Balancing selection maintains polymorphisms at neurogenetic loci in field experiments

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Most variation in behavior has a genetic basis, but the processes determining the level of diversity at behavioral loci are largely unknown for natural populations. Expression of arginine vasopressin receptor 1a (*Avpr1a*) and oxytocin receptor (*Oxtr*) in specific regions of the brain regulates diverse social and reproductive behaviors in mammals, including humans. That these genes have important fitness consequences and that natural populations contain extensive diversity at these loci implies the action of balancing selection. In *Myodes glareolus*, *Avpr1a* and *Oxtr* each contain a polymorphic microsatellite locus located in their 5′ regulatory region (the regulatory region-associated microsatellite, RRAM) that likely regulates gene expression. To test the hypothesis that balancing selection maintains diversity at behavioral loci, we released artificially bred females and males with different RRAM allele lengths into field enclosures that differed in population density. The length of *Avpr1a* and *Oxtr* RRAMs was associated with reproductive success, but population density and the sex interacted to determine the optimal genotype. In general, longer *Avpr1a* RRAMs were more beneficial for males, and shorter RRAMs were more beneficial for females; the opposite was true for *Oxtr* RRAMs. Moreover, *Avpr1a* RRAM allele length is correlated with the reproductive success of the sexes during different phases of reproduction; for males, RRAM length correlated with the numbers of newborn offspring, but for females selection was evident on the number of weaned offspring. This report of density-dependence and sexual antagonism acting on loci within the arginine vasopressin–oxytocin pathway explains how genetic diversity at *Avpr1a* and *Oxtr* could be maintained in natural populations.

Avpr1a | *Oxtr* | sexual conflict | density-dependent selection | *Myodes glareolus*

Most variation in behavior has a substantial genetic basis. Identifying loci that underpin the expression of behavior is central to our understanding of the evolution and adaptive significance of behavioral diversity (1, 2). Although many studies have found an association between genotype and behavior (2–4), few have quantified the eco-evolutionary dynamics of these genetic polymorphisms. A corollary of the diversity of behaviors exhibited in wild populations is the action of balancing selection (3, 5), a general term for mechanisms that promote fitness of alternate genotypes, including density-dependent selection (1), negative frequency-dependent selection (6), heterozygote advantage (7), and sexual antagonism (8, 9). Density- and frequency-dependent selection, for example, can maintain polymorphisms at the foraging gene in laboratory populations of *Drosophila melanogaster* (1, 10). However, the lack of evidence for the conditions that drive balancing selection on behavioral loci in natural settings creates a challenge to behavioral genetics in understanding the dynamics of behavioral loci in real-world scenarios. Genes within the arginine vasopressin–oxytocin pathway present a classic opportunity to meet this challenge; its constituent loci have been subject to extensive study because they exert major effects on animal behavior (5, 11, 12).

The neurotransmitters vasopressin and oxytocin are evolutionarily conserved, with the vasopressin–oxytocin pathway regulating social and reproductive behaviors in many mammals including humans (5, 11, 13, 14). The behaviors associated with vasopressin and oxytocin are often mediated by the density of their receptors, notably arginine vasopressin receptor 1a (V1aR) and oxytocin receptor (OTR), in specific regions of the brain (5, 11–13). The genetic basis of the variation in V1aR density and its concomitant effect on behavior has been studied comprehensively in microtine voles (5, 15–17). In the prairie vole *Microtus ochrogaster*, arginine vasopressin receptor 1a (*Avpr1a*) expression and V1aR density in specific regions of the brain correlate with allele length at a regulatory region-associated microsatellite (RRAM) located in the 5′ regulatory region of the *Avpr1a* gene (15, 16), and longer *Avpr1a* RRAM alleles are associated with greater partner preference and male parental care in the laboratory (15). This intraspecific pattern of an association between *Avpr1a* RRAM allele length and V1aR expression in the brain and/or socio-reproductive behavior extends to other mammals. In chimpanzees, genetic diversity at one *Avpr1a* RRAM locus is associated with sociality (18). In humans, allele length at the *Avpr1a* RRAM locus RS3 is correlated with gene expression in the hippocampus (19) and with male pair bonding (14), altruism (19), and maternal behavior (20), whereas allele length at a second *Avpr1a* RRAM locus (RS1) correlates with autism and promoter activity (21). An association between RRAM allele length and transcriptional activity is not unique to *Avpr1a* and has been shown in other genes and in diverse taxa (22, 23).

