JYVÄSKYLÄ STUDIES IN BIOLOGICAL AND ENVIRONMENTAL SCIENCE
327

Eija Lönn

Selection on Two Behavioral Genes

Fitness Effects of Receptor Genes for Arginine Vasopressin 1a and Oxytocin in the Bank Vole *Myodes glareolus*

JYVÄSKYLÄN YLIOPISTO

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Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston Konneveden tutkimusasemalla
helmikuun 10. päivänä 2017 kello 14.

Academic dissertation to be publicly discussed, by permission of
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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2017

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Publishing Unit, University Library of Jyväskylä

Jyväskylä Studies in Biological and Environmental Science

Permanent link to this publication: http://urn.fi/URN:ISBN:978-951-39-6969-1

URN:ISBN:978-951-39-6969-1
ISBN 978-951-39-6969-1 (PDF)

ISBN 978-951-39-6968-4 (nid.)
ISSN 1456-9701

Jyväskylä University Printing House, Jyväskylä 2017

ABSTRACT

Lönn, Eija
Selection on two behavioral genes: fitness effects of receptor genes for arginine vasopressin 1a and oxytocin in the bank vole *Myodes glareolus*
Jyväskylä: University of Jyväskylä, 2017, 42 p.
(Jyväskylä Studies in Biological and Environmental Science
ISSN 1456-9701; 327)
ISBN 978-951-39-6968-4 (nid.)
ISBN 978-951-39-6969-1 (PDF)
Yhteenveto: Valintamekanismit käyttäytymistä säätelevien geenien taustalla: arginiini vasopressiini 1a- ja oksitosiinireseptorigeenien kelpoisuusvaikutukset metsämyyrällä (*Myodes glareolus*)
Diss.

Most variation in behavior is regulated by genes; nevertheless the mechanisms behind maintenance of genetic diversity at behavioral loci have remained mainly elusive in natural populations. I studied in my thesis selection mechanisms of two genes associated with socio-sexual behavior, arginine vasopressin receptor 1a (*Avpr1*) and oxytocin receptor (*Oxtr*) in bank vole (*Myodes glareolus*). Expression of *Avpr1a* and *Oxtr* in specific regions of the brain regulates diverse social and reproductive behaviors such as parental care, aggression, sexual behavior, social recognition as well as pair and parent-offspring bonding in mammals. In addition, there is a link between the length of a regulatory region associated microsatellite (RRAM) of *Avpr1* and receptor density in the brain in some species. Bank voles have highly polymorphic RRAM in *Avpr1a* and *Oxtr*. My first study revealed that different length variants of RRAM allele in *Avpr1* and *Oxtr* genes are maintained by balancing selection; more precisely the selection is sex-specific and dependent on population density. Different allele lengths were favored in males compared to females indicating that *Avpr1a* and *Oxtr* are sexually antagonistic (SA) genes. My second study showed that intrasexual competition in both males and females drives selection of *Avpr1a* RRAM lengths. My third study evidenced that bank voles bias their offspring sex ratio to mitigate sexual conflict caused by the SA *Oxtr* gene. My fourth study showed that individuals with the extreme lengths of *Avpr1* and *Oxtr* RRAMs experience constraints in breeding success indicating stabilizing selection. In conclusion, my thesis provides evidence for diverse mechanisms acting on genetic diversity of behavioral genes.

Keywords: *Avpr1a*; bank vole; behavioral genes; *Myodes glareolus*; *Oxtr*; selection.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV. I am the first author in papers I and II and carried out a large portion of planning and data collection in all the papers. In addition, I conducted substantial portion of statistical analyses of papers I-III.

I Lonn E., Koskela E., Mappes T., Mokkonen M., Sims A.M. & Watts P.C. 2017. Balancing selection maintains polymorphisms at neurogenetic loci in field experiments. Submitted manuscript.

II Lonn E., Koskela E., Mappes T., Mokkonen M. & Watts P.C. 2017. Intrasexual competition drives the pattern of balancing selection at arginine vasopressin receptor 1a. Manuscript.

III Mokkonen M., Koskela E., Lonn E., Mappes T. & Watts P.C. 2017. Cryptic bias of a sexually antagonistic locus in a small mammal. Manuscript.

IV Watts P.C., Kallio E., Koskela E., Lonn E., Mappes T. & Mokkonen M. 2017. Stabilising selection on microsatellite allele length. Manuscript.

1 INTRODUCTION

1.1 Social behavior

1.1.1 Diversity in social behavior regulated by genes

Social behavior describes all activities that are directed towards members of the same species - in the other words, activities become social when they involve interactions among conspecifics (Robinson *et al.* 2008). Individual social behaviors are common, and important for success, in sexual reproduction. Social interactions related to reproduction may manifest as aggression, competition, communication, dominance and parental care.

For a long time, there has been a debate about the extent to which social behaviors have a genetic basis (Robinson 2004, Shaw and Wiley 2010). Nevertheless, a large body of evidence indicates that social behavior has genetic basis, (Rankin 2002, Robinson *et al.* 2005, Robinson *et al.* 2008, Smith *et al.* 2008, Gadau and Hunt 2009, Shaw and Wiley 2010). Moreover, it appears that many social behaviors, for example foraging behavior (Sokolowski *et al.* 1997), aggressive behavior (Cases *et al.* 1995) or mate recognition (Enard *et al.* 2002), are regulated by one or few loci (Table 1).

Inter- and intra-specific diversity in social behavior may be driven by genetic changes that alter protein structure or function, and/or by genetic or epigenetic changes that affect gene expression (Preuss *et al.* 2004, Li *et al.* 2016). Genetic regulation of social behavior is a taxonomically diverse phenomenon; it is shown in species from yeast and worms to rodents and humans (Robinson *et al.* 2008). For instance, in *Caenorhabditis elegans* a one nucleotide change in the *npr-1* neuropeptide receptor gene alters protein structure and determines whether an individual is a solitary or social feeder (De Bono and Bargmann 1998). Conversely, in humans, the length of a microsatellite in the regulatory region of the *NOS1* gene is associated with impulsive behavior (Rife *et al.* 2009, Retz *et al.* 2010).

It is clear that many social behaviors have a genetic underpinning and that there is growing evidence that certain genes exert a profound influence on social behavior in field and lab populations.

TABLE 1 Examples of social behaviors that are regulated by a single gene.

Behavior	Gene	Organism	Reference
Rover versus sitter phenotype	*foraging*	*Drosophila melanogaster*	(Sokolowski *et al.* 1997)
Solitary versus social feeding	*npr-1* (neuropeptide receptor family 1)	*Caenorhabditis elegans*	(De Bono and Bargmann 1998)
Aggression	*MAOA* (monoamine oxidase A)	*Mus musculus domesticus*	(Cases *et al.* 1995)
Cooperative behavior, cell adhesion	*FLO1*	*Saccharomyces cerevisiae*	(Smukalla *et al.* 2008)
Vocal learning; vocalization	*FOXP2* (winged helix/forkhead protein)	*Taeniopygia guttata; Homo sapiens*	(Enard *et al.* 2002)
Male courtship	*period*	*Drosophila melanogaster*	(Wheeler *et al.* 1991)
Impulsive behavior	*NOS1* (nitric oxide synthase 1)	*Homo sapiens*	(Retz *et al.* 2010)

1.1.2 *Avpr1a* and *Oxtr* regulate social behavior

Concerning genes that affect social behavior, there are genes of two hormones and their receptors that have become extensively studied; they are vasopressin and oxytocin (and their homologs: vasotocin and isotocin in non-mammalian species). Vasopressin, oxytocin and their receptors have a common decent, having arisen from duplication event approximately over 100 million years ago (Yamashita and Kitano 2013). Vasopressin is highly conserved in mammalian species (Donaldson and Young 2008) whereas oxytocin has six different variants found in primates and tree shrews (Vargas-Pinilla *et al.* 2015, Ren *et al.* 2015). In the brain, evolutionarily conserved vasopressin and oxytocin hormones operate through their receptor genes arginine vasopressin 1a (*Avpr1a*) and oxytocin receptor (*Oxtr*), respectively. Vasopressin also has two additional receptors, arginine vasopressin receptor 1b (V1bR) and vasopressin 2 receptor (V2R) (Caldwell and Young III 2006). V2R is not linked to behavior or located in the brain but V1bR is located in the brain and this receptor has behavioral implications related, for example, to aggression and social memory

in rodents (Roper *et al.* 2011). In addition, there is potential cross-reaction so that vasopressin hormone binds to oxytocin receptor (OTR) at some affinity and oxytocin binds to V1aR also at low affinity (Åkerlund *et al.* 1999).

