



# Regulatory Variation in Functionally Polymorphic Globin Genes of the Bank Vole: A Possible Role for Adaptation

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Interaction between gene expression and protein-coding genetic variation is increasingly being appreciated as an important source of adaptive phenotypic variation. In this study, we used reverse transcription-qPCR to test for gene expression variation in two  $\beta$ -globin paralogs (HBB-T1 and HBB-T2) of the Eurasian bank vole (*Myodes glareolus*), which both display the same structural polymorphism Ser52Cys responsible for variation in Cys-based antioxidant capacity of red blood cells (RBCs). We first demonstrated that HBB-T1 is the major expressed adult HBB gene in the bank vole accounting for ~85% of total hemoglobin. We then measured the relative expression of the two homozygous genotypes in each gene and found that when present in HBB-T1, the oxidative-stress resistant Cys52 allele is significantly associated with higher expression ratio HBB-T1:HBB-T2. The results further indicated that the Cys52 allele present in HBB-T1 was associated with higher normalized expression of that gene compared to the Ser52 allele, although this difference was statistically significant only when using one reference gene but not the other. We argue that, altogether, our results indicate the presence of a *cis*-acting regulatory genetic variation modulating the expression of the two alleles in HBB-T1. Previous studies indicated that the resistant RBC phenotype is likely beneficial under conditions conducive to oxidative stress. The duplicate HBB genes of the bank vole thus may represent a novel example of gene-regulatory genetic variation interacting with a well-defined protein-coding variation to control an adaptive trait.

**Keywords:** cysteine, gene expression, hemoglobin, *Clethrionomys glareolus*, oxidative stress, regulatory variation

## INTRODUCTION

Non-synonymous nucleotide variation altering the amino acid sequence of functionally important protein domains (e.g., ligand-binding sites) is a well-recognized source of adaptive phenotypic variation (Hoekstra et al., 2004; Wheat et al., 2006; Mitchell-Olds et al., 2007; Yokoyama et al., 2008; Storz et al., 2009). However, the phenotypic effect of a gene can also be modified through genetic variation in levels of gene expression (Harrison et al., 2012); for example, when this affects the concentration and hence, activity level of the resulting protein product (Steiner et al., 2007). Interaction between both types of variation can then yield complex

patterns of phenotypic variation, where regulatory variants differentially modulate the functional effect of protein variants by changing their absolute or relative abundance in the cell. Therefore, a regulatory genetic variant can enhance or reduce the functional effects of a protein-coding variants, which can result in a stronger or weaker phenotypic effect than expected, if only the protein-coding variation was present (Dimas et al., 2008). When a regulatory variant is in linkage disequilibrium (*cis*-acting) with a coding variant, then selection on specific allelic combinations can result in adaptive expression differences between functionally-distinct protein alleles (Signor and Nuzhdin, 2018).

Variation in gene expression is common between species and within populations (Fraser, 2013). However, while genome-wide patterns have suggested that genetic interactions between regulatory (*cis*- as well as *trans*-acting) and protein-coding variation (i.e., epistasis; Dimas et al., 2008) may be a common cause of genetically complex phenotypes, it is not clear how much of such variation is adaptive, with well-characterized examples being limited to a few species and genetic systems (e.g., Laurie et al., 1991; Steiner et al., 2007). Here, we present the first report on gene expression variation in a pair of duplicate  $\beta$ -globin genes in wild populations of a Eurasian rodent, the bank vole (*Myodes glareolus*), as a potential candidate system with a red blood cell (RBC) phenotype controlled by both protein-coding and regulatory variation (Kotlík et al., 2014; Stráznická et al., 2018).

Although primarily known as an oxygen carrier, hemoglobin (Hb) is a multifunctional molecule with structural variation conveying functional adaptation to environmental stress in diverse organisms and circumstances (Reischl et al., 2007; Storz, 2019). However, duplicate Hb genes are also well-known for their ontogenetic regulatory switching, with the expression of a fetal gene being replaced with the expression of an adult gene, as well as for their expression variation between paralogous copies encoding each of the  $\alpha$  (Storz et al., 2010; Marková et al., 2014) and  $\beta$  subunits (Weich et al., 1988; Kotlík et al., 2014) of the tetrameric Hb. Therefore, Hb represents a suitable system to look for the presence of both coding and regulatory variation. The bank vole shows striking Hb variation where individuals from across Europe may carry one of two structurally and functionally distinct adult Hb types (Kotlík et al., 2014; Stráznická et al., 2018), originally detected by protein electrophoresis and designated Hb S (slow) and Hb F (fast) (Hall, 1979). A recent study showed that Hb F homozygotes have significantly higher antioxidant capacity of the red blood cells (RBCs) than Hb S homozygotes, which is due to a single amino acid substitution of serine (Ser) with cysteine (Cys) at position 52 in the  $\beta$  subunit of Hb F (Kotlík et al., 2014). The explanation is that the thiol (-SH) group of the side chain of Cys52 in Hb F takes part in the RBC protein redox system involved in detoxification of reactive oxygen species (ROS) (Rossi et al., 1998; Storz et al., 2012; Kotlík et al., 2014; Petersen et al., 2017). Previous studies found evidence suggesting that the resistant RBC phenotype is advantageous under environmental conditions conducive of oxidative stress (Kotlík et al., 2014; Stráznická et al., 2018). The allele frequency variation across populations may therefore

