Master of Science Thesis

Epigenetic mechanisms behind phenotypic changes in different population densities in bank voles (*Myodes glareolus*)

Elina Sorri

University of Jyväskylä

Department of Biological and Environmental Science

Ecology and Evolutionary Biology

17.5.2017

UNIVERSITY OF JYVÄSKYLÄ, Faculty of Mathematics and Science

Department of Biological and Environmental Science
Ecology and Evolutionary Biology

Sorri, E:	Epigenetic mechanisms behind phenotypic changes in different population densities in bank vole (*Myodes glareolus*)
Master Thesis:	26 p.
Supervisors:	PhD Angela Sims, PhD Esa Koskela
Inspector:	Prof. Anneli Hoikkala
May 2017	

Key Words: epigenetics, CpG island, methylation, oxytocin, population density

ABSTRACT

Epigenetic mechanisms play a fundamental role in normal gene expression activity and can be seen as a system of molecular processes that react to environmental cues and regulate gene expression without changing the original sequences. DNA methylation is a commonly studied epigenetic mechanism which typically occurs at CpG-sites when a methylgroup is added to the 5'-position of a cytosine followed by a guanine and this can inhibit the transcription of the associated genes. Regions of DNA sequence that are dense in CG-dinucleotides, called CpG islands, are located in gene promoter regions and their methylation can alter gene expression and phenotype. These methylation markers have the potential to be transgenerational and thus epigenetics mechanism could provide a pathway to adapt to varying selection pressures. Changes in population density are predicted to cause changes in epigenetic regulation of genes coding hormones and/or their receptors associated with density-dependent behaviour. In the bank vole *(Myodes glareolus*) population density correlates with several phenotypic traits such as: behaviour, breeding activity and infanticide. The oxytocin hormone has a crucial role in reproduction and social behaviour, such as maternal care, stress and anxiety. The main question of my thesis is how population density affects the methylation of CpG islands located in the promoter region of the oxytocin receptor gene (*Oxtr*). I hypothesize that in a high population density, methylation is decreased to enable more transcription of the *Oxtr* enhancing the responsivity of the individual to the effects of oxytocin in order to endure the stressful social environment. This was studied from DNA samples extracted from tissues of the F1 bank vole offspring whose prenatal and early postnatal development took place in either a high or a low population density. The methylation levels were significantly lower in the high density samples in the liver, lung, heart, muscle and kidney tissues, whereas no significant differences were found in the brain, blood and gonad samples. These results indicate that the expression of oxytocin receptor gene, *Oxtr*, is positively density-dependent and therefore individuals in higher densities are more responsive to oxytocin effects. Whether this is beneficial for the reproductive success or survival of bank vole individuals and further on the process of adaptive evolution remains to be studied.

JYVÄSKYLÄN YLIOPISTO, Matemaattis-luonnontieteellinen tiedekunta
Bio- ja ympäristötieteiden laitos
Ekologia ja evoluutiobiologia

Sorri, E: Metsämyyrän (*Myodes* glareolus) fenotyyppisten muutosten epigeneettiset mekanismit eri populaatiotiheyksissä
Pro gradu tutkielma: s. 26
Työn ohjaajat: PhD Angela Sims, PhD Esa Koskela
Tarkastaja: Prof. Anneli Hoikkala
Toukokuu 2017

Hakusanat: epigenetiikka, CpG-saari, metylaatio, oksitosiini, populaatiotiheys

TIIVISTELMÄ

Epigeneettisilla mekanismeilla on perustava rooli normaalissa geenien ekspression aktiivisuudessa ja ne voidaan nähdä molekulaaristen prosessien systeeminä, joka reagoi ympäristötekijöihin ja säätelee geenisäätelyä muuttamatta alkuperäistä DNA-sekvenssiä. DNA-metylaatio on eniten tutkittu epigeneettinen mekanismi jolla on tärkeä rooli geenien säätelyssä normaalin solujen kehityksen aikana. Metylaatio ilmenee tyypillisesti CpG-dinukleotideissa, joissa metyyliryhmä kiinnittyy 5'-sytosiiniin (C) jota seuraava emäs on guaniini (G). Tämä voi estää näihin CpG-paikkoihin liittyvien geenien ilmentymisen. DNA-sekvenssin alueet joissa CpG-dinukleotideja esiintyy runsaasti, GpG-saaret, sijaitsevat geenien promoottorialueella ja näiden metylaatio voi muuttaa geenin ilmentymistä ja yksilön fenotyyppiä. Tällä metylaatiokuviolla on potentiaali olla sukupolvien yli periytyvä ja siten epigeneettiset mekanismit voisivat olla keino sopeutua muuttuviin valintapaineisiin. Populaatiotiheyden muutosten on arveltu vaikuttavan epigeneettiseen säätelyyn erityisesti niissä geeneissä, jotka koodaavat hormoneja ja/tai reseptoreissa, jotka vaikuttavat populaatiotiheydestä riippuvaan käyttäytymiseen. Metsämyyrällä *(Myodes glareolus),*populaatiotiheys korreloi stressiherkkyyden, aggressiivisuuden, lisääntymisaktiivisuuden, poikuekoon ja omiin jälkeläisiin kohdistuvan aggressiivisuuden kanssa. Näihin käyttäytymispiirteisiin vaikuttavat useimmiten myös useat hormonit. Oksitosiinilla on keskeinen rooli lisääntymisesä ja sosiaalisessa käyttäytymisessä, kuten emon hoivakäyttäytyminen, stressi ja ahdistuneisuus. Tutkielmani keskeisin tutkimuskysymys on kuinka populaatiotiheys vaikuttaa oksitosiinihormonin reseptorigeenin promoottorialueen CpG-saarien metylaatioon. Hypoteesini on, että korkeassa populaatiotiheydessä metylaatio on vähäisempää, mikä mahdollistaa oksitosiinin reseptorigeenin transkription. Tämä edistäisi yksilön vastaanottokykyä oksitosiinin vaikutuksille. Metylaatioastetta tutkittiin eri kudoksista DNA-näytteistä metsämyyrien F1-sukupolven jälkeläisistä, joiden kehitys ennen ja jälkeen synnytyksen tapahtui korkeassa tai matalassa populaatiotiheydessä. Metylaatiotasot olivat merkitsevästi matalammat korkean tiheyden näytteissä maksan, keuhkojen, sydämen ja munuaisten kudosnäytteissä. Tulokset viittaavat oksitosiinin reseptorigeenin korkeampaan ilmentymiseen ja yksilöiden korkeampaan herkkyyteen oksitosiinin vaikutuksille korkeassa populaatiotiheydessä.

Contents

1. INTRODUCTION

Natural selection favours individuals that are best adapted to their environment. In general, in a constant environment many generations within a population under selection may reach the optimal phenotype of certain traits in that environment (Via & Lande 1985). Adapting to environmental changes usually requires genetic variation (Haldane 1937, Reed & Frakham 2003). However, in rapidly and frequently changing environment the evolutionary rate of genetic adaptation may not be fast enough for individuals to adapt (Dudley & Schmitt 1996). Therefore, phenotypic plasticity, the ability of a single genotype to produce more than one form of morphological, behavioural and/or physiological trait, often plays an important role in adaptation, niche shift, evolutionary diversification and population dynamics (West-Eberhard 1989, Agrawal 2001).

Multiple studies have recorded examples of environmentally induced phenotypes and measured changes in gene transcription (Schmid & Dolt 1994, Chen *et al.* 2003, Kurashige & Agrawal 2005). For example in bank vole (*Myodes glareolus*) males, changes in the environmental conditions maintained variation in testosterone levels and dominance advertisement (Mills *et al.* 2007). However, the mechanisms that regulate gene transcription activity in response to environmental changes are not yet fully understood (Richards *et al.* 2010). In general, gene transcription is regulated by genetic and epigenetic mechanisms. Genetic regulation consists of complex interactions between genes that encode products that can repress the expression of other genes. Changes to this regulation pattern usually require a change in the sequence of the gene that encodes the transcription repressing product. Epigenetic gene regulation occurs without alteration to the sequence (Bird 2002). DNA methylation is the most studied epigenetic mechanism with several categories: 1) hypomethylation of CpG dinucleotides in the promoter allows binding of the transcription factors, 2) hypermethylation of CpGs inhibits the transcription factors from binding and 3) methylcytosine-binding proteins bound to CpGs in promoter region blocks access of transcription factors (Attwood *et al.* 2002). The evidence is increasing that epigenetic mechanisms can react to environmental changes and can vary gene expression in an individual and its offspring (Mousseau & Fox 1998, Bossdorf *et al.* 2008, Novikova *et al.* 2008, Widiker *et al.* 2010). Therefore, epigenetics can provide a new insight on the interaction between environment and phenotypic variability (Bossdorf *et al.* 2008, Ledón-Rettig *et al.* 2013).

1.1. Epigenetic gene regulation

Epigenetic mechanisms play a fundamental role in normal gene expression activity and can be seen as a system of molecular processes that react to environmental cues and regulate gene expression without changing the original sequences (Frésard *et al.* 2013). Across different taxa, the main mechanisms are DNA methylation, chromatin modification, covalent modification of histone tails, DNA packaging around nucleosomes and regulatory function of non-coding RNA (Bird 2002, Jaenisch & Bird, 2003, Frésard *et al.* 2013). Here, I focus on DNA methylation which is a commonly studied epigenetic process that occurs in most living organisms (Angers *et al.* 2010). It is a time- and tissue-specific mechanism, which has important roles in normal cell differentiation and imprinting, X-chromosome inactivation, maintenance of chromosomal stability, and gene expression regulation (Lee *et al.* 2013). The role and genome site for methylation varies between taxa, species and individuals (Angers *et al.* 2010).