Vasopressin and its receptor arginine vasopressin 1a (V1aR), as well as oxytocin and the OTR, are involved in processing social information in the brain and translating it into social behavior (Insel 2010). Variation in the distribution and density of V1aR and OTR within and between species affect social behavior (Caldwell and Young III 2006, Donaldson and Young 2008, Ophir *et al.* 2012). Different brain areas are linked to different social behaviors including parental care, aggression, sexual behavior, social recognition and pair and parent-offspring bonding in rodents and primates including humans (Caldwell and Young III 2006). Oxytocin can affect how much affiliation an individual provides (Feldman 2012) but also how much it receives. Marmoset females that were treated with oxytocin received more affiliative behavior than females treated with saline (control) from their partners (Cavanaugh *et al.* 2015). This result suggests that oxytocin can influence individuals to give delicate signals that induce their partner to show more affiliative behavior, for instance, grooming.

The genetic basis of V1aR density variation and its associated effect on behavior has been intensively studied in microtine voles (Hammock and Young 2005, Ophir *et al.* 2008, Donaldson and Young 2013, Okhovat *et al.* 2015). The gene-brain-behavior model shows how variation in the regulatory region associated microsatellite (RRAM) in the *Avpr1a* gene affects gene expression of this gene in specific brain regions and confers concomitant behavior. There is an association of longer *Avpr1a* RRAM alleles, a higher level of gene expression in a reporter gene assay, and V1aR density in the olfactory bulb and rostral forebrain with partner preference and male parental care in prairie voles (Hammock and Young 2004, Hammock and Young 2005). A previous study argued that the presence of the *Avpr1a* RRAM allele might have led to a monogamous mating strategy in voles (Young *et al.* 1999). However, more recent investigation of the *Avpr1a* locus in various species of vole and in species of deer-mice (*Peromyscus* sp.) has not found any connection between absence or presence of *Avpr1a* RRAM allele and monogamy (Fink *et al.* 2006, Turner *et al.* 2010). *Avpr1a* RRAM allele length affects reproductive success of rodents in the laboratory (Castelli *et al.* 2011) and in some (Solomon *et al.* 2009, Harris *et al.* 2014), but not all (Ophir *et al.* 2008), field experiments. Contrary to *Avpr1a*, no RRAM in the oxytocin receptor gene (*Oxtr)* has been associated with behavior in any species so far, however the area about 1 kbp upstream of *Oxtr* is crucial in the regulation of the expression of this gene (Inoue *et al.* 1994). However, many single nucleotide polymorphisms (SNPs) in introns or the 3' untranslated region of *Oxtr* are linked to social behavior, especially in humans (Skuse *et al.* 2013, King *et al.* 2015).

Previously, vasopressin and oxytocin were thought to have sex-specific effects so that vasopressin affects only behaviors important for males and oxytocin only for behaviors important for females. Nevertheless, subsequent studies have revealed that the effects of vasopressin and oxytocin can be either

opposing between the sexes, stronger in one of the sexes or the same for both sexes (Dumais and Veenema 2015). Gonadal hormones play role in conferring differences in effects between the sexes. Estrogens regulate synthesis of oxytocin and its receptor and androgens regulate synthesis of vasopressin and its receptor (Skuse and Gallagher 2009). An example of an opposing effect between the sexes is the manipulation of the vasopressin system in the lateral septum that produced opposite effects on social play behavior in rats (Bredewold *et al.* 2014). Sex-specific effects of vasopressin have been shown in a study where V1aR blockade impaired partner preference in male voles only (Winslow *et al.* 1993, Insel and Hulihan 1995). However, another study found that an administered V1aR antagonist in the lateral septum deteriorated social recognition in both sexes of adult rats (Veenema *et al.* 2012).

1.2 Microsatellites in gene promoters

Promoters regulate gene expression by initiating transcription which is the first stage of gene expression (Alberts *et al.* 2008). Many eukaryotes have microsatellites in their promoters (Sawaya *et al.* 2013, Abe and Gemmell 2014). For example, approximately 25 % of *Saccharomyces cerevisiae* yeast genes contain a microsatellite in their promoter (Vinces *et al.* 2009), while in humans, microsatellites are situated in promoters more often than expected by chance (Sawaya *et al.* 2013).

Microsatellites are regions of DNA where a short sequence motif (between 2-6 bp) is repeated in tandem (Jarne and Lagoda 1996, Ellegren 2000, Chambers and MacAvoy 2000, Selkoe and Toonen 2006), typically for some 10s to 100s of bp in total. Microsatellites are rarely situated in coding regions (i.e. they are rarely transcribed, but there are exceptions such the locus responsible for Huntington's Disease) (Gemayel *et al.* 2010, Labbadia and Morimoto 2013), and thus have been traditionally considered to be largely nonfunctional, so-called "junk" DNA. However, this view is over-simplistic and outmoded since new evidence shows that various microsatellites have genomic functions (Fondon III *et al.* 2008, Gemayel *et al.* 2010). There is an association between microsatellites and gene regulatory regions, and notably transcription factor binding sites (TFBSs) as enhancers and silencers. This implies that microsatellite loci may modulate levels of gene expression, for example, by the length of the allele that determines the position and thus, efficacy of the TFBS (Schmidt and Anderson 2006, Sawaya *et al.* 2013). In this way, microsatellite loci are hypothesized to function as "tuning knobs" of gene expression providing a large range of different phenotypes, potentially enabling efficient adaptation (King *et al.* 2006).

That microsatellites located in promoter regions affect gene expression has been widely reported (Vinces *et al.* 2009, Sawaya *et al.* 2012). In bacteria, the length of alleles at promoter-associated microsatellites can switch genes on and off in a length dependent manner (Moxon *et al.* 2006, Gymrek *et al.* 2015). In tilapia fish, variation in the length of the promoter microsatellite in the prolactin

gene regulates salt tolerance (Streelman and Kocher 2002), while an $(AG)_n$ microsatellite upstream of the gene encoding for malic enzyme affects gene expression in the chicken (Xu and Goodridge 1998). In *Saccharomyces cerevisiae* yeast, the length of a microsatellite in the promoter is intricately linked to the level of gene expression (Vinces *et al.* 2009). Gene expression was highest with the intermediate lengths of those microsatellites and the lowest with the extreme microsatellite lengths. In humans, a change in the length of the promoter microsatellite in the neural genes *NOS1* and *PAX6* results in a change in gene expression (Sawaya *et al.* 2012), and longer alleles at both promoter-associated microsatellite loci increase gene expression in reporter gene assays (Rife *et al.* 2009). *NOS1* encodes nitric-oxidase synthase that generates the signaling molecule nitric oxide (NO). PAX6 is related to eye, nose and neural development (Grindley *et al.* 1995, Larsen *et al.* 2010). Furthermore, pathological effects of genetic variation at these loci are apparent and consistent with changes in gene expression. Short microsatellite alleles at *NOS1* are linked with impulsivity, including hyperactive and aggressive behaviors, supposedly because of a decrease in NO signaling in the brain (Retz *et al.* 2010), whereas longer *PAX6* promoter microsatellites predispose individuals to myopia (Ng *et al.* 2009).

Details of mechanisms underlying the association between allele length and gene expression remain largely unknown, though in theory a number of processes may allow regulatory region-associated microsatellites to modulate gene expression. Alteration of chromatin structure is thought to be a common mechanism (Morris *et al.* 2010). In addition to allele length, nucleotide composition of the microsatellite sequence appears to impact gene expression (Li *et al.* 2002, Sawaya *et al.* 2013). In particular, GA repeats are able to change DNA structure from normal double helix to triple helix (H-DNA) and to bind GAGA transcription factors (TFs). H-DNA structure allows chromatins to open and enables TFs to bind to neighboring regulatory sequences (Morris *et al.* 2010). Expansion and contraction of microsatellites causes TFBSs located in these DNA sequences to change their location and spacing. The specific location and distance between TFBSs is important in the interaction of these sequences in the regulation of gene expression (Vardhanabhuti *et al.* 2007), thus, this is suggested to be one mechanism how microsatellite length affects gene expression.

Given that many factors can have an effect on how microsatellites affect gene expression, it is clear that regulation of gene expression is often unpredictable based on length per se, but rather depends on cell-type, location of microsatellite and characters of TFs involved in expression of each gene. Thus, gene expression levels can increase (Okladnova *et al.* 1998, Rife *et al.* 2009) or decrease (Nowling *et al.* 2008, Morris *et al.* 2010) when microsatellite allele length increases or gene expression can decrease in one cell-type but decrease in the other one (Hammock and Young 2004, Hammock and Young 2005). In addition, the expression pattern can be nonlinear when compared to microsatellite length (Uhlemann *et al.* 2004, Vinces *et al.* 2009).