In contrast to *Avpr1a*, no genetic polymorphism in the 5′ regulatory region of the oxytocin receptor (*Oxtr*) that associates with variation in OTR density in the brain has been identified. Nonetheless, the region ∼1–5 kbp upstream of *Oxtr* is important for the

Significance

Arginine vasopressin receptor 1a (*Avpr1a*) and oxytocin receptor (*Oxtr*) are evolutionarily conserved loci that affect socio-reproductive behavior in many animals. That these loci affect fitness and exhibit substantial genetic variation in wild populations raises questions about the processes that maintain genetic variation at these loci. We show that the length of microsatellites located in the 5′ regulatory regions of *Avpr1a* and *Oxtr* are associated with reproductive success and gene expression in the brain. Crucially, balancing selection through sexually antagonistic fitness effects and density-related social influences is capable of maintaining microsatellite length polymorphisms at both genes. The action of sex and population density operating at two loci indicates that balancing selection may maintain diversity at many other behavioral loci.

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regulation of this gene's expression (24, 25), and other studies have found an association between behavior and SNPs within an intron or in the 3′ UTR of *Oxtr* (26, 27). Moreover, there is appreciable variation in OTR density within and among rodents (12, 28). Variation of OTR density in the nucleus accumbens is associated with partner preference and maternal care in prairie vole females, such that individuals with higher OTR density show more alloparental care than individuals with lower OTR densities (29). In short, many studies have provided convincing evidence that variation in *Oxtr* expression has a prominent role in regulating social and sexual behavior in many animals (30, 31).

Polymorphisms at the *Avpr1a* RRAM are associated with the reproductive success of rodents in the laboratory (32) and in some (5, 33, 34), but not all (17), field experiments. There have been few attempts to quantify the fitness consequences of polymorphisms at the *Oxtr* regulatory region, or indeed the natural levels of genetic diversity, at this locus. Nonetheless, the extensive variation in OTR density in the brains of male prairie voles presumably impacts fitness, because the distribution of OTR density in the brain predicts male mating success (12). Directional selection is expected to erode fitness-associated genetic diversity toward an optimum value (35), but wild rodent populations contain extensive standing genetic variation, at least at *Avpr1a*. For example, wild prairie vole populations have more than 15 alleles at the *Avpr1a* RRAM locus (15, 17, 33, 34), and Okhovat et al. (5) identified an excess of intermediate-frequency alleles at nucleotide sites within *Avpr1a* as compared with putative neutral loci. A combination of putative fitness effects and extensive genetic and phenotypic diversity at *Avpr1a* and *Oxtr* are compelling evidence for the action of balancing selection. However, experimental manipulations that explicitly test this prediction are lacking (5).

In line with a well-established gene–brain–behavior model in rodents (5, 11, 15, 16) and in primates (19, 36), the bank vole *Myodes glareolus* presents a good model to study selection operating on polymorphisms in the regulatory regions of *Avpr1a* and *Oxtr* (*SI Materials and Methods*). Here, we quantify the roles of sex and population density in determining the fitness of bank voles with different genotypes at *Avpr1a* and *Oxtr*. Assessing the role of sex follows the predominantly sexually divergent roles adopted by loci within the vasopressin–oxytocin pathway: variation in V1aR density in the brain typically is associated with the expression of behaviors in males that include spatial memory, mating behavior, offspring care, and aggressiveness (5, 15, 37), whereas variation in OTR density in the brain is associated more with female behaviors, such as maternal aggression, mother–infant bonding, and same-sex social interactions (30, 38, 39). Quantifying whether there is an interaction between genotype and population density is an extension of the ecology of many species, notably the prairie vole (40) and the bank vole (41), whose populations naturally experience periodic fluctuations in density that alter the extent of intraspecific competition, e.g., for food and breeding territories (41). Bank voles experience a decrease in reproductive success and survival probability as population density increases, and thus environmental heterogeneity can favor alternate genotypes (42). To determine whether balancing selection can maintain high standing genetic variation, we first used artificial selection to create sufficient numbers of bank voles with distinct genotypes at RRAM loci for both *Avpr1a* and *Oxtr*. Next, we allowed animals to compete naturally for territories and mates, and then we quantified the fitness components of different genotypes.

Results

Genetic Diversity and Gene Expression. Both *Avpr1a* and *Oxtr* RRAM loci exhibit high levels of genetic variation in natural bank vole populations. After genotyping 325 individuals, we observed 31 alleles at the *Avpr1a* RRAM that varied between 460 and 528 bp in length and that had a qualitatively normal distribution around the most frequent alleles, which were between 496 bp and 502 bp long (Fig. S1*A*). At the *Oxtr* RRAM, we uncovered 24 alleles that varied between 264 and 310 bp in length, with the most frequent alleles being between 286 and 290 bp in length (Fig. S1*B*).