The normal methylation pattern is usually maintained throughout the lifespan of the organism, but certain environmental factors such as toxins or diet can change it (Burdge *et al.* 2007, Mousseau & Fox 1998, Novikova *et al.* 2008, Widiker *et al.* 2010) e.g. mice (*Mus musculus*) eating high-fat diets exhibited decreased methylation of the melanocortin-4 receptor gene that has a crucial role in regulating appetite and body-weight and its activation reduces food intake (Widiker *et al.* 2010). Social environment can also affect methylation patterns: in rats (*Rattus norvegicus*) a high level of maternal care decreased methylation in CpG-sites of the hippocampal exon 1_7 glucocorticoid receptor gene in the offspring, thus causing higher responsivity to stress (Weaver *et al.* 2005). Similar evidence of the social environment being able to change methylation pattern was found by Champagne *et al.* (2006), where extensive maternal care (licking and grooming) of the offspring reduced methylation and increased expression of the estrogen receptor gene in rats. Results like these suggest that methylation patterns can be prone to change when organisms are exposed to a wide variety of environmental changes and therefore methylation (together with other epigenetic mechanisms) may have an important role as an adaptive strategy under varying selection pressures.

Methylation patterns in mammals have been found to be heritable over generations (Burdge *et al.* 2007). Because the methylation pattern is copied accurately to the next cell at the time of DNA replication, any environmentally induced changes will also be transmitted (Rossiter 1996, Mousseau & Fox 1998). Evidence of environmentally induced epigenetic regulation and inheritance to the next generation was found in mice (*M. musculus*) in which maternal exposure to cocaine altered DNA methylation and gene expression of hippocampal neuron cells of the offspring (Novikova *et al.* 2008). In a study by Burdge *et al.* (2007) a protein-restricted diet for pregnant female rats altered the methylation patterns of a peroxisomal proliferator-activated receptor and glucocorticoid receptor promoters in the adult offspring of the first and second generation.

The adaptive function of a maintained and heritable DNA methylation pattern is still not completely understood. The amount of CpGs varies greatly between taxa; for example the genome of nematode worm *Caenorhabtidis elegans* has no detectable methylation site cytosines whereas many other invertebrate genomes have relatively high levels of CpG-sites (Bird *et al.* 2002). The importance and evolutionary potential of methylation may depend on the ecology of the organism. One possibility is that a certain methylation pattern, best adapted to the present environment, is heritable enough to benefit the offspring to survive and reproduce in the same environment that the parent is adapted to (Sachser *et al.* 2011). According to the theory of behavioural epigenetics, the fetus can make adaptations through programming in response to environmental cues in order to prepare for the postnatal environment (Lester *et al.* 2013). As many organisms develop only once, the changing development in response to environmental conditions is generally an irreversible process. After the developmental phase however, epigenetic mechanisms enable the organism to adapt to a current environment flexibly with no additive effect of changing methylation pattern. Therefore, epigenetic mechanisms enable an organism to adapt to environmental conditions that prone to sudden change (i.e. density induced social stress) reacting through simple changes in homeostasis, which would otherwise require multiple generations to adapt to via genetics adaptations (Lester *et al.* 2013).

The environment affects the development of the fetus as gene expression in the placenta is subject to environmental regulation and the placenta links the developed maternal physiological state with that of the developing fetus (Wilhelm-Benartzi *et al.* 2012). In their study DNA methylation profiles in the placenta had significant and strong associations with infant growth. In the prenatal phase, the interactions between mothers and the fetus are crucial for development and growth (Champagne 2008). According to

Champagne (2008), variation in these interactions, such as prenatal stress and maternal malnutrition, are likely to have lifelong consequences to the health and physiology of the offspring. Maternal malnutrition and prenatal stress are the best studied mechanisms that alter the mother's neuroendocrine system and physiology, producing a shift in fetal neurodevelopment. So far a little of evidence has been found for epigenetic transmission of behavioural experience, such as maternal care in rats changed epigenetic patterns in offspring (Weaver *et al.* 2004, Lester *et al.* 2013). Here I am to provide necessary evidence from a wild small mammal whether high population density of conspecifics / social stress could cause differential DNA methylation pattern in the offspring.

1.2. Mechanism of DNA methylation

The most studied epigenetic mechanism is methylation of the fifth position of cytosine (Smith & Meissner 2013). In this reaction a methyl group is attached to promoter regions of genes (Bird 2002). Methyl groups prevent transcription proteins from attaching to the sequence and hence the expression of the gene is disabled (Jones & Takai 2001). A methyl group is attached to 5'-cytosines that are followed by guanine in the sequence and these cytosines, with potential for methylation, are called CpG-sites (Bird 2002). DNA methylation can be increased (hypermethylation) or decreased (hypomethylation) at the CpG-site but hypermethylation in particular is associated with promoter regions and gene transcription regulation (Clark & Melki 2002).

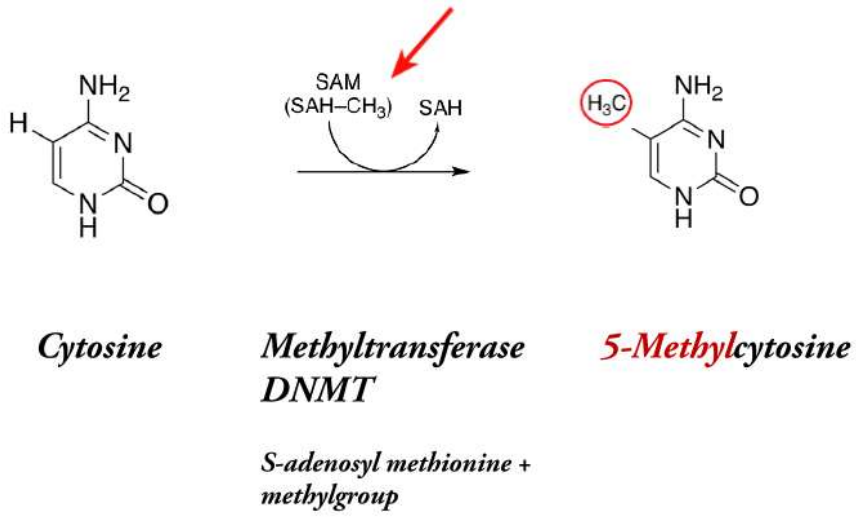

Figure 1. Mechanism of methylation (Afanas'ev 2014).

Sites for potential methylation are distributed across all types of DNA sequences, such as promoters, genes, intragenic regions and transposable elements (Antequera 2003). However, important regions for epigenetic gene regulation are short 0.2-3 kb long regions called CpG islands (CGIs), which contain a high density of CpG-sites (over 60 %) and they often span the promoter region of genes (Gardiner-Garden & Frommer 1987, Illingworth & Bird 2009). These sites usually remain nonmethylated and when hypermethylated, they contribute to transcriptional repression in most tissues (Razin 1998, Saxonov *et al.* 2006). In vertebrate genomes, over half of the promoter regions contain CGIs and the rest of the genome is depleted for CpGs (Gardiner-Garden & Frommer 1987, Antequera & Bird 1993, Jones 2012). CpG-sites are underrepresented in the genome (around 20-% of the expected frequency) due to the spontaneous deamination of methylated cytosines into thymine and

later on into uracil (Antequera 2003). However, the association between genes and CGIs is strong enough so that gene promoters in humans can be mapped using these islands and promoters can be categorized by the number of CpG islands (Ioshikhes & Zhang 2000, Antequera 2003). The role and definition of CGIs is still under discussion because there are also regions with intermediate CpG densities and therefore the studies have focused on CGIs in the transcriptional start sites (TSS) (Takai & Jones 2002, Jones 2012). Methylation of CGIs in the TSS is linked to long-term silencing of genes, as in X-chromosome inactivation and imprinting, but CGIs in gene bodies are occasionally methylated in a tissue-specific manner (Jones 2012). Whether non-CGI methylation has a role in silencing is not yet known.

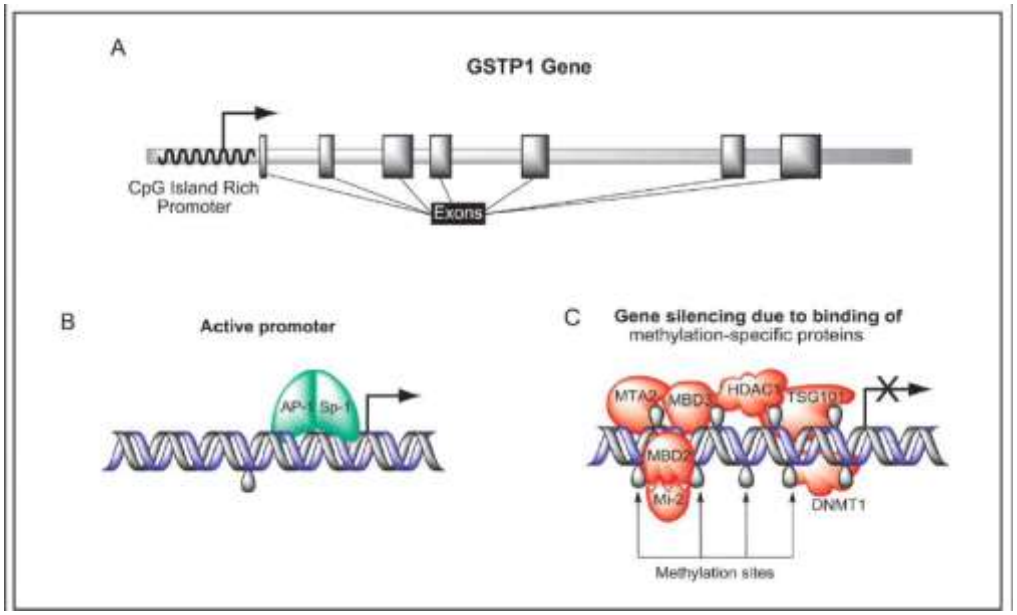

Figure 2. A) Example of location of CpG. on the promoter region of gene Glutathione S-Transferase Pi 1 B) Active promoter, gene expressed. C) Promoter methylated, gene silence (Maxwell *et al.* 2009).