reflect the strength of local environmental selection (Stráznická et al., 2018).

Due to the history of frequent inter-paralog gene conversion (Stráznická et al., 2018), the same allelic variation Ser52Cys is shared by both gene duplicates encoding the  $\beta$ -globin in the bank vole, HBB-T1 and HBB-T2 (Kotlík et al., 2014; Stráznická et al., 2018). However, a preliminary comparison of the relative expression of the two genes using high-throughput RNA sequencing (RNA-seq) from three individuals suggested that HBB-T1 is the major  $\beta$ -globin gene expressed in adult RBCs, accounting for  $\sim$ 95% of total Hb (Kotlík et al., 2014). The phenotypic effect of the Cys52 allele is therefore dependent on whether it is present in the HBB-T1 (major) or HBB-T2 (minor) gene. Expression of HBB-T1 and HBB-T2 can be regulated independently of one another in rodents (Whitney, 1977). Therefore, there is a possibility that regulatory variation in the expression ratio of the two genes (Thompson et al., 2014; Sandkam et al., 2015a,b), but also of the two alleles within the same gene (Dimas et al., 2008), could add an additional dimension to variation in the RBC phenotype and contribute to evolutionary adaptation by helping to fine-tune the RBCs phenotype in response to selection pressures (Stráznická et al., 2018).

The aim of the present study was to test for gene expression variation as such variation potentially could modulate the functional effect of the two  $\beta$ -globin alleles in bank vole RBCs. Hence, we compare the expression levels of the two HBB paralogs as measured by quantitative reverse transcription PCR (RT-qPCR) between voles possessing different genotypes and originating from different populations across Europe, as evidenced by their mitochondrial DNA (mtDNA) clade membership (Filipi et al., 2015; Stráznická et al., 2018). As the result of an expansion from multiple isolated glacial refugia at the end of the last glaciation, the present range of the bank vole is partitioned into multiple clades, designated according to the glacial refugium from which the clade is derived (Filipi et al., 2015). Clade memberships thus serves as a proxy for historically separate population. We tested for the association of the protein-coding genotype (Ser52 or Cys52) with the expression level of the gene in which it is present (HBB-T1 or HBB-T2), as well as with the expression ratio between the two genes (HBB-T1:HBB-T2).

## MATERIALS AND METHODS

### Samples and RNA Preparation

The study used 47 samples selected among the set previously studied by Stráznická et al. (2018) for which the Ser52Cys genotype in HBB-T1 and HBB-T2 had been determined by either pyrosequencing or by Sanger sequencing (Stráznická et al., 2018). The samples originated from eight localities across Europe [Germany ( $n = 1$ ), Netherlands ( $n = 7$ ), United Kingdom–England ( $n = 16$ ), United Kingdom–Scotland ( $n = 4$ ), Sweden ( $n = 4$ ), Denmark ( $n = 5$ ), Italy ( $n = 5$ ), and Norway ( $n = 5$ ); see **Table S1**], as described by Stráznická et al. (2018). All samples were spleen tissue, an erythropoietic organ in rodents (Brodsky et al., 1966), stored in RNA later (Qiagen, Valencia, CA) at  $-20^{\circ}\text{C}$ . Only homozygotes Cys52Cys and Ser52Ser were

**TABLE 1** | Numbers of voles analyzed from each population (mtDNA clade) and HBB-T1 and HBB-T2 genotype.

Population	HBB-T1	HBB-T2	Two locus genotype	n
Calabrian	Ser52Ser	Ser52Ser	Ser52Ser/Ser52Ser	5
Carpathian	Cys52Cys	Cys52Cys	Cys52Cys/Cys52Cys	2
		Ser52Ser	Cys52Cys/Ser52Ser	9
	Ser52Ser	Ser52Ser	Ser52Ser/Ser52Ser	10
Eastern	Ser52Ser	Ser52Ser	Ser52Ser/Ser52Ser	5
Western	Cys52Cys	Cys52Cys	Cys52Cys/Cys52Cys	7
		Ser52Ser	Cys52Cys/Ser52Ser	9
Total				47

Population names refer to glacial refugium from which the population derives and not necessarily its present-day distribution. n = sample size.

included in this study (Table S1). The sample sizes of the two homozygotes in each gene and of each of the three commonly occurring two-locus combinations are given in Table 1, which lists the genotypes by mtDNA clade (Deffontaine et al., 2005; Filipi et al., 2015; Strážnická et al., 2018) as a proxy for historically separate population (Avisé, 2000).