In *Oxtr* RRAM loci, gene expression was sex-specific in different regions of the brain ($P = 0.019$) (Fig. 1, Fig. S2, and Tables S1 and S2), and the association between *Oxtr* RRAM allele lengths and gene expression interacted with brain regions ($P = 0.004$) (Fig. 1, Fig. S2, and Tables S1 and S2). Longer *Oxtr* RRAM alleles were associated with increased gene expression in the olfactory bulbs (Fig. 1*A*) and in the midbrain of females (Fig. 1*B*). At *Avpr1a* RRAM loci, expression differed in brain regions in females ($P < 0.001$) (Fig. 1, Fig. S2, and Tables S1 and S2): Longer *Avpr1a* RRAM alleles were associated with increased gene expression in the caudal forebrain (Fig. 1*C*) and decreased gene expression in the midbrain (Fig. 1*D*).

Effect of *Avpr1a* and *Oxtr* RRAM Genotype upon Fitness. We released more than 300 mature bank voles (*Avpr1a*, $n = 180$; *Oxtr*, $n = 138$) with different *Avpr1a* and *Oxtr* RRAM genotypes into experimental field populations (*Avpr1a*, $n = 13$; *Oxtr*, $n = 16$) that contained an equal number of voles of each genotype. These animals were allowed to compete and reproduce at high and low population densities (*SI Materials and Methods*, *Field Experiments*). We observed that RRAM allele length at both loci had a significant effect on reproductive success that was contingent on both sex and population density (Figs. 2 and 3, Table 1, and Table S3).

Females with longer *Avpr1a* RRAM alleles produced significantly more offspring that survived to recruitment (weaned offspring) at high population density ($P = 0.009$ allele length × density) (Table 1 and Table S3); more precisely, an ∼20 bp increase in *Avpr1a* RRAM allele length corresponds to an additional recruitment of one offspring per female at high population

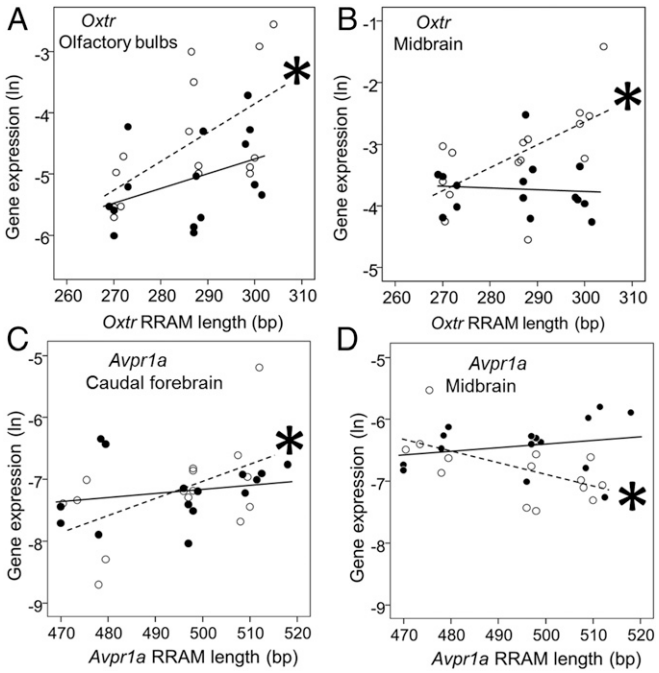

Fig. 1. Significant associations between gene expression (ln-transformed) of *Oxtr* (*A* and *B*) and *Avpr1a* (*C* and *D*) and RRAM allele lengths in the olfactory bulbs (*A*), the midbrain (*B* and *D*), and the caudal forebrain (*C*) of the bank vole. White circles and dashed lines represent females, and black circles and solid lines represent males. See Fig. S2 and Tables S1 and S2 for full details of statistical tests of gene expression in all brain regions and Fig. S4 for illustration of brain regions. Significant sex-specific associations according to linear models (Table S2) are indicated by asterisks.