In the mammalian genome, the methylation status of most CpG dinucleotides remains stable even through mitosis, but some sites are dynamically regulated (Smith & Meissner 2013). Methylation in CpG-sites other than CGIs is more dynamic and tissue-specific than CGI methylation (Jones 2012). Dynamic regulation occurs by oxidative modification of methylation sites and epigenetic information can be reversibly created and erased through a cyclic cytosine-modifying cascade by demethylation activity (Wu & Zhang 2014). Methylation is established and maintained by families of DNA methyltransferase enzymes, mainly DNMT3 and DNMT1 groups (Angers *et al.* 2010). The DNMT3A and DNMT3B methyltransferases establish the methylation patterns and DNMT1 maintain the methylation marks through DNA replication with the ongoing participation of DNMT3 methyltransferases (Ponger & Li 2005, Jones & Liang 2009). Methyltransferases are essential for setting up the methylation patterns in early development phases and during early development of the organism the genome is globally methylated at CpG-sites (Jones 2012). The regulatory regions of genes consist of short modules to which activator and repressor factors bind enabling or suppressing the expression of the gene (Arnone & Davidson 1997). Methylated CGIs located in the promoter area within the regulatory region attract MeCP and MBD families of proteins to bind to these sites. These bound proteins recruit histone deacetylases and transcriptional corepressors, forming a complex that inhibits the transcription of the gene (Jones *et al.* 1998, Lemon & Tjian 2000).

However, the extent of methylation can result in differences in gene expression and thus result in variation in the phenotype. Epialleles, the alternative methylation states of the same gene, have been linked to differences in individual morphology, physiology, development and behaviour (Weaver *et al.* 2005, Weber *et al.* 2005, Dolinoy 2008, Kumsta *et al.* 2013). For example in mice the phenotype of the agouti pigment pattern depends on the methylation state of the pigment encoding gene A^{vy} (Wolff *et al.* 1998). In humans the differences in the degree of methylation in the promoter region of the oxytocin receptor gene was associated with childhood disorders (Kumsta *et al.* 2013). In addition, epiallelic variants can differ in their relative dominance, for example, mice, that are homozygous for an allele that encodes yellow coat colour, can have one hypomethylated and one hypermethylated allele, leading to the one allele being dominant over the latter (Rakyan *et al.* 2002). Differences in epigenetic regulation can also be caused by dysfunction of DMNT1 methyltransferase or interactions between other epigenetic mechanisms and genetic regulation (Bird 2002, Martienssen & Colot 2001). In rats, nurturing maternal behaviour can lead to postnatal modification of the alleles in offspring in glucocorticoid receptor gene and this created a hypomethylated epiallele that can last until adulthood, whereas offspring with low nurturing mothers had a silenced GR gene epiallele (Weaver *et al.* 2004).

2. EFFECTS OF POPULATION DENSITY ON PHENOTYPE

Population density can create frequent and fast-changing, intense selection pressure on individuals by intensifying the competition for food, territories and mating opportunities. Thus population density can affect several morphological, behavioural and life-history traits (Barnes & Siva-Jothy 2000, Korpela *et al.* 2011, Svensson & Sinervo 2000). For example in side-blotched lizards (*Uta stansburiana*) egg size varied in different population densities (Svensson & Sinervo 2000). In red deer, increasing population density and winter rainfall decreased the number of male offspring because the environmental factors were associated with nutritional stress during pregnancy (Kruuk *et al.* 1999). This change in sex ratio corresponded to reductions in fecundity. A decrease in fecundity during high density season is found from other mammal species (Christian 1971b). Taken together this suggests that individuals may be able to adapt to temporary environmental conditions via modifications during development. Population density can affect individuals at several life-history stages, increasing selection pressure on traits crucial for fitness. According to Yokozawa and Hara (1995) traits that alter according to density can allow the individuals to exhibit strategies that enable them to live under conditions of dense population or to move away from too dense populations into less crowded ones. This would suggest that traits that are most important for enhancing the individual's fitness in varying population densities are under natural selection.

The first evidence of a genetic basis for density-dependent phenotype was discovered in fruit flies (*Drosophila melanogaster*) where selection at the larval stage of development and the allele expressed varied depending on the population density (Sokolowski *et al.* 1997). Similarly, density-dependent gene regulation was found in the bacteria *Bradyrhizobium japonicum* where the expression of two nodulation genes was suppressed in low population densities (Loh *et al.* 2002). Population density could therefore affect some fitness traits that have a genetic basis. An epigenetic regulation on top of this would allow a population to reach a local optimum faster than just by genetic natural selection.

2.1. Population density and changes in phenotypic traits in rodents

In mammals, characteristics affected by density include behavioural traits. Small rodent species are known for their varying population densities that can have multiannual phases of high densities followed by a significant collapse in the number of individuals. In many studies, varying population densities have altered the phenotype of rodent species (Clarke 1953, Christian 1971b, Johannesen 2003, Korpela *et al.* 2011). For example, condition and fitness of individual, body size, litter sex ratio, proportion of maturating young and the timing of reproduction was associated with fluctuations in population density in several rodent species (Norrdalh & Korpimäki 2002, Johannesen 2003, Inchausti & Ginzburg 2009). However, the effects of population density may be species–specific as, for example, dietary quality/quantity and social organization systems of the species could be crucial in determining the intensity with which population density would affect individuals. For example, body mass correlated with population density in bank voles (*M. glareolus)* and body mass was associated with survival (Johannesen & Andreassen 2008). The study compared two species, bank voles and grey-sided voles (*Myodes rufocanus*) and density did not affect body mass or survival of grey-sided voles therefore suggesting that population density would affect individuals species-specifically depending on its life-history.

Traits such as aggressiveness and reproduction appear to be particularly sensitive to population density changes (Norrdahl & Korpimäki 2002). Changes in behaviour and reproduction may occur under different densities, determining fitness because in high densities it would be beneficial to avoid the crowded locations or to be a strong competitor by behaving aggressively. High densities may also favour less active reproduction and smaller litter sizes by increasing survival and reducing reproductive cost. These phenotypic changes were found in field voles (*Microtus agrestis)* that had shorter breeding seasons in dense populations than those from less dense ones (Clarke 1953). Moreover, females from dense populations were less fertile and performed more frequent aggressive behaviour resulting in wounding (Clarke 1953). Studies with bank voles (*M. glareolus*) revealed similar results: increasing density decreased breeding activity, enhanced infanticide (killing a conspecific young by adult individual) and reduced litter size (Oksanen *et al.* 2007, Korpela *et al.* 2011). In the house mouse *(Mus musculus*) high densities delayed reproductive maturation (Van Zegeren 1980). In open–field experiments, male prairie voles (*Microtus ochrogaster*) and meadow voles (*M. pennsylvanicus*) behaved more aggressively in high density compared to the lower densities (Krebs 1970). Therefore it is evident that in several rodent species the phenotypes of certain traits (e.g. body size, number and size of offspring) alter depending on the population density, potentially through e.g. food availability, stress, predation and pathogen pressure, scarcity of suitable habitat and/or direct interaction between individuals. There is evidence for a genetic basis for some of these traits (Schroderus *et al.* 2010, 2012) but how epigenetic regulation mechanisms affects them is not yet known.

3. DNA METHYLATION IN VARYING POPULATION DENSITY

Identifying the genes behind quantitative traits is challenging because of several genetic interaction mechanisms such as: locus heterogeneity, epistasis, low penetrance, variable expressivity and pleiotropy (Glazier *et al.* 2002). Epigenetic regulation brings another level of complexity to the genotype–to–phenotype expression, because epigenetic mechanisms are still mostly unknown and they are affected by the environment, which makes them more unpredictable to study than genetic regulation. This is why finding genes behind

complex density–dependent traits, solving the role of methylation in the expression of them and understanding the density–dependency of methylation needs a large amount of further research in genetics and behavioural ecology. Therefore it may be more justifiable to focus on the epigenetic regulation of single genes that are known to encode for products associated with the density–dependent traits.

In species with cyclic population densities reproductive characteristics such as rate of sexual maturation, breeding season, weight at first breeding, number of breeding females, winter breeding and litter size may vary according to the density (Gustafsson *et al.* 1983). Population density can cause changes in the food availability and social environment. For example in bank voles, high density can suppress reproduction as establishing a territory is a prerequisite for reproduction and lower food supplement causes later initiation of reproduction (Kawata 1985, Eccard and Ylönen 2001).

3.1. Density–dependent DNA methylation

The association between population density and DNA methylation has not been studied in rodents, but there are indications that some density–dependent traits are affected according to the altered methylation state. In mice, postnatal stress of infants altered DNA methylation in the germline and these patterns were maintained to the next generation in the male germline (Franklin *et al.* 2010). In high densities, increased competition for food resources can force individuals to change their diet. This could trigger the methylation changes as found by Widiker *et al.* (2010) that an altered diet changed the methylation and expression of melanocortin–3 receptor that affects body–weight regulation in mice (*M. musculus*). Population density can be a strong environmental force requiring fast hormonal responses not only in the individual but also in the offspring when they are developing and growing in those conditions.

Studying the epigenetic regulation of hormone receptor genes that contribute to density–dependent traits gives insight to the links between DNA methylation and population density. Here it is more beneficial to focus on genes encoding the hormone receptors, as the receptors indicate the function of the hormone in the tissue and the amount of receptors may indicate the response more accurately than just the amount of encoded hormone. For example, maternal care alters the epigenetic regulation of genes that regulate hormonal stress responses and this effect seems to be associated with the expression of the oxytocin receptor gene (Meaney 2001). In rat, high pup licking and grooming caused significantly higher oxytocin receptor levels in the lateral septum, medial preoptic area, central nucleus of the amygdala, paraventricular nucleus of the hypothalamus and the bed nucleus of the stria terminalis in the brain (Champagne *et al.* 2001). As high maternal care is linked to offspring showing less anxiety related behaviour, this could be the link between maternal care and the behavioural phenotype, mediated by an epigenetic regulation of certain hormone receptor genes.

