Does methylation of the brain promoter region of *Cyp19* impact the development of sex-typical psychological traits?

by

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Abstract

While humans demonstrate sexually differentiated psychological and behavioural traits, such as aggression, there is a vast amount of within-sex variation for these traits. Further, the effect size of these sex differences is relatively small and there is a large amount of overlap between males and females on each measure. The mechanisms underlying these differences may operate, in part, prenatally. For neurodevelopment, estrogen serves to masculinize and defeminize the male brain. The aromatization hypothesis suggests that the conversion of testosterone to estrogen in the brain facilitates this process. Consequently, the degree of expression of *Cyp19*, the aromatase producing gene, may influence the extent to which the brain is sexually differentiated. This study assessed *Cyp19* methylation levels to determine a general sex difference for the epigenetic marker and investigate the influence of the genes productivity on sex typical cognitive phenotypes. I hypothesized that males will have lower methylation levels, allowing for greater aromatase expression to masculinize and defeminize the brain. Thus, I also hypothesized that this difference would be reflected in the psychological measures, whereby lower methylation levels would be associated with more male-typical results. Aggression, mental rotation ability, and autism traits were assessed in 41 female and 36 male undergraduate psychology students, who also provided saliva samples for genomic processing. A sex difference in overall methylation levels was found, but this did not extend to the psychological measures.

Preface

This thesis is an original work by Nathan Thomas Bartlett. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Personality and genetic polymorphism in humans", No. 00015728, 10/2/2013. Some of the research conducted for this thesis forms part of a research collaboration, including Professors B. J. Crespi and G. Prefontaine at Simon Fraser University, with Professor P. L. Hurd being the lead collaborator at the University of Alberta. Genomic data was processed by S. Read, S. Massah, and G. Prefontaine.

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Introduction

Males and females differ on numerous psychological measures. For example, men tend to be more aggressive than women. This is particularly true for direct aggression, such as physical confrontations (Buss & Perry, 1992). Men are also more likely to exhibit traits associated with autism, a disorder with a male incidence rate over four times greater compared to women (Christensen et al., 2012). Autistic-like characteristics include a preference for objects over people, an attraction to systems, and reduced social perception ability (Baron-Cohen, 2002). While exceptionally prominent in autistic populations, these traits also show variation in non-clinical samples with a distinct sex difference of attenuation in females. Related to these traits are visuo-spatial skills, such as mental rotation ability. Like aggression and autism, this is another case where men tend to score higher than women (Maeda & Yoon, 2013). While these sex differences are found on average, there is a great deal of overlap between sexes. The stereotypes may have some support in reality, but referring to these traits as sexually dichotomous would be erroneous; the within-sex variation for them is greater than it is between. There is more difference, on average, between any two men or any two women than there are between the average man and the average woman.

The relative size of sex differences compared to within-sex variation can be measured using Cohen's d (1). This measure calculates the effect size for a between-group difference and is calculated as the ratio of the difference of the two groups' means to their pooled standard deviation:

$$d = (M_1 - M_2) / SD_{pooled} \tag{1}$$

A Cohen's d value lower than 1 for a measure indicates the pooled standard deviation of that measure is greater than the between-group difference of means. The individual variation would

then overshadow the between-group differences. This is the case for all the aforementioned traits. For example, despite the male population having a greater assessment score for aggression, a large percentage of women exceed the average male on the same measure. This is represented by the moderate effect sizes of the sex differences for each trait: aggression = 0.42; autism = 0.49; mental rotation = 0.56 (Archer, 2004; Ruzich et al., 2015; Voyer et al., 1995). What causes the between- and within-sex differences for these behaviours and are they caused by the same thing?

There are four types of explanations for the occurrence of any given behaviour: function, phylogeny, ontogeny, and mechanism (Tinbergen, 1963). I am particularly interested in exploring the mechanisms that leads to sex differences. One needs to understand the mechanistic processes (e.g., hormonal actions) that are allowing the behaviour to manifest. To understand why females and males behave differently for a trait, I will ask what is occurring biologically in one sex that is, on average, different in the other. Additionally, I will ask how this mechanism allows for such a large amount of individual variation regardless of sex.

Three forms of results demonstrate that a sex difference has a biological basis: the effect can be exhibited at young ages, it can be replicated in other animals, and it is susceptible to early hormone manipulations (Overman, Bachevalier, Schuhmann, & Ryan, 1996). If an effect meets all these criteria, ontogeny/socio-cultural influences are suggested to have a reduced weight on the expression of the trait. This is demonstrated in newborn female rodents treated with androgens, where aggressive behaviours and visuo-spatial ability are both increased, presenting comparably to untreated male rodents (Cohen-Bendahan, Van De Beek, & Berenbaum, 2005). Similarly, a greater attraction to objects than to people, a trait that is correlated with autism, can arguably be represented by toy choice preference (Baron-Cohen, 2002). Male children tend to

choose mechanical objects (e.g., toy trucks) while females are typically more attracted to plush toys (e.g., dolls) (Liss, 1981). Like aggression and visuo-spatial ability, this preference difference also meets Overman's criteria for the impact of biological factors (Berenbaum & Hines, 1992; Hassett, Siebert, & Wallen, 2008). Further support for a biological basis to a sexually differentiated behaviour can be garnered if the difference is consistent across cultures, as is the case for the above-mentioned traits (Archer, 2004; Peters, Lehmann, Takahira, Takeuchi, & Jordan, 2006; Wakabayashi, Baron-Cohen, Wheelwright, & Tojo, 2006).

The Sry gene on the Y chromosome initiates a cascade for male development, causing the undifferentiated gonads to develop as testes, which then begin releasing testosterone and anti-mullerian hormone. The latter primarily serves to masculinize the gonads while testosterone and its metabolites have more wide-ranging effects and acts on various tissue types, including gonadal tissue and the brain. The testes begin producing testosterone at 9 weeks of fetal age, with a surge of production occurring for the following 9 weeks (Finegan, Bartleman, & Wong, 1989). This is a critical period for neurodevelopment, as testosterone is used for sexual differentiation of the fetus during this timeframe and acts to masculinize and defeminize the male brain by protecting neurons or inducing apoptosis depending on the region (Finegan et al., 1989; McCarthy & Arnold, 2011).

During this critical period, equivalent to the middle trimester, testosterone production and absorption in the hypothalamus is at its peak, but the brain has not yet started expressing androgen receptors (Abramovich, Davidson, Longstaff, & Pearson, 1987). It is primarily estrogen, a metabolite of testosterone, causing this differentiation (McCarthy & Nugent, 2015). Consequently, the availability of aromatase, the enzyme responsible for converting testosterone to estrogen, may be a mediating factor for how much organizational influence these hormones

have on sexual differentiation of the brain. Further, differences in expression of aromatase at the individual level may also be part of the reason for such great individual variation on sex-typical traits. For example, males who have attenuated expression of the enzyme may then have less estrogen available in the brain for sexual differentiation, resulting in more female-typical cognitive development.

Expression of a gene can be affected by numerous processes, particularly in relation to epigenetic mechanisms. Methylation of DNA is a prominent example of this. Methyl groups have a high affinity for areas in DNA where a cytosine nucleotide is followed by a guanine (CpG sites). Methyl attaches to the fifth carbon of the cytosine ring, forming 5-methylcytosine, and subsequently attenuates the productivity of the gene. Methylation of a region is influenced by numerous factors, including genetic imprinting from the parents' epigenome, sex of the individual, stress, social interaction, and availability of CpG sites (Jones, Goodman, & Kobor, 2015). Therefore, gene expression can be variable across individuals depending on their epigenome. If aromatase availability is a significant component in sex differentiation of the human brain, between-sex and individual variation in methylation levels may be the mechanism that allows for such a diverse spread of sex-typical traits across the population. Importantly, the variation of methylation levels for a given site is low between cell types. Methylation for CpG sites in buccal cells obtained from saliva samples show a particularly strong resemblance to that found for the same sites in brain cells (Smith et al., 2015). Because of this, buccal cells can be used as a less invasive proxy for measuring methylation of brain cells.

Aromatase in sex differentiation mechanisms has proven to be a key enzyme across taxa (Balthazart & Ball, 2012). Aromatase expression has been found to be positively correlated with the development of ovaries, while suppression of the enzyme leads to testes development

(Piferrer et al., 1994). In red-eared slider turtles (*Trachemys scripta elegans*), a species with temperature-dependent sex determination, aromatase expression is found to be dramatically higher in females compared to males (Matsumoto, Buemio, Chu, Vafaee, & Crews, 2013). It was shown that this difference in production is due to higher temperatures causing demethylation of *Cyp19*, allowing for greater expression of the enzyme and, which then results in the development of ovaries. In the protandrous black porgy (*Acanthopagrus schlegeli*), researchers found that the male-to-female transformation could be prevented by feeding the fish aromatase inhibitors (Lee, Yueh, Du, Sun, & Chang, 2002).

The conversion of androgens to estrogens occurs in multiple steps. For each stage, aromatase is the only enzyme involved (Santen, Brodie, Simpson, Siiteri, & Brodie, 2009). Testosterone is first hydroxylated to form 19-hydroxyandrostenedione, removing the angular methyl group and allowing aromatization of the A ring of the steroid (Meyer, 1955). This metabolite is then converted to 19-oxoandrostenedione and, subsequently, estrogen (Morato, Hayano, Ralph, & Axelrod, 1961). The exact processes underlying the metabolic pathway for 19-oxoandrostenedione conversion to estrogen remains less understood than the steps upstream in the assay (Santen et al., 2009).

Aromatase and Brain Sex Differentiation

Humans show sex-typical personality dimensions, but these dimensions are also extremely variable at the individual level. Traits like aggression, autism, and mental rotation have a sex difference on average, but not in a way that is dichotomous. The overlap found between sexes for these traits may be associated with early brain development. In particular, the critical stage of development, when the fetus first begins sexual differentiation and males express testosterone in the absence of testosterone receptors in the brain, may be a vital period for where

these differences (and variation in these differences) first arise. Because it is estrogen that masculinizes and defeminizes the brain, the availability of aromatase, the enzyme responsible for converting testosterone to estrogen in brain cells, is vital for masculinization and defeminization of the brain. The expression of aromatase can be mediated by epigenetic mechanisms, such as a methylation. Consequently, methylation levels for the brain promoter region of the aromatase gene could be accounting for some of these between- and within-sex differences.