EVOLUTION

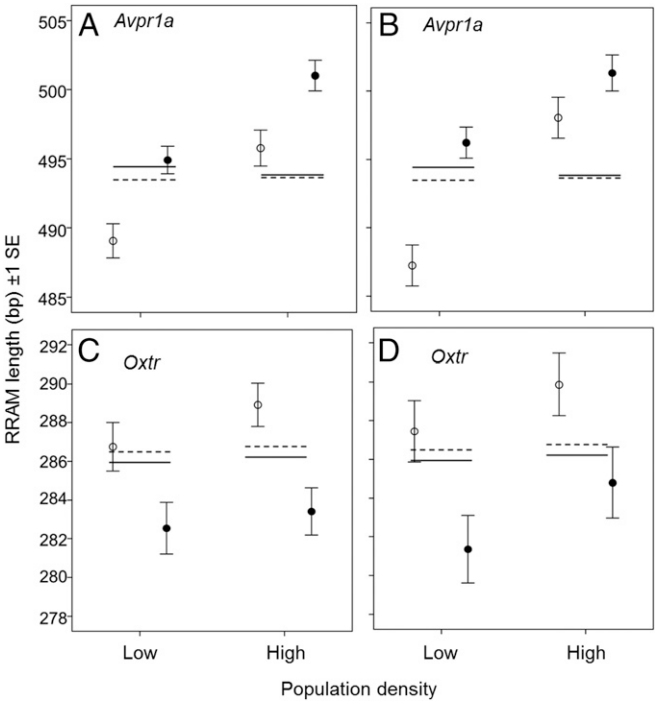

Fig. 2. Sex-specific and density-dependent selection of *Avpr1a* and *Oxtr* loci in the field. Mean lengths (bp ± 1 SE) of the paternally (black circles) and maternally (white circles) derived alleles at *Avpr1a* (*A* and *B*) and *Oxtr* (*C* and *D*) RRAM loci in bank vole offspring produced at high and low population densities. Newborn offspring are shown in *A* and *C*; recruited offspring are shown in *B* and *D*. Reproductively successful males had significantly longer *Avpr1a* alleles than females, whereas the opposite pattern was observed for *Oxtr* allele lengths. Furthermore, increasing population density selected for longer *Avpr1a* alleles in both sexes. The mean *Avpr1a* (*A* and *B*) and *Oxtr* (*C* and *D*) RRAM allele lengths in the parental generation are shown as solid (males) and dashed (females) lines.

density. Male reproductive success also showed an interaction of population density × *Avpr1a* RRAM allele length, but with the main effect operating on the number of sired offspring (newborn animals) ($P = 0.011$) (Table 1 and Table S3), not the number of recruited offspring ($P = 0.130$) (Table 1 and Table S3). At high density, an ~30 bp increase in *Avpr1a* RRAM allele length in males corresponded to the production of one more newborn offspring, but *Avpr1a* genotype had no apparent fitness effect at low density. *Oxtr* had a significant impact only on male reproductive success at low population density; males with shorter alleles sired more offspring ($P < 0.001$ for allele length; $P = 0.011$ for allele length × density) (Fig. 3*D*, Table 1, and Table S3) and achieved more recruited offspring ($P < 0.001$ for allele length; $P = 0.002$ for allele length × density) (Fig. 3*E*, Table 1, and Table S3). In effect, reducing a male *Oxtr* genotype by about 20 bp corresponds to an increase in fitness of one additional recruited offspring at the low population density.

Additional evidence for sex-specific optima was apparent by quantifying the lengths of the maternal and paternal alleles in the offspring (Fig. 2). There was a trend to produce offspring with longer RRAM alleles at a high population density (Fig. S3), although this density effect was significant only for *Avpr1a* (Table S4). At both high and low population densities, offspring inherited significantly longer *Avpr1a* alleles from males than from females ($P < 0.001$ for origin of allele) (Fig. 2 *A* and *B* and Table S4). At *Oxtr*, we found the opposite pattern, with offspring inheriting significantly longer RRAM alleles from their mothers than from their fathers ($P = 0.004$ for origin of allele) (Fig. 2 *C* and *D* and Table S4). These results indicate sex-specific fitness optima for both *Avpr1a* and *Oxtr* alleles.

Discussion

Genes within the arginine vasopressin–oxytocin pathway provide some of the best-studied models of the link from gene to brain to socio-sexual behavior (5, 11–14), but the mechanisms that can maintain high phenotypic and genetic variation in these loci are not known (5). Our field experiments show how RRAM genotypes at both *Avpr1a* and *Oxtr* affect reproductive success, in agreement with some work on the *Avpr1a* RRAM in the prairie vole (33, 34), and provide insight into the dynamics of the *Oxtr* locus. The major advance in understanding the eco-evolutionary dynamics of the arginine vasopressin–oxytocin pathway is that both loci have sex- and population density-specific fitness optima. Genetic diversity at these loci thus has adaptive relevance in natural settings and is likely maintained by balancing selection.