For behavioural traits that changed according to population density (e.g. stress, reproduction, aggression) it is most informative to research methylation on hormones that have a crucial role in affecting these traits. Neurohormones such as oxytocin have a strong correlation with the social behaviour and stress (Neumann 2008). Oxytocin is a nonapeptide, synthesized by magnocellular neuron in the hypothalamus (Barberis *et al.* 1998). In females, oxytocin is crucial for normal reproductive physiology. It is released in the central nervous system and functions as a birth and lactation hormone but it also has a role as stress–related gene and in social behaviour (Carter *et al.* 2009, Neumann 2002, Meyer–Lindenberg *et al.* 2011). In addition to nervous system, oxytocin is also synthesized in peripheral tissues such as gonads and heart (Gimpl & Fahrenholz 2001). According to Meyer–Lindenberg *et al.* (2011), oxytocin has a crucial role in regulating social cognition

and behaviour, such as attachment, social recognition, anxiety and stress–related behaviours. However, it can attach to other receptors besides the oxytocin receptor and therefore the effects of oxytocin are not straightforward (Landgraf & Neumann 2004). Oxytocin's role in inducing species–specific social and reproductive behaviour is evolutionarily conserved (Insel & Young 2000). This suggests that oxytocin has an evolutionarily important role and reduced reproduction, alterations in social behaviour and elevated stress in females in high densities could therefore be related to deficiencies or dynamic regulation in the function of oxytocin or its receptors. In high density, it may be more beneficial to have higher sensitivity to oxytocin in order to enhance stress tolerance, have stronger partner preference, induce more explorative behaviour and show higher maternal care under more stressful environment. The effects of oxytocin also differ between centrally and peripherally administered oxytocin. For example in prairie voles, centrally administered oxytocin in the brain tissue facilitates social behaviour and peripherally administered oxytocin is generally ineffective in influencing these processes (Cushing & Carter 2000). However, in a study where prairie voles received peripheral oxytocin in pulses, the females showed significant pair-bonding behaviour by favouring their partner and spending longer time with them (Cushing & Carter 2000). This raises the question of whether epigenetic oxytocin receptor regulation differs in the brain or peripheral tissue and does this correlate with population density, which can act as a social stressor and induce change in social behaviour.

In this study the focus is on how the oxytocin receptor gene *Oxtr* methylation pattern reacts to the different population densities. The hypothesis is that in higher densities, the promoter region of the oxytocin receptor gene in mothers is less methylated and therefore expression of the *Oxtr* is elevated in the offspring. This would indicate that the offspring could be better able to cope with a socially stressful environment.

4. MATERIALS AND METHODS

4.1. Study animals

The bank vole (*Myodes glareolus*) is a small microtine species which is distributed across Europe and Siberia (Kotlík *et al.* 2006). Small rodent populations in Fennoscandia show cyclic trends in their density, with some non-cyclical periods (Steen *et al.* 1990). Vole population dynamics are regulated both by extrinsic factors, such as food resources and especially predation, and intrinsic factors such as spacing behaviour and territoriality (Korpimäki *et al.* 2005, Radchuk *et al.* 2016).

Experimental voles for the study originated from enclosed populations located in Konnevesi Research Station of the University of Jyväskylä.Three high density populations of 10 males and 10 females and three low density populations of 5 males and 5 females were released in six field enclosures (size 40 m x 50 m) where they freely mated. After 14 days, the populations were monitored using Ugglan live traps and females were taken to into laboratory conditions to give birth. The females and their pups were released back to the enclosures within a few days after labour. The adults were removed from the enclosures when all pups were at least 15 days old and thus weaned from their mothers. The early growth conditions of the offspring took place in the enclosure without parents until they were 30 days old, after which time they were recaptured and euthanized. In total, the DNA from 5 male and 5 female offspring from high and low density populations was extracted (total N=60 individuals).

4.2. DNA extraction and bisulfite conversion

The DNA was extracted with high salt method as follows (Aljanabi & Martinez 1997). The tissues from liver, lung, gonads, brain, blood, muscle, heart and kidney were homogenized in 400 µl of sterile salt homogenizing buffer (05. M NaCL 10 mM Tris–HCl pH 8.0 and 2 mM EDTA pH 8.0), using a Polytron Tissue Homogenizer, for 10–15 s. Then 40 µl of 20 % SDS and 8 µl of 20 mg/ml proteinase K was added and mixed. The samples were incubated at 55–65^{o}C for at least 1 h, after which 300 µl of 6 M NaCl was added to each sample. Samples were vortexed for 30 min at 10 000 g. The supernatant was transferred to fresh tubes and equal volume of isopropanol was added to each sample, mixed and left to incubate at –20 ^{o}C for 1 h. Samples were then centrifuged for 20 min, 4 ^{o}C, at 10 000 g. The pellet was washed with 70% ethanol, dried and resuspended in 300–500 µl sterile dH_2O. Genomic DNA (5–15 ng) in 10 µl of ddH_2O was used for random amplified polymorphic DNA (RAPD) PCR–amplification of genomic DNA.

Table 1. Primer pairs designed, tested and amplified.

Gene receptor	Hormone	Primer pairs designed and tested	Amplifying pairs in test-PCR	Sequencing primers designed	Functioning pairs in pyrosequencing
Oxtr	Oxytocin	5	2	2	1
Ins	Insulin	5	0	0	0
NR3C1	Glucocorticoid	5	0	0	0
Avpr1a	Vasopressin	5	2	2	0
Pgr	Progesterone	5	0	0	0
Esr1	Estrogen	5	1	1	0

Designing primers was tested with several genes encoding receptors for hormones vasopressin (Avpr1), estrogen (Esr), progesterone (Pgr), testosterone (Ar), insulin (Ins) and oxytocin (Oxtr). Functioning primers for both amplifying and pyrosequencing stages were found for oxytocin. Studied gene sequences were found by blasting mRNA reference sequences of the gene of interest against the whole genome of the bank vole (Watts *et al.,* unpublished). The gene sequences used for the study were from the related species prairie vole (*Microtus ochrogaster*). mRNA sequences were downloaded from NCBI–database (http://www.ncbi.nlm.nih.gov/). Sequences of mRNA were used to avoid unnecessary intron regions within the gene that could lead to biased blast results. Sequences were blasted against a local nucleotide database of the bank vole genome, uploaded to CSC Taito Servers, using an optimized e–value of 0.1^{-7}. The gene sequences with e–value of 0.0 were then blasted against a rodent–database by using blastx to confirm the identification of sequences. Promoter regions upstream of blast hits were identified by the percentage of CG–sites (at least 60%) with a minimal length of 200 bp. Gene sequences were also compared to literature for information about the location of exons and introns within the gene.

The primers for pyrosequencing were designed using the SEQUENOM Epidesigner–program (www.epidesigner.com). The parameters were optimized with every primer search. Primers were then analyzed with NetPrimer (http://www.premierbiosoft.com/netprimer/) for self-dimers, hairpins, poly-A or poly-T tails, repeats and stability. Five of the best primer pairs were then selected and tested for each gene. The best working primers were selected by agarose gel performance. For the pyrosequencing stage, sequencing primers were designed by hand for the primer pairs the

performed the best. For oxytocin gene, the primers were designed for two positions that had CpG-sites in the promoter region of oxytocin.

DNA samples were bisulfite converted with EZ DNA Methylation™ Lightning Kit (Zymo Research, United States, distributor Nordic BioSite Oy, Tampere, Finland) according to the protocol provided. The bisulfite conversion was performed in order to detect methylated cytosines from the unmethylated ones by converting unmethylated cytosines into thymine. All DNA samples were amplified by PCR with the normal forward and biotinylated reverse primers. The PCR conditions were optimized by analysing the optimum amplifying temperature using a temperature gradient PCR and analysing the results with agarose gel electrophoresis. In the gel the desired result was to gain clear bands with no dimers. Further optimization was done by optimizing the PCR mix proportions and reaction preparation technique.

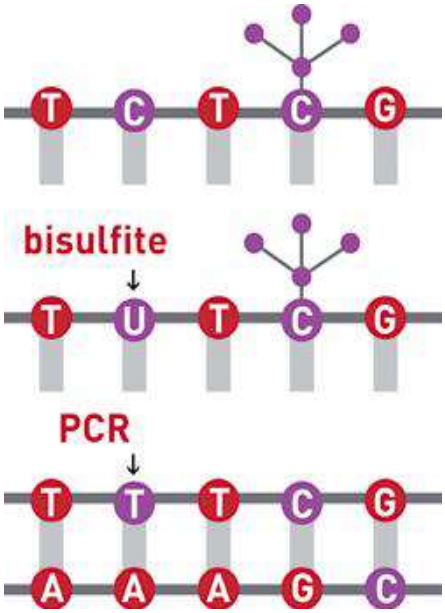

Figure 3. Bisulfite conversion of DNA. Methylated cytosine remains cytosine while unmethylated cytosines turn uracil in bisulfite conversion and thymine in PCR amplification (Diagenode, Inc. (US) 2016. Website of Epigenetics DNA Methylation, Bisulfite conversion. https://ww.diagenode.com/applications/dna-bisulfite-conversion/read 12.2.2015).

4.3. Pyrosequencing

Pyrosequencing was performed to distinguish methylated cytosines from the rest of the amplified sequence by detecting nucleotides with a light reaction (method explained in the following section). As unmethylated cytosines are converted into thymine during bisulfite conversion, cytosines in the converted sequence can be recognized as methylated. The PCR products of the bisulfite converted samples were analysed, using a Pyromark Q24 pyrosequencer available in the Central Finland Central Hospital.

Pyrosequencing is based on light reaction. Each cycle releases a pyrophosphate (PPi) in a quantity equivalent to the amount of attached nucleotide. AtP sulfurylase converts PPi to ATP in the presence of adenosine 5'phosphosulfate (APS). The ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by charge coupled device (CCD) camera and seen as a peak proportional to the number f nucleotides incorporated in the raw data output (pyrogram).