Cyp19 Genomic Organization in Humans

Most mammals only show aromatase expression in the brain and gonads. In primates, however, the enzyme is found in a wider array of tissue types (Simpson et al., 1994). The versatile expression of aromatase is primarily due to the large number of promoter regions on the gene. Spanning over 123 kilobasepairs (kb) in humans, the remarkably complex *Cyp19* gene is located on chromosome 15q21.2, has a 30 kb coding region, and a 90 kb 5' flanking region (Bulun et al., 2003). This 5' area contains promoter sites that correspond to expression in multiple types of tissue, including brain, gonad, bone, skin, fetal, placental, endothelial, and adipose tissue (promoters I.f, PII, I.6, I.4, I.5, I.1, I.7, and I.3, respectively) (Bulun et al., 2003). Some of these sites have garnered a significant amount of attention, particularly in regards to breast cancer tissue (Knower, To, Simpson, & Clyne, 2010).

Little consideration has been given to the brain promoter region and its potential effects on neurodevelopment in humans. Located 33kb upstream from the first coding region and exon II, I.f is 875 bp in length and has a total of 14 CpG sites (GenBank accession no. D29757.1). Three of these sites are located downstream of the two TATA boxes and the exon boundary. For the purpose of isolating the region, the 5' end was extended by 300 bp and by 20 bp on the 3' end. The added 5' basepairs contain 2 additional CpG sites, one of which was included in my

analyses, providing a total of 15 sites available for methylation (added basepairs are italicized, analyzed CpG sites are underlined, TATA boxes are in bold, and the exon boundary is marked with an asterisk):

GAGTCACAGAGACCTTGAAGAATGGAGGAGGTAGAAAAAGGAGAAATGCAGGAAGGAGG
TGTGTTATGGAGTTATCCAGTGGAGGCTCGCATCCAGCTTTATTTTGCCTCCAAAGATCTT
GCATCCTACCTGTTAAGAAGCTTGGAATGCCACTATTCCATTAAATCTTAAAGGAACTTGAG
TCTTTCATTTAAAAATGTGTTCCTAAAATGTAAATGTCCCTATGTGGGACAGTATTTAGCCG
*ACAGTAAATTTGAGAGGAGGGTCTCAAGGGGCAGTGTCACCAGGAAAAGAGAGAAA*GGCT
CCTCTCCCCAAGTCAAACCTTACCTTACTTAACCGATTGTATTTCCTCCCTCAGAGGA
TGCCATATCTCAGTACAGGAGAGAAAATAGAAGGTAGAGAGACTTTTACCCAAGCA
CCCCCTGAACCCCAGGTGTACACAACTGAACCTGATGCAGTGACAATCACGTTCACA
CATAAAACATCTGGCTAAAGGCTAAGATCACTTCGGATTTCCGACATACATTTTCCT
AAGCAGTGCATTTTTCTTTAATTTTCCTTAGAAAAAGACTGTAAAGTAGCCCCACAA
TTCCCACATCTTCATACTCCACCCTGCATTCAAGTTTTCCTGGGACAGGTATCTATGT
GTGTGCATGAATCTATTTTTACGGCATATGTCTAGGACCCCCTACGAGGAGCCAAAG
TTTCAGAGAGCCCAGCAACTATGTAACTCCATGGAAGGGAGGCATGATATTACTCTC
TGTTCACAGGAGCGTACGCACAGATCTTTTCTCCTCCTCATGGTCAGTTTTCTATTTG
TGATTAGTAATTAGCTTCTCTTGGTACGCTACGATCTATTACAAAAGCCAAACATTC
AGGGGGCGAGCTGAAATGACAAAATTTGGC**TATA**ATTTATGTTGGCCCCTGACA**TA**
TATATTTTTTTAATGGTTTGGTCTCTAAGCAACTGATCTCTTAGCAACAA*GAAGCAC
CTTTATAAAAGATGGCACACGAAGAGTGATTGCCAGAAAAGCCACCTGGTTCTTAA
ACAGCCGCGCATCATTAGCAAAACTCACCATCTTCAAGAGTCCAAAAACTAGAAGT
GACCAGCAGACCCAGGTAACCTTGATATTTGCACATTTTCCTGGGAAAAAAAAAA
AAAAAAAAAAAAACTCAT*GCAACTATTGAAGAGAACAA*

Prenatal Testosterone Exposure as a Personality Dimension

In addition to psychological differences, humans also demonstrate a myriad of sex differences in morphology that can be informative of hormonal activity both across the lifespan and for specific periods. Many of these traits are obvious, such as primary and secondary sex characteristics, but others are more subtle. One such example is the proportional length of the index finger (2D) to the ring finger (4D), typically treated in research settings as a ratio of 2D:4D. This measure is, on average, smaller in males and is used as a proxy for prenatal androgen exposure (Lutchmaya, Baron-Cohen, Raggatt, Knickmeyer, & Manning, 2004; Manning, Scutt, Wilson, & Lewis-Jones, 1998). That is, the smaller the value, the greater levels

of testosterone to which the fetus was likely exposed. The sex difference can be observed by the second trimester and remains constant for the life of the individual (Hönekopp, Bartholdt, Beier, & Liebert, 2007). Further support for this proxy is shown by masculinized digit ratios in females with congenital adrenal hyperplasia (CAH) who, as a consequence of their disorder, were exposed to greater levels of testosterone in utero (Brown, Hines, Fane, & Breedlove, 2002). Females with CAH also demonstrate greater levels of aggression than their unaffected sisters, a difference that can be seen by early childhood (Pasterski et al., 2007).

Digit ratios are associated with sex-typical psychological traits. In males, it has been found that aggression negatively correlates with digit ratio, with trait aggression being higher for more masculinized digit ratios (Turanovic, Pratt, & Piquero, 2017). Similar relationships have been found for mental rotation ability and autism in males and females (Manning, Baron-Cohen, Wheelwright, & Sanders, 2001; Peters, Manning, & Reimers, 2007). These findings indicate the importance of testosterone for neurodevelopment and suggest that digit ratios can potentially be used as a proxy for prenatal testosterone exposure and the degree of brain sex differentiation.

Brain Development and Differentiation

One brain region that has received a relatively large amount of attention regarding sex differences is the hypothalamus, in part due to its role in reproductive behaviour (Swaab, 1995). There are numerous nuclei in the hypothalamus that show sex differences in mammals. In rodents, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is a prominent example. Males' SDN-POA is over three times larger than females' (Gorski, Gordon, Shryne, & Southam, 1978). The analogous region in humans, called the third interstitial nucleus of the anterior hypothalamus (INAH3), is similarly differentiated, with males' INAH3 being twice the size of females' (Swaab & Fliers, 1985). Lesions to the SDN-POA in male rodents alter their

sexual behaviour, causing a reduction in mounting and intromission attempts (DeBold & Clemens, 1978). In homosexual men, the INAH3 is smaller, reflective of the female-typical size, suggesting the nucleus plays a role in reproductive behaviour in humans (LeVay, 1991). The suprachiasmatic nucleus (SCN), a region associated with circadian rhythm and sexual behaviour, is also different between males and females, specifically in regards to shape. Males have a spherical SCN while females' is elongated (Swaab, 1995). Like the INAH3, the shape of the SCN is more female-typical in homosexual men, again suggesting a role in reproductive behaviour (D. F. Swaab & Hofman, 1990).

The beginning of brain sex differentiation is part of a critical period for neurodevelopment, lasting from gestational week (GW) 16 to GW18 (Abramovich & Rowe, 1973; Finegan et al., 1989). Testosterone production begins at GW8 and is expressed at peak levels by GW16 (Finegan et al., 1989). However, despite uptake of testosterone by the hypothalamus, androgen receptors have yet to be expressed in this brain region (Abramovich et al., 1987). Testosterone is absorbed by neurons and metabolized by aromatase (aromatization) to produce estrogen. At this stage of development, estrogen receptors are present and estrogen acts at different locations to either protect neurons and connections or to remove them (Morris, Jordan, & Breedlove, 2004). Thus, it is estrogen that is sexually differentiating the hypothalamus. This phenomenon has a rich history in research and has become known as the aromatization hypothesis.

The notion that aromatase may play a role in sexual development of the brain was conceived in the mid-twentieth century after researchers began to realize that sex-typical hormones were not quite as typical as initially perceived (Naftolin, 2012). That is, while parsimonious, the idea that androgens influence male behaviour and estrogens female behaviour

was incomplete. For example, it could not account for the studies showing that female rats treated with testosterone subsequently induced feminine sexual responses or that the same treatment in both female and male humans resulted in increased levels of urinary estrogens (Beach, 1942; Nathanson & Jones, 1939). This led to the understanding of testosterone as a prohormone, which can be metabolized by aromatase to form estrogens (Dorfman & Ungar, 1953). Validation of the aromatization hypothesis started with the prediction that androgens which could not be aromatized, such as dihydrotestosterone (DHT), chlorotestosterone acetate, or androsterone, would fail to reinstate typical sexual behaviour. This prediction was upheld in studies using castrated rats and ovariectomized rabbits treated with testosterone or DHT (McDonald et al., 1970; McDonald, Beyer, & Vidal, 1970). The overarching conclusion was that nonaromatizable androgen treatment in these animals failed where testosterone succeeded. The hypothesis was further supported when Naftolin and colleagues (Naftolin, Ryan, & Petro, 1971) found that conversion of androstenedione to estrone and estrogen was occurring in hypothalamic tissue of male fetuses.