That sex and population density interact to vary the fitness optima for alleles at *Avpr1a* and *Oxtr* RRAM loci provides plausible mechanisms for the maintenance of genetic diversity at these loci (33, 34). Apparent functional divergence between sexes can maintain polymorphisms by generating different optimal trait values between the sexes via sexual antagonism (8, 9). A taxonomically widespread influence of sexually antagonistic alleles is supported by empirical studies on quantitative traits (e.g., testosterone level, body size) (6, 43) and at specific loci (44–46). Some authors have argued that sexual antagonism alone may be insufficient to account for most natural patterns of genetic diversity (6) but that instead some interaction with changes in social environment, such as fluctuation in population density, is required (6, 42).

Changes in population density (40, 41) can impact components of fitness through intraspecific competition (e.g., for food, mates, and territories). Competitive interactions for resources are often resolved by an individual's level of aggression, a behavior regulated by *Avpr1a* and *Oxtr* (13, 47). Interestingly, male prairie voles with divergent *Avpr1a* genotypes enjoy similar overall fitness that is achieved via different mechanisms, being associated with either an apparent capability to monopolize a female partner or increased extra-pair fertilization (5). Okhovat et al. (5) suggested that population density could dictate the strength and direction of selection acting on divergent *Avpr1a* genotypes, with population density cycles thus maintaining genetic diversity. Therefore it is relevant that we observed an interaction between population density and *Avpr1a* RRAM allele length in bank voles, in contrast to a field study on prairie voles in which males with shorter *Avpr1a* RRAM alleles enjoyed greater reproductive success irrespective of density treatment (33), likely a response to the greater competition at high population density. More generally, high population density selects for longer alleles at both loci and in both sexes of the bank vole (Fig. 2). By analogy, these results imply selection for increased gene expression (15, 17, 19, 21), raising the possibility that the optimum female *Avpr1a* genotype at high population density represents a shift toward the male optimum genotype, and the male allelic optimum for *Oxtr* at high population density represents a shift toward the female optimum (Fig. 2). Conversely, there is the possibility of sex-specific gene expression associated with genotype (e.g., *Avpr1a* in the midbrain) (Fig. 1*D*) and for still further fine-scale variation in V1aR and OTR receptor density in the brain (12, 15). Indeed, no association between genotype and behavior was identified in female prairie voles at *Avpr1a* (15). Processes such as the activation of hormone receptors can drive sex-specific gene expression; for example, estrogen receptor mediates the transcriptional activity of many genes, including the expression of *Oxtr* (48). Nonetheless, examining several processes in tandem demonstrates how intralocus sexual conflict can be dynamic through an interrelationship with the social environment (6, 42). Interactions between different mechanisms of balancing selection have a fundamental role in maintaining diversity.

Intraspecific interactions determine reproductive success, but the severity and timing of competition often differ between sexes (6, 35,

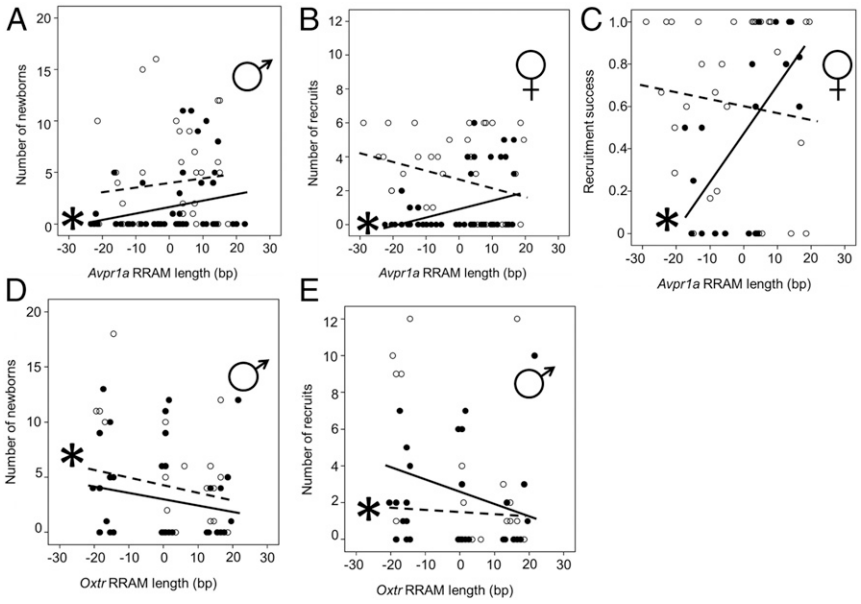

Fig. 3. Effect of sex and population density (solid trend lines and black circles indicate high density; white circles and dashed trend lines indicate low density) upon variation in fitness components of different RRAM genotypes at *Avpr1a* (*A*–*C*) and *Oxtr* (*D* and *E*). (*A*) Longer *Avpr1a* RRAM alleles are associated with more newborn offspring in males at high population density. (*B* and *C*) Longer *Avpr1a* RRAM alleles are associated with more recruited offspring (*B*) and greater recruitment success (the ratio of the number of recruited offspring to the number of newborn offspring) (*C*) in females. (*D* and *E*) For *Oxtr*, shorter RRAM alleles were associated with greater numbers of newborn (*D*) and recruited (*E*) offspring in males at low population density. Allele lengths are shown as population-centered values of the mean length for each sex. Symbols indicate the sex that is included in the plot; only data with a significant interaction between allele length and density are shown (see Table 1 and Table S3). Significant effects of allele length in densitywise GLMM analyses are indicated by asterisks.