Total RNA was extracted from ~0.05 mg of tissue by using the RNeasy Protect Mini Kit with RNase-Free DNase I treatment (Qiagen). The RNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific) and its integrity was verified by gel electrophoresis. RNA in each sample was diluted in RT-PCR Grade Water (Invitrogen) to a final concentration of 10 ng/μl and stored at -80°C.

## Reverse Transcription–qPCR Design

Primers for the RT-qPCR assays were designed using the Oligo 7 software (Colorado, USA). To make assays specific to each of the two HBB paralogs (HBB-T1 and HBB-T2), each assay used a gene-specific reverse amplification primer located within the 3' untranslated region (UTR) showing consistent sequence differences between HBB-T1 and HBB-T2 (Kotlík et al., 2014). The forward primer was common to both assays and the reverse primers were positioned such that the amplicon length was similar between the two assays (Table S2). Primers were also designed for a selection of candidate reference genes for normalizing the RT-qPCR data, taking advantage of the previously available bank vole transcriptome assembly (Kotlík et al., 2018). Potential reference genes were selected based on literature reports from the bank vole and other mammals (Swiergosz-Kowalewska et al., 2007; Ren et al., 2010; Zarzycka et al., 2016; Eissa et al., 2017; Li et al., 2017). A total of seven genes, namely those encoding β-actin (*Actb*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), peptidyl-prolyl isomerase A (*Ppia*), 18S ribosomal RNA (*Rn18s*), 60S acidic ribosomal protein P0 (*Rplp0*), TATA box binding protein (*Tbp*), and ubiquitin-conjugating enzyme E2D 2A (*Ube2d2a*) were considered. To evaluate the expression stability of the candidate reference genes, the expression level of each gene was determined across eight individuals selected from the total sample of 47 specimens,

which varied in genotype. Suitable reference genes were selected following the recommendations of Pfaffl et al. (2004).

RT-qPCR amplification was performed in a Rotor Gene 3000 (Corbett Research, Australia) cyclor using the OneStep RT-PCR Kit (Qiagen) with EvaGreen dye (Biotium, California, US). The reactions were set up in a total volume of 10 μl and contained 2 μl 5× reaction buffer, 0.4 μl dNTP mix (10 mM stock of each), 0.2 μl of both reverse and forward primers (0.02 mM stock), 0.4 μl Qiagen One Step RT-PCR enzyme mix, 4.6 μl RNase free water and 2 μl of RNA. All reactions were carried out in three replicates. Amplifications were performed using 0.1 ml strip tubes and caps (Qiagen) placed in the 72-well rotor of the Rotor Gene instrument, which allowed the HBB-T1 and HBB-T2 assays for each vole to be run simultaneously, in addition to negative controls. Run conditions were as follows: a cDNA synthesis at 50°C for 30 min and a pre-denaturation at 94°C for 15 min, followed by cycles consisting of a denaturation at 94°C for 3 s, an annealing at the temperature specific for each set of primers (Table S2) for 20 s and an extension at 72°C for 20 s, followed by a final extension at 72°C for 5 min. The specificities of the RT-qPCR assays were verified by a melting curve analysis tool of the Rotor-Gene Q Series Software, version 2.3.1. The amplification of the desired PCR product was confirmed by Sanger sequencing (Macrogen, Amsterdam, Netherlands) and BLAST comparison against the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>) database.