Present Study

With this research, I aim to explore a potentially fundamental mechanism for sexual differentiation of the brain and its impact on sexually differentiated traits. Specifically, I analyzed methylation levels on the brain promoter region of the aromatase gene in healthy adults to infer its association with prenatal development and subsequent psychological phenotypes. Based on evidence provided by the aromatization hypothesis, I expect that a mediator on aromatase expression in the brain, such as methylation, would differ between sexes. Because males require the presence of estrogen in the brain for normal development, it is logical to postulate that the expression of aromatase in that region, which is essential for testosterone-to-

estrogen conversion, would be higher than females. Thus, the first hypothesis is that males would exhibit hypomethylation relative to females, which would indicate greater estrogen availability. The second hypothesis is that the extent of methylation for this region will account for some of the variation seen in sexually differentiated traits. That is, hypermethylated individuals will show more female-typical responses on my measures and those with lower methylation levels will be more male-typical.

Methods

Participants

Participants were introductory psychology students at the University of Alberta who were compensated with course credit for their time. The data for this study was collected as part of a larger project. Participation requirements included English as a first language due to the use of questionnaires and Caucasian and Asian ethnicity to minimize genetic variability. For inclusion in this particular study, I used the data from individuals who expressed extreme right handed digit ratios relative to the rest of the sample (full project sample size = 1126; 392 males and 734 females). More specifically, I included males and females who either had the highest (extremely feminized) or lowest (extremely masculinized) digit ratio scores relative to the rest of the subjects in the larger project. This provided me with a cohort of 36 males and 41 females that had presumably been exposed to either high or low levels of prenatal testosterone exposure, depending on their digit ratio score. Participants entered the testing room in groups of 20 for a one-hour testing period and were given a brief description of the study before providing written consent.

Procedures and Measures

Genomic data. Saliva samples were collected using the protocol outlined by Lum and Le Marchand (1998). Participants were provided with 50 ml centrifuge tubes containing 30 ml of Scope brand mouthwash, which they swished in their mouths for 60 seconds. The samples were immediately placed in an ice cooler until the end of the testing period, at which time they were stored at -40 °C.

Isolation of genomic DNA and bisulfite sequencing. Genomic DNA was isolated using the methodology from Hurd, Vallaincourt, & Dinsdale (2011) and Leach, Prefontaine, Hurd, &

Crespi (2014), which was based on Lum and Le Marchand (1998). After thawing, 10 ml aliquots from each sample were spun at 2700 rpm for 15 minutes. The supernatant was discarded and, after adding 10 ml of TE buffer, the samples were spun again. 700 µl of Lifton buffer was used to re-suspend the pellet, 35 µl of proteinase K was added, and the solution was subsequently incubated at 58°C for 2 h. As outlined by Lum and Le Marchand (1998), phenol-chloroform extractions were performed and the samples air dried in a fume hood before being re-suspended in water and stored at -20°C.

Thawed DNA was treated with bisulfite using the Imprint DNA Modification Kit (Sigma, Cat. # MOD50-1KT). This process converts all unmethylated cytosine bases into uracil (Supplementary Information Part 1). PCR and pyrosequencing procedures followed as Massah, et al. (Massah et al., 2014). The PCR primers were designed using the PyroMArk Assay Design software 2.0 from Qiagen. Four primary and nine secondary PCR reactions were performed covering the total of 15 CpG sites. The primer designs and relative positions of primary primers to CpG sites are depicted in Supplementary Information Part 2. Following binding to streptavidin Sepharose beads (GE Healthcare Cat. # 17-5113-01), 10 ul of each sample was used in each sequencing reaction. Samples were sequenced using PyroMark Q24 pyrosequencer. The present methylation at cytosines is measured by comparing the intensity of signal for cytosines versus thymines.

Following saliva collection, questionnaires and tasks (including those for the aforementioned larger study) were administered to each participant.

Mental rotation. Mental rotation ability (MRT) was assessed using the mental rotation task, a test developed by Vandenberg and Kuse (1978). The task provides participants with an image of an object as a reference point and gives four possible variants of how the object may

look if it were rotated in space, only two of which are correct. Each correct response receives a score of 1 and incorrect responses receive a 0. Total scores for this measure are obtained by summing the correct responses.

Aggression. Direct aggression was measured using Buss and Perry's (1992) aggression questionnaire, a tool that has been found to have high test-retest validity and consistent sex differences (Archer, 2004). The questionnaire contains 29 questions that assess four subtypes of aggression, verbal, physical, hostility, and anger, as well as a total aggression score. Aggression scores are obtained by summing the 1-5 Likert-based responses, where greater scores indicate higher levels of aggression. Total direct aggression (DA) scores are the sum of each sub-component. An example item from this questionnaire is: "I have become so mad that I have broken things".

Autism. The Autism-Spectrum Quotient was used to measure traits associated with autism (Baron-Cohen, Wheelwright, Skinner, Martin, & Clubley, 2001). The questionnaire, consisting of 50 items, was developed as a self-report tool for measuring the degree to which an individual falls on the autism spectrum, if at all, and has been validated for both clinical and non-clinical populations (Ruzich et al., 2015). Participants respond using a 1-4 Likert scale, with 1 referring to "definitely agree", 2 to "slightly agree", 3 to "slightly disagree", and 4 to "definitely disagree" for each statement. Half of the questions are specific to the presence of autistic traits (e.g., "I prefer to do things the same way over and over again"), while the other half are related to the absence of autistic traits ("In a social group, I can easily keep track of several different people's conversations"). Question types are randomly dispersed in the questionnaire. Items related to the absence of autistic traits are reverse coded during analysis and all responses are coded in a binary fashion: a response of either "strongly agree" or "slightly agree" will have a

score of 1 for that question and a response of “slightly disagree” or “strongly disagree” will score a 0. The questionnaire produces five subcomponents (10 questions each): social skills, communication, attention to detail, attention switching, and imagination. A total autism quotient score (AQ) is calculated for each individual by summing the scores of all subcomponents.

Digit ratio. While completing the questionnaires, individuals were called by subject number to the front of the room to have their hands photographed. A black felt pen was used to mark the basal crease for each finger. Participants then placed both hands on a clear table, under which a camera was used to photograph their hands. Lengths of the index and ring fingers were later measured using GNU Image Manipulation Program (GIMP) to create digit ratio variables (R2D4D for the right hand, L2D4D for the left).

Several participants did not fully complete the Aggression Questionnaire or the Autism-Spectrum Quotient, resulting in a slightly diminished sample relative to the rest of the variables (34 males and 38 females for DA, 36 and 39 for AQ). Upon completing all requested tasks, participants were subsequently debriefed. Saliva samples were shipped to Simon Fraser University in Burnaby, British Columbia for genomic processing.

Results

Descriptives

I compared mean scores between men and women on each measure (Table 1). Both DA ($t = 4.10$, $p < 0.001$) and AQ ($t = 2.39$, $p = 0.02$) scores were shown to be significantly higher for males. I also analyzed all subcomponents of these two measures, the results of which did not differ from their total scores. While non-significant, MRT and L2D4D differed between sexes in the expected direction, with males having higher MRT scores and smaller L2D4D values. No difference was found between R2D4D, but this is likely due to my use of this variable in the sampling criteria. By using the highest and lowest R2D4D values as selection criteria, we are using outliers for the measure. Consequently, the variance of these extreme scores is increased, reducing the likelihood of a sex difference. Males and females did not differ significantly in age.

Methylation

Methylation of the *Cyp19* brain promote region was generally high across all 15 sites (Figure 1). Pairwise comparisons using t-tests with pooled standard deviation were used to investigate any differences among methylation levels of the sites. While there were various significant differences between many of the sites, CpG.14 was unique in that it had significantly higher methylation levels than all other sites. Conversely, CpG.3 was significantly hypomethylated compared to all other sites. When split by sex, males consistently showed hypomethylation relative to females at most sites, though not to the extent of statistical significance (Figure 2).

The mean methylation levels across all sites was calculated, referred to as total methylation, with a value of 88.30% ($SD = 3.05$). When split by sex, males were 87.53% ($SD = 3.06$) methylated and females 88.97% ($SD = 2.92$). A Welch's two sample t-test showed that

males had significantly lower levels of total methylation (t = -2.11, p = 0.038, d = 0.45; Figure 3). Cohen's d was obtained for each site (figure 4), showing relatively consistent effects. While CpG.3 methylation was low and CpG.14 high, neither of these sites showed a sex difference effect markedly dissimilar from any other site. I conducted a principal component analysis using the 15 CpG sites to investigate potential patterns that may be underlying the spread of methylation levels, but none of the components appeared to be accounting for a disproportionate amount of variance (Table 2). There was no association between age and overall methylation levels for either sex.

Methylation and Sexually Differentiated Traits

I tested for effects of total methylation on sexually differentiated traits using Analyses of Covariance (ANCOVA) including sex as a cofactor. I did not find any significant main effects for total methylation on DA (F(1,68) = 0.11, p = 0.74), MRT (F(1, 73) = 0.03, p = 0.86), AQ (F(1, 71) = 0.04, p = 0.85), R2D4D (F(1, 73) = 0.39, p = 0.54), or L2D4D (F(1, 73) = 0.15, p = 0.70). Similarly, I found no interaction effect between total methylation and sex on these measures (DA (F(1, 68) = 0.09, p = 0.77); MRT (F(1,73) = 0.19, p = 0.67); AQ (F(1, 71) = 0.06, p = 0.80); R2D4D (F(1, 73) = 0.31, p = 0.58); L2D4D (F(1, 73) = 0.01, p = 0.94); Figures 5, 6, 7, 8, 9, respectively).

I repeated the same analyses for each individual CpG site for all sexually differentiated traits. This provided 15 p-values for CpG site main effects and 15 for sex by site interactions for the five outcome phenotypes. I then used a one-sample Kolmogorov-Smirnov to test whether the distribution of p-values deviated from the uniform distribution predicted by the null hypothesis. None of the traits showed a significant skew towards small p-values for methylation main effects or for sex interactions (Table 3). While 9 of the 150 p-values showed significant effects, this was

approximately the rate under the null. Main effects on DA (D = 0.22, p = 0.80), MRT (D = 0.24, p = 0.33), AQ (D = 0.25, p = 0.28), R2D4D (D = 0.22, p = 0.43), and L2D4D (D = 0.16, p = 0.80) were all confirmed to be uniform. This was also the case for site by sex interactions on DA (D = 0.18, p = 0.64), MRT (D = 0.16, p = 0.76), AQ (D = 0.27, p = 0.19), R2D4D (D = 0.17, p = 0.74), and L2D4D (D = 0.21, p = 0.50).