Microsatellites tend to mutate very frequently (10^{-2} to 10^{-6} times per locus per generation) (Ellegren 2000), even up to ten thousand times faster than single nucleotides, leading to rapid evolution (Rando and Verstrepen 2007, Kelkar *et al.* 2008, Bhargava and Fuentes 2010, Kelkar *et al.* 2011). Microsatellites expand or contract by DNA slippage or recombination which cause deletions or insertions of repeats (Fan and Chu 2007, Gemayel *et al.* 2010).

1.3 Selection

1.3.1 How selection functions

Selection changes the frequency of alleles in a population depending on the fitness effects on the individuals. Selection acts on the individual's phenotype which is determined by a combination of alleles – the genotype. Selection can decrease variation in allele frequency by stabilizing or directional selection or variation can be increased by balancing selection (Bell 2008). There are various mechanisms of balancing selection such as density-dependent selection (Sokolowski *et al.* 1997), sexual antagonism (Chippindale *et al.* 2001, Wedell *et al.* 2006), negative frequency-dependent selection (Mokkonen *et al.* 2011) and heterozygote advantage (Penn *et al.* 2002). Nevertheless, this thesis concentrates on two of these mechanisms: density-dependent selection and sexually antagonistic selection.

1.3.2 Density-dependent selection

Population density is regulated by reproduction, survival and movement of individuals; these processes are generally the result of social interactions, where population density has an effect on frequency of encounters and the intensity of competition (Alonzo and Sheldon 2010). Competition for resources or mates varies according to the population density, which impacts individual fitness (Sutherland 1996, Grant 1997, Sutherland and Norris 2002).

In density-dependent selection (Pimentel 1961, Travis *et al.* 2013), genotypes that are favored at low population density differ from those favored at high population density (Travis *et al.* 2013). Field (Sinervo *et al.* 2000, Levitan and Ferrell 2006, Mappes *et al.* 2008, Bassar *et al.* 2013, Farkas and Montejo-Kovacevich 2014) and laboratory studies (Mueller 1997, Borash *et al.* 1998) provide evidence of density-dependent selection on different genotypes.

Density-dependent selection may give rise to a feedback loop between ecological and evolutionary processes (Schoener 2011). The Chitty hypothesis (also called the self-regulation or the genetic control hypothesis) proposes that specific genotypes are favored and become common in a low population density which gives rise to population increase (Chitty 1967). In a high population density, different genotypes are favored and increase in frequency, which might contribute to ecological conditions that lead to a population crash

and low density, where in turn low density genotypes are favored (Sinervo, Svensson & Comendant 2000, Mappes et al. 2008). The level of aggression is one example of behavior that is selected differentially in high versus low population densities (Knell 2009). At low density, aggressive behavior is not beneficial, but in a high density, male aggressiveness becomes beneficial when encounters with other males are more frequent and lead to greater male-male competition to monopolize females (Emlen and Oring 1977). Density dependent selection also operates at individual loci that underpin behavior. In the fruit fly *Drosophila melanogaster,* the *foraging* gene controls locomotor activity in larvae in the presence of food, and has two polymorphisms, 'sitter' and 'rover' (Sokolowski *et al.* 1997). Individuals with the sitter phenotype move less during foraging and are positively selected in low density populations. Conversely, rover phenotypes move more and are favored in high density populations.

Although the importance of environmental aspects, for instance population density, in studies of sexual selection are well identified (Kokko and Rankin 2006, Candolin and Heuschele 2008), such effects have been largely neglected in studies of sexual conflict. Environmental aspects, for example population density, need to be taken into account when studying sexual conflict because the ecological context defines whether conflict occurs, as well as the associated costs and benefits of conflict (Arnqvist and Rowe 2005, Fricke *et al.* 2009, Mokkonen *et al.* 2011).

1.3.3 Sexually antagonistic selection and sexual conflict

Many traits are shared between the sexes due to almost identical genome (Pennell and Morrow 2013), but those traits may have different selection pressures in females and males because of divergent reproductive or survival strategies between the sexes (Trivers 1972, Arnqvist and Rowe 2005, Rice and Gavrilets 2014). These traits are selected by sexually antagonistic selection, which means that each sex selects for their own optimum trait value, thereby creating opposing selection between the sexes (Parker 1979, Arnqvist and Rowe 2005, Cox and Calsbeek 2009). Therefore, selection acting on one sex can prevent the other sex to reach its phenotypic optimum, simultaneously reducing its fitness (Lande 1980). Sexually antagonistic selection gives rise to sexual conflict (Parker 1979), which can be separated to intralocus (IASC) and interlocus (IRSC) sexual conflict. In IASC, also known as sexual antagonism, the conflict occurs over the alleles at the same locus between females and males, while in IRSC, the conflict occurs between different genes (Parker and Partridge 1998). An example of IASC is human height. Men of average height have the highest reproductive success and conversely shorter women have the highest reproductive success (Stulp *et al.* 2012). Thus, neither of the sexes can reach their optimum in human height. Though the most intense IASC appears when selection on a trait is opposite for the sexes, IASC exists every time when selection differs between females and males, as in the previous example, because selection on one sex restricts adaptive evolution in the other sex (Bonduriansky and Chenoweth 2009). Characteristic for IRSC is that there is

conflict over the outcome of female-male interactions such as mating rate, parental care and remating behavior. (Parker 1979). Bed bugs' (*Cimex lectularius*) mating provides an example of IRSC (Reinhardt *et al.* 2003). Males copulate by penetrating female's abdomen -the action called traumatic insemination that is deleterious to females. As a counterstrategy to mitigate immunological costs that result in traumatic insemination females have evolved a specific organ called spermalege in their abdomen.

1.3.4 Genetic basis of sexually antagonistic selection

In contrast to the many examples of phenotypic aspects that are sexually antagonistic (Fedorka and Mousseau 2004, Arnqvist and Rowe 2005, Foerster *et al.* 2007, Mokkonen *et al.* 2011, Mills *et al.* 2012), identifying one or more candidate genes associated with sexual antagonism has proven quite elusive (Pennell and Morrow 2013). Studies on *Drosophila melanogaster* have found candidate genes related to IASC (Innocenti and Morrow 2010), but specific sexually antagonistic genes are largely unknown. Indeed, several authors have claimed that it is probably too laborious to find specific, underlying single genes of sexually antagonistic traits (Fry 2010, Jordan and Charlesworth 2012, Mullon *et al.* 2012).

Despite these assertions, there are recent reports of sexually antagonistic genes related to IASC and IRSC. *Distal-less* gene, related to IRSC, in water strider males affects development of grasping appendages that are used for defeating female's resistance to mating (Khila *et al.* 2012). In addition, there are two examples of genes related to IASC in Drosophila melanogaster. Dean et al. (2012) created artificially sexually antagonistic alleles that were located on the X-chromosome; Rostant et al. (2015) identified a naturally occurring DDT-resistance allele in the *Cyp6g1* gene that decreased male fitness but increased female fecundity and survival of their offspring. Both of these aforementioned studies showed the efficiency of sexually antagonistic selection in maintaining alleles that confer opposing fitness effects for males and females. To date, there is only one corresponding finding of a SA locus in a vertebrate - the vestigial-like family member 3 gene (*VGLL3*) that has a sexually antagonistic effect on maturation age in salmon. There are two different SNP polymorphisms in *VGLL3*, which are associated with early or late maturation age (Barson *et al.* 2015).