41, 49, 50). Quantifying the numbers of offspring from birth to recruitment revealed sex-specific timing of selection acting on *Avpr1a*. Bank vole males do not perform parental care, and male reproductive success is determined primarily by intrasexual competition for mating opportunities when males establish a dominance hierarchy (41). Female bank voles compete intrasexually for breeding territories and then protect and care for their young (41). Consistent with this sexual dichotomy in natal care, *Avpr1a* RRAM allele length affects the outcome of competition for mating opportunities in males (i.e., newborn offspring), whereas for females the critical period of selection on the *Avpr1a* genotype occurs during maternal care (i.e., weaning). *Avpr1a* genotypes associate with aggressive behavior (13), but we do not know if this association is the mechanism by which *Avpr1a* genotype affects intrasexual competition in bank voles. For example, female choice may determine male reproductive success, because laboratory studies indicate that female prairie voles prefer to mate with males that have longer *Avpr1a* alleles (32). In females, our data imply a role for the expression of behaviors associated with the protection of offspring, e.g., from infanticide by intruders (51), and/or mother–offspring social dynamics rather than intrasexual competition for territories. In bank voles, the *Avpr1a* and *Oxtr* genotype impacts the outcome of sexual interactions in nature, but with the timing and mechanisms differing between sexes indicating pleiotropy.

Variation in *Oxtr* expression is associated typically with maternal affiliative behavior (30, 38) and aggression (39). Despite sex-specific fitness optima for *Oxtr*, the nonsignificant association between female *Oxtr* RRAM allele length and reproductive success indicates that other factors, e.g., environmental plasticity or epistatic interactions of gene networks, have a greater role in driving female reproductive success in the wild. Nonetheless, our data indicate that *Avpr1a* has a prominent role underlying female reproductive success, counter to laboratory studies on prairie voles (15) but consistent with work on other rodents (39) and in humans (20). Conversely, the significant role of *Oxtr* in determining male bank vole reproductive success adds support to evidence that OTR density in the brain predicts mating tactics and reproductive success in male prairie voles (12). Indeed, greater oxytocin induces partner-preference formation (30). However, in bank voles, longer *Oxtr* alleles appear costly for males, especially at low population density; this difference presumably reflects the promiscuous and monogamous mating systems of bank voles and prairie voles, respectively. In short, we find no evidence that this gene has stereotyped sex-limited fitness effects.

That genetic diversity at both RRAM loci affects fitness shows how polymorphisms in microsatellite allele length can represent important functional genetic variation. This finding is consistent with evidence that microsatellites often are associated with gene-regulatory elements (52). Changes in microsatellite allele length may alter the position of regulatory DNA motifs, such as transcription factor-binding sites. Positional changes in regulatory motifs can alter transcriptional activity (23, 53) and represent one mechanism by which changes in microsatellite length can affect gene expression. Microsatellite allele length is associated with the level of expression of many genes (4, 23), including *Avpr1a* in several vertebrates (17, 21, 54). Of course, regulation of gene expression extends to genomic features beyond the action of microsatellite allele length, even at *Avpr1a*. For example, Turner et al. (55) did not find a correlation between RRAM allele length and V1aR brain expression in an interspecific comparison of deer mice (*Peromyscus*) species. More specifically, a detailed functional genetic analysis of *Avpr1a* in the prairie vole uncovered how SNPs and methylation of CpG sites affect gene expression of V1aR in the brain and associated male socio-sexual behaviors (5). Multifaceted control of gene expression, both genetic and epigenetic, may explain the failure to establish a link between *Avpr1a* RRAM length and behavior, such as fidelity, in some field studies on prairie voles (17, 33, 56) and the variation in gene expression among similar RRAM genotypes (Fig. 1 and Fig. S2). Indeed, the failure to maintain a significant association between candidate genetic polymorphisms and behavior across different populations highlights the substantial challenge in quantifying evolutionary dynamics at behavioral loci in wild populations (57).