Apyrase is present in the reaction so that unused nucleotides continuously degrade, setting the stage for the type of nucleotide to be added. Addition of dNTPs is performed sequentially. Deoxyadenosine alfa-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is used by the DNA polymerase without being recognized by the luciferase. With the process continuing, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.

4.4. Statistical analysis

As there were multiple fixed factors, as well as a random factor, to consider, a generalized linear mixed model (GLMM) was chosen as the appropriate statistical test. As the dependent variable methylation level is proportional, it was arcsine square root transformed for the analyses. In the GLMM-analyses, density (1, 2), sex (1, 2), tissue (1, 8) and CpG-site position (1, 2) were used as fixed factors, and the individual as a random factor. In the preliminary analysis, there was no significant difference in the methylation level between the sexes ($p > 0.10$). As the primary aim was to study differences between densities, the only interaction term entered in the model was between density and tissue. Thus, the final model included the factors density, tissue, position and the interaction between tissue and density as fixed factors as well as individual as a random factor. The pairwise post-hoc comparisons between densities within each tissue were performed using Mann-Whitney U test available in the SPSS Staticstics Standard-statistical software Version 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

5. RESULTS

Out of the six genes tested I was only able to design working primers for the *Oxtr* promoter and sequence one part successfully (Table 1). This might be due to for example primer dimers, degradation of the DNA in bisulfite conversion or primers attaching at the wrong location in the sequence. Consequently, my results on the methylation level differences between low and high density include only one gene out of the 6 originally aimed at (Table 1).

Table 2. GLMM analysis to quantify the effects of population density, tissue and CpG site position on methylation percentages of bank vole offspring. Data is presented in Figure 4.

Source	F	df	df	p-value
Intercept	96.32	16	229	< 0.001
Density	22.25	1	229	< 0.001
Tissue	20.76	7	229	< 0.001
Position	1335.57	1	229	< 0.001
Density by Tissue	6.82	7	229	< 0.001

Table 3. Pair-wise Mann-Whitney U tests to quantify the effects of density on methylation percentages in different tissues of bank vole offspring. The two CpG site positions analysed separately.

Tissue	Parameter	Position 1	Position 2
Liver	N	17	17
	Mann–Whitney U	66	56,5
	p–value	0.002 < 0.05	0.046 < 0.05
	Higher methylation %	**Low density**	**Low density**
Lung	N	18	18
	Mann–Whitney U	64	63
	p–value	0.040 < 0.05	0.05
	Higher methylation %	**Low density**	**Low density**
Gonads	N	15	15
	Mann–Whitney U	18	19
	p–value	0.281 > 0.05	0.336 > 0.05
	Higher methylation %	No sig. difference	No sig. difference
Muscle	N	13	13
	Mann–Whitney U	37	34
	p–value	0.022 < 0.05	0.073 > 0.05
	Higher methylation %	**Low density**	No sig. difference
Heart	N	15	15
	Mann–Whitney U	44,5	53
	p–value	0.054 > 0.05	0.002 < 0.05
	Higher methylation %	No sig. difference	**Low density**
Kidney	N	14	14
	Mann–Whitney U	47	46
	p–value	0.001 < 0.05	0.003 < 0.05
	Higher methylation %	No sig. difference	**Low density**
Brain	N	18	18
	Mann–Whitney U	31	31,5
	p–value	0.436 > 0.05	0.436 > 0.05
	Higher methylation %	No sig. difference	No sig. difference
Blood	N	13	13
	Mann–Whitney U	15	11
	p–value	0.524 > 0.05	0.222 > 0.05
	Higher methylation %	No sig. difference	No sig. difference

The two CpG sites differed statistically significantly in methylation levels (Table 2). There was a significant interaction between the density and tissue showing that the density has tissue specific effects on methylation level (Table 2) which then allows tissue-wise analyses of the data. In the Mann-Whitney U-tests, the methylation level differed significantly ($p < 0.05$) in high density situations causing hypomethylation in five out of eight tissues: liver, lungs, muscle, hearth and kidney. This could indicate a high expression of *Oxtr* in the high density situations.

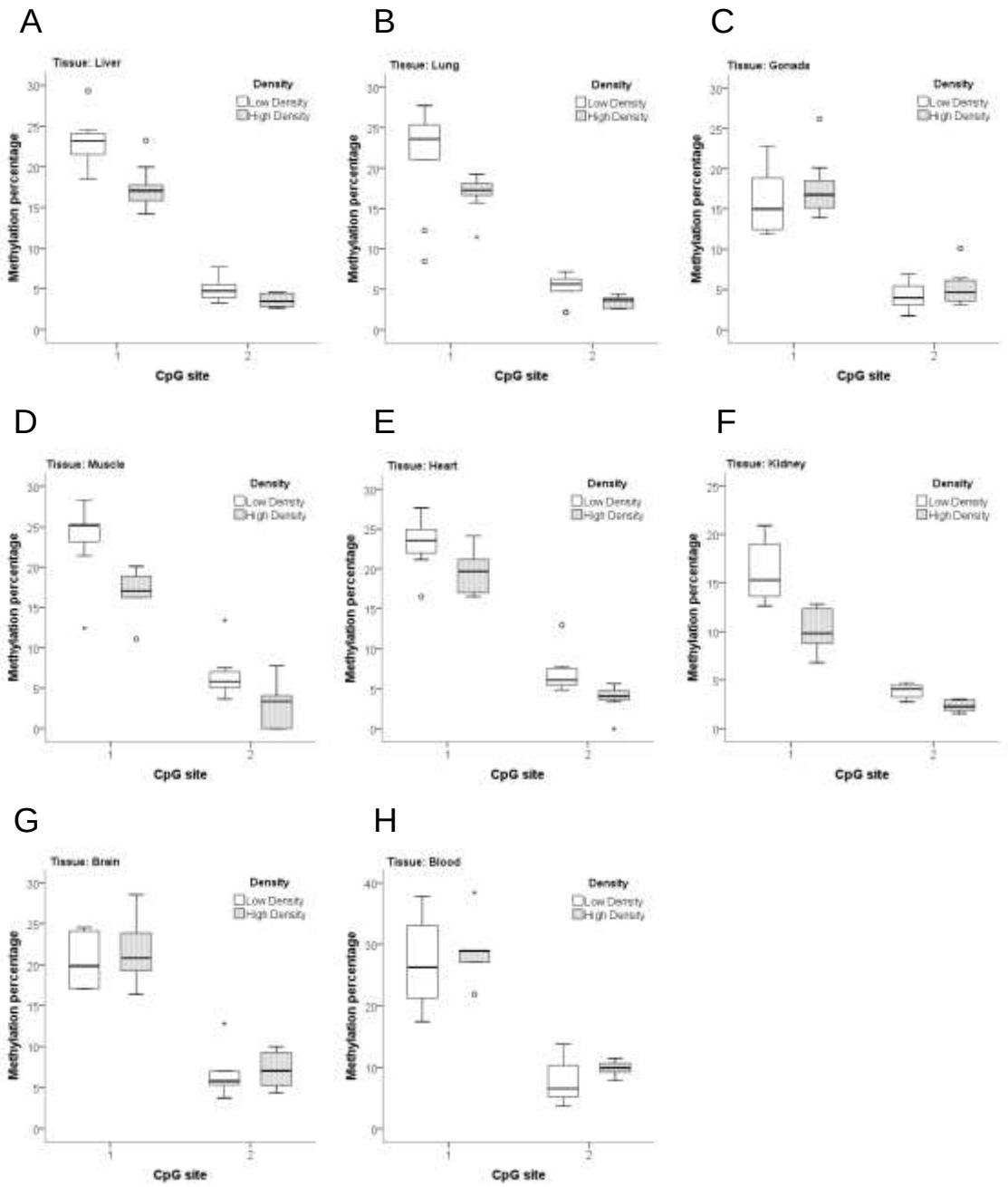

Figure 4. Tissue-specific differences (box plot) in methylation percentages of bank vole offspring originating from low and high density. Methylation studied from DNA extracted from A) liver, B) lung, C) gonads, D) muscle, E) heart, F) kidney, G) brain and H) blood. The two CpG site positions are shown separately. Higher average methylation percentages are mainly found in low density samples from liver, lung, kidney and muscle tissues. For pairwise statistics see Table 3.

Methylation levels were significantly higher ($p < 0.05$) on average in low density samples in tissue samples from liver, kidney, lung, muscle and heart on either the first or second CpG-site in the amplified regions. There were no statistically significant differences found in samples of blood, brain or gonads (Table 3).

DISCUSSION

Higher methylation levels were found in samples from liver, lung, heart, kidney and muscle from individuals that were conceived and raised in low density populations. This suggests that in high densities the methylation pattern is decreased causing an increasing expression of more oxytocin receptors and thus increasing oxytocin responsivity. These results do not support nor reject the hypothesis of methylation being higher in low densities in brain, blood or gonad tissues but instead they support the idea of oxytocin having a larger role in more peripheral tissues. This may indicate that oxytocin has a dynamic regulation pattern in these tissues according to population density. However, in high density the changes in methylation pattern might also be caused by e.g. quality and quantity of food instead of behavioural experience.

No differences were found from brain tissues and therefore support for behavioural adaptation through oxytocin receptor regulation requires further research. Perhaps no differences were found because the brain samples were looked at as one pooled sample and were not from any particular region of the brain. While brain oxytocin is an important regulator of physiological and behavioural stress, and induces maternal care, aggressive behaviour, pair bonding, social memory and support, anxiety-related behaviour, stress coping and sexual behaviour (Neumann 2008), it is also related to non-behaviour related traits such as appetite, body weight and insulin regulation (Dumais &Veneema 2016).