1.3.5 Reduction of fitness costs caused by sexual conflict

Given that the sexes experience strongly divergent reproductive strategies that cause sex-specific selection on many shared traits, it is beneficial evolutionarily to favor sexual dimorphism by sex-specific expression to mitigate IASC (Hedrick and Temeles 1989, Bonduriansky and Chenoweth 2009, Pennell and Morrow 2013, Ingleby *et al.* 2014). Though, the resolution is not always easy to implement (Harano *et al.* 2010). Sex-specific expression of autosomal genes occurs via sex-specific TFs (Williams and Carroll 2009) or alternative splicing

mechanisms that are used for example in the sex-determination pathway (Lopez 1998, McIntyre *et al.* 2006). It has also been hypothesized that IASC can be resolved by gene duplication (Connallon and Clark 2011, Wyman *et al.* 2012) but it is still uncertain whether gene duplication is able to resolve IASC entirely (Hosken 2011). When genes are duplicated, the function of both duplicated genes is the same initially; however, one locus may subsequently adopt a new role as selection is relaxed (because both of the genes are not needed to fulfill the initial gene function) and this locus may accumulate mutations. In this way, the mutated gene may then adopt a sex-specific expression pattern. In *Drosophila melanogaster*, male-biased gene expression is shown more than female-biased gene expression in duplicated genes (Wyman *et al.* 2012). Other mechanisms to decrease sexual conflict include genomic imprinting that determines whether paternally or maternally inherited genes are expressed in the offspring (Day and Bonduriansky 2004, Patten and Haig 2008) and condition-dependence (Bonduriansky *et al.* 2005, Wyman *et al.* 2010). The most recent mechanism for resolving sexual conflict is dominance reversal, whereby a dominant allele in one sex is recessive in the other sex (Barson *et al.* 2015).

An alternative mechanism to reduce the costs of sexually antagonistic genes is to adjust the offspring sex ratio (Alonzo and Sinervo 2007, Fawcett *et al.* 2007, Patten and Haig 2009, Cox and Calsbeek 2010b). Under offspring sex ratio alteration, the sex ratio of the offspring can be biased based on the maternal or paternal genotype. This mechanism improves the chance that the sexually antagonistic alleles are transmitted to the sex they benefit. Theoretical models support the idea that this mechanism can partially alleviate fitness costs associated with IASC when sex-limited gene expression cannot be implemented (Blackburn *et al.* 2010). In the brown anole lizard, females produce more sons when they mate with large mates and produce more daughters when they mate with small mates (Calsbeek and Bonneaud 2008). This is consistent with the fact that in this species larger males confer higher fitness whereas females with intermediate size have the highest fitness (Cox and Calsbeek 2010a). Experimental manipulations revealed that the fitness of daughters is independent of sire size, implying that offspring sex ratio bias has not evolved to avoid the production of low fitness daughters but to confer genetic benefits that are sex-specific to sons (Cox and Calsbeek 2010b). Comparable variation in offspring sex ratios related to differential fitness effects between sexes have been reported in side blotched lizards (Calsbeek and Sinervo 2004), *Drosophila* flies (Connallon and Jakubowski 2009) and barn owls (Roulin *et al.* 2010). Conversely, in the broad-horned flour beetle, females adjust their offspring sex according to her own fitness rather than fitness of her mate; lower fitness females produced opposite-sex offspring, while females with higher fitness produced more daughters (Katsuki *et al.* 2012).

1.4 Aims of the thesis

My aim in this thesis was to study the selection of polymorphisms in two behavioral genes, *Avpr1a* and *Oxtr*, in the bank vole (*Myodes glareolus*). These genes regulate important socio-reproductive behaviors in mammals (Caldwell and Young III 2006, Donaldson and Young 2008, Walum *et al.* 2008).

The study questions per study were the following:

I. Is the maintenance of high diversity in *Avpr1a* and *Oxtr* polymorphisms maintained by density-dependent and sexually antagonistic selection in the field?

II. Is the selection of *Avpr1a* polymorphisms dependent upon intrasexual competition between males and females?

III. Does the bank vole adjust its offspring sex ratio based on the *Oxtr* polymorphism?

IV. Are the *Avpr1a* and *Oxtr* polymorphisms associated with breeding success?

Avpr1a and *Oxtr* genes have been widely studied in rodents and primates due to their effect on important socio-reproductive behaviors including parental care, aggression, sexual behavior, social recognition and pair and parent-offspring bonding. (Caldwell and Young III 2006, Donaldson and Young 2008, Walum *et al.* 2008). Nevertheless, there is a lack of knowledge about how high phenotypic and genotypic variation in these loci are maintained. I studied how variation in polymorphisms (i.e. length of regulatory region associated microsatellite –RRAM) in *Avpr1a* and *Oxtr* is maintained. I established field experiments to test if polymorphisms in these genes are maintained by density-dependent selection and sexually antagonistic selection. In addition, given the essential role of *Avpr1a* in reproductive behaviors (Hammock and Young 2005, Caldwell and Young III 2006), I studied whether the selection of *Avpr1a* RRAM allele length is dependent upon intrasexual competition between males and females in the field. Furthermore, I studied whether bank voles can adjust their offspring sex ratio associated with Oxtr genotype to mitigate sexually antagonistic fitness costs in the field. This phenomenon has not been studied much in mammalian species, and to my knowledge, there is only one field study carried out in red deer (Gomendio *et al.* 2006) in this taxon. In addition, there is a lack of knowledge of specific genes behind the control of offspring sex ratio biasing. Finally in study IV, I bred male-female pairs and assessed whether the length of their *Avpr1a* or *Oxtr* RRAM alleles had an impact on their breeding success. My aim was to explore if microsatellite allele length can be associated with constraints in breeding.

2 MATERIALS AND METHODS

2.1 Study species

The bank vole is among the most common mammalian species in Europe and it inhabits forests and fields (Hansson 1979, Stenseth 1985). Females are philopatric and defend their territories while males are more dispersive and their home ranges overlap with many female territories. Males do not care for their offspring and both sexes mate multiply (Mills *et al.* 2007, Mills *et al.* 2014). As most small rodents, bank voles experience fluctuations in population densities that change the level of competition over food and mating opportunities (Korpela *et al.* 2013). These factors have fitness consequences so that reproductive success is lowered when the population density is increased (Mappes *et al.* 2008). The trappability of the bank vole is high (>80 %) (Kallio *et al.* 2007), and they are not sensitive to handling, thus they are an optimal species for research conducted both in laboratory and in the field (Kallio *et al.* 2007, Mappes *et al.* 2008).

2.2 Regulatory region associated microsatellites in *Avpr1a* and *Oxtr* in bank vole

The bank vole has microsatellite loci in the 5′ regulatory regions (*i.e.* RRAM) of both *Avpr1a* and *Oxtr.* At *Avpr1a,* the RRAM is located about 700 bp upstream of the presumed transcription starting site and is formed of $(CA)_n$ and $(GA)_n$ dinucleotides. The bank vole *Avpr1a* RRAM positionally corresponds to the RRAM isolated from prairie voles (Hammock and Young 2005, Donaldson and Young 2013), msat1 of deer mice (*Peromyscus* sp.) (Turner *et al.* 2010) and RS1 microsatellite in primates including humans (Babb *et al.* 2010). The *Oxtr* RRAM in bank voles is composed of $(CT)_n$ and $(GA)_n$ dinucleotides which are located approximately 10 bp upstream of the *M. musculus Oxtr* transcript variant X1

(accession number XM_006505723) and 1,448 bp upstream of the *Oxtr* transcription start site in *M. musculus* (accession number NM_001081147).

Primer walking was used to find the 5′ regulatory regions of the *Avpr1a* and *Oxtr* of the bank vole. Two different pairs of primers were designed; first primers were created for the conserved areas of *Avpr1a* and *Oxtr* genes based on the data of other rodent species (prairie vole *Microtus ochrogaster*; accession number HQ156469; montane vole *Microtus montanus,* accession number GU954414.1; house mouse *Mus musculus,* accession number NM_016847.2; Norway rat *Rattus norvegicus,* accession number CM000237.2) found from Genbank database; the final primers were designed based on the specific bank vole sequence produced from the previous sequencing. The sequences were generated by Sanger sequencing using Big Dye chemistry on an ABI3100 (Applied Biosystems). Primer3 programme (Rozen and Skaletsky 2000, Koressaar and Remm 2007) was used to produce following primers to amplify *Avpr1a* and *Oxtr* RRAMs; *Avpr1a*_RRAM_forward 5′- AGC TCC TAG TTT AAA AGC CC-3′ and *Avpr1a*_RRAM_reverse 5′-GAA CCA GTG AGG ATG ACA GG-3′ and *Oxtr*_RRAM_forward 5′-AAG ATT TCT CTC AGG GTT GGT G-3′ and *Oxtr*_RRAM_reverse 5′-CTC TCA GAG ATG TAG GAA CCT TG-3′.

Total genomic DNA was extracted from tissue samples using Kingfisher magnetic particle processor (Thermo Fisher Scientific, U.S.). PCR analyses were carried out in 10 µl reactions that were comprised of 1.5 µg DNA (5-50 ng in total), 2 mM each dNTP, 1×DreamTaq Buffer (Fermentas), 0.3 µM reverse primer and 0.3 µM labelled forward primer (*Avpr1a* primers had VIC dye and *Oxtr* primers had NED dye) and 0.05 U DreamTaq (Fermentas). The thermocycling conditions for the reactions were 95°C for 3 min and then 35 cycles of 95°C for 30s, 50°C for 30 s and 72°C for 30 s.