Uncovering the mechanism(s) by which the *Oxtr* and *Avpr1a* RRAMs might affect gene expression in the bank vole and identifying the influence of other potential modifiers of gene expression require detailed functional genomic analyses. Nonetheless, an analogous influence of sex and population density on the two genes *Oxtr* and *Avpr1a* is convincing support of a direct influence of allele length. What is most relevant for our understanding of the eco-evolutionary dynamics of *Avpr1a* and *Oxtr* in natural bank vole populations is that, although these loci having duplicated and then adopted more specialized roles in mammals over roughly 100 Mya (58), both density-dependent selection and sexual antagonism act on both loci.

Materials and Methods

Model Species. The bank vole, *M. glareolus*, is a small rodent that inhabits forests and fields in the Palearctic; its distribution extends from Europe into

Table 1. GLMMs to quantify effects of sex, population density, and *Avpr1a* and *Oxtr* RRAM allele lengths on three different components of reproductive success: number of newborn offspring, number of recruited (weaned) offspring, and recruitment success in the bank vole *M. glareolus*

Fitness component, locus–sex combination	Allele length		Population density		Allele length × population density	
	Estimate	*P*	Estimate	*P*	Estimate	*P*
Number of newborn offspring						
Avpr1a male	−0.007	0.422	−0.419	0.093	0.050	**0.011**
Avpr1a female	0.000	0.950	−0.257	0.070	0.011	0.370
Oxtr male	−0.024	**<0.001**	−0.011	0.936	0.023	**0.011**
Oxtr female	−0.001	0.850	−0.062	0.630	0.001	0.900
Number of recruited offspring						
Avpr1a male	−0.003	0.800	−0.518	0.160	0.046	0.130
Avpr1a female	0.007	0.325	−0.776	**0.006**	0.053	**0.009**
Oxtr male	−0.037	**<0.001**	−0.240	0.463	0.045	**0.002**
Oxtr female	0.002	0.840	−0.119	0.520	0.002	0.900
Recruitment success						
Avpr1a male	0.035	0.051	0.131	0.853		
Avpr1a female	0.127	**0.002**	0.669	0.289	−0.146	**<0.001**
Oxtr male	0.006	0.354	0.431	0.481	−0.036	0.169
Oxtr female	0.007	0.869	0.516	0.442	−0.011	0.629

Allele length × population density refers to the interaction between allele length and population density. Significant ($P < 0.05$) effects are highlighted in bold.

western Siberia (41). Female bank voles are philopatric and defend their breeding territories; males are more dispersive and do not make provision for their young; both sexes mate multiply (41).

***Avpr1a* and *Oxtr*.** *M. glareolus* contains microsatellite loci in the 5′ regulatory region (i.e., RRAM) of both *Avpr1a* and *Oxtr* (*SI Materials and Methods*, *Sequencing the Coding Sequence and 5′ Regulatory Region of Avpr1a and Oxtr in the Bank Vole* and Table S5). At *Avpr1a*, the RRAM consists of (CA) and (GA) dinucleotide motifs and is located ∼920 bp upstream of *Avpr1a* exon 1. The *Avpr1a* RRAM appears conserved in many rodents; e.g., a RRAM that also is rich in (CA) and/or (GA) motifs is located some 903, 963, 965, and 980 nt upstream of *Avpr1a* exon 1 in the prairie vole (15, 16), mouse, Norway rat, and in eight species of deer mice (55), respectively. The *Oxtr* RRAM in the bank vole comprises a mixture of predominantly $(CT)_n/(GA)_n$ dinucleotide motifs that are located immediately (∼10 bp) upstream of the oxytocin receptor transcript variant X1 and 1,448 bp upstream of the oxytocin receptor transcription start site in *Mus musculus*.

To quantify natural levels of polymorphisms in the *Avpr1a* and *Oxtr* RRAM loci, we caught 325 wild bank voles from central Finland from 20 trapping locations that were scattered over an area of ∼100 km^2. All animals were genotyped using the primers and PCR conditions described in *SI Materials and Methods*, *Genotyping of Avpr1a and Oxtr RRAM in the Bank Vole*. The use of the animals followed the principles of Directive 2010/63/EU (License no. ESAVI/3834/04.10.03/2011) as well as all the institutional guidelines for animal research in Finland.