Stress can have several ways of affecting the offspring. First, exposure to stress can alter the brain development of the foetus by changing the methylation patterns during the development (Schneider *et al.* 2002). Postnatal maternal care may later change methylation patterns causing changes in the expression of genes, therefore e.g. regulating the functioning of hypothalamic pituitary adrenal (HPA) axis in the brain, causing behavioural effects (Schneider *et al.* 2002). Oxytocin has a crucial role in regulating heart rate, blood pressure and cardiovascular response to stress (Schneider *et al.* 2002). Previously oxytocin has been characterized as a female reproductive and behavioural hormone, thereby having a role in the gonads, blood and brains of individuals. However, oxytocin receptor also has a significant, tissue-specific role in more peripheral organs (Kimura & Ivell 1999). Oxytocin receptor expression has been found in uterine myometrium, mammary gland, endometrium, decidua, gonads, epididymis, vas deferens, thymus, heart and kidney and the brain (Kimura & Ivell 1999, Gimpl and Fahrenholz 2001). In addition, the oxytocin receptor gene is dynamically regulated in the heart and kidney in which oxytocin affects the function of these organs and has a complex dynamic regulation pattern that is specific for the developmental stage and tissue (Kimura *et al.* 2003). For example in rat's hearts, all four heart compartments and large vessels synthesize oxytocin receptors (Gutkowska *et al.* 2000). Gutkowska *et al.* (2000) hypothesized that oxytocin receptor production is physiologically relevant in the heart because activation of the oxytocin receptor slows the heart by inducing a negative chronotropic effect. This could be a vital trait in order for small sized species such as bank voles to endure stressful conditions, especially during pregnancy. In humans, the oxytocin receptor methylation levels were significantly lower after a stress induction by The Trier Social Stress Test including tasks of public speaking and mental arithmetic performed in front of an audience, therefore suggesting oxytocin has a role in psychosocial recovery processes (Kirschbaum *et al.* 1993, Heinrichs *et al.* 2003). In the presence of continuous and varying social stressors such as high density, it would be beneficial to have the recovering effect of oxytocin consistently in order to continuously maintain good physiological condition. The oxytocin system regulates blood pressure and

volume, cardiovascular homeostasis, heart rate and cardiovascular response to stress, which all aid the individual to cope with the physiological effects of long–term stress (Gimpl and Fahrenholtz 2001). Stress responses may have short or long term duration and stressors such as attacks from conspecifics can activate the response in individuals (Girolami *et al.* 1996).

Whether densities the mother experienced had an effect on the mothers or the offspring requires further research as there can be various different factors affecting methylation patterns in different life stages. Any adaptive effect found may not be limited only to the maternal care, but also the hormonal activity during pregnancy. Stressors experienced by the mother during pregnancy caused high anxiety behaviour in the offspring (Valleé *et al.*1997). In rats, high population density during the pregnancy and/or lactation resulted in higher anxiety levels and lower locomotion which is less explorative behaviour in open locations (Batuev *et al.* 1999). In guinea pigs, offspring showed sex–specific differences in behaviour, brain development and endocrine system depending on whether the mother had experienced a stable or an instable social environment during pregnancy and lactation (Kaiser and Sachser 2005). In the study by Kaiser and Sachser (2005), the social stressor was caused by changing the group composition frequently, which reflects the social stress experienced in high density. Mothers respond to the changed environment with hormonal change which affects the embryonic endocrine state (Sachser *et al.* 2011). Change in the stress hormone corticosterone levels caused by unstable social environment has been found in rats (Stefanski *et al.* 2005). In guinea pigs, daughters were more masculinized and sons infantilized when their mother experienced a high density (Kaiser & Sachser, 2005). Kaiser and Sachser (2005) suggested that the traits could be more beneficial in high–density populations, as the more masculine females gain dominant social positions and more access to resources in high density situation. This trait however has trade–off costs as the resources are not used only for reproduction but also for gaining and defending resources: therefore in a low density it is more beneficial to have less masculine features.

In mammals, the effects of oxytocin may be especially vital during the prenatal period when the offspring are particularly vulnerable to stress. Prenatal stress can have long term consequences to the individual. For example, in rats, prenatal stress increased blood pressure and changed activity within the hypothalamic–pituitary–adrenal axis in adulthood (Barker 1998). In another study, a series of unpredictable stresses to pregnant female rats in their last week of pregnancy generated social withdrawal and molecular changes similar to schizophrenia in the offspring (Lee *et al.* 2007). However, in prenatally stressed male rats, there was less oxytocin mRNA in the paraventricular nucleus but increased oxytocin receptor binding in the central amygdala and therefore reversed the social withdrawal of the prenatally stressed individuals. (Lee *et al.* 2007). Therefore, oxytocin can play a significant role in protecting the offspring from stress. Similar result was found by Sohlström *et al.* (2000) when decreases in corticosterone in response to postnatal oxytocin treatment were significant in rats that experienced prenatal stress. How oxytocin can affect individuals in the early life stages like this is not entirely known, but this suggests oxytocin having a role in the evolution of complex social behaviour as it is an almost uniquely mammalian hormone (Acher *et al.* 1994).

As an adaptive strategy, higher expression of oxytocin receptor according to population density may have not only short term advantages but could also be an evolutionary adaptative mechanism that is specific for females. Females change their mating behaviour in high densities according to environmental cues that signal future

breeding and survival (Mappes & Ylönen 1997). Even though stronger reactions such as higher stress level is costly to the pregnant females, the individual will still gain higher fitness by producing offspring that are well adapted to their environment. For males, the same level of reacting could be too costly with no benefit to their fitness. However, this phenomenon may be species–specific, especially because the ecology and responses to different population densities vary across mammals. In bank voles, reproductive parameters such as litter size, are reduced in high densities (Christian 1971b). This may be partly regulated by epigenetic mechanisms, especially for hormones with a crucial role in reproduction, such as oxytocin. The expression of the receptor genes would be one of the most effective systems as receptors regulate the effect of the hormone in certain tissues. The regulation of the hormone receptor gene expression may affect the reproductive behaviour before mating, during pregnancy and after birth. In epigenetic studies, the effect of maternal care is well demonstrated (Francis *et al.* 2002). In most mammalian species the females do not express spontaneous maternal care without pregnancy hormones priming the brain so the role of oxytocin may be especially important a stressful environment (Keverne 1995). In rats, high maternal care increases the oxytocin receptor binding and expression in the brain tissue of female offspring which can be ultimately translated in how female offspring will care for their own offspring (Insel & Young 2000, Francis *et al.* 2002). Also, offspring receiving high levels of licking and grooming, are more exploratory, which may be a beneficial trait in an environment where food and territories are scarcer (Francis 1999).

Previous epigenetic studies have focused on the causes of epigenetic changes, but further research is required to study the role of epigenetics in an eco-evolutionary perspective. In bank voles the cyclic population density changes may act as a frequent but predictable changing natural selection pressure. Feinberg and Irizarry (2010) constructed an evolutionary model of genetically inherited stochastic variation in evolution. Results showed that the variation potential of a single phenotype would increase fitness by increasing variability of a genotype, not by changing the mean phenotype. Therefore epigenetic gene regulation mechanisms may have a significant role in adaptation processes. Oxytocin affects bank vole in so many reproductive and behavioural traits it would be an advantage if the epigenetic regulation of its receptors was an adaptation mechanism that has developed over a long period of time. Tissue-specific methylation across species has been reported and localization near certain genes would provide specificity to the effect of variation in methylation (Feinberg and Irizarry 2010). In humans, tissues are characterized by methylation patterns specific to those tissues and variation between tissues is higher than between individuals (Lokk *et al.* 2014). Therefore, as in my study the differences in methylation levels of *Oxtr* were found in peripheral tissues, the methylation may actually have a different role in these tissues than in the brain, blood and gonads where oxytocin usually affects behavioural traits and reproduction. Why and how oxytocin can affect bank voles in these tissues remains to be studied.

Possible areas of improvement in this study are the field experiment conditions and increasing the data gained from tissues and from more genes. Also, population density is a very variable situation and species–specific factor, which may have not only varying effects but also varying responses. It is not known, what amount of individuals will be the threshold for bank voles to experience social stress caused by population number but as a territorial species by the females it is likely that bank voles are relatively sensitive to multiple conspecifics living near their territory. Methylation is also a fast changing mechanism that may react to multiple stressors, such as catching and handling the

individuals. The two methylation positions amplified and sequenced are a fairly faint evidence of the methylation status of the complete CpG-island. For example a large study with 10, 20 or more CpG-sites would give stronger results on the actual methylation trends. However, the amplified product is necessary to keep under 150 bp as the bisulfite conversion damages the DNA and the product cannot be pyrosequenced successfully. Further studies could be performed for more stress and behaviour related genes and the tissue samples could be taken from both parents and the offspring. Also varying the density over a long period of time could give a better insight on how the methylation is correlating with density.

ACKNOWLEDGEMENTS

I thank my supervisors PhD Angela Sims and PhD Esa Koskela for their supervision and comments throughout the work. Special thanks to also PhD Joannes Van Cann and Prof. Anneli Hoikkala for comments on the thesis and seminars. I also want to thank Central Finland Central Hospital for letting me use their pyrosequencing equipment for this study.

REFERENCES

Acher R., Chauvet J., & Chauvet M.T. 1994. Man and the chimaera. Selective versus neutral oxytocin evolution. *Advances in experimental medicine and biology*, 395, 615–627.

Afanas'ev I. 2014. New nucleophilic mechanisms of ros–dependent epigenetic modifications: comparison of aging and cancer. *Aging and disease*, 5(1), 52–62.

Agrawal A.A. 2001. Phenotypic plasticity in the interactions and evolution of species. *Science*, 294(5541): 321−326.

Aljanabi S.M. & Martinez I. 1997. Universal and rapid salt–extraction of high quality genomic DNA for PCR–based techniques. *Nucleic acids research*, 25(22), 4692–4693.

Angers B., Castonguay E. & Massicotte R. 2010. Environmentally induced phenotypes and DNA methylation: how to deal with unpre0dictable conditions until the next generation and after. *Mol Ecol*, 19(7): 1283−1295.

Antequera F. & Bird A. 1993. Number of CpG islands and genes in human and mouse. *PNAS*, 90(24): 11995−11999.

Antequera F. 2003. Structure, function and evolution of CpG island promoters. *CMLS*, 60(8): 1647−1658.