The amplified fragments were detected with an ABI3100 (Applied Biosystems) using LIZ600 as a standard and analyzed with GENEMAPPER (Applied Biosystems) version 5.0.

2.3 Breeding

Altogether 325 wild bank voles were trapped from Central Finland (62°37'N, 26°20'E) using Ugglan Special multiple-capture live traps (Grahnab, Sweden) to establish the laboratory breeding colony. The trapped bank voles were kept in the Experimental Animal Unit, at the University of Jyväskylä in 43x26x15 cm standard Makrolon cages with sawdust and hay as bedding. Water and food (Labfor 36; Lactamin AB, Sweden) were provided *ad libitum*. The room temperature was 22 °C and photoperiod 16:8 light/dark. All animals were implanted with electronic identification microchips (Trovan Unique™) with antiseptic techniques.

Avpr1 and *Oxtr* RRAMs are highly variable in length in wild bank vole populations. I observed 31 alleles at *Avpr1a* RRAM whose length ranged from

460 bp and 528 bp the most abundant alleles extending from 496 bp to 502 bp. I divided *Avpr1a* RRAM alleles into three categories: short (S=460-484 bp), medium (M=485-504 bp) and long (L=505-529 bp). I discovered 24 alleles at the *Oxtr* RRAM that ranged from 264 bp to 310 bp which were distributed around the most abundant alleles, whose lengths were 286 bp and 290 bp. Based on that information, I divided the *Oxtr* RRAM lengths into three categories: as short (S=264-274 bp), medium (M=286-290 bp) and long (L=298-310bp).

Since the short and long *Avpr1a* and *Oxtr* RRAM alleles were scarce, I had to breed captured animals to obtain more individuals with suitable genotypes. I paired wild-caught bank voles with known genotypes and also used their offspring for further breedings. I made sure that all the mating pairs were outbred. The animals were bred so that a male and female were kept in the same cage for 14 days, after which I checked whether the female was pregnant by assessing the size of her abdomen. The females who were not pregnant were paired again with a new male. Eventually I achieved an appropriate number of animals with two short alleles (SS), two intermediate alleles (MM), two long alleles (LL) or combination of these alleles (SM and LM).

The study followed the laws and ethical guidelines for animal research in Finland.

2.4 Field experiments

The field experiments (studies I, II and III) were conducted in Central Finland (62°37'N, 26°20'E) in large outdoor enclosures (40m x 50m). The fences were approximately 1m high and prevented the study animals from escaping the enclosure but did not hinder predators, such as weasels (*Mustela nivalis*), stoats (*Mustela erminea*) and avian predators, from accessing the enclosures. The fences were submerged ~0.5 m into the soil to prevent bank voles digging out under the fence. The data for studies I and III were gained from the same enclosure populations. The *Avpr1a* and *Oxtr* experiments were conducted with separate enclosure populations. To manipulate the amount of competition and resources, I established low and high population density treatments. Population density manipulation is relevant for bank vole because they experience naturally periodic fluctuations in population density (Korpela *et al.* 2013) which tends to change level of competition between individuals within populations (Mills *et al.* 2014). In the *Avpr1a* experiment, I established low population density enclosures (N=8 enclosures) by releasing 5 females and 5 males per enclosure, and high population density enclosures (N=5 enclosures) by releasing 10 females and 10 males per enclosure. There were the same amount of individuals carrying each genotype (i.e. SS, SM, MM, LM and LL) in each enclosure. In the *Oxtr* experiment, I established low population density enclosures (N=9 enclosures) by releasing 3 females and 3 males per enclosure and high population density enclosures (N=7 enclosures) by releasing 6 females and 6 males per enclosure. There were the same amount of individuals carrying

each genotype (i.e. SS, MM and LL) in each enclosure. The amount of individuals differed between the *Avpr1a* and *Oxtr* experiments due to constraints in producing enough heterogeneous (SM, ML) animals for the *Oxtr* populations. In study II, I established enclosures with 5 males/10 females (N=7) and compared these with enclosures of 10 males/10 females and 5 males/5 females that were used in study I. Thus, the sex ratio (ASR) and density of males and females differed in these field populations.

The study animals were trapped out of the enclosures approximately 16 days after releasing and carried to the animal care unit where the females were monitored for births every 24 hours. The pups (*Avpr1a* study I: N=242, study II: N=358, *Oxtr* study I: N=244) were sexed by measuring the anogenital distance, while a DNA sample was taken from the tip of tail for genotyping and paternity analysis. The pups and the mother were taken back to the same enclosure within 3 days of birth. The mothers were trapped out of the enclosures when the pups were approximately 20 days old, and furthermore, the pups were trapped out when they were approximately 30 days old, thereby providing information of offspring recruitment (weaning of the pups).

Paternity of the pups was assessed using 6 microsatellite markers (MSCg10A11; 6-FAM dye, MSCg13G2; 6-FAM dye, MSCg15F7; VIC dye, MSCg16E2; NED dye, MSCg17E9; PET dye and MSCg6G11; NED dye) with PCR conditions used by Rikalainen et al. (2008). The PCR fragments were run in capillary electrophoresis on an ABI3100 using LIZ600 (Applied Biosystems) as a size standard. Paternities were assigned with 95 % statistical confidence using Cervus v.3.0 (Kalinowski *et al.* 2007) using the simulation and procedure of "most likely candidate with known mother" with LOD scores. Offspring maternity was known since the pups were born in the laboratory.

2.5 RT-qPCR

To assess whether the length of *Avpr1a* and *Oxtr* RRAMs are linked to variation in gene expression of these genes in the bank vole brain, I carried out RT-qPCR (reverse transcriptase quantitative polymerase chain reaction). I dissected brains from a total of 60 animals so that I had 30 *Oxtr* and 30 *Avpr1a* animals, consisting of equal numbers of both sexes and genotypes (SS, MM and LL). The brains were dissected into five regions (olfactory bulbs, rostral forebrain, caudal forebrain, midbrain and hindbrain, Fig. 1). The brain samples were preserved in RNAlater (Qiagen) immediately after dissection, and then stored at -80 °C until analysed (~3 months later). RNA was extracted with Trizol/RNeasy kit followed with RNA purification using a PureLink RNA Micro Kit (Ambion) for the small olfactory bulbs or PureLink Mini Kit (Ambion) for the rest of the brain regions. RNA quantity was assessed by Qubit fluorometer (Invitrogen) that makes use of target-specific fluorescence detection.

The volume of RT-qPCR reactions was 10 µl consisting ~75 ng RNA, 0.08 µl RT enzyme mix (Applied Biosystems), 5 µl power SYBR® Green master mix

(Applied Biosystems), 1.5 μM forward primer and 0.25 μM reverse primer (or to amplify the beta-actin housekeeping gene using 2 μM beta-actin primer mix) (Ambion). Forward and reverse *Avpr1a* primers were 5′-GCC TAC GTG ACC TGG ATG AC-3′ and 5′-CGC CAG ATG TCG TAG CAG AT-3′ respectively; *Oxtr* primers were 5′-GTC ACA TGG ATC ACG CTT GC-3′ and 5′-CGT CTT GAG TCC CAG GTT CT-3′ respectively. We amplified beta-actin gene using primers originally designed for mouse (Steuerwald *et al.* 1999), but which are conserved in *Myodes glareolus*, forward primer 5′-TGC GTG ACA TCA AAG AGA AG-3′ 129 and reverse primer 5'-GAT GCC ACA GGA TTC CAT A-3′. The reactions were carried out on a 7500 Fast Real-Time PCR System (Applied Biosystems) with the following thermal cycling conditions; 30 min at 48°C, followed by 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. A melting curve analysis was conducted to look at the specificity of PCR product. The amount of *Avpr1a* or *Oxtr* cDNA, normalized to the amount of the reference gene beta-actin cDNA, was calculated by comparative CT method (Livak and Schmittgen 2001) using 7500 Software for 7500 Fast Real-Time PCR v.2.0.6 (Applied Biosystems).