Selective Breeding of Animals with Distinct *Avpr1a* and *Oxtr* Genotypes. Individuals with short (i.e., ≤484 bp and ≤274 bp in *Avpr1a* and *Oxtr*, respectively) and long (i.e., ≥504 bp and ≥298 bp in *Avpr1a* and *Oxtr*, respectively) alleles at the *Avpr1a* and *Oxtr* RRAM were rare in natural populations (Fig. S1). We therefore used selective breeding to produce sufficient unrelated animals with short and long alleles (as well as animals with medium-length alleles) at both loci (see *SI Materials and Methods*, *Breeding for* Avpr1a *and* Oxtr *Genotypes* for details). This procedure allowed us to balance each field enclosure with contrasting genotypes, i.e., animals with short (S) alleles (*Avpr1a*: 460–484 bp; *Oxtr*: 264–274 bp), medium (M) (*Avpr1a*: 486–504 bp *Oxtr*: 286–290), or long (L) alleles (*Avpr1a*: 504–528 bp; *Oxtr*: 298–310 bp) as well as individuals with a combination of S and M (SM) alleles or L and M (LM) alleles.

Effect of *Avpr1a* and *Oxtr* RRAM Genotypes upon Reproductive Success. We determined the relative effects of *Avpr1a* and *Oxtr* RRAM genotype, sex, and population density on reproductive success under seminatural conditions in outdoor enclosures at the Konnevesi Research Station, University of Jyväskylä (62°37′ N, 26°20′ E) (see *SI Materials and Methods*, *Field Experiments* for details). To manipulate the degree of breeding selection among individuals, we established higher- and lower-population-density treatments. Animals of opposite sex with a common ancestor in the selective breeding pedigree were not released into the same enclosure to avoid possible inbreeding-avoidance effects. For *Avpr1a*, the lower-population-density treatment ($n = 8$ populations) contained five females and five males per enclosure, and the higher-population-density treatment ($n = 5$ populations) contained 10 females and 10 males per enclosure; each genotype (i.e., SS, SM, MM, LM, or LL) was equally represented in each enclosure, so that there were one male and one female of each genotype at the lower density and two males and two females of each genotype at the higher density. For *Oxtr*, the lower-population-density treatment ($n = 9$ populations) contained three females and three males per enclosure, and the higher-population-density treatment ($n = 7$ populations) contained six females and six males per enclosure; again each of three genotypes (SS, MM, and LL) was equally represented in each enclosure. The number of individuals differed in the *Avpr1a* and *Oxtr* experiments because of constraints in producing enough heterogeneous (SM or ML) animals for the *Oxtr* populations.

Animals were allowed to move, establish territories, and reproduce. After 16 d we began to trap animals on a regular trapping grid to identify breeding females. All trapped animals were measured in the laboratory, where the pregnant females were maintained and monitored until they gave birth; females and pups were returned to the enclosures within 3 d after birth (*SI Materials and Methods*, *Field Experiments*). We determined the parentage of all pups (*Avpr1a*, $n = 241$; *Oxtr*, $n = 243$; see details in Table S6) at birth using microsatellite genotyping (*SI Materials and Methods*, *Genotyping of* Avpr1a *and* Oxtr *RRAM in the Bank Vole*) and followed their survival to recruitment. Thus, our variables of reproductive success (see *Statistical Analyses*) combine data for both breeding and fecundity selection as well as survival selection.

Statistical Analyses. We used generalized linear mixed models (GLMMs) to analyze the effect of *Avpr1a* and *Oxtr* RRAM genotype on (*i*) the number of newborn offspring, (*ii*) the number of recruited offspring, and (*iii*) the recruitment success (the ratio of the number of recruited offspring to the number of newborn offspring) (Table 1 and Table S3). Sexes were examined separately. The GLMMs quantified whether the numbers of newborn or recruited offspring (dependent variables) could be predicted by the independent variables of allele length (centered value of the mean length of *Avpr1a* or *Oxtr* RRAM alleles), population density (high or low), and their interaction. Variation between years and enclosures in the *Avpr1a* experiment and replicates and enclosures in the *Oxtr* experiment were accounted for by including them as random factors. Numbers of newborn and recruited offspring were examined using a zero-inflated negative binomial model (ZINB) with a Poisson distribution, using glmmadmb in R v. 3.1.1 (R Development Core Team 2014). Recruitment success was examined using GLMM (events-trials, binomial distribution, and logit link function) in SPSS (IBM SPSS Statistics 22). The difference in the length of maternally and paternally derived *Avpr1a* and *Oxtr* RRAM alleles (Table S4) was

analyzed using population density and origin of allele (maternal or paternal RRAM allele) and their interactions as independent variables. Offspring ID nested within litter and experimental enclosure was included as a random effect. We used linear models with R v.3.1.1 (R Development Core Team 2014) to analyze the effects of allele length, sex, brain region, and their interactions on the expression of *Avpr1a* and *Oxtr* (Table S1). *Avpr1a* and *Oxtr* expression also was analyzed separately for each brain region (Table S2).

Ethical Approval. Use of study animals followed the ethical guidelines for animal research in Finland.

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