Discussion

With this research, I sought to explore a potentially fundamental mechanism for sexual differentiation of the brain and its impact on sex-typical psychological traits. Specifically, I analyzed methylation levels on the brain promoter region of the aromatase gene in healthy adults to infer its association with prenatal development. Based on evidence provided by the aromatization hypothesis, I expected that a mediator on aromatase expression in the brain, such as methylation, would differ between sexes. Because males require the presence of estrogen in the brain for normal development, it is logical to postulate that the expression of aromatase in that region, which is essential for testosterone-to-estrogen conversion, would be higher. Furthermore, *Cyp19* contains an androgen-response element which allows testosterone to up-regulate aromatase expression (Abdelgadir et al., 1994; Honda, Harada, & Takagi, 1994). So, the first hypothesis was that males would exhibit hypomethylation relative to females, which would indicate greater estrogen availability. The second hypothesis was that the extent of methylation for this region would account for some of the variation seen in sexually differentiated traits. That is, hypermethylated individuals would show more female-typical responses on my measures and those with lower methylation levels would be more male-typical.

I discovered a sex difference in methylation levels on the brain promoter region of *Cyp19*, with men being hypomethylated relative to women. This is consistent with *Cyp19* being an important mechanism in sexual differentiation of the brain. My results suggest that hypomethylation of this region for males would allow for greater expression of aromatase enzyme in the brain and, consequently, more testosterone-to-estrogen synthesis. Placed in the framework of the aromatization hypothesis, this implies that males have more access to estrogen during prenatal development.

The fact that only the total methylation level of the region demonstrated a sex difference indicates that the individual CpG sites within this region are acting in concert. While methylation levels moderately varied across the 15 sites, the sex difference also varied in a proportional manner (both males and females would show similar increases and decreases across sites). If methylation of this region is impacting sexual differentiation of the brain, I expect that it would be due to the overall methylation rather than any individual CpG site.

I also found that methylation levels were generally high across all CpG sites in the region. Nugent et al. (2015) performed whole-genome bisulfite sequencing on POA tissue in rat pups and tested for sex differences of methylation levels at individual CpG sites. They found that across the entire genome, ~84% of the sites showing a sex difference were in intergenic regions, whereas only ~2% were in promoter regions. Based on these values, a sex difference in a single promoter region should be relatively rare. This significant sex effect I found in the brain promoter region further supports the idea that it plays a role in sexual differentiation of the brain.

I did not find any relationship between overall methylation levels and sexually differentiated traits. This was also the case for sex by CpG site interactions for the phenotypes. While some of my analyses uncovered significant effects for various CpG sites, these effects manifested at a rate approximately expected by chance (6%) and no specific site was particularly laden with effects.

Aggression is the most closely linked variable to reproductive behaviour in this study that has also been assessed in past research in the context of the aromatization hypothesis. In general, across many species, manipulation of steroid expression and action (such as castration or use of aromatase inhibitors) in males attenuates aggression in addition to normal reproductive behaviours (Ubuka & Tsutsui, 2014). There are several possible explanations for why I did not

find an effect of methylation on aggression. It may simply be that methylation does not impact aggression (which could also be the case for MRT and autism traits). However, the nature of how I assessed the trait may be a significant factor. From an evolutionary perspective, a large role for aggression is to assert dominance and ensure the capability of reproducing (Buss & Shackelford, 1997). In this study, I measured aggression with a questionnaire. For testing this trait in rats, the subjects do not have the means to ruminate over their projected aggressive tendencies. Further, this highlights the potential for response bias. While at times useful, aggression, especially physical aggression, is a behaviour that receives a great deal of scrutiny in human culture with possible consequences extending beyond retaliation by the defender (e.g., incarceration or ostracism). The participants may have been hesitant, consciously and/or subconsciously, about providing the extreme scores that they may actually represent. These possibilities could be investigated using more ecological measures of aggression.

One of the primary benefits to this study is the identification of a biomarker: the presence of a sex difference in methylation of the *Cyp19* brain promoter. This offers numerous avenues for future research regarding the implications of the aromatization hypothesis and estrogen as a brain masculinizing and defeminizing hormone for males. It is also important to explore the properties of this sex difference further. Due to the malleable nature of methylation across the lifespan, it would be prudent to assess methylation levels in a longitudinal study by obtaining samples collected during early life-stages, beginning at the first testosterone surge. I did not find a relationship with age and methylation levels in my cohort, suggesting that levels likely stabilize by adulthood. However, determining if this is true and exactly when stabilization occurs is beyond the scope of this study.

A possible explanation for the lack of influence of methylation on sexually differentiated traits is that the types of phenotypes I examined may be unaffected by this mechanism. Since its inception, the majority of research surrounding the aromatization hypothesis has focused on reproductive behaviors in various animals (often rodents). A general paradigm would be to perform gonadectomies on experimental subjects, treating some with steroids, and comparing behaviours in adulthood. For rats and mice, this would typically entail counting the number of times the animal engaged in lordosis, attempted to mount a conspecific, and, for males specifically, intromitted and ejaculated (Roselli, Liu, & Hurn, 2009). Other behaviours that have been assessed, while not directly sexual, are still linked to reproduction. For example, castration can impact song production in songbirds, but singing is a usually vital component for their reproductive success (Schlinger & Balthazart, 2012). For the aromatization hypothesis, it may be that estrogen is not masculinizing and defeminizing the male brain in general, but more specifically, brain regions regulating reproductive behaviors.

Measuring sexual behaviours in humans could be a fruitful direction for this concept, particularly incorporating non-heterosexual subjects. Gay men often have a preference regarding the extent to which they prefer being the penetrator (tops) or receiver of penetration (bottoms) from their partner during anal sex (Rosenberger et al., 2011). This could be a relatively straightforward model for relating rodent behaviours such as lordosis and intromission to human sexual behaviour. If the mechanism is primarily tied to reproductive behaviours, the aromatization hypothesis suggests that gay male tops would have experienced greater masculinizing and defeminizing effects in the brain via estrogen activity than gay male bottoms. These effects might then be reflected in methylation levels of the brain promoter region of *Cyp19*, with tops being hypomethylated relative to bottoms. There are at least two additional

benefits to using gay men for exploring this hypothesis. First, many gay men do not actually practice anal sex (Rosenberger et al., 2011). This group could add valuable data to this construct as their sexual practices are, practically speaking, further removed from reproductive behaviour than actual penetrative sex. Second, for men that do engage in anal sex, a large portion identify as “versatile”, whereby they enjoy both topping and bottoming. This population’s preference could be reflected by a proportional trend in methylation levels based on where each individual falls in the top-bottom spectrum.

Conclusion

With this study, I sought to explore a potential mechanism for brain sex differentiation in humans. Specifically, I examined how methylation of the brain promoter of the aromatase producing gene differs between sexes and if methylation differences of this region impact the expression of sex-typical traits. My first hypothesis, that males would be hypomethylated compared to females, was confirmed. This finding suggests that males, on average, express more aromatase in the brain. My second hypothesis, that lower methylation levels would be associated with more masculinized responses on sex-typical measures, was not found in my study. The lack of effect on sex-typical measures may be a consequence of the variables under study. The sex difference for methylation levels implies a functional difference in neurodevelopment between sexes and would benefit from further scrutiny with a larger sample size and a focus on reproductive and/or sexual behaviour.

Table 1. *Descriptive Data for Sex-typical Measures and Sex Differences*.

	N		Mean		SD		Range		*T*	*p*	Cohen's *d*
	M	F	M	F	M	F	M	F			
Age	36	41	19.86	19.44	2.25	2.19	17-27	18-26	0.83	0.41	0.19
DA	34	38	72.65	59.29	14.58	12.90	51-111	37-91	4.10	<0.001	0.48
MRT	36	41	12.64	10.71	5.26	2.06	0-23	0-22	1.64	0.11	0.37
AQ	36	39	17.47	14.74	5.46	4.29	8-33	7-25	2.39	0.02	0.56
R2D4D	36	41	0.99	0.99	0.06	0.06	0.90-1.07	0.90-1.09	-0.22	0.83	0.05
L2D4D	36	41	0.99	1.00	0.04	0.06	0.92-1.07	0.91-1.15	-1.26	0.21	0.28

Note. Comparisons made using a Welch's two-sample t-test. DA = direct aggression; MRT = mental rotation; AQ = autism quotient; R2D4D = right digit ratio; L2D4D = left digit ratio; N = sample size; SD = standard deviation; M = Males; F = Females.

Table 2. *Principal Components One, Two, and Three Loadings Across CpG Site Methylation Levels*

CpG Site	**PC1 (19.2%)**	**PC2 (15.1%)**	**PC3 (11.5%)**
CpG.1	-0.18	0.33	0.34
CpG.2	-0.02	-0.32	0.05
CpG.3	-0.22	-0.20	0.32
CpG.4	-0.27	0.38	0.16
CpG.5	0.18	0.28	0.23
CpG.6	-0.04	-0.18	-0.42
CpG.7	-0.35	0.40	-0.05
CpG.8	-0.45	0.02	-0.23
CpG.9	-0.45	-0.09	-0.17
CpG.10	-0.39	-0.05	-0.01
CpG.11	-0.21	-0.31	0.19
CpG.12	-0.13	-0.31	-0.09
CpG.13	-0.03	-0.17	0.52
CpG.14	-0.16	-0.31	0.34
CpG.15	-0.21	0.05	0.06

Note. PC = Principal Component.