## Quantification of HBB-T1 and HBB-T2 Expression

The expression ratio HBB-T1:HBB-T2 compares two genes in the same sample (rather than the same gene in two different samples normalized to a reference gene) and a simple mathematical model  $(1+E_1)^{-Cq1}/(1+E_2)^{-Cq2}$  therefore applies (Thompson et al., 2014; Sandkam et al., 2015a,b), where *Cq* is the number of cycles each gene (1 and 2) takes to cross a fluorescence threshold, and *E* is the PCR amplification efficiency of that gene (Pfaffl, 2001). The term  $(1+E)^{-Cq}$  therefore represents the input quantity of each gene relative to the quantity of that gene at the quantification cycle (Rieu and Powers, 2009). Such an approach has previously been applied to study expression ratios of gene duplicates (Thompson et al., 2014; Sandkam et al., 2015a,b), pairs of single genes (Ma et al., 2004; Goetz, 2006), and splice variants of the same gene (Camacho Londoño and Philipp, 2016). For a valid ratio calculation, the PCR amplification efficiencies of the two genes must be approximately equal, or a bias-correction needs to be applied (Thompson et al., 2014; Sandkam et al., 2015a,b). We validated the applicability of this approach to our system by assessing the mean amplification efficiency of each RT-qPCR assay using the *Cq* slope method, as recommended by Bustin et al. (2009) and Livak and Schmittgen (2001). We constructed a calibration curve for each gene from seven sequential dilution points (12.5, 3, 0.3, 0.1, 0.03, 0.003, and 0.0003 ng/μl). The slope of the calibration curve ( $-\log(E+1)$ ) plotting the values of *Cq* against the logarithm of the initial template concentration (Bustin et al., 2009) was -3.312 for HBB-T1 and -3.315 for HBB-T2, indicating the assays were approximately

equally ( $\Delta E < 0.5\%$ ) and near 100% efficient. The applicability of the model was further supported by a near-zero slope (0.0497) of a calibration curve plotting the difference in  $Cq$  values ( $\Delta Cq$ ) between the two genes on the  $y$  axis (Livak and Schmittgen, 2001).

Despite the results suggesting near optimal and equal mean efficiencies of both assays, we applied the efficiency calibrated model above in order to account for any reaction-specific PCR efficiency variation (McCurdy et al., 2008). The amplification efficiency of each individual reaction was calculated with the comparative quantitation tool of the Rotor-Gene Q Series Software (McCurdy et al., 2008), which uses the second derivative of raw fluorescence values (the slope of the amplification curve) to calculate both the efficiency ( $E$ ) of each reaction and the take-off point ( $Cq$ ) at which the exponential phase of amplification begins (McCurdy et al., 2008; Camacho Londoño and Philipp, 2016).

In order to compare gene expression levels between voles carrying different genotypes, we normalized the expression of each gene relative to the two reference genes meeting the criteria for consistent expression levels (Pfaffl et al., 2004) (see Results). Calculations that consider reaction-specific PCR efficiencies were performed according to Pfaffl (2001).

Therefore, we obtained altogether nine measures of HBB expression for each of the 47 voles; specifically, the non-normalized expression  $(1+E)^{-Cq}$  of each of HBB-T1 and HBB-T2, the ratio HBB-T1:HBB-T2, and the expression of each of HBB-T1 and HBB-T2 normalized to each of the two selected reference genes (see Results) and to their mean.

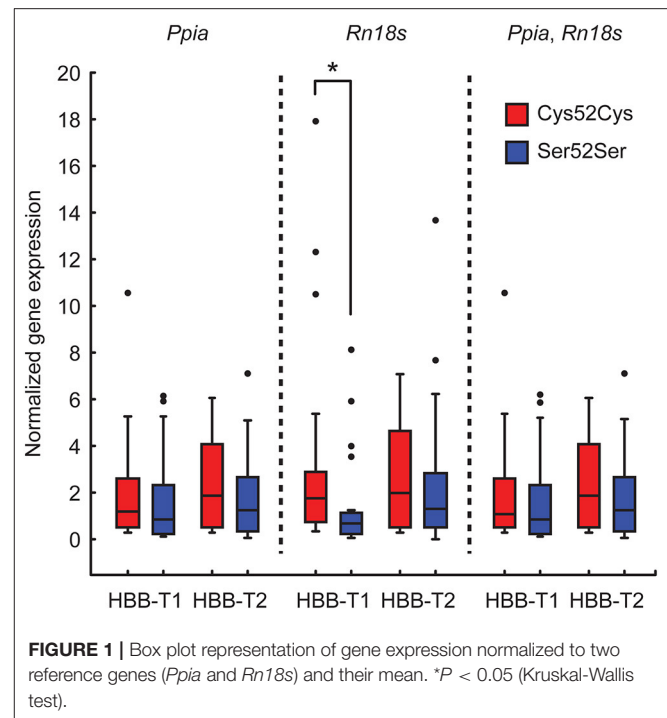
## Statistical Analyses

We analyzed the data with SAS software, version 8.2 (SAS Inst. Inc., Cary, NC). The assumption of normality was tested for each variable using the Shapiro-Wilk  $W$  test. The null hypothesis of a normal distribution was not rejected for the ratio HBB-T1:HBB-T2 and therefore, ANOVA was used to test the differences between groups. The effect of population on gene expression differences between genotypes was tested by a two-way ANOVA with HBB-T1:HBB-T2 ratio as the dependent variable and genotype and mtDNA clade as explanatory variables, followed by Tukey-Kramer multiple comparison test. The values of  $(1+E)^{-Cq}$  and the normalized expression values of the individual genes did not follow a normal distribution and therefore Kruskal-Wallis (non-parametric) test was applied.

## RESULTS

### Difference in the Expression Between HBB-T1 and HBB-T2

The ratio HBB-T1:HBB-T2 ranged from 1.4- to 14.9-fold (mean  $\pm$  SD,  $6.69 \pm 3.24$ ), indicating higher expression level of HBB-T1 than HBB-T2 for all individuals. The Kruskal-Wallis test indicated the difference in the expression between HBB-T1 and HBB-T2 was significant ( $\chi^2 = 24.6$ ,  $P < 0.0001$ ).



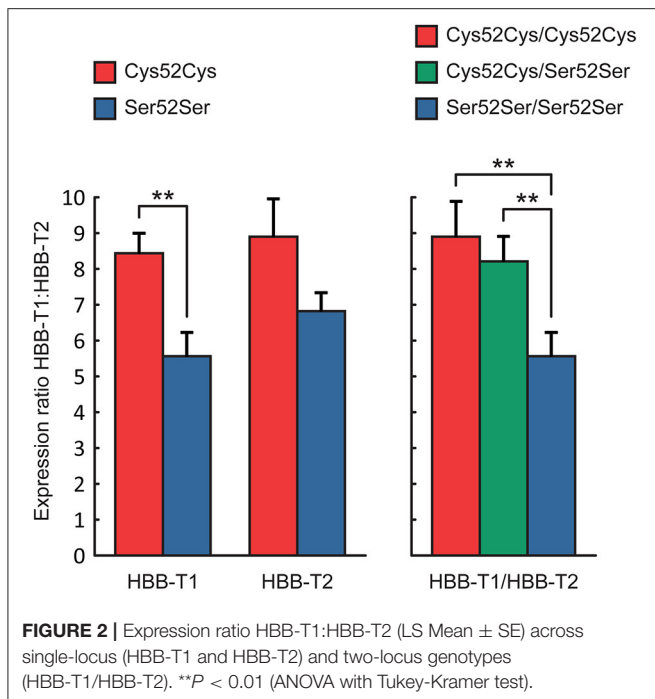
### The Effect of Ser52Cys SNP on Individual HBB Gene Expression

The expression levels for the candidate reference genes *Actb*, *Gapdh*, *Rplp0*, *Tbp*, and *Ube2d2a* varied substantially within datasets (maximum fold difference exceeded 2). *Ppia* and *Rn18s* showed the lowest coefficients of variance (13.8 and 23.3%, respectively) and the lowest standard deviations of the average  $Cq$  values (0.25 and 0.123), which indicated consistent expression levels (Pfaffl et al., 2004). Therefore, in order to compare the expression of each HBB-T1 and HBB-T2 paralog between individuals homozygous for different alleles in that gene, we normalized the expression of each paralog relative to, alternatively, *Ppia*, *Rn18s* and their mean (Figure 1). HBB-T1 homozygotes Cys52Cys showed on an average 1.7-fold higher gene expression compared to homozygotes Ser52Ser. However, the difference was only significant when *Rn18s* was used for normalization (Kruskal-Wallis test,  $\chi^2 = 5.91$ ,  $P < 0.05$ ), but not when *Ppia* was used (Figure 1). A similar difference in expression is also observed in HBB-T2, but is never statistically significant (Figure 1).

### The Effect of Ser52Cys SNP on HBB-T1:HBB-T2 Ratio

We then tested for association of HBB-T1 genotypes, HBB-T2 genotypes and different two-locus genotype combinations (HBB-T1/HBB-T2) with the (non-normalized) expression ratio HBB-T1:HBB-T2. HBB-T1 genotype Cys52Cys was associated with significantly higher ratio (8.5 vs. 5.6) compared to HBB-T1 genotype Ser52Ser (ANOVA,  $F = 11.06$ ,  $P < 0.01$ ;





**Figure 2).** There was no significant difference in HBB-T1:HBB-T2 ratio between the two-locus genotypes Cys52Cys/Cys52Cys and Cys52Cys/Ser52Ser, but they were each associated with a significantly higher ratio (8.9 and 8.2 vs. 5.6, respectively) when compared to the two-