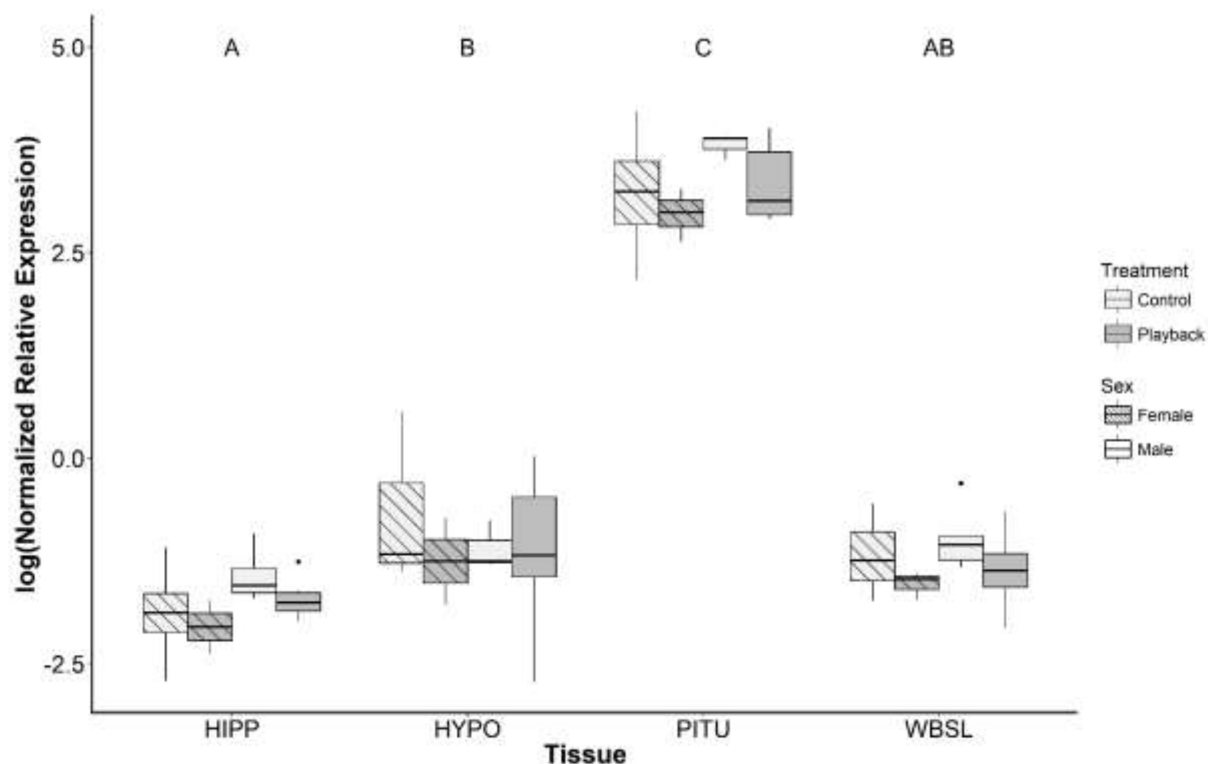

Figure 3.4. Normalized Relative Expression analysis for the expression of Pomc in red squirrel yearlings. Gene expression significantly differed between tissues (signified by letters), however was not significantly different between the sexes or significantly affected by the maternal playback treatment. Data separated by tissue, sex (dashed = female, open = male), and treatment (light grey = control, dark grey = playback). Sample sizes vary between 2 and 6 based on interaction group of tissue, sex, and treatment. HIPP = hippocampus, HYPO = hypothalamus, PITU = pituitary gland, WBSL = whole brain slice homogenate.

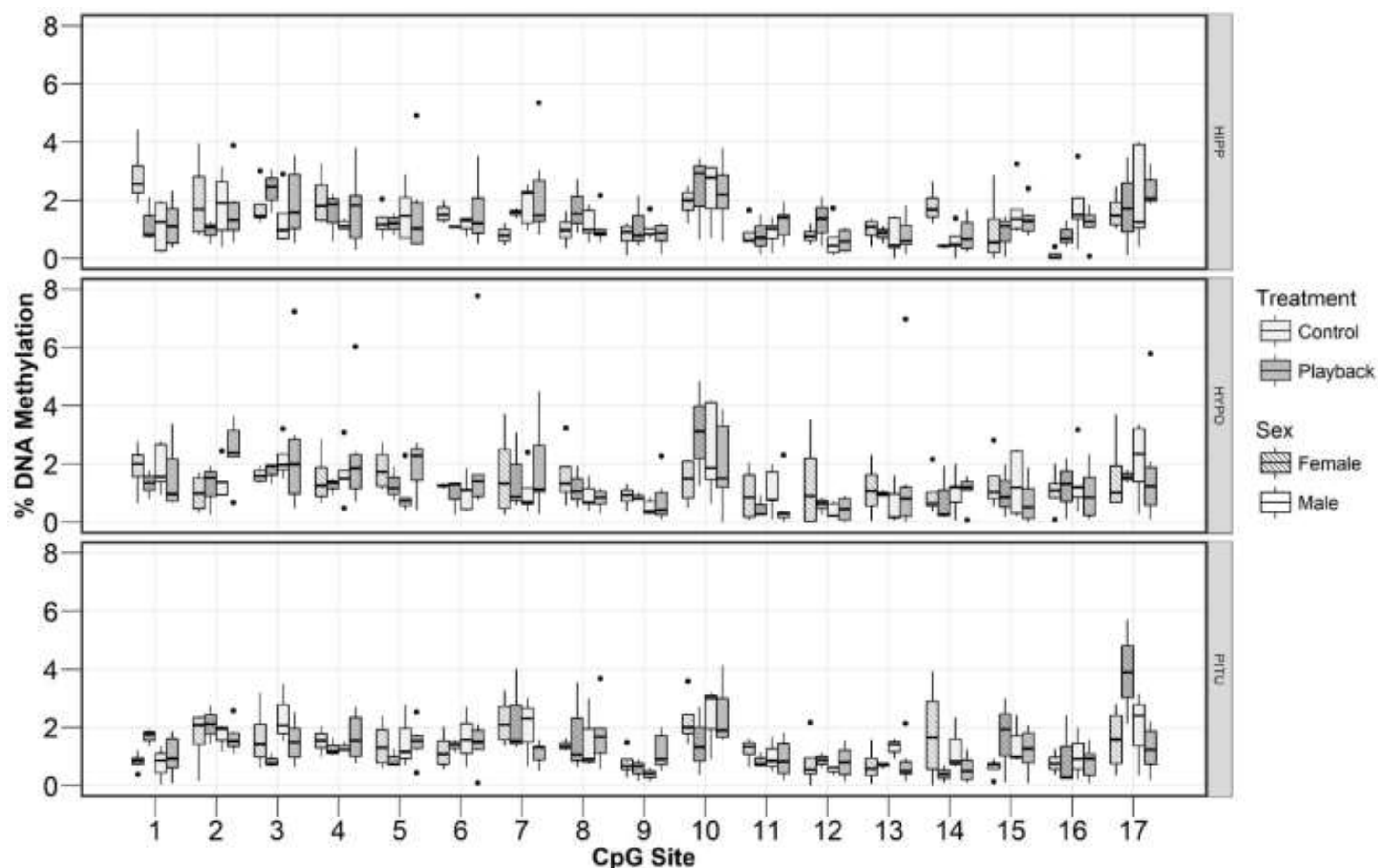

Figure 3.5. Site-specific DNA methylation levels at CpG Island Upstream of Nr3c1 in North American red squirrel yearlings. CpG sites (regions of the DNA sequence with a 5' to 3' pairing of a cytosine and a guanine) were found to significantly differ from each other, however tissue, maternal playback treatment, and sex did not have a significant effect on DNA methylation. Data broken down by tissue, sex (dashed = female, open = male), and treatment (light grey = control, dark grey = playback). Sample sizes vary between 2 and 6 based on interaction group of tissue, sex, and treatment. HIPP = hippocampus, HYPO = hypothalamus, PITU = pituitary gland.

Chapter 4: Epilogue

Until recently, the study of the maternal effects of stress remained largely constrained within laboratory settings and our understanding of these effects in natural environments has remained limited. With this thesis, I have integrated the expertise of both molecular biologists and ecophysiologists into an analysis of our current knowledge of epigenetic maternal effects of stress and have expanded our knowledge on these effects in a complex ecological environment. In **Chapter Two**, I have identified key gaps relating to our understanding of the ecological and evolutionary role that epigenetic maternal effects play in free-living mammals and discovered critical concerns related to the biases present within the laboratory literature ecophysiologists use to inform their research. In **Chapter Three**, I have presented a validated methodological framework which integrates cutting edge molecular genetic techniques with an innovative ecological field manipulation for studying epigenetic maternal effects of stress in a free-living population of red squirrels. As a result, I identified interesting and important sex-specific differences in glucocorticoid receptor expression and, although limited by statistical power, identified promising trends which suggest red squirrel offspring exhibit sex-specific expression of *Nr3c1* mRNA, but exhibit no change in *Nr3c1* or *Pomc* expression or *Nr3c1* DNA methylation in response to ecologically-relevant increases of perceived social density and elevated maternal stress. Together, these studies highlight the value of using an integrative approach to testing trends from laboratory data in more natural environments, and the importance of further expanding our understanding of the epigenetic maternal effects of stress in free-living populations of animals. Further field studies testing trends in parental effects observed in

laboratory studies in a variety of taxa, environments, and physiological stress conditions will be needed to fully understand the depth of complexity the addition of ecological contexts brings to the study of parental effects.

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SUPPLEMENTARY INFORMATION

Supplement 1. Comparison of Overall and Absolute Analyses

Analysis of all 264 data points found that there is a significant positive standardized mean difference in effect size for change in offspring DNA methylation percentage as a result of maternal effects. Analysis of absolute mean difference also found a significant standardized mean difference in effect size as a result of maternal effects, which is a 4.48-fold change in effect size when eliminating directional biases in the overall data (Table S1). The use of an absolute standardized mean difference allows for the evaluation of magnitude of effect size without losing information due to the simultaneous analysis of context-dependent increases and decreases in DNA methylation. The occurrence of a 4.48-fold increase in overall effect as a result of assessing an absolute measurement suggests that strong effects occur with both the increase and decrease of DNA methylation as a result of maternal effects and the untransformed analysis highlights that conditions which result in an increase in DNA methylation occur more strongly (than those which decrease DNA methylation) when transmitted from mother to offspring.

Table S1. Comparison of overall and absolute analyses of effect sizes for maternal effects on offspring DNA methylation percentage born in traditional laboratory model mammals.

	Mean Effect Size ± SEM	Confidence Interval	Sample Size	p-value
Overall	0.176 ± 0.075	(0.030, 0.323)	264	0.018
Absolute	0.789 ± 0.049	(0.692, 0.885)	264	< 0.001

Supplement 2. Reference List of Literature Included for Analysis

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Supplement 3. qRT-PCR Assay Design and Optimization

Primers and probes were initially designed for six different genes; two targets (*Nr3c1*, *Pomc*) and four reference genes (*Rn18s, Fau, Rpl13a, Rps23*). Reference genes were selected as previous studies have shown that their expression levels do not significantly vary as a result of experimental stress treatments (Choudhary et al., 2016; Mitter et al., 2009). For *Nr3c1*, a previously published forward primer (Pan et al., 2014) was used to target the appropriate region. A reverse primer was designed using PrimerBLAST (Ye et al., 2012), using available human and rodent gene sequences (Table S2). Candidate primer sets were validated to target conserved *Nr3c1* sequences using an alignment of previously described sequences in MEGA v6, using the MUSCLE alignment algorithm (Tamura et al., 2013) and were additionally validated for target specificity using NCBI BLAST to identify possible amplification of non-target gene fragments. All other primer sets had novel forward and reverse primer sequences synthesized following this protocol. Similarly, major groove binding (MGB) probes were designed to be located near either the forward or reverse primer, to have a melting temperature (T_m) 6-8˚C greater than the primers, an annealing temperature no more than 5˚C below either primer T_m, to have a GC% composition between 35-65%, and to have no complementarity or secondary structure interference. Probes were designed with a 5’ FAM dye fluorophore reporter and a 3’ IowaBlack fluorescent quencher (IDT). Primer and probe sequences are summarized in Table S3. Due to a lack of target specificity, *Rps23* was excluded as a reference gene for further analysis.

Each individual assay was optimized for ideal primer and probe concentrations to allow for earliest amplification. Probe concentrations for each assay were tested at concentrations ranging from 1 to 4 µM, keeping forward and reverse primer concentrations constant at 5 µM each. Forward and reverse primer concentrations were tested using an 8x8 matrix of

concentration combinations ranging from 1 to 8 µM each using the previously determined optimized probe concentrations for each assay. Probe optimizations were conducted simultaneously for all assays, while primer optimizations were conducted in individual runs for each assay. Optimized concentrations were selected based on reactions which displayed the earliest crossing of the threshold cycle (C_T), and are summarized in Table S4. Amplification efficiency for each assay was calculated using Formula 1, where E represents percentage amplification efficiency and m represents the slope of the standard curve generated.

Formula 1. $$E = 10^{-\frac{1}{m}} * 100$$

Optimized amplification efficiency was determined to be appropriate for further use for all assays except *Rpl13a* and this assay was not used for downstream analyses (Table S5). Samples were included onto plates semi-randomly, alternating between all tissues (hypothalamus, hippocampus, pituitary, or whole brain slice homogenate) of a treatment or a control squirrel to ensure at least one squirrel of each treatment group was included on each plate.

Each reaction replicate run was adjusted for interplate variance using a plate-specific correction value determined through the use of the IPCs (interplate controls) and calculated using Formula 2,

Formula 2. $$C_{q_i}^{corrected} = C_{q_i}^{uncorrected} - C_{q_i}^{IPC} + \frac{\sum_{i=1}^{\#\,of\,plates} C_{q_i}^{IPC}}{\#\,of\,plates}$$

where *C* represents the cycle number of amplification for a reaction, *q* represents the replicate number, *i* represents the plate number, and C_{qi}^{IPC} represents the mean cycle number of amplification for the IPC of a specific plate run (TATAA Interplate Calibrator User Manual).

Table S2. Reference sequence accession numbers for qRT-PCR assay primer design.

Gene Target	Species	Accession Number(s)
Nr3c1	*Ictidomys tridecemlineatus*	XM_013358150.1, XM_013358148.1, XM_013358149.1, XM_005324221.2, XM_005324220.2
	Marmota marmota marmota	XM_015485722.1
	Rattus norvegicus	NM_012576.2
	Mus musculus	NM_008173.3
	Homo sapiens	NM_000176.2
Pomc	*I. tridecemlineatus*	XM_005322582.2
	M. marmota marmota	XM_015479490.1
	R. norvegicus	NM_139326.2
	M. musculus	NM_001278581.1
Rn18s	*R. norvegicus*	NR_046237.1
	M. musculus	NR_003278.3
	Cricetulus griseus	NR_045132.1
Rpl13a	*I. tridecemlineatus*	XM_005336677.2, XM_005336677.2
	M. marmota marmota	XM_015496551.1
	R. norvegicus	NM_173340.2
	M. musculus	NM_009438.5
	H. sapiens	NM_012423.3, NM_001270491.1
	C. griseus	XM_003508651.3
	Castor canadensis	XM_020159956.1
Fau	*I. tridecemlineatus*	XM_005333454.1
	M. marmota marmota	XM_015478247.1
	R. norvegicus	NM_001012739.1
	M. musculus	NM_001160239.2
	H. sapiens	NM_001160239.2
	C. canadensis	XM_020164775.1
Rps23	*I. tridecemlineatus*	XM_005315656.2
	M. marmota marmota	XM_015497862.1
	R. norvegicus	NM_078617.3
	M. musculus	NM_024175.3
	H. sapiens	NM_001025.4
	C. canadensis	XM_020178029.1

Table S3. Primer and probe sets designed for prospective target and reference genes for use in qRT-PCR.

Gene Target	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe Sequence (5'-3')	Fragment Length (bp)
Nr3c1	TTCAAAAGGCTCTGTAAGCCA TGC†	TCCCCAGAGGAAAGGCTGAT	TCTGTCCAAAGCAG TTTCACTCTCAAT	153
Pomc	CGTGTTATAGCCGCTCAGGG	CTTTCCGTGGTGAGGTCCTG	TGGTGTCTAGAGAG CACCCAG	112
*Rn18*s	CCATTCGAACGTCTGCCCTA	ATGTGGTAGCCGTTTCTCAGG	AACTTTCGATGGTA GTCGCCGT	121
Rpl13a	TGCGACTGAAGCCAACAAGA	TCTGCCTGTTTTCGTAGCCT	TGGAGGAGAAAAG GTGGGAGAA	166
Fau	GCCCTCGCTTCTTCCTCTTT	GTCTCCTGGCCGGTTACTTC	TCGACTCCATCTTC ACGGTAGC	132
Rps23	TTGTGGCTCCTTCCTGTGTC	GGTGACTACGGAGCTTCCTG	*N/A*	106

† Previously designed by Pan et al., 2014

Table S4. **Summary of optimized reaction conditions for all qRT-PCR assays.**

	Optimized Reagent Concentrations (µM)		
Gene Target	**Probe**	**Forward Primer**	**Reverse Primer**
Nr3c1	4	3	4
Pomc	4	8	5
Rn18s	4	1	8
Rpl13a	4	3	8
Fau	4	7	8

Table S5. Amplification efficiency and coefficients of determination of qRT-PCR assays.

Gene Target	Equation of the Line (y = mx + b)	Amplification Efficiency (%)	R^2
Nr3c1	y = -3.561x + 31.538	90.892	0.9990
Pomc	y = -3.417x + 34.555	96.164	0.9992
Rn18s	y = -3.38x + 19.618	97.612	0.9991
Rpl13a	y = -3.694x + 38.801	86.507	0.9625
Fau	y = -3.57x + 36.622	90.581	0.9990

Supplement 4. **Sodium Bisulfite Sequencing Assay Design and Validation**

Identification of the best candidate CpG island was conducted using the UCSC Genome Browser (Kent et al., 2002) and Ensemble release 91 (Zerbino et al., 2018) within the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) genome (Ensembl ENSSTOG00000016527). One CpG island was noted in close proximity to the relevant *Nr3c1* transcript and was selected for downstream use (Position JH393314:10959864-10961733 forward strand). Candidate primers were designed for use with bisulfite sequencing PCR (BSP) using MethPrimer 2.0 (Li and Dahiya, 2002) under the following criteria: 1) amplicon length of 100-250 bp with optimal length of 150 bp; 2) primer lengths of 20-30 bp each with no more than five maximally repeated A,C, or G bases or with eight or more T bases in a row contained within the primers; 3) primer T_m between 50-60°C; and 4) minimum of four CpG sites within target amplicon. Two primer sets targeting unique sections of the CpG island were synthesized (Table S6) and amplicons were validated by gel electrophoresis of PCR amplicons generated from sodium bisulfite treated red squirrel DNA. Primer set RSNr3c1Met2 was selected for downstream analysis due to the more successful amplification of target amplicon under PCR protocols used for NGS library preparation.

Table S6. Primer sets designed for amplification of high-density CpG site fragments upstream of Nr3c1 with sodium-bisulfite treated DNA.

Primer Set	Forward Primer (5'-3')	Reverse Primer (5'-3')	Fragment Length (bp)
RSNr3c1Met1	TTCAAAAGGCTCTGTAAGCCATGC	TCCCCAGAGGAAAGGCTGAT	153
RSNr3c1Met2	CGTGTTATAGCCGCTCAGGG	CTTTCCGTGGTGAGGTCCTG	112

Supplement 5. Next-generation sequence data

Table S7. Next-generation sequence numbers before and after bioinformatic processing.

Sample	Tissue	Sex	Treatment	Paired Unedited Reads	Mapped Reads
BT B1.	HIPP	Male	Playback	262016	3952
	HYPO	Male	Playback	429390	1051
	PITU	Male	Playback	2713266	2259
BT B3.	HIPP	Male	Playback	257258	2054
	HYPO	Male	Playback	233476	948
	PITU	Male	Playback	158496	1661
BT E3.	HIPP	Male	Control	181124	4524
	HYPO	Male	Control	271526	5533
	PITU	Male	Control	231262	2576
BT F1	HIPP	Female	Control	22024	250
	HYPO	Female	Control	175976	2523
	PITU	Female	Control	58602	586
BT G1	HIPP	Female	Control	121964	2287
	HYPO	Female	Control	365084	3708
	PITU	Female	Control	277754	15749
BT J3	HIPP	Female	Control	216310	888
	HYPO	Female	Control	101828	3124
	PITU	Female	Control	422876	1542
BT P0	HIPP	Male	Control	358006	2288
	HYPO	Male	Control	278108	4040
	PITU	Male	Control	468068	1474
RR -112	HIPP	Male	Playback	218332	3275
	HYPO	Male	Playback	431870	2593
	PITU	Male	Playback	479680	14776
RR AA4	HIPP	Male	Control	103646	1000
	HYPO	Male	Control	296656	1932
RR B2	HIPP	Male	Control	163314	2346
	HYPO	Male	Control	148748	2920
RR C12	HIPP	Female	Playback	659844	3518
	HYPO	Female	Playback	280892	2338
	PITU	Female	Playback	186408	2311
RR CC.6	HIPP	Male	Control	425808	1755
	HYPO	Male	Control	64134	10763
	PITU	Male	Control	265198	3037
RR E.0	HIPP	Male	Playback	87224	1382
	HYPO	Male	Playback	191500	1489
	PITU	Male	Playback	112122	1958
RR EE7	HIPP	Female	Playback	143966	5899

	HYPO	Female	Playback	150918	54307
	PITU	Female	Playback	276662	1923
RR F5.	HIPP	Female	Control	229772	4015
	HYPO	Female	Control	233034	1829
	PITU	Female	Control	225824	6718
RR F6	HIPP	Male	Playback	703016	906
	HYPO	Male	Playback	94818	90
	PITU	Male	Playback	158800	1028
RR H.1	HIPP	Female	Playback	919364	2068
	HYPO	Female	Playback	298098	3576
	PITU	Female	Playback	226756	4036
RR I0	HIPP	Male	Playback	562524	1341
	HYPO	Male	Playback	300656	1779
	PITU	Male	Playback	150656	1132

Supplement 6. Meta-analysis raw data

Table S8. Raw data extracted from articles included in maternal effects epigenetics meta-analysis

Citation	Species	Type of Effect	Behaviour Type	Diet Supplement	Timing	Post Natal Tx°	PN TCA[Δ]	Sex	Gene Target	CpG Site	Tissue	Tx Sample Size	Tx Mean[†]	Tx SD[•]	Ctrl[+] Sample Size	Ctrl Mean	Ctrl SD
Schaible et al 2011	Mus musculus	Diet	N/A	Methyl Donor	Prenatal	N/A	30	N/A	Ptpn22	N/A	Colon	9	9	2.1	8	12.4	0.848528137
Schaible et al 2011	Mus musculus	Diet	N/A	Methyl Donor	Prenatal	N/A	90	N/A	Ptpn22	N/A	Colon	5	8	0.223606798	4	12.8	0.4
Schaible et al 2011	Mus musculus	Diet	N/A	Methyl Donor	Prenatal	N/A	30	N/A	Ppara	N/A	Colon	10	2.1	0.632455532	10	3.4	1.897366596
Schaible et al 2011	Mus musculus	Diet	N/A	Methyl Donor	Prenatal	N/A	90	N/A	Ppara	N/A	Colon	8	0.5	0.791959595	8	1.85	0.848528137
Marjonen et al 2015	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	28	M	Vmn2r64	N/A	Hippocampus	8	90.96	2.82	8	90.96	3.39
Marjonen et al 2015	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	28	M	Vmn2r64	N/A	Hippocampus	7	70.51	10.25	7	76.54	6.89
Marjonen et al 2015	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	28	M	Olfr110	N/A	Hippocampus	7	89.27	1.68	7	89.44	4.58
Marjonen et al 2015	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	28	M	Vpreb2	N/A	Hippocampus	6	89.86	2.99	6	88.28	2.31
Marjonen et al 2015	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	28	M	Olfr601	N/A	Hippocampus	8	95.07	2.24	10	95.97	1.85
Marjonen et al 2015	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	28	M	Olfr601	N/A	Olfactory Epithelium	5	94.01	1.23	5	92.21	4.43
McCoy et al 2016	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	14	350	M	GFI1	N/A	Hippocampus	10	0.18	0.031622777	10	0.01	0.015811388
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	M	CNNM1	N/A	T Cells	6	1.37	0.220454077	5	0.458	0.178885438
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	F	CNNM1	N/A	T Cells	3	0.9	0.017320508	3	0.77	0.08660254
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	M	CNNM1	N/A	T Cells	5	0.36	0.02236068	4	0.48	0.04
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	F	CNNM1	N/A	T Cells	2	1.66	0.212132034	2	0.25	0.028284271
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	M	CRHBP	N/A	T Cells	6	0.85	0.122474487	5	0.29	0.089442719
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	F	CRHBP	N/A	T Cells	3	0.53	0.034641016	3	0.75	0.207846097
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	M	CRHBP	N/A	T Cells	5	0.76	0.223606798	4	0.16	0.04
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	F	CRHBP	N/A	T Cells	2	0.28	0.113137085	2	0.37	0.113137085
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	M	FKBP5	N/A	T Cells	6	0.53	0.514392846	5	1	0.089442719
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	F	FKBP5	N/A	T Cells	3	0.7	0.138564065	3	0.26	0.103923048
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	M	FKBP5	N/A	T Cells	5	0.69	0.134164079	4	0.98	0.08
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	F	FKBP5	N/A	T Cells	2	1.04	0.268700577	2	0.63	0.155563492

Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	M	Nr3c1	N/A	T Cells	6	0.0193	0.007348469	5	0.155	0.049193496
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	F	Nr3c1	N/A	T Cells	3	0.195	0.04330127	3	0.0751	0.011085125
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	M	Nr3c1	N/A	T Cells	5	0.177	0.01118034	4	0.114	0.01
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	F	Nr3c1	N/A	T Cells	2	0.0869	0.022768838	2	0.172	0.04384062
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	M	SMAD3	N/A	T Cells	6	1.01	0.685857128	5	0.38	0.02236068
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	14	F	SMAD3	N/A	T Cells	3	0.53	0.017320508	3	0.55	0.017320508
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	M	SMAD3	N/A	T Cells	5	1.81	0.245967478	4	0.95	0.18
Massart et al 2016	Macaca mulatta	Behaviour	Seperation	N/A	Post-natal	0	730	F	SMAD3	N/A	T Cells	2	0.96	0.113137085	2	1.11	0.155563492
Kalpachidou et al 2016	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	0	13	M	Adrb1	N/A	Prefrontal Cortex	7	102.76	48.12621635	7	100	47.0150008
Kalpachidou et al 2016	Rattus norvegicus	Behaviour	Maternal Care	N/A	Post-natal	0	13	M	Adrb1	N/A	Prefrontal Cortex	7	43.01	44.95131477	7	100	47.0150008
Mansell et al. 2016	Homo sapiens	Behaviour	Anxiety	N/A	Prenatal	0	0	N/A	IGF2/H19	N/A	Cord Blood	111	39.77	7.23	465	42.23	5
Barua et al. 2015	Mus musculus	Diet	N/A	Folic Acid	Prenatal	0	1	M	Genomic	N/A	Cerebral Hemisphere	4	1	0.0358	4	1	0.0752
Barua et al. 2015	Mus musculus	Diet	N/A	Folic Acid	Prenatal	0	1	F	Genomic	N/A	Cerebral Hemisphere	4	0.9305	0.0224	4	1	0.0582
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Genomic	N/A	White adipose tissue	15	11.71	7.784696526	15	10.3	2.323790008
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	7	White adipose tissue	15	31.9	2.788548009	15	36.55	2.517439175
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	8	White adipose tissue	15	41.39	3.059656844	15	46.43	4.841229183
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	9	White adipose tissue	15	40.28	3.292035844	15	44.86	4.066632514
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	10	White adipose tissue	15	35.57	2.517439175	15	37.99	3.292035844
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	11	White adipose tissue	15	33.27	3.059656844	15	36.22	3.563144679
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	12	White adipose tissue	15	37.27	2.982197177	15	39.1	3.795523679
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	13	White adipose tissue	15	40.08	2.556169008	15	43.29	3.292035844
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	14	White adipose tissue	15	40.67	2.556169008	15	44.4	3.834253513
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	15	White adipose tissue	15	43.32	3.446955178	15	45.78	4.066632514
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	16	White adipose tissue	15	46.11	3.292035844	15	48.53	4.066632514
Jousse et al. 2011	Mus musculus	Diet	N/A	Low Protein	Prenatal	0	210	M	Leptin	17	White adipose tissue	15	45.91	3.059656844	15	48.53	4.299011514
Chen et al. 2017	Rattus norvegicus	Diet	N/A	High Sugar	Prenatal	N/A	1	M	Genomic	N/A	Dental Epithelium	3	1.48	0.08	3	1	0
Janssen et al 2017	Homo sapiens	Behaviour	N/A	Nicotine	Prenatal	N/A	1	N/A	CYP1A1	1	Placenta	62	34.75	12.57430849	255	33.59	13.19863544
Janssen et al 2017	Homo sapiens	Behaviour	N/A	Nicotine	Prenatal	N/A	1	N/A	CYP1A1	2	Placenta	62	40.19	10.46519924	255	40.42	21.22373168

Janssen et al 2017	Homo sapiens	Behaviour	N/A	Nicotine	Prenatal	N/A	1	N/A	CYP1A1	3	Placenta	62	53.74	6.508108549	255	58.96	13.19863544
Janssen et al 2017	Homo sapiens	Behaviour	N/A	Nicotine	Prenatal	N/A	1	N/A	CYP1A1	4	Placenta	62	38.24	8.376176744	255	37.64	16.98713265
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	1	Hippocampus	6	32	15.54	6	0	0
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	2	Hippocampus	6	48.46	16	6	1.83	4.11
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	3	Hippocampus	6	55.31	10.29	6	17.14	4.8
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	4	Hippocampus	6	41.83	9.6	6	10.29	6.17
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	5	Hippocampus	6	56.59	26.84	6	34.97	4.8
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	6	Hippocampus	6	50.29	21.71	6	23.77	16
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	7	Hippocampus	6	65.37	10.29	6	29.94	12.57
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	8	Hippocampus	6	66.97	13.26	6	19.89	6.62
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	9	Hippocampus	6	61.94	21.95	6	16.91	13.72
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	10	Hippocampus	6	58.74	19.43	6	26.47	18.79
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	11	Hippocampus	6	68.57	23.77	6	26.74	20.35
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	12	Hippocampus	6	61.94	19.2	6	26.74	19.43
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	13	Hippocampus	6	60.34	20.35	6	25.14	8.46
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	14	Hippocampus	6	65.6	15.77	6	5.26	8.45
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	15	Hippocampus	6	66.97	15.77	6	18.51	13.26
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	16	Hippocampus	6	96.91	7.78	6	15.09	10.51
Zhu et al 2017	Rattus norvegicus	Behaviour	Seperation	N/A	Post-natal	21	90	N/A	Nr3c1	17	Hippocampus	6	96.91	4.8	6	78.4	7.54
van Dijk et al. 2016	Homo sapiens	Diet	N/A	Docosahexaenoic acid	Prenatal	N/A	1	F	Genomic	N/A	Blood	257	1.56	0.64	232	1.5	0.48
van Dijk et al. 2016	Homo sapiens	Diet	N/A	Docosahexaenoic acid	Prenatal	N/A	1	M	Genomic	N/A	Blood	260	1.39	0.49	242	1.36	0.42
van Dijk et al. 2016	Homo sapiens	Diet	N/A	Docosahexaenoic acid	Prenatal	N/A	1825	N/A	Genomic	N/A	Blood	342	0.99	0.15	325	0.99	0.16
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-16.5	N/A	GLUT3	N/A	Placenta	15	7.15	6.34	15	4.48	2.77
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-16.5	N/A	LINE-1	N/A	Placenta	15	66.04	5.62	15	68.98	8.02
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-16.5	N/A	IAP	N/A	Placenta	15	75	6.12	15	73.67	7.71
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-19.5	N/A	GLUT3	N/A	Placenta	15	6.51	4.16	15	3.89	8.37
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-19.5	N/A	LINE-1	N/A	Placenta	15	67.38	11.76	15	69.52	9.89
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-19.5	N/A	IAP	N/A	Placenta	15	77.13	7.98	15	76.33	7.18

Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-16.5	N/A	GLUT3	N/A		Placenta	15	7.09	7.47	15	4.48	2.77
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-16.5	N/A	LINE-1	N/A		Placenta	15	69.52	5.88	15	68.98	8.02
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-16.5	N/A	IAP	N/A		Placenta	15	75.8	13.3	15	73.67	7.71
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-19.5	N/A	GLUT3	N/A		Placenta	15	9.39	3.3	15	3.89	8.37
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-19.5	N/A	LINE-1	N/A		Placenta	15	72.19	9.36	15	69.52	9.89
Xu et al. 2016	Mus musculus	Diet	N/A	Cadmium	Prenatal	N/A	-19.5	N/A	IAP	N/A		Placenta	15	77.13	5.32	15	76.33	7.18
Mansell et al. 2016	Homo sapiens	Behaviour	Anxiety	N/A	Prenatal	N/A	0	N/A	Nr3c1		47	Cord Blood	77	1.763	0.246	368	1.518	0.156
Mansell et al. 2016	Homo sapiens	Behaviour	Depression	N/A	Prenatal	N/A	0	N/A	Nr3c1	12 + 13		Cord Blood	88	5.236	0.355	357	4.527	0.218
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	Igf2	N/A		Liver	11	66.43	2.421136097	10	66.07	2.877672671
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	H19Cbs2	N/A		Liver	11	51.21	2.387969849	10	53.75	2.846049894
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	H19Cbs4	N/A		Liver	11	51.93	3.018128559	10	51.75	2.308462692
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	H19PP	N/A		Liver	11	84.89	0.63015871	10	85.99	0.569209979
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	IG-DMR	N/A		Liver	11	36	3.018128559	10	35.82	3.44688265
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	SnrpnICR	N/A		Liver	11	39.39	2.122639866	10	39.12	2.877672671
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	59.5	N/A	Grb10DMR	N/A		Liver	10	45.32	2.308462692	10	51.7	5.186135363
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	Igf2	N/A		Sperm	11	90.83	1.492481156	10	91.37	0.758946638
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	H19Cbs2	N/A		Sperm	11	93.38	2.620133584	10	94.92	1.043551628
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	H19Cbs4	N/A		Sperm	11	92.99	3.416123534	10	94.14	0.664078309
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	H19PP	N/A		Sperm	11	88.26	0.829156198	10	88.59	0.537587202
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	IG-DMR	N/A		Sperm	11	88.45	6.301587102	10	90.93	2.877672671
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	SnrpnICR	N/A		Sperm	11	4.14	2.454302345	10	3.24	1.264911064
Xue et al. 2016	Mus musculus	Diet	N/A	Vitamin D deficiency	Prenatal	N/A	56	M	Grb10DMR	N/A		Sperm	10	6.13	2.023857703	10	5.1	1.4862705
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Crf		232	Hypothalamus	12	65.7	7.967433715	7	71.9	3.174901573
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Crf		226	Hypothalamus	12	67.9	2.078460969	7	70.2	3.174901573
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Crf		212	Hypothalamus	12	76.5	2.078460969	7	74.2	3.174901573
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Crfr1	N/A		Hippocampus	7	0.00623	0.003042614	7	0.01269	0.004286117
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Crfr1	N/A		Amygdala	7	0.00756	0.005159215	7	0.02268	0.010344888
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Pomc	N/A		Hypothalamus	7	0.155	0.118000508	7	0.0484	0.046300648

Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Pomc	238	Hypothalamus	7	19.71	2.751581364	7	15.87	1.852025918
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Pomc	224	Hypothalamus	7	26.86	1.375790682	7	21.8	2.301803641
Jabbar et al. 2016	Rattus norvegicus	Diet	N/A	Ethanol	Prenatal	N/A	23	M	Pomc	216	Hypothalamus	7	19.88	1.852025918	7	17.79	2.830953903
Ganguly et al 2016	Mus musculus	Diet	N/A	Calorie Restriction	Prenatal	N/A	-3	N/A	glut3	KDM3A	Placenta	9	397.55	474.84	3	100	56.32629226
Fabian et al 2015	Mus musculus	Diet	N/A	Calorie Addition	Prenatal	N/A	-21	M	Genomic	N/A	Zygote (PN3)	37	71.87	13.01711181	28	70.76	24.12925196
Fabian et al 2015	Mus musculus	Diet	N/A	Calorie Addition	Prenatal	N/A	-21	M	Genomic	N/A	Zygote (PN4)	36	74.75	16.62	35	61.33	18.57649052
Fabian et al 2015	Mus musculus	Diet	N/A	Calorie Addition	Prenatal	N/A	-21	M	Genomic	N/A	Zygote (PN3)	33	72.98	21.31232742	28	70.76	24.12925196
Fabian et al 2015	Mus musculus	Diet	N/A	Calorie Addition	Prenatal	N/A	-21	M	Genomic	N/A	Zygote (PN4)	32	74.49	11.93596247	35	61.33	18.57649052
Cho et al 2012	Rattus norvegicus	Diet	N/A	Lipotropes	Prenatal	N/A	22	F	Genomic	N/A	Mammary Tissue	4	99	9.9	4	100	17
Cho et al 2012	Rattus norvegicus	Diet	N/A	Lipotropes	Prenatal	N/A	22	F	Genomic	N/A	Mammary Tissue	4	97.7	15.1	4	100	9.9
Ly et al 2011	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	196	F	Genomic	N/A	Mammary Tissue	70	55000	13210.86182	70	53421	13210.86182
Zheng et al 2011	Rattus norvegicus	Diet	N/A	Low Protein	Prenatal	N/A	38	F	p16	CDKN2A, Hha1 digested	Mammary Tissue	5	101.66	7.93804132	5	100	18.0227079
Zheng et al 2011	Rattus norvegicus	Diet	N/A	Low Protein	Prenatal	N/A	38	F	p16	CDKN2A, Aci1 digested	Mammary Tissue	5	100	5.791416062	5	100	5.791416062
Novikova et al 2008	Mus musculus	Diet	N/A	Cocaine	Prenatal	N/A	3	M	Genomic	N/A	Hippocampus	10	70.6	22.54703972	10	98.44	7.051879182
Novikova et al 2008	Mus musculus	Diet	N/A	Cocaine	Prenatal	N/A	30	M	Genomic	N/A	Hippocampus	10	131.55	38.89601522	10	97.72	11.890164
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	Genomic	N/A	Liver	16	24.93	8.64	16	37.46	9.8
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	1	N/A	Genomic	N/A	Liver	10	32.13	12.77560175	10	19.31	14.13538114
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	28	N/A	Genomic	N/A	Liver	16	20.61	8.64	16	18.01	8.08
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	188	N/A	Genomic	N/A	Liver	16	15.27	10.96	16	19.6	8.64
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	Genomic	N/A	Liver	16	30.84	9.2	16	37.46	9.8
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	1	N/A	Genomic	N/A	Liver	10	23.78	11.82691845	10	19.31	14.13538114
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	28	N/A	Genomic	N/A	Liver	16	16.86	5.2	16	18.01	8.08
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	188	N/A	Genomic	N/A	Liver	16	11.38	9.8	16	19.6	8.64
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	Genomic	N/A	Skeletal Muscle	16	30.43	10.28	16	34	8.56
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	1	N/A	Genomic	N/A	Skeletal Muscle	10	35.86	5.850213671	10	31.86	5.850213671
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	28	N/A	Genomic	N/A	Skeletal Muscle	16	25.71	9.16	16	31	10.28
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	188	N/A	Genomic	N/A	Skeletal Muscle	16	25.29	11.4	16	35	12.56
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A		N/A	Genomic	N/A	Skeletal Muscle	16	30.29	10.84	16	34	8.56

Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	1	N/A	Genomic	N/A	Skeletal Muscle	10	30.57	6.32455532	10	31.86	5.850213671
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	28	N/A	Genomic	N/A	Skeletal Muscle	16	26.29	6.84	16	31	10.28
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	188	N/A	Genomic	N/A	Skeletal Muscle	16	28.43	11.44	16	35	12.56
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	NCAPG	N/A	Liver	16	3.52	0.6	16	4.3	0.32
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	NCAPG	N/A	Liver	16	3.78	0.48	16	4.3	0.32
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	NCAPG	N/A	Skeletal Muscle	10	4.48	0.284604989	10	2.59	0.316227766
Altmann et al 2012	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	NCAPG	N/A	Skeletal Muscle	10	2.72	0.221359436	10	2.59	0.316227766
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	1	N/A	CYP2C34	-349	Liver	10	73.86	11.51069068	10	69.55	10.75174404
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	1	N/A	CYP2C34	-137	Liver	10	38.41	24.44440631	10	3.41	25.1401074
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	1	N/A	CYP2C34	-112	Liver	10	58.41	32.35010046	10	16.36	29.47242779
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	1	N/A	CYP2C34	-349	Liver	10	72.95	11.51069068	10	69.55	10.75174404
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	1	N/A	CYP2C34	-137	Liver	10	25.45	28.77672671	10	3.41	25.1401074
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	1	N/A	CYP2C34	-112	Liver	10	44.09	30.89545274	10	16.36	29.47242779
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	PGC1alpha	-214	Liver	16	3.62	4.28	16	3.85	3.92
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	PGC1alpha	-21	Liver	16	14.52	2.6	16	12.53	2.24
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	PGC1alpha	-17	Liver	16	18	2.8	16	16.19	2.8
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	PGC1alpha	5	Liver	16	15.73	3.36	16	13.97	3.52
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	PGC1alpha	-214	Liver	16	2.51	5	16	3.85	3.92
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	PGC1alpha	-21	Liver	16	13.04	3.16	16	12.53	2.24
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	PGC1alpha	-17	Liver	16	16.8	3.16	16	16.19	2.8
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	PGC1alpha	5	Liver	16	14.76	3.88	16	13.97	3.52
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	-95	N/A	Nr3c1	N/A	Liver	16	3.57	0.8	16	4.47	0.6
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	-95	N/A	Nr3c1	N/A	Liver	16	3.67	0.8	16	4.47	0.6
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	Low Protein	Prenatal	N/A	1	N/A	Ppara	N/A	Liver	20	3.15	1.296919427	20	2.05	1.520526225
Altmann et al 2013	Sus scrofa domesticus	Diet	N/A	High Protein	Prenatal	N/A	1	N/A	Ppara	N/A	Liver	20	2.29	1.073312629	20	2.05	1.520526225
Smith et al 2014	Rattus norvegicus	Diet	N/A	Low Calorie	Prenatal	N/A	2	M	IGFBP2	N/A	Liver	10	3.25	0.031622777	10	3.45	0.758946638
Smith et al 2014	Rattus norvegicus	Diet	N/A	High Fat	Prenatal	N/A	2	M	IGFBP2	N/A	Liver	10	3.28	0.537587202	10	3.45	0.758946638
Blaze et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	90	M	Bdnf IV	N/A	Prefrontal Cortex	7	11.79	2.010770996	6	3.99	1.420704051

Blaze et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	90	F	Bdnf IV	N/A		Prefrontal Cortex	7	4.76	1.508078247	6	9.7	6.981045767
Cai et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	PSEN1	N/A		Liver	8	0.85	0.67882251	8	1	0.480832611
Cai et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	STAT3	N/A		Liver	8	1.66	0.707106781	8	1	0.311126984
Castillo et al 2012	Rattus norvegicus	Diet	N/A	Cadmium	Prenatal	N/A	-1	M	Nr3c1			Liver	6	185	51.4392846	6	100	0
Castillo et al 2012	Rattus norvegicus	Diet	N/A	Cadmium	Prenatal	N/A	-1	F	Nr3c1			Liver	6	62.2	26.94438717	6	100	0
Chen et al 2018	Mus musculus	Diet	N/A	Nicotine	Prenatal	N/A	1	F	Genomic			Lung	3	8	0.848704896	3	3.22	3.204293994
Chen et al 2018	Mus musculus	Diet	N/A	Vapour	Prenatal	N/A	1	F	Genomic			Lung	3	15.61	1.004589468	3	3.22	3.204293994
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-606	Liver	5	29.35	4.852267511	9	29.35	11.94
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-606	Liver	6	44.2	4.433576434	9	39.13	16.29
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-508	Liver	5	28.65	0.178885438	9	27.03	13.77
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-508	Liver	6	39.19	4.629535614	9	26.76	18.63
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-440	Liver	5	40.27	6.037383539	9	30.27	20.28
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-440	Liver	6	34.05	84.7278502	9	25.95	6.48
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-248	Liver	5	14.32	6.842368011	9	13.22	4.92
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-248	Liver	6	13.88	6.148219254	9	15.52	2.94
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-129	Liver	5	18.32	4.718103433	9	18.97	12.66
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-129	Liver	6	19.14	1.984086692	9	22.86	15.57
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-81	Liver	5	14.92	3.309380607	9	15.86	9.69
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-81	Liver	6	14.92	2.302520358	9	9.54	11.7
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	M	PEPCK		-90	Liver	5	15.32	3.622430124	9	15.86	9.27
Hoile et al 2012	Rattus norvegicus	Diet	N/A	Folic Acid	Prenatal	N/A	86	F	PEPCK		-90	Liver	6	14.38	0.979795897	9	17.34	9.27
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	Stra13	N/A		Heart	5	5.58	1.542886904	5	4.37	0.447213595
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	PPARG	N/A		Heart	5	10.67	2.124264579	5	8.47	1.364001466
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	E2F	N/A		Heart	5	3.5	1.095673309	5	4.19	1.296919427
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	Egr-1	N/A		Heart	5	7.89	2.146625258	5	3.38	2.01246118
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	MTF1-1	N/A		Heart	5	6.1	1.02859127	5	3.56	1.162755348
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	SP1-1	N/A		Heart	5	4.8	2.839806331	5	3.59	1.095673309
Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	SP1-2	N/A		Heart	5	2.31	0.849705831	5	2.08	0.916787871

Lawrence et al 2012	Rattus norvegicus	Diet	N/A	Nicotine	Prenatal	N/A	-2	N/A	MTF1-2	N/A		Heart	5	3.21	1.94537914	5	2.72	0.827345152
Leung et al 2017	Rattus norvegicus	Diet	N/A	Bisphenol A	Prenatal	N/A	21	F	Car7	N/A		Mammary Tissue	11	42.83	41.52414238	11	30.71	37.51102638
Leung et al 2017	Rattus norvegicus	Diet	N/A	Bisphenol A	Prenatal	N/A	21	F	Kcnv2	N/A		Mammary Tissue	11	89.38	25.47167839	11	94.58	23.97919723
Li et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	IGF2	DMR1		Hippocampus	6	1.31	0.269443872	6	1	0.048989795
Li et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	IGF2	DMR2		Hippocampus	6	1.24	0.122474487	6	1	0.122474487
Li et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	IGF2	DMR3		Hippocampus	6	1.17	0.171464282	6	1.05	0.489897949
Li et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	IGF2/H19	IGF1R		Hippocampus	6	1.08	0.09797959	6	1	0.293938769
Li et al 2015	Sus scrofa domesticus	Diet	N/A	Betaine	Prenatal	N/A	1	M	IGF2/H19	IGF2R		Hippocampus	6	1.14	0.612372436	6	1.04	0.465403051
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		1	Embryo	5	0.29	0.01	5	0.3	0.01
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		2	Embryo	5	0.33	0.04	5	0.35	0.01
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		4	Embryo	5	0.38	0.4	5	0.39	0.01
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		8	Embryo	5	0.32	0.01	5	0.32	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		9	Embryo	5	0.3	0.02	5	0.31	0.01
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		10	Embryo	5	0.37	0.02	5	0.36	0.03
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2/H19		11	Embryo	5	0.38	0.02	5	0.37	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2		1	Embryo	5	0.34	0.02	5	0.35	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2		2	Embryo	4	0.53	0.04	5	0.54	0.03
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2		3	Embryo	4	0.52	0	5	0.52	0.03
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2		4	Embryo	4	0.43	0.03	4	0.44	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	IGF2		5	Embryo	5	0.25	0.01	5	0.24	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		1	Embryo	5	0.35	0.03	5	0.34	0.03
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		2	Embryo	5	0.37	0.03	5	0.36	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		3	Embryo	5	0.34	0.02	5	0.33	0.05
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		6	Embryo	5	0.42	0.02	5	0.4	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		7	Embryo	5	0.42	0.02	5	0.41	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		11	Embryo	5	0.41	0.04	5	0.4	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		12	Embryo	5	0.43	0.06	5	0.42	0.03
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	SnrpnICR		13	Embryo	5	0.41	0.03	5	0.4	0.03

Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	1	Embryo	3	0.42	0.01	5	0.4	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	2	Embryo	5	0.41	0.02	5	0.4	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	3	Embryo	5	0.39	0.02	4	0.4	0.01
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	4	Embryo	3	0.37	0.03	4	0.34	0.01
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	5	Embryo	5	0.4	0.02	3	0.38	0
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	11	Embryo	4	0.44	0.03	5	0.43	0.02
Marjonen et al 2018	Mus musculus	Diet	N/A	Ethanol	Prenatal	N/A	-12	N/A	Peg3	13	Embryo	4	0.4	0.03	5	0.41	0.03
McKay et al 2011	Mus musculus	Diet	N/A	Folic Acid	Prenatal	N/A	-1	N/A	Esr1	N/A	Intestine	16	8.72	2.68	18	8.83	3.309259736
McKay et al 2011	Mus musculus	Diet	N/A	Folic Acid	Prenatal	N/A	-1	N/A	Igf2	N/A	Intestine	16	52.5	6.32	18	52.5	3.351686143
McKay et al 2011	Mus musculus	Diet	N/A	Folic Acid	Prenatal	N/A	-1	N/A	Slc39a4	CGI 1	Intestine	16	66.99	6	18	71.36	2.03646753
McKay et al 2011	Mus musculus	Diet	N/A	Folic Acid	Prenatal	N/A	-1	N/A	Slc39a4	CGI 2	Intestine	16	63.27	10.44	18	63.27	4.412346315
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	36.12	2.522974435	6	35.6	4.433576434
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	25.89	3.478275435	6	27.7	4.752010101
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	18.38	5.070443768	6	21.49	6.025744767
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	18.12	5.070443768	6	15.53	6.025744767
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	30.29	6.025744767	6	27.18	2.547469332
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	27.44	2.229035666	6	27.06	1.886107102
St-Cyr et al 2017	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	0	N/A	FKBP5	N/A	Amygdala	6	21.62	0.636867333	6	22.91	1.592168333
Tian et al 2014	Rattus norvegicus	Diet	N/A	Nicotinamide	Prenatal	N/A	-1	N/A	Genomic	N/A	Liver	18	1.49	0.509116882	16	2.36	0.72
Tian et al 2014	Rattus norvegicus	Diet	N/A	Nicotinamide	Prenatal	N/A	-1	N/A	Genomic	N/A	Liver	19	0.97	0.78460181	16	2.36	0.72
Xu et al 2014	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	38	M	CRH	N/A	Hypothalamus	9	58.16	10.71	10	71.09	9.8663063
Xu et al 2014	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	38	F	CRH	N/A	Hypothalamus	9	56.6	180.51	8	68.86	7.551900423
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Genomic	N/A	Hippocampus	5	0.77	0.134164079	5	1.21	0.290688837
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	F	Genomic	N/A	Hippocampus	5	0.37	0.089442719	5	0.52	0.111803399
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-913	Hippocampus	5	2.22	0.894427191	5	2.52	1.095673309
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-909	Hippocampus	5	4.52	1.274558747	5	3.21	1.498165545
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-883	Hippocampus	5	1.83	1.699411663	5	2.27	1.162755348
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-833	Hippocampus	5	0	0	5	6.2	1.811215062

Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-831	Hippocampus	5	3.28	0.827345152	5	2.79	1.386362146
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-829	Hippocampus	5	3.21	1.207476708	5	2.89	1.050951949
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-809	Hippocampus	5	2.79	0.782623792	5	3.28	1.162755348
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	-5	Hippocampus	5	4.84	0.715541753	5	4.49	1.94537914
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	6	Hippocampus	5	2.54	0.514295635	5	2.1	0.715541753
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	9	Hippocampus	5	4.27	0.894427191	5	4.79	1.319280107
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	13	Hippocampus	5	0	0	5	5.31	1.319280107
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	21	Hippocampus	5	2.37	0.559016994	5	2.49	0.447213595
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	24	Hippocampus	5	6.57	1.542886904	5	6.67	2.414953416
Xu et al 2018	Rattus norvegicus	Behaviour	Stress	N/A	Prenatal	N/A	63	M	Crhr1	38	Hippocampus	5	2.79	0.782623792	5	2.37	0.715541753
Zhang et al 2018	Mus musculus	Diet	N/A	Nitrogen Dioxide	Prenatal	N/A	1	M	Genomic	N/A	Heart	6	0.79	0.048989795	6	1	0.09797959
Zhang et al 2018	Mus musculus	Diet	N/A	Nitrogen Dioxide	Prenatal	N/A	7	M	Genomic	N/A	Heart	6	0.89	0.073484692	6	1.08	0.171464282
Zhang et al 2018	Mus musculus	Diet	N/A	Nitrogen Dioxide	Prenatal	N/A	21	M	Genomic	N/A	Heart	6	1.03	0.171464282	6	1.21	0.09797959
Zhang et al 2018	Mus musculus	Diet	N/A	Nitrogen Dioxide	Prenatal	N/A	1	F	Genomic	N/A	Heart	6	1.04	0.269443872	6	1	0.220454077
Zhang et al 2018	Mus musculus	Diet	N/A	Nitrogen Dioxide	Prenatal	N/A	7	F	Genomic	N/A	Heart	6	0.85	0.024494897	6	0.79	0.146969385
Zhang et al 2018	Mus musculus	Diet	N/A	Nitrogen Dioxide	Prenatal	N/A	21	F	Genomic	N/A	Heart	6	0.84	0.09797959	6	0.83	0.220454077
Zhong et al 2015	Rattus norvegicus	Behaviour	Maternal Care	N/A	Post-natal	51	51	N/A	Genomic	N/A	Amygdala	55	9.58	1.58	49	5.32	0.84

° Tx = treatment group
Δ PN TCA = Post-natal tissue collection age, in days
† Mean percentage DNA methylation
● SD = Standard deviation
+ Ctrl = Control group