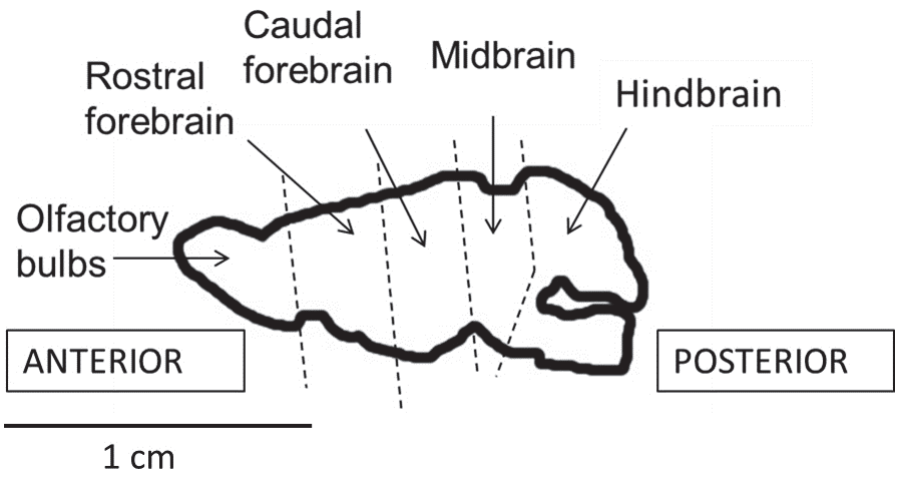

FIGURE 1 The lateral view of bank vole brain regions chosen for gene expression analyses of *Avpr1a* and *Oxtr*. Five brain regions for the analyses are olfactory bulbs, rostral forebrain, caudal forebrain, midbrain and hindbrain starting from anterior to posterior end.

3 RESULTS AND DISCUSSION

3.1 Maintenance of genetic diversity in *Avpr1a* and *Oxtr* (I)

There is a large body of evidence that *Avpr1a* and *Oxtr* affect socio-reproductive behavior in mammals (Young and Wang 2004, Caldwell and Young III 2006, Donaldson and Young 2008, Walum *et al.* 2008, Ophir *et al.* 2012). Both of these genes have highly variable regulatory region associated microsatellite (RRAM) alleles in wild bank vole populations that raises the question: what are the mechanisms that maintain this genetic diversity? I released bank voles with different lengths of *Avpr1a* and *Oxtr* RRAM alleles into semi-natural enclosures with low and high population densities and looked at how the length of these RRAM alleles is associated with reproductive success of the bank voles and whether the effect is population density- or sex-specific.

The results show that the length of *Avpr1a* and *Oxtr* RRAMs were associated with variation in reproductive success but the effect was sex-specific and shown only in high population density for *Avpr1a* and only in low population density for *Oxtr*. I found that, in general, longer *Avpr1a* RRAMs were beneficial for males, while shorter ones benefited females. The opposite, non-significant trend, was found with *Oxtr*: longer *Oxtr* RRAMs were beneficial for females and shorter alleles were beneficial for males. In addition, the length of the *Avpr1a* RRAM was associated with reproductive success in different phases of reproduction in females and males, which was evidenced in high density only such that males with longer *Avpr1a* RRAMs produced more newborn offspring whereas females with longer *Avpr1a* RRAMs produced more recruited (weaned) offspring. The *Oxtr* RRAM correlated only with male reproductive success at low population density; males with shorter alleles produced more newborns and recruited offspring.

I found additional evidence of sex-specific optima in selection of different length of *Avpr1a* and *Oxtr* RRAMs when I looked at the maternal and paternal alleles in the offspring. Furthermore, there tended to be a trend to produce offspring with longer RRAM alleles at high population density, though this was significant only for *Avpr1a*. The offspring inherited significantly longer *Avpr1a*

alleles from the males than from the females at both densities. Conversely at *Oxtr,* the offspring inherited significantly shorter alleles from females than males. These results show that there are sex-specific optima for both *Avpr1a* and *Oxtr* RRAM allele lengths.

I found that the length of both *Avpr1a* and *Oxtr* RRAMs was associated with gene expression of these genes in the bank vole brain but the association depended on the brain region and sex. Longer *Avpr1a* RRAM alleles were correlated with higher gene expression in the caudal forebrains and decreased gene expression in the midbrain. The effect in both brain regions was shown only in females. Correspondingly, the longer *Oxtr* RRAM alleles were associated with higher gene expression in olfactory bulbs and in the midbrain and the effect was shown only in females.

These results imply that balancing selection is behind the maintenance of genetic diversity in *Avpr1a* and *Oxtr* genes. Furthermore, the selection operating on *Avpr1a* polymorphisms acted in different phases of reproduction for males and females. For males *Avpr1a* RRAM allele length affected the outcome of competition for mating opportunities (i.e. newborn offspring), while for females the critical period of selection on the *Avpr1a* genotype occurred during maternal care (i.e. weaning). In addition, gene expression of these genes is associated with *Avpr1* and *Oxtr* polymorphisms but the effect is sex- and brain region specific. This indicates a possible role of brain region- and sex-specific transcription factors (TFs) in the regulation of *Avpr1a* and *Oxtr* gene expression.

3.2 Role of intrasexual competition in selection of *Avpr1a* RRAM (II)

Population density is very relevant to the evolution of diversity in behavioral polymorphism (Sokolowski *et al.* 1997, Sinervo *et al.* 2007). However, many selection pressures often exhibit covariation with alteration of population density, thus observing density-dependence in the optima of a certain trait (and underlying behavioral gene) does not automatically reveal the relevant driver of the density selection. Thus, even if study I evidenced that *Avpr1a* RRAM length is dependent on population density, that study could not tell what is the relevant driver of the density selection. Therefore, in study II the effects of (1) overall population density, (2) male and (3) female density or (4) male-male and (5) female-female competition were separated. I compared field populations from study I (treatment A: 10 males and 10 females and treatment B: 5 males and 5 females) with populations with 5 males and 10 females (treatment C). Male-male competition reduces with overall density and is the lowest when adult sex ratio (ASR) is biased towards females (treatment C) where the number of females is so high that males do not need to compete for them fiercely. Conversely, females compete with each other over territories and therefore

density of females affects female-female competition rather than density of males.

By quantifying the parental origin of the *Avpr1a* alleles in the offspring of all three populations, I found that different levels of intrasexual competition and male/female density favored different allele lengths in mothers and fathers. The extent of intrasexual competition is associated with selection of *Avpr1a* alleles in both sexes; more specifically, males in the populations with expected weaker male-male competition (treatment C) were under selection for shorter *Avpr1a* alleles, while significantly longer alleles were favored in the populations expected to experience an increasing degree of male-male competition (treatments A & B). The same effect of intrasexual selection was evidenced for females: when the expected female-female competition (and female density) was higher (treatments A, C), longer *Avpr1a* alleles were favored; when female-female competition was lower (treatment B) shorter *Avpr1a* alleles were favored. Therefore, the results did not support the overall population density or male density to be the driving force of selection of *Avpr1a* allele, rather intrasexual competition in both sexes was shown to be the driving force of selection of *Avpr1a* allele.

3.3 Effect of *Oxtr* RRAMs on offspring sex ratio (III)

The aim of this study was to test whether the bank vole can alleviate fitness costs caused by sexually antagonistic alleles by biasing the offspring sex ratio in the field. In this mechanism, males and females preferentially produce the offspring sex that is expected to minimize sexually antagonistic fitness costs.

I found that females and males with shorter *Oxtr* RRAM alleles had male-biased offspring sex ratio. In addition, the effect was density-dependent so that for females, the effect was shown only in low population density and conversely for males, only at high population density. Shorter *Oxtr* RRAM alleles are more beneficial for males (study I), so when either of the parents have shorter *Oxtr* alleles, bank voles are able to alleviate fitness costs caused by *Oxtr* alleles by producing more sons. These population density- and sex-specific effects on sex-ratio can have implications for bank vole population cycles by the alteration of adult sex ratio.

Furthermore, I assessed the lengths of *Oxtr* RRAM alleles derived from mothers and fathers within male and female offspring, and observed a significant difference in the transmission pattern of *Oxtr* RRAM alleles to male and female offspring. The length between maternally and paternally derived *Oxtr* RRAM alleles in male offspring but not in female offspring differed from each other. In male offspring, the maternally-derived allele was longer than paternally-derived allele. Together with effect of offspring sex ratio bias, this mechanism shows that *Oxtr* alleles are passed on to the offspring that confers the greater fitness advantage, which additionally gives rise to greater allelic variance in sons compared to daughters at the population level.

3.4 Effect of *Avpr1a* and *Oxtr* RRAMs on breeding success (IV)

The aim in this study was to determine why the long and short *Avpr1a* and *Oxtr* RRAM alleles exist at low frequencies in natural bank vole populations. Therefore, I assessed effect of *Avpr1a* and *Oxtr* RRAM length on breeding success in laboratory conditions. Over 1300 breeding trials were conducted with pair of randomly-chosen female and randomly-chosen male bank voles.

I found that breeding success was reduced with long and short RRAM alleles of both loci. Females with an intermediate *Avp1a* genotype had the highest breeding success. Correspondingly, males with a larger difference between lengths of their *Avpr1a* RRAM alleles had the lowest breeding success. Conversely with *Oxtr*, breeding success was lower when females and males of the breeding pair had dissimilar *Oxtr* genotypes between each other.

These results show that *Avpr1a* and *Oxtr* RRAM alleles are under stabilizing selection meaning that selection is operating against extreme allele lengths in both loci. In addition, reduced breeding success associated with dissimilar *Oxtr* allele lengths between females and males hints that bank voles can experience assortative breeding related to *Oxtr* allele length. Our results show how microsatellites can affect fitness to provide a better understanding of the mechanisms that can determine microsatellite allele length distributions. In addition, these results confer more insights into fitness effects of genetic diversity at *Avpr1a* and *Oxtr*.

4 CONCLUSIONS

Avpr1a and *Oxtr* are two of the best studied examples of the connection from gene to brain to socio-sexual behavior (Caldwell and Young III 2006, Donaldson and Young 2008, Walum *et al.* 2008, Ophir *et al.* 2012), nevertheless mechanisms that maintain genetic diversity in these genes, like in other behavioral genes, have remained elusive. In this thesis I have shown that selection of polymorphisms in *Avpr1a* and *Oxtr* genes is dependent on sex and environmental factors, including population density (I,III) and degree of intrasexual competition (II). I have generated information of how the genetic diversity in these behavioral genes is maintained by sexually antagonistic and density-dependent selection (study I). In addition, study II showed how different alleles of *Avpr1a* RRAMs are favored when intrasexual competition between males and females is manipulated. Furthermore, study III revealed how intralocus conflict caused by sexually antagonistic selection can be alleviated by biasing the offspring sex ratio towards the sex that benefits more from the inherited alleles (study III). And finally in study IV, I looked at how the length of *Avpr1a* and *Oxtr* RRAMs is correlated with breeding success.

In study I, I evidenced that genetic variation in behavioral genes is maintained by balancing selection. That result has been observed before under laboratory conditions in invertebrate species (Sokolowski *et al.* 1997, Fitzpatrick *et al.* 2007), whereas my study utilized a vertebrate species in semi-natural settings. In addition, specific genes underlying sexually antagonistic selection have widely remained a mystery; there are only two studies on *Drosophila melanogaster* (Dean *et al.* 2012, Rostant *et al.* 2015) and only one study on vertebrate species, salmon (*Salmo salar*) (Barson *et al.* 2015) that have discovered specific sexually antagonistic genes. Furthermore, in earlier studies no functional microsatellite, linked with behavior, in the regulatory region of *Oxtr* has been found in any species. I found functional regulatory region associated microsatellites from both *Avpr1a* and *Oxtr* genes in the bank vole to be associated with sex-specific variation in reproductive success with density-dependence and also brain region-specific gene expression. In addition, study II proved intrasexual competition to be the driving force behind balancing

selection and not population density *per se*. That can indicate that different lengths of *Avpr1a* RRAMs confer a different amount of V1aR receptors in specific brain regions responsible for variation in aggressive behavior, which can confer a selective advantage to individuals with longer RRAMs in an environment where aggressive behavior against other females or males provides a higher reproductive success.

Study III confirmed the results of an earlier study (Mills *et al.* 2012) which hinted that bank voles can bias the sex ratio of their litters based on the sexually antagonistic traits of the parents in the laboratory. In my study, I found that bank voles can decrease fitness costs caused by the sexually antagonistic *Oxtr* gene by biasing the transmission of alleles to the sex that benefits them the most in the field.

Study IV gave new insights into what constrains individuals with extreme microsatellite lengths can experience and how microsatellite length distributions can be formed. Extreme RRAM lengths of *Avpr1a* and *Oxtr* were associated with lower probability of breeding and in addition individuals showed assortative mating pattern associated with *Oxtr* polymorphisms.

To sum up, my thesis provides new evidence for balancing selection to be behind maintenance of genetic diversity in behavioral genes. I predict that balancing selection may maintain diversity at many other behavioral loci and that would be relevant topic for future studies. I conclude that my thesis provides new insights into sexual conflict and how individuals cope with it in mammals. In addition, the thesis provides a new perspective for studying sexually antagonistic alleles at the genetic level, which enables future studies to genetically manipulate sexually antagonistic alleles.

Acknowledgements

First of all, I'd like to thank all my four supervisors Esa Koskela, Tapio Mappes, Mikael Mökkönen and Phillip Watts. I couldn't have hoped for better supervisors than you. Thank you for giving me an opportunity to work with such an intriguing research topic. I'm grateful for all the advice and guidance you have given me and for showing me how to 'make science'. We've been laughing so much together between hard work.

I've been grateful to have opportunity to work with such an excellent people in my research group. Without all these people, I couldn't have made it. I have spent so many amazing weeks with Sirpa Huttunen, Taru Niittynen, Anja Siukkola, Susanne Varjola, Meeri Väätäinen in Konnevesi doing field work. Eliisa Kiukkanen and Aija Leppänen have helped me to take care of voles in the Animal Research Centre. Sami Kyröläinen and Juho Niva have done valuable work in DNA lab with vole samples. I thank also Elina Virtanen and Sari Viinikainen for helpful guidance in DNA lab. I want to thank my current office mates Claire Cayol and Joannes Van Cann, and former office mate Angela Sims for great international company. I also want to thank other members of our vole group, Heikki Helle, Kati Kivisaari, Kikka Kallio, Zbyszek Boratynski, Kaisa Rikalainen and Eero Schroderus for important support during these years. I thank all my voles, "Myyrä Myyräset", for being so adorably cute and awesome, and so damn annoying from time to time.

I also want to thank my support group members Emily Knott and Juan Galarza for helpful advice during my PhD studies. I thank all the people in ECO section for creating a warm atmosphere - it has been pleasure to work there. I owe a great deal to Jari Haimi for editing my thesis, and to Toni Laaksonen and Gerald Heckel for examining the thesis, and to Craig Primmer for engaging to be my opponent. I want to thank the staff of Konnevesi Research station for providing me the opportunity to work in a beautiful environment and for providing me everything that I needed to complete my research.

The warmest thanks go to my family and friends for all the support, and patience with thousands of vole videos I've been showing to you.

I'm thankful to the Biological Interactions Doctoral Programme for funding me and to the Academy of Finland for funding our project.

YHTEENVETO (RÉSUMÉ IN FINNISH)

Valintamekanismit käyttäytymistä säätelevien geenien taustalla: arginiini vasopressiini 1a- ja oksitosiinireseptorigeenien kelpoisuusvaikutukset metsämyyrällä (*Myodes glareolus*)

Suurin osa käyttäytymispiirteistä on geneettisesti säädeltyjä, mutta on olemassa vain vähän tietoa mekanismeista, jotka ylläpitävät geneettistä monimuotoisuutta näissä geeneissä luonnonpopulaatioissa. Väitöskirjassani tutkin erilaisia valintamekanismeja kahden sosiaalista käyttäytymistä säätelevän geenin, arginiinivasopressiini 1a- (*Avpr1a*) ja oksitosiinireseptorin (*Oxtr*) monimuotoisuuden taustalla. Tutkimuslajina käytin metsämyyrää (*Myodes glareolus*), joka on yleinen pikkunisäkäs Pohjois-Euroopassa ja jonka elinkiertoekologia on hyvin tunnettu. *Avpr1a-* ja *Oxtr*–geenien ilmentämien reseptorien määrä ja sijainti aivoissa on yhteydessä nisäkkäiden sosiaaliseen ja lisääntymiskäyttäytymiseen, kuten aggressiivisuuteen, seksuaaliseen käyttäytymiseen, jälkeläisten hoitoon ja kiintymyssuhteisiin koiras-naaras-parien sekä vanhemman ja jälkeläisen välillä. Yleinen geneettistä monimuotoisuutta ylläpitävä valintamekanismi on balansoiva valinta, joka suosii samanaikaisesti useita erilaisia populaatiossa esiintyviä genotyyppejä. Populaatiotiheydestä riippuva valinta ja vastakkainen valinta sukupuolten välillä (seksuaaliantagonistinen valinta) ovat tärkeitä esimerkkejä balansoivan valinnan toiminnasta. Seksuaaliantagonismi syntyy, jos valinta suosii saman geenin eri alleeleita koirailla kuin naarailla. Koska koiraat ja naaraat jakavat samat geenit keskenään, toinen sukupuoli voi kasvattaa kelpoisuuttaan lisääntymiseen liittyvien ominaisuuksien suhteen toisen sukupuolen kustannuksella, ja lopputuloksena on, että kumpikaan sukupuoli ei saavuta ihannekelpoisuutta. Useat mekanismit voivat lieventää seksuaaliantagonismin aikaansaamaa kelpoisuuden heikkenemistä. Yksi keino on tuottaa jälkeläisissä enemmän sitä sukupuolta, joka hyötyy enemmän vanhemmilta perityistä seksuaaliantagonistisen geenin alleeleista. Esimerkiksi tietyt matelijanaaraat, jotka parittelevat isokokoisen koiraan kanssa, tuottavat poikueen, jossa on enemmän koiras- kuin naaraspoikasia, koska isokokoisuudesta on hyötyä koiraille. Seksuaaliantagonismia on tutkittu suurelta osin vain fenotyyppitasolla ja ilmiön taustalla olevia geenejä ei juuri tunneta.

Ensimmäisessä osatutkimuksessa tutkin, mitkä mekanismit pitävät yllä vaihtelua *Avpr1a-* ja *Oxtr*-geenien säätelyalueilla sijaitsevissa mikrosatelliiteissa. Erityisesti tutkin populaatiotiheydestä riippuvan ja seksuaaliantagonistisen valinnan vaikutusta. Tutkin myös, onko edellä mainittujen mikrosatelliittien pituuden ja *Avpr1a-* ja *Oxtr*-geenien ilmentymisen välillä yhteyttä aivoissa. Toisessa osatutkimuksessa tutkin kuinka koiras-koiras- ja naaras-naaraskilpailu vaikuttaa *Avpr1a*-mikrosatelliittiin kohdistuvaan valintaan. Kolmannessa osatutkimuksessa selvitin miten eri sukupuolet reagoivat seksuaaliantagonistisesta valinnasta johtuvaan kelpoisuuden vaihteluun. Viimeisessä osatutkimuksessa

selvitin *Avpr1a*- ja *Oxtr*-mikrosatelliitin pituuden yhteyttä metsämyyrien lisääntymistodennäköisyyteen.

Ensimmäinen osatutkimukseni paljasti, että *Avpr1a*- ja *Oxtr*-geeneihin kohdistuu vastakkaissuuntaista valintaa sukupuolten välillä. Yleisesti ottaen valinta suosii pidempiä *Avpr1a*-mikrosatelliitteja koirailla ja lyhyempiä naarailla, sitä vastoin *Oxtr*-mikrosatelliitin lyhyempiä muotoja suositaan koirailla ja pidempiä muotoja naarailla. Lisäksi *Avpr1a*-mikrosatelliitin vaikutukset lisääntymismenestykseen ilmenivät koirailla ja naarailla lisääntymisen eri vaiheissa siten, että vaikutus näkyi koirailla vastasyntyneiden poikasten määrässä ja naarailla vieroitettujen poikasten määrässä. Nämä vaikutukset tulivat esiin kummallakin sukupuolella vain tiheämmässä populaatiotiheydessä; pidemmän *Avpr1a*-mikrosatelliitin omaavat koiraat saivat enemmän vastasyntyneitä poikasia ja saman mikrosatelliitin omaavat naaraat vieroittivat enemmän poikasia verrattuna lyhyemmän *Avpr1a*-mikrosatelliitin omaaviin yksilöihin. Tämä viittaa siihen, että koirailla vaikutus kohdistui kilpailuun parittelukumppaneista ja naarailla poikasten hoitoon. Tulosten perusteella voidaan päätellä, että balansoiva valinta, ja tarkemmin sanottuna seksuaaliantagonistinen ja populaatiotiheydestä riippuva valinta, ovat mekanismeja, jotka voivat ylläpitää monimuotoisuutta sekä *Avpr1a*- ja *Oxtr*-mikrosatelliittien pituudessa ja tämän myötä myös lisääntymiskäyttäytymisessä. Tämän lisäksi havaitsin, että *Apr1a*- ja *Oxtr*-mikrosatelliitin ja näiden geenien ilmentymisen välillä on yhteys, mutta se on sukupuolesta ja aivoalueesta riippuvainen.

Toinen osatutkimukseni osoitti *Avpr1*-mikrosatelliitin osalta, että ensimmäisessä osatutkimuksessa havaittua balansoivaa valintaa ohjaa sukupuolten sisäinen kilpailu resursseista ennemmin kuin populaatiotiheydestä riippuva valinta itsessään. Tämän lisäksi tutkimus osoitti, että sukupuolijakauman ja sen myötä aiheutuva koiras-koiras- ja naaras-naaraskilpailun muutos kääntää *Avpr1a*-mikrosatelliitin optimin koiraiden ja naaraiden välillä päinvastaiseksi. Populaatioissa, joissa naaraita oli enemmän kuin koiraita, *Avpr1*-mikrosatelliitin optimipituus oli naarailla pidempi kuin koirailla, mutta jos naaraita ja koiraita oli populaatiossa saman verran, *Avpr1*-mikrosatelliitin optimipituus koirailla olikin pidempi kuin naarailla.

Kolmas osatutkimukseni puolestaan paljasti, että koirasvaltaisten poikueiden tuottaminen on yhteydessä isän tai äidin *Oxtr*-mikrosatelliitin pituuteen. Tämä löydös/tulos riippui kuitenkin populaatiotiheydestä siten, että lyhyemmän mikrosatelliitin omaavat naaraat (matala kelpoisuus) harvemmassa populaatiossa ja vastaavan mikrosatelliitin omaavat koiraat (korkea kelpoisuus) tiheämmässä populaatiossa tuottivat suhteessa enemmän koiras- kuin naaraspoikasia. Toisin sanoen metsämyyrillä näyttäisi olevan kyky tuottaa enemmän sitä sukupuolta, jota vanhemman genotyyppi hyödyttää enemmän. Tämä mekanismi mahdollistaa sen, että seksuaaliantagonistiset alleelit pystytään siirtämään seuraavassa sukupolvessa sille sukupuolelle, jota ne enemmän hyödyttävät.

Neljäs osatutkimukseni osoitti, että sekä erittäin lyhyet että pitkät *Avpr1a*- ja *Oxtr*-mikrosatelliitit huonontavat yksilöiden lisääntymistodennäköisyyttä.

Erittäin pitkiä tai lyhyitä mikrosatelliittipituuksia karsitaan, joten *Avpr1a*- ja *Oxtr*-geenit ovat stabiloivan valinnan alla. Lisäksi tulokseni antaa lisätietoa siitä, mitkä mekanismit vaikuttavat mikrosatelliittialleelien pituusjakaumaan. Lisäksi lisääntymistodennäköisyys laski sitä mukaan, mitä isompi ero *Oxtr*-mikrosatelliittien pituudessa oli koiraan ja naaraan välillä, mikä viittaa assortatiiviseen pariutumiseen.

Väitöskirjantutkimukseni tulokset viittaavat siihen, että useat eri valintamekanismit pitävät yllä monimuotoisuutta käyttäytymistä säätelevillä geenialueilla. Stabiloiva valinta karsii geenin säätelyalueen mikrosatelliittien ääripituuksia ja balansoiva valinta määrittää optimipituuden, joka voi erota sukupuolten ja populaatiotiheyden välillä. Väitöskirjani antaa myös uutta tietoa seksuaaliantagonistisista geeneistä ja niiden aiheuttamien kelpoisuuskustannusten pienentämisestä nisäkkäillä.

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ORIGINAL PAPERS

I

BALANCING SELECTION MAINTAINS POLYMORPHISMS AT NEUROGENETIC LOCI IN FIELD EXPERIMENTS

by

Eija Lonn, Esa Koskela, Tapio Mappes, Mikael Mokkonen, Angela M. Sims & Phillip C. Watts

Submitted manuscript

II

INTRASEXUAL COMPETITION DRIVES THE PATTERN OF BALANCING SELECTION AT ARGININE VASOPRESSIN RECEPTOR 1A

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III

CRYPTIC BIAS OF A SEXUALLY ANTAGONISTIC LOCUS IN A SMALL MAMMAL

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IV

STABILISING SELECTION ON MICROSATELLITE ALLELE LENGTH

by

Phillip C. Watts, Eva R.K. Kallio, Esa Koskela, Eija Lonn, Tapio Mappes & Mikael Mokkonen

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