Arnone M.I. & Davidson E.H. 1997. The hardwiring of development: organization and function of genomic regulatory systems. *Development*, 124(10): 1851−1864.

Attwood J.T., Yung R.L. & Richardson B.C. 2002. DNA methylation and the regulation of gene transcription. *Cellular and Molecular Life Sciences CMLS*, 59(2), 241–257.

Barberis C., Mouillac B. & Durroux T. 1998. Structural bases of vasopressin/oxytocin receptor function. *Journal of Endocrinology*, 156(2), 223–229.

Barker D.J.P. 1998, In Utero programming of chronic disease. *Clin. Sci*. 95, 115−128.

Barnes A.I. & Siva−Jothy M.T. 2000. Density–dependent prophylaxis in the mealworm beetle *Tenebrio molitor L*.(Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proc R Soc Lond Biol*, 267(1439):177−182.

Batuev A.S., Poliakova O.N. & Aleksandrov A.A. 1999. The effect of" social stress" during rat pregnancy on the anxiety level of the progeny. Zhurnal vysshei nervnoi deiatelnosti imeni IP Pavlova, 50(2), 281–286.

Bird A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev*, 16(1): 6−21.

Bossdorf O., Richards C.L. & Pigliucci M. 2008. Epigenetics for ecologists. *Ecol Lett*, 11(2): 106−115.

Burdge G.C., Slater−Jefferies J., Torrens C., Phillips E.S., Hanson M.A., & Lillycrop K.A. 2007. Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation

of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *Br J Nutr*, 97e (03), 435−439.

Carter C.S., Boone E.M., Pournajafi−Nazarloo H. & Bales K.L. 2009. Consequences of early experiences and exposure to oxytocin and vasopressin are sexually dimorphic. *Dev Neurosci*, 31(4): 332−341.

Champagne F.A. 2008 "Epigenetic mechanisms and the transgenerational effects of maternal care." *Frontiers in neuroendocrinology* 29.3: 386–397.

Champagne F.A., Weaver I.C., Diorio J., Dymov S., Szyf M. & Meaney M.J. 2006. Maternal care associated with methylation of the estrogen receptor−α1b promoter and estrogen receptor−α expression in the medial preoptic area of female offspring. *Endocrinology*, 147(6): 2909−2915.

Christian J.J. 1971a. Fighting, maturity, and population density in *Microtus pennsylvanicus*. *J Mammal*: 556−567.

Christian J.J. 1971b. Population density and reproductive efficiency. *Biology of Reproduction*, 4(3), 248–294.

Clark S.J. & Melki J. 2002. DNA methylation and gene silencing in cancer: which is the guilty party?. *Oncogene*, 21(35): 5380−5387.

Clarke J.R. 1953. The effect of fighting on the adrenals, thymus and spleen of the vole (*Microtus agrestis*). *J Endocrinol*, 9(1): 114−126.

Cushing B.S. & Carter C.S. 2000. Peripheral pulses of oxytocin increase partner preferences in female, but not male, prairie voles. Hormones and behavior, 37(1), 49–56.

Dolinoy D.C. 2008. The agouti mouse model: an epigenetic biosensor for nutritional and environmental alterations on the fetal epigenome. *Nutr Res*, 66(s1): S7−S11.

Dudley S.A. & Schmitt J. 1996. Testing the adaptive plasticity hypothesis: density− dependent selection on manipulated stem length in Impatiens capensis. *Amer Nat*, 445−465.

Dumais K.M. & Veenema A.H. 2016. Vasopressin and oxytocin receptor systems in the brain: sex differences and sex–specific regulation of social behavior. Frontiers in neuroendocrinology, 40, 1–23.

Eccard J.A. & Ylönen H. 2001. Initiation of breeding after winter in bank voles: effects of food and population density. *Canadian Journal of Zoology*, 79(10), 1743–1753.

Feinberg A.P. & Irizarry R.A. 2010. Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease. *Proceedings of the National Academy of Sciences*, 107(suppl 1), 1757–1764.

Francis D., Diorio J., Liu D. & Meaney M.J. 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science, 286(5442), 1155–1158.

Francis D.D., Young L.J., Meaney M.J. & Insel T.R. 2002. Naturally occurring differences in maternal care are associated with the expression of oxytocin and vasopressin (V1a) receptors: gender differences. Journal of neuroendocrinology, 14(5), 349–353.

Franklin T.B., Russig H., Weiss I.C., Gräff J., Linder N., Michalon A. ... & Mansuy I.M. 2010. Epigenetic transmission of the impact of early stress across generations. *Biol Psychiatry*, 68(5): 408−415.

Frésard L., Morisson M., Brun J.M., Collin A., Pain B., Minvielle F. & Pitel F. 2013. Epigenetics and phenotypic variability: some interesting insights from birds. *Genetics Selection Evolution*, 45(1): 16.

Gardiner−Garden M. & Frommer M. 1987. CpG islands in vertebrate genomes. *J Mol Biol*, 196(2): 261−282.

Gimpl G. & Fahrenholz F. 2001. The oxytocin receptor system: structure, function, and regulation. *Physiological reviews*, 81(2), 629–683.

Girolami L., Fontani G., Lodi L. & Lupo C. 1996. Hippocampal cholineacetyltransferase activity, agonistic behaviour and social stress in male rabbits. Behavioural processes, 36(1), 77–84.

Glazier A.M., Nadeau J. H., & Aitman T.J. 2002. Finding genes that underlie complex traits. *Science*, 298(5602): 2345−2349.

Gustafsson T.O., Andersson C.B. & Westlin L.M. 1983. Reproduction in laboratory colonies of bank vole, Clethrionomys glareolus, originating from populations with different degrees of cyclicity. *Oikos*, 182–188.

Gutkowska J., Jankowski M., Mukaddam–Daher S., & McCann S.M. 2000. Oxytocin is a cardiovascular hormone. *Brazilian Journal of Medical and Biological Research*, 33(6), 625–633.

Kaiser S. & Sachser N. 2005. The effects of prenatal social stress on behaviour: mechanisms and function. *Neuroscience & Biobehavioral Reviews*, *29*(2), 283–294.

Kawata M. 1985. Mating system and reproductive success in a spring population of the red–backed vole, Clethrionomys rufocanus bedfordiae. *Oikos*, 181–190.

Kimura T. & Ivell R. 1999. The oxytocin receptor. *Regulatory Peptides and Cognate Receptors,* 135–168. Springer Berlin Heidelberg.

Kimura T., Saji F., Nishimori K., Ogita K., Nakamura H., Koyama M. & Murata Y. 2003. Molecular regulation of the oxytocin receptor in peripheral organs. Journal of molecular endocrinology, 30(2), 109–115.

Kirschbaum C., Pirke K.M. & Hellhammer D.H. 1993. The 'Trier Social Stress Test'–a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology*, 28(1–2), 76–81.

Haldane J.B.S. 1937. The effect of variation of fitness. *American Naturalist*: 337−349.

Heinrichs M., Baumgartner T., Kirschbaum C. & Ehlert U. 2003. Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress. *Biological psychiatry*, 54(12), 1389–1398.

Illingworth R.S. & Bird A.P. 2009. CpG islands–'a rough guide'. *FEBS Lett*, 583(11): 1713−1720.

Inchausti P. & Ginzburg L.R. 2009. Maternal effects mechanism of population cycling: a formidable competitor to the traditional predator–prey view. *Philos Trans R Soc London [Biol]*, 364(1520): 1117−1124.

Insel T.R. & Young L.J. 2000. Neuropeptides and the evolution of social behavior. *Current opinion in neurobiology*, 10(6), 784–789.

Ioshikhes I.P. & Zhang M.Q. 2000. Large−scale human promoter mapping using CpG islands. *Nature Gen*, 26(1): 61−63.

Jaenisch R. & Bird A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Gen*, 33: 245−254.

Johannesen E. 2003. Intra−and interspecific density dependence in the survival and recruitment of grey−sided (*Clethrinomys rufocanus*) and bank voles (*C. glareolus*). *In Annales Zoologici Fennici* 40 (1): 35−44.

Johannesen E. & Andreassen H.P. 2008. Density–dependent variation in body mass of voles. *Acta theriologica*, 53(2), 169–173.

Jones P.A. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, 13(7), 484–492.

Jones P.A. & Takai D. 2001. The role of DNA methylation in mammalian epigenetics. *Science*, 293(5532): 1068−1070.

Jones P.A. & Liang G. 2009. Rethinking how DNA methylation patterns are maintained. *Nature Reviews Genetics*, 10(11), 805–811.

Jones P.A. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, 13(7), 484–492.

Jones P.L., Veenstra G.C.J., Wade P.A., Vermaak D., Kass S.U., Landsberger N., ... & Wolffe A. P. 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet*, 19(2): 187−191.

Keverne E. 1995. Neurochemical changes accompanying the reproductive process: their significance for maternal care in primates and other mammals. *Motherhood in Human and Nonhuman Primates: Biosocial Determinants* (pp. 69–77).

Korpela K., Sundell J. & Ylönen H. 2011. Does personality in small rodents vary depending on population density?. *Oecologia*, 165(1): 67−77.

Korpimäki E., Norrdahl K., Huitu O. & Klemola T. 2005. Predator–induced synchrony in population oscillations of coexisting small mammal species. Proceedings of the Royal Society of London B: Biological Sciences, 272(1559), 193–202.

Kotlík P., Deffontaine V., Mascheretti S., Zima J., Michaux J.R. & Searle J.B. 2006. A northern glacial refugium for bank voles (Clethrionomys glareolus). *Proceedings of the National Academy of Sciences*, 103(40), 14860–14864.

Krebs C.J. 1970. Microtus population biology: behavioral changes associated with the population cycle in *M. ochrogaster* and *M. pennsylvanicus. Ecology*: 34−52.

Kruuk L.E., Clutton–Brock T.H., Albon S.D., Pemberton J.M. & Guinness F.E. 1999. Population density affects sex ratio variation in red deer. *Nature*, 399(6735), 459–461.