Table 3. *P-values from Analyses of Covariance of Sex-typical Traits Main Effects and Sex Interactions for CpG Site Methylation*

CpG Site	DA	MRT	AQ	R2D4D	L2D4D
Main Effects					
CpG.1	0.95	0.63	0.97	0.10	0.05
CpG.2	0.42	0.31	0.49	0.49	0.64
CpG.3	0.55	0.31	0.22	0.33	0.88
CpG.4	0.72	0.56	0.78	0.42	0.46
CpG.5	0.17	0.12	0.44	0.01	0.11
CpG.6	0.68	0.37	0.72	0.79	0.38
CpG.7	0.78	0.66	0.71	0.22	0.26
CpG.8	0.43	0.70	0.70	0.57	0.79
CpG.9	0.61	0.62	0.43	0.47	0.10
CpG.10	0.26	0.13	0.94	0.88	0.58
CpG.11	0.71	0.95	0.38	0.73	0.31
CpG.12	0.10	0.23	0.92	0.58	0.21
CpG.13	0.48	0.50	0.38	0.30	0.65
CpG.14	0.89	0.19	0.66	0.18	0.20
CpG.15	0.69	0.04	0.28	0.55	0.77
Sex Interaction Effects					
CpG.1	0.86	0.78	0.51	0.83	0.46
CpG.2	0.67	0.56	0.09	0.51	0.10
CpG.3	0.89	0.12	0.02	0.68	0.59

CpG Site	DA	MRT	AQ	R2D4D	L2D4D
CpG.4	0.52	0.63	0.78	0.79	0.80
CpG.5	0.52	0.54	0.23	0.79	0.89
CpG.6	0.65	0.99	0.17	0.34	0.45
CpG.7	0.77	0.84	0.69	0.22	0.21
CpG.8	0.03	0.53	0.79	0.31	0.28
CpG.9	0.20	0.03	0.84	0.04	0.06
CpG.10	0.52	0.50	0.75	0.76	0.08
CpG.11	0.60	0.02	0.77	0.46	0.53
CpG.12	0.87	0.06	0.60	0.69	0.50
CpG.13	0.24	0.27	0.64	0.03	0.01
CpG.14	0.30	0.90	0.77	0.23	0.80
CpG.15	0.31	0.83	0.98	0.12	0.22

Note. DA= direct aggression; MRT = mental rotation; AQ = autism quotient scores; R2D4D = right hand digit ratio; L2D4D = left hand digit ratio.

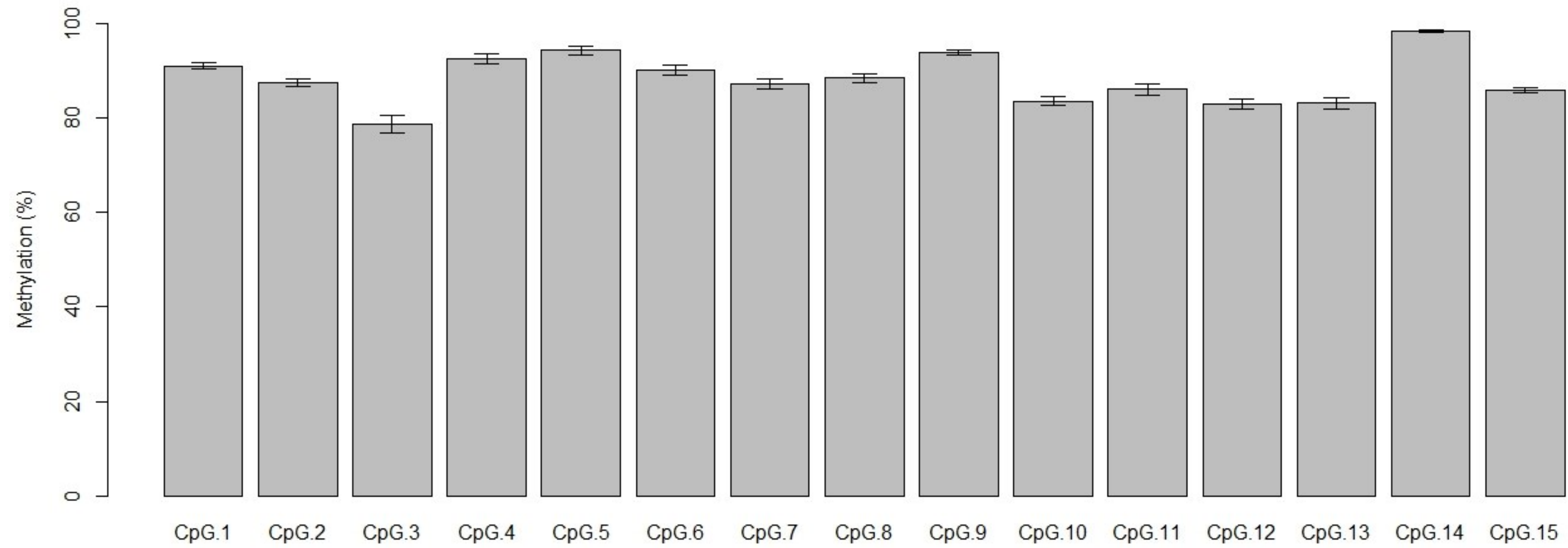

Figure 1. Methylation Levels for Each CpG Site

Note. Error bars represent standard error of the mean.

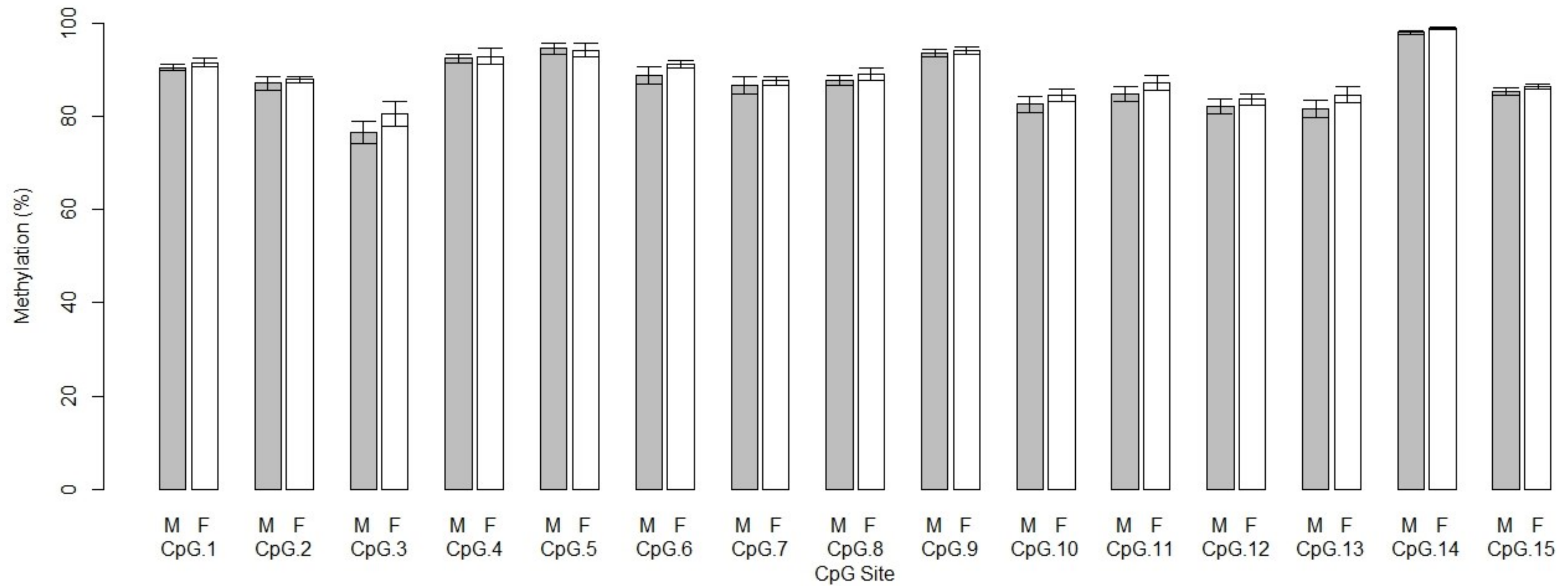

Figure 2. Methylation Levels for Each CpG Site Split by Sex

Note. M= Males; F = Females; error bars represent standard error of the mean.

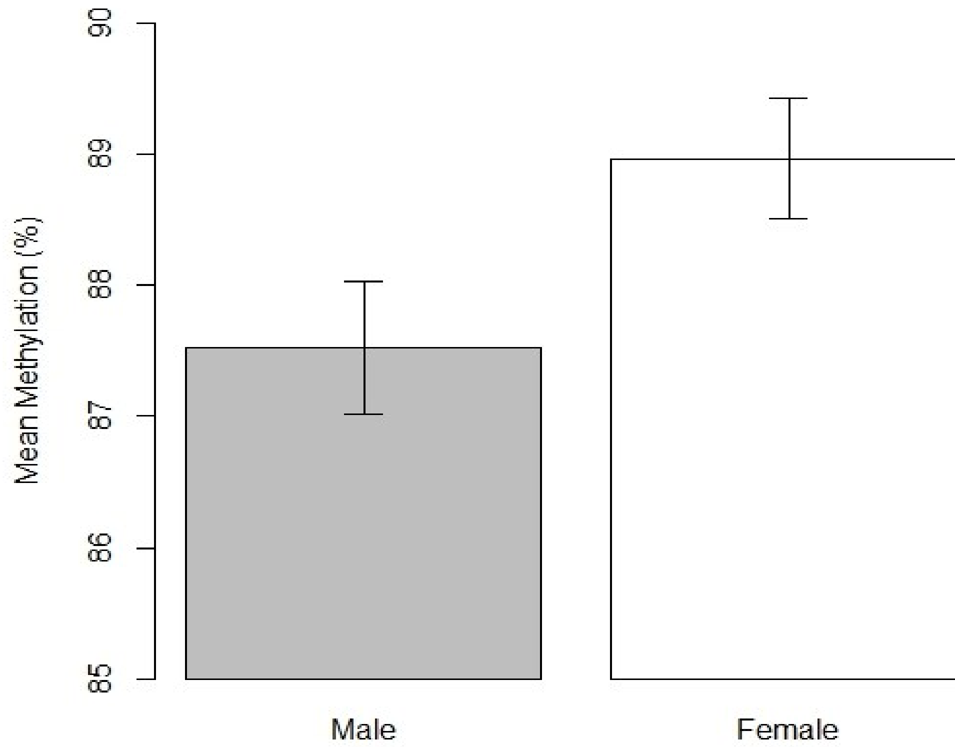

Figure 3. Total Methylation Levels Split by Sex (t = -2.11, p = 0.038, d = 0.45).

Note. Error bars represent standard error of the mean.

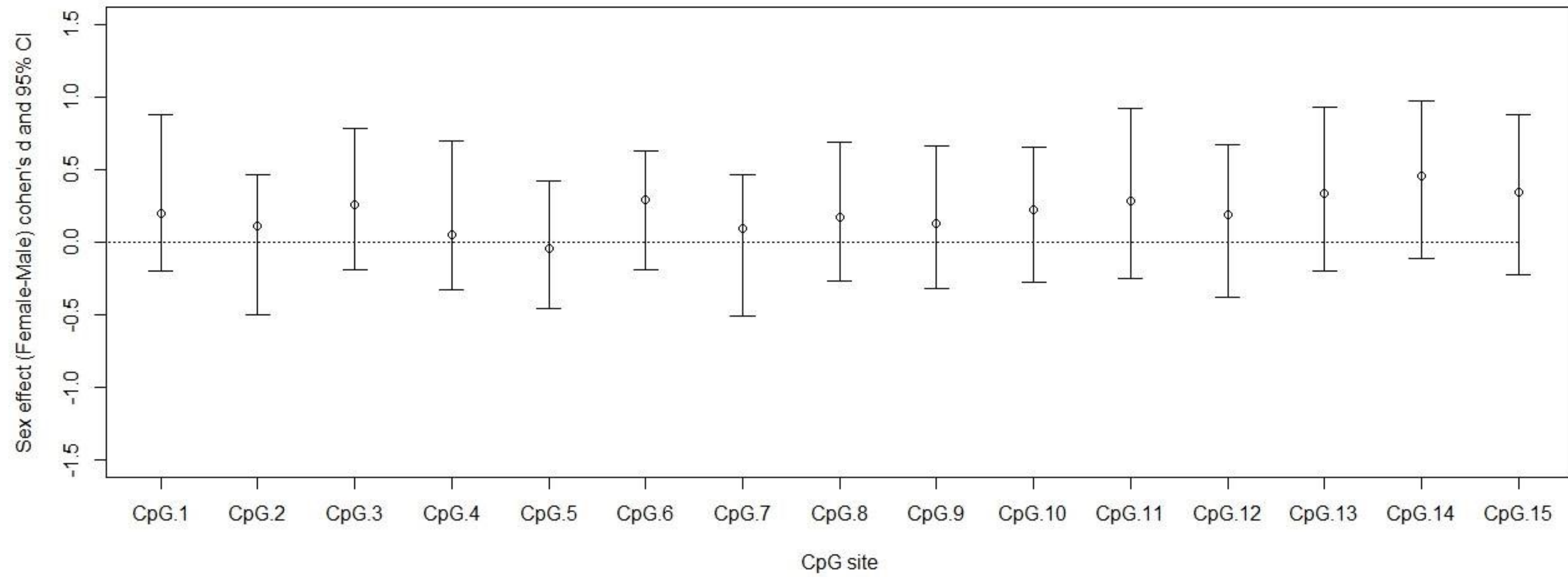

Figure 4. Cohen’s d for Sex Differences at Each CpG Site

Note. Error bars represent 95% confidence intervals.

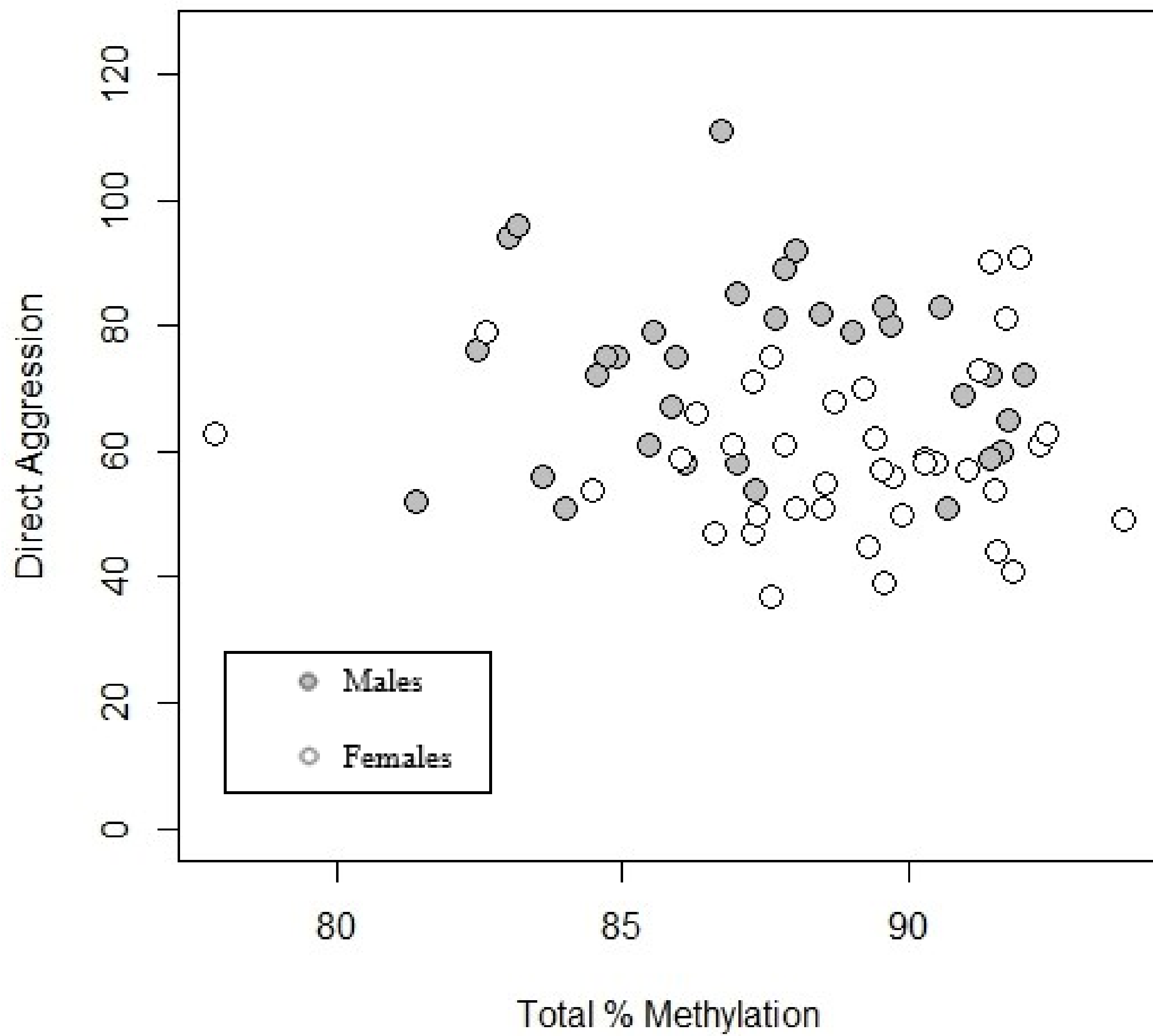

Figure 5. Scatterplot Comparing Methylation Levels Against Direct Aggression.

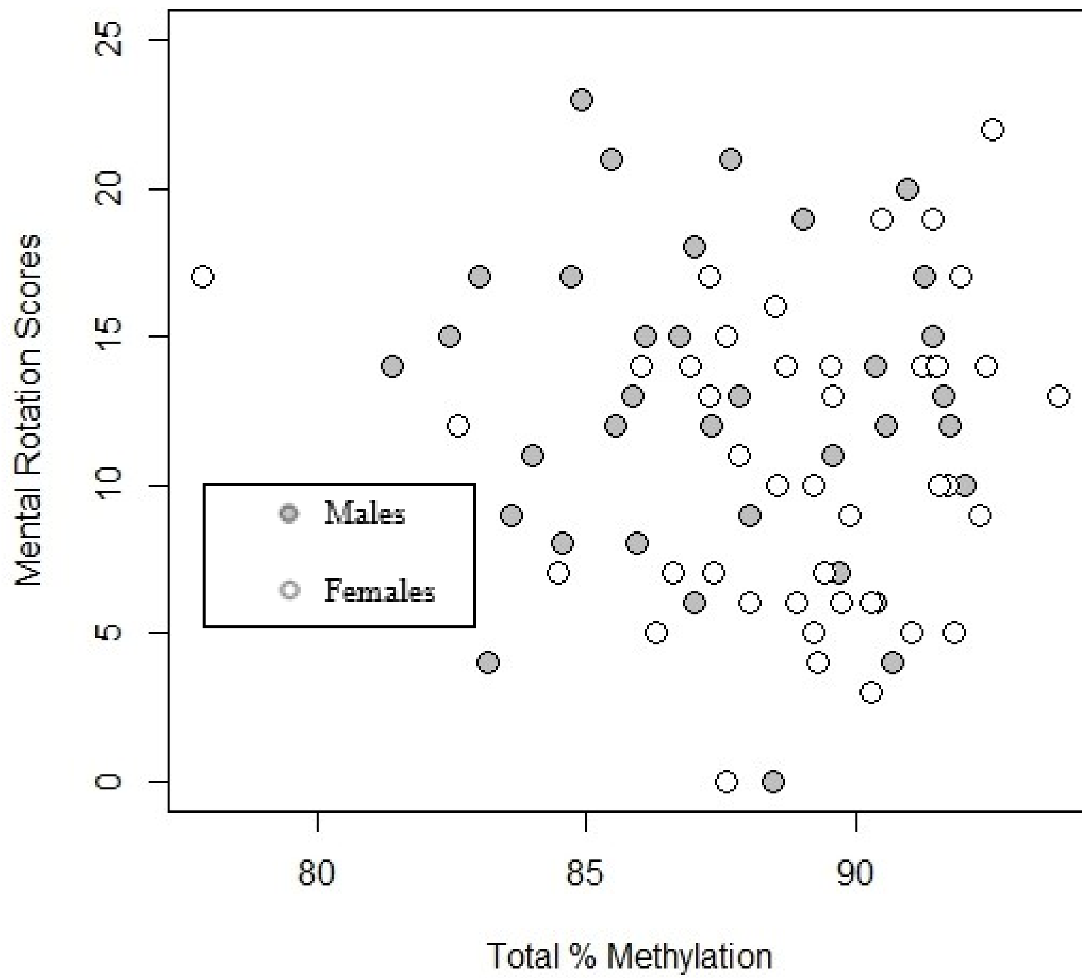

Figure 6. Scatterplot Comparing Methylation Levels Against Mental Rotation Scores

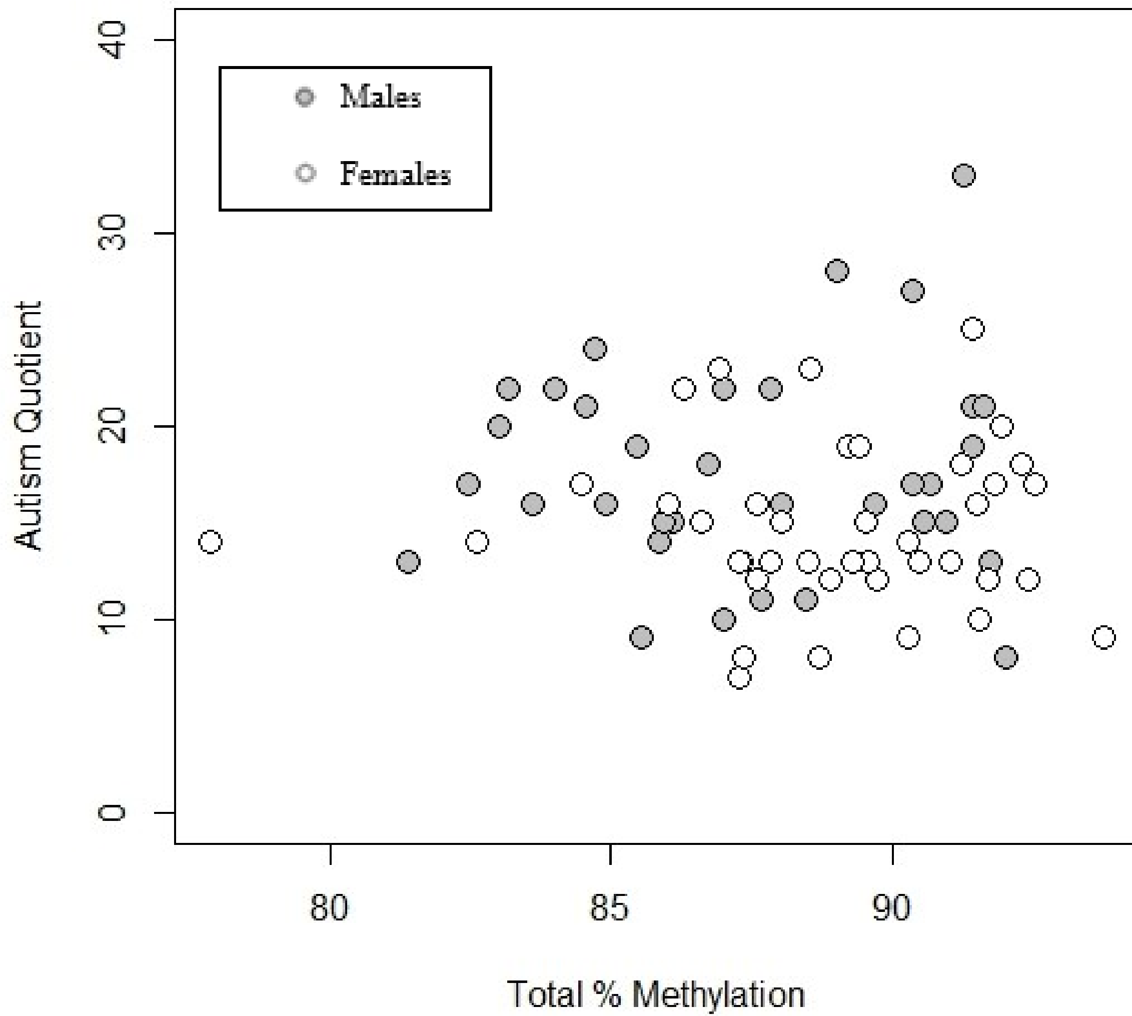

Figure 7. Scatterplot Comparing Methylation Levels Against Autism Quotient Scores

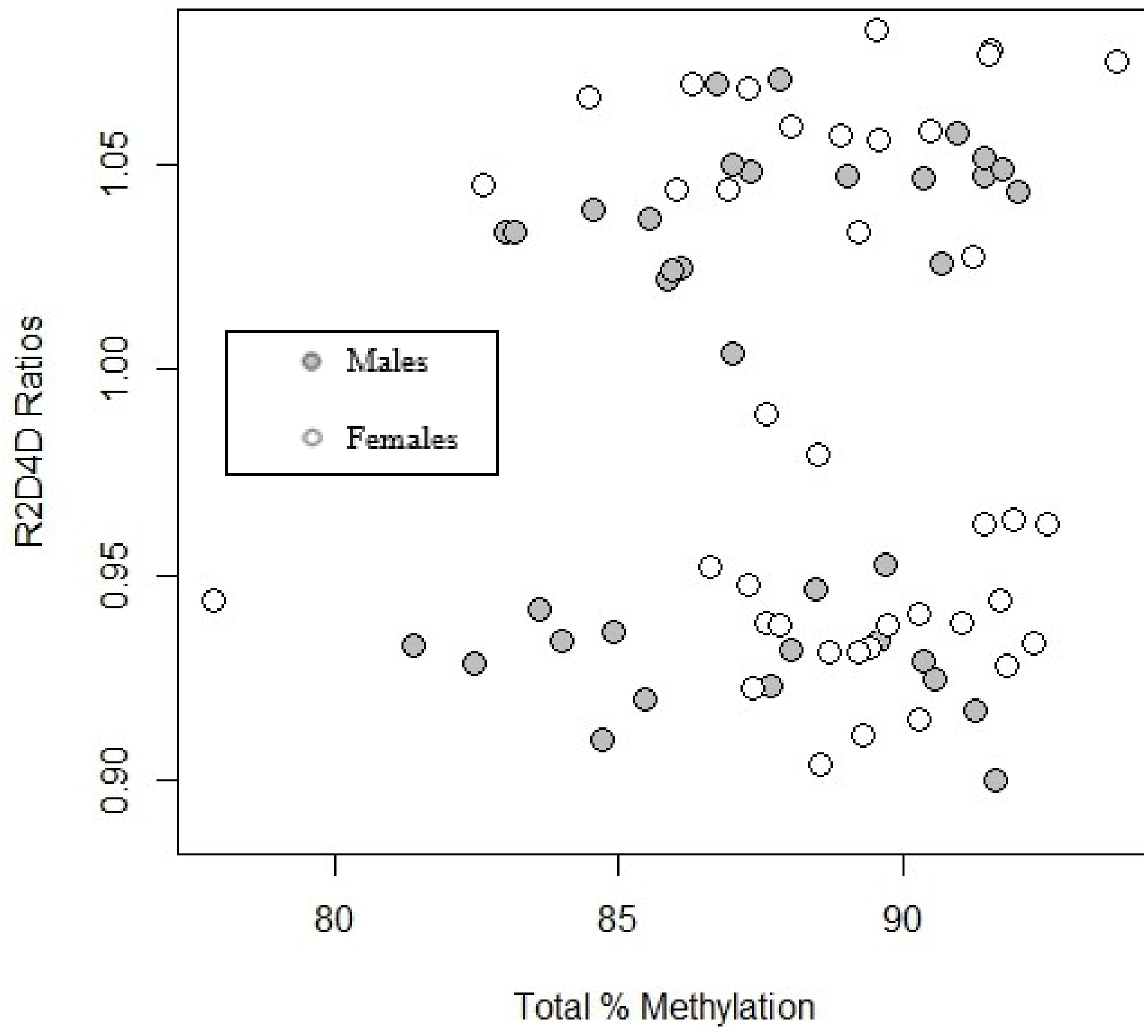

Figure 8. Scatterplot Comparing Methylation Levels Against Right Digit Ratio Scores

Note. R2D4D = Right digit ratio.

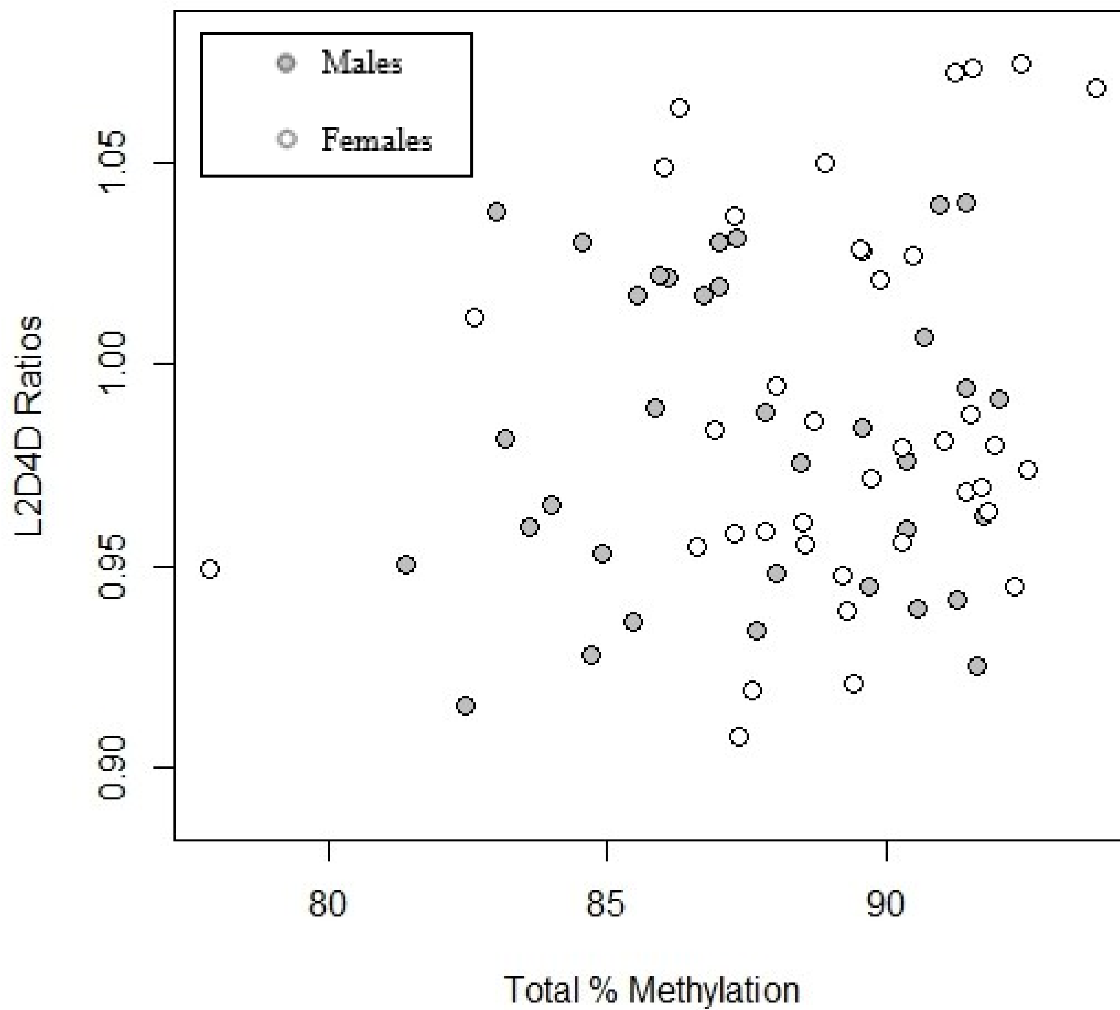

Figure 9. Scatterplot Comparing Methylation Levels Against Left Digit Ratio Scores

Note. L2D4D = Left digit ratio.

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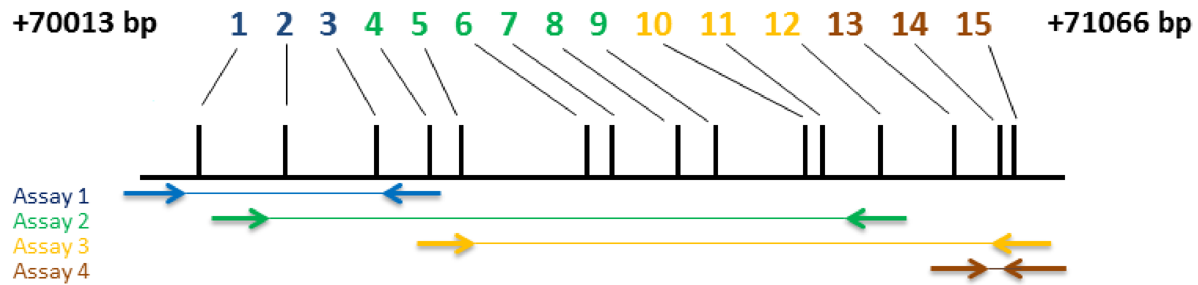
Appendix A

Bisulfite Sequencing

DNA Modification Solution was mixed with DNA Modification Powder, incubated at 65°C for 2 minutes, and vortexed with Balance Solution. 110 µl of this mixture was added to 10 µl of DNA, vortexed, and incubated at 99°C for 6 minutes. For post-modification clean up, the modified DNA solution was added to 300 µl of Capture Solution in a Spin Column placed in a Capless Collection Tube and spun at 12, 000 x g for 20 seconds. The flow-through was discarded and the product was then spun with Ethanol-diluted Cleaning Solution. Next, Balance/Ethanol Wash Solution was added to the bottom of the spin column, incubated at room temperature for 8 minutes, and spun for 20 seconds. The flow-through was discarded before adding 200 µl of 90% ethanol to the spin column and spinning the solution again for 20 seconds, after which the flow-through was again discarded. Another 200 µl of 90% ethanol was added to the spin column and was spun for 40 seconds, after which the Capless Collection Tube was removed and the Spin Columns were placed into 1.5 ml Collection Tubes. Last, 12 µl of Elution solution was added to the bottom of the spin column, incubated for 1 minute, and spun for 20 seconds. The eluted solution, which contains the modified DNA, was then stored at -20°C until needed for PCR and pyrosequencing.

Appendix B

Primer Design



Assay 1

Primary primer (CpG1-CpG3)

Nested forward (Tm=57.98): TTTTATTTGTTAAGAAGTTTGGAATGT

Nested reverse (Tm=60.03): CCGAAATAATCTTAACCTTTAACCAA

Secondary primers

CpG1 forward (Tm=58.90): ATGTAAATGTTTTTATGTGGGATAGTAT

CpG1 reverse (biotinylated; Tm=58.20): TTTCTCTCTTTTCCTAATAACACTACC

CpG1 sequence (Tm=44.10): GTTTTTATGTGGGATAGTATTTA

CpG1 analyze: GTYGATAGTAAATTTGAGAGGAGGGTTT

CpG2 forward (biotinylated; Tm=61.60): GATAGTAAATTTGAGAGGAGGGTTTTAAGG

CpG2 reverse (Tm=59.50): CTCTACCTTCTATTTTCTCTCCTATAC

CpG2 sequence (Tm=45.60): ACATCCTCTAAAAAAAAAAATACA

CpG2 analyze: ATCRATTAAATAAAATAAAATTTAACTTA

CpG3 forward (Tm=58.00): AGGAGAGAAAATAGAAGGTAGAGA

CpG3 reverse (biotinylated; Tm=59.40): TATCATACCTCCCTTCCATAAAATTACA

CpG3 sequence (Tm=44.20): ATAATTGAATTTGATGTAGTGA

CpG3 analyze: TAATTAYGTTTATATATAAAATATTTGGTTAA

Assay 2

Primary primer (CpG4-CpG9)

Nested forward (Tm=58.17): GTGTTATTAGGAAAAGAGAGAAAGGTT

Nested reverse (Tm=60.79): ATTTCAACTCGCCCCCTAAA

Secondary primers

CpG4-5 forward (Tm=58.00): AGGAGAGAAAATAGAAGGTAGAGA

CpG4-5 reverse (biotinylated; Tm=61.10): ATCATACCTCCCTTCCATAAAATTACA

CpG4-5 sequence (Tm=44.10): ATTTGGTTAAAGGTTAAGATTA

CpG4-5 analyze: TTTYGGATTTTYGATATATATTTTTTAAGTAGTGTA

CpG6-7 forward (Tm=60.30): TTGGGATAGGTATTTATGTGTGTGTA

CpG6-7 reverse (biotinylated; Tm=60.20): CATACCTCCCTTCCATAAAATTACA

CpG6-7 sequence (Tm=40.60): TTATGTGTGTGTATGAA

CpG6-7 analyze: TTTATTTTTAYGGTATATGTTTAGGATTTTTTAYGAGGAGTTAAAGT
TTTAGAGAGTTT

CpG8-9 forward (Tm=59.00): GTAATTTTATGGAAGGGAGGTATGAT

CpG8-9 reverse (biotinylated; Tm=58.30): AAAATCAATTACTTAAAAACCAAACCATTA

CpG8-9 sequence (Tm=42.80): ACCATAAAAAAAAAAAAAAATCTAT

CpG8-9 analyze: ACRTACRCTCCTATAAACAAAAAATAATATCA

Assay 3

Primary primer (CpG10-CpG13)

Nested forward (Tm=60.03): TTGGTTAAAGGTTAAGATTATTTCGG

Nested reverse (Tm=58.29): ACGCGACTATTTAAAAACCAAAT

Secondary primers

CpG10-12 forward (biotinylated; Tm=60.20): TATGTAATTTTATGGAAGGGAGGTATGAT

CpG10-12 reverse (Tm=58.00): ACCAACATAAATTATAACCAAATTTTATCA

CpG10-12 sequence (Tm=44.20): ACCAAATTTTATCATTTCAAC

CpG10-12 analyze: TCRCCCCCTAAATATTTAACTTTTATAATAAATCRTAACRTACCAA
AAAAAACTAATTACTAATC

CpG13 forward (Tm=58.60): AGGGGGAGAGTTGAAATGATAAAAT

CpG13 reverse (biotinylated; Tm=59.50): ACCAAATAACTTTTCTAACAATCACTC

CpG13 sequence (Tm=43.20): GAAGTATTTTTATAAAAGATGGTAT

CpG13 analyze: AYGAAGAGTGATTGTTAGAAAAGTTAT

Assay 4

Primary primer (CpG14-CpG15)

Nested forward (Tm=59.78): AAAAGATGGTATACGAAGAGTGATTGT

Nested reverse (Tm=60.00): TTCCCATCAAATAAAAACCTACAAA

Secondary primers

CpG14-CpG15 forward (Tm=58.40): AAGATGGTATAAGAAGAGTGATTGTTAGAA

CpG14-CpG15 reverse (biotinylated; Tm=58.50): ATTATTCCCCTCCTTCCATT

CpG14-CpG15 sequence (Tm=44.00): GAAAAGTTATTTGGTTTTTAAATAG

CpG14-CpG15 analyze: TYGYGTATTATTAGTAAAATTTATTATTT