Kumsta R., Hummel E., Chen F.S. & Heinrichs M. 2013. Epigenetic regulation of the oxytocin receptor gene: implications for behavioral neuroscience. *Frontiers in neuroscience*, 7.

Kurashige N.S. & Agrawal A.A. 2005. Phenotypic plasticity to light competition and herbivory in *Chenopodium album* (Chenopodiaceae). *Am J Bot*, 92(1): 21−26.

Landgraf R. & Neumann I.D. 2004. Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. *Front Neuroendocrinol*, 25(3): 150−176.

Ledón−Rettig C.C., Richards C.L. & Martin L.B. 2013. Epigenetics for behavioral ecologists. *Behav Ecol*, 24(2): 311−324.

Lee P.R., Brady D.L., Shapiro R.A., Dorsa D.M. & Koenig J.I. 2007. Prenatal stress generates deficits in rat social behavior: reversal by oxytocin. *Brain research*, 1156, 152–167.

Lee E., Luo J., Wilson J., Shi H. 2013. Analyzing the cancer methylome through targeted bisulfite sequencing. *Cancer Lett* 340: 171−178.

Lemon B. & Tjian R. 2000. Orchestrated response: a symphony of transcription factors for gene control. *Genes Dev*, 14(20): 2551−2569.

Lester B.M., Conradt E. & Marsit C.J. 2013. Epigenetic basis for the development of depression in children. *Clinical obstetrics and gynecology*, 56(3), 556.

Loh J., Lohar D.P., Andersen B. & Stacey G. 2002. A two−component regulator mediates population−density−dependent expression of the *Bradyrhizobium japonicum* nodulation genes. *J Bacteriol*, 184(6): 1759−1766.

Lokk K., Modhukur V., Rajashekar B., Märtens K., Mägi R., Kolde R., ... & Tõnisson N. 2014. DNA methylome profiling of human tissues identifies global and tissue–specific methylation patterns. *Genome biology*, 15(4), 3248.

Mappes T. & Ylönen H. 1997. Reproductive effort of female bank voles in a risky environment. *Evolutionary Ecology*, *11*(5), 591–598.

Martienssen R.A. & Colot V. 2001. DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science*, 293(5532): 1070−1074.

Maxwell A., McCudden C.R., Wians F. & Willis M.S. 2009. Recent advances in the detection of prostate cancer using epigenetic markers in commonly collected laboratory samples. *Lab Medicine*, 40(3), 171–178. http

Meaney M.J. 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annual review of neuroscience*, 24(1), 1161–1192.

Meyer–Lindenberg A., Domes G., Kirsch P. & Heinrichs M. 2011. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. *Nature Reviews Neuroscience*, 12(9), 524–538.

Mills S.C., Alatalo R.V., Koskela E., Mappes J., Mappes T. & Oksanen T. A. 2007. Signal reliability compromised by genotype-by-environment interaction and potential mechanisms for its preservation. *Evolution*, 61(7): 1748−1757.

Mousseau T.A. & Fox C.W. 1998. The adaptive significance of maternal effects. *Trends Ecol Evolut*, 13(10): 403−407.

Neumann I.D. 2002. Involvement of the brain oxytocin system in stress coping: interactions with the hypothalamo–pituitary–adrenal axis. *Prog Brain Res* 2002; 139: 147–162.

Neumann I.D. 2008. Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. *Journal of neuroendocrinology*, *20*(6), 858–865.

Norrdahl K., & Korpimäki E. 2002. Changes in population structure and reproduction during a 3-yr population cycle of voles. *Oikos*, 96(2), 331–345.

Novikova S.I., He F., Bai J., Cutrufello N.J., Lidow M.S. & Undieh A.S. 2008. Maternal cocaine administration in mice alters DNA methylation and gene expression in hippocampal neurons of neonatal and prepubertal offspring. *PloS one*, 3(4).

Oksanen T.A., Koivula M., Koskela E. & Mappes T. 2007. The cost of reproduction induced by body size at birth and breeding density. *Evolution*, 61(12): 2822−2831.

Ponger L. & Li W.H. 2005. Evolutionary diversification of DNA methyltransferases in eukaryotic genomes. *Mol Biol Evol*, 22(4): 1119−1128.

Radchuk V., Ims R.A. & Andreassen H.P. 2016. From individuals to population cycles: the role of extrinsic and intrinsic factors in rodent populations. *Ecology*, 97(3), 720–732.

Rakyan V.K., Blewitt M.E., Druker R., Preis J.I. & Whitelaw E. 2002. Metastable epialleles in mammals. *Trends in Genetics*, 18(7), 348–351.

Razin A. 1998. CpG methylation, chromatin structure and gene silencing—a three-way connection. *The EMBO journal*, 17(17): 4905−4908.

Reed D.H. & Frankham R. 2003. Correlation between fitness and genetic diversity. *Conserv Biol*, 17(1): 230−237.

Richards C.L., Bossdorf O. & Verhoeven K. J. 2010. Understanding natural epigenetic variation. *New Phytol*, 187(3): 562−564.

Rossiter M. 1996. Incidence and consequences of inherited environmental effects. *Annu Rev Ecol Syst*: 451−476.

Sachser N., Hennessy M.B. & Kaiser S. 2011. Adaptive modulation of behavioural profiles by social stress during early phases of life and adolescence. *Neuroscience & Biobehavioral Reviews*, 35(7), 1518–1533.

Saxonov S., Berg P. & Brutlag D.L. 2006. A genome−wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *PNAS*, 103(5): 1412−1417.

Schmid B. & Dolt C. 1994. Effects of maternal and paternal environment and genotype on offspring phenotype in *Solidago altissima L. Evolution*: 1525−1549.

Schneider M.L., Moore C.F., Kraemer G.W., Roberts A.D. & DeJesus O.T. 2002. The impact of prenatal stress, fetal alcohol exposure, or both on development: perspectives from a primate model. *Psychoneuroendocrinology*, 27(1), 285–298.

Schroderus E., Jokinen I., Koivula M., Koskela E., Mappes T., Mills S.C., ... & Poikonen T. 2010. Intra-and intersexual trade-offs between testosterone and immune system: implications for sexual and sexually antagonistic selection. *Am Nat*, 176(4): E90−E97.

Schroderus E., Koivula M., Koskela E., Mappes T., Oksanen T.A. & Poikonen T. 2012. Can number and size of offspring increase simultaneously?−a central life−history trade−off reconsidered. *BMC Evol Biol*, 12(1): 44.

Smith Z.D. & Meissner A. 2013. DNA methylation: roles in mammalian development. Nature Reviews Genetics, 14(3), 204–220.

Sohlström A., Carlsson C. & Uvnäs–Moberg K. 2000. Effects of oxytocin treatment in early life on body weight and corticosterone in adult offspring from ad libitum–fed and food–restricted rats. *Neonatology*, 78(1), 33–40.

Sokolowski M.B., Pereira H.S. & Hughes K. 1997. Evolution of foraging behavior in *Drosophila* by density−dependent selection. *PNAS*, 94(14): 7373−7377.

Steen H., Yoccoz N.G. & Ims R.A. 1990. Predators and small rodent cycles: an analysis of a 79–year time series of small rodent population fluctuations. *Oikos*, 115–120.

Stefanski V., Raabe C. & Schulte M. 2005. Pregnancy and social stress in female rats: influences on blood leukocytes and corticosterone concentrations. *Journal of neuroimmunology*, 162(1), 81–88.

Svensson E. & Sinervo B. 2000. Experimental excursions on adaptive landscapes: density-dependent selection on egg size. *Evolution*, 54(4): 1396−1403.

Takai D. & Jones P.A. 2002. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl Acad. Sci.* USA 99, 3740–3745.

Vallee M., Mayo W., Dellu F., Le Moal M., Simon H. & Maccari S. 1997. Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress–induced corticosterone secretion. *Journal of neuroscience*, 17(7), 2626–2636.

Van Zegeren K. 1980 Variation in aggressiveness and the regulation of numbers in house mouse populations. *Netherlands JournaI of Zoology* 30: 635−770.

Via S. & Lande R. 1985. Genotype−environment interaction and the evolution of phenotypic plasticity. *Evolution*: 505−522.

Weaver I.C., Cervoni N., Champagne F.A., D'Alessio A.C., Sharma S., Seckl J.R., ... & Meaney M.J. 2004. Epigenetic programming by maternal behavior. *Nature neuroscience*, 7(8), 847–854.

Weaver I.C., Champagne F.A., Brown S.E., Dymov S., Sharma S., Meaney M.J. & Szyf M. 2005. Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *J Neurosci*, 25(47): 11045−11054.

Weber M., Davies J.J., Wittig D., Oakeley E.J., Haase M., Lam W.L. & Schuebeler D. 2005. Chromosome−wide and promoter−specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nature Genet*, 37(8): 853−862.

West−Eberhard M.J. 1989. Phenotypic plasticity and the origins of diversity. *Annu Rev Ecol Syst* 20: 249−278.

Widiker S., Kärst S., Wagener A. & Brockmann G.A. 2010. High−fat diet leads to a decreased methylation of the Mc4r gene in the obese BFMI and the lean B6 mouse lines. *Journal of applied genetics*, 51(2): 193−197.

Wilhelm–Benartzi C.S., Houseman E.A., Maccani M.A., Poage G.M., Koestler D.C., Langevin S.M., ... & Marsit C.J. 2012. In utero exposures, infant growth, and DNA methylation of repetitive elements and developmentally related genes in human placenta. *Environmental health perspectives*, 120(2), 296.

Wolff G.L., Kodell R.L., Moore S.R. & Cooney C.A. 1998. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. *The FASEB Journal*, 12(11): 949−957.

Wu H. & Zhang Y. 2014. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell*, 156(1), 45–68.

Yokozawa M. & Hara T. 1995. Foliage profile, size structure and stem diameter–plant height relationship in crowded plant populations. *Annals of Botany*, *76*(3), 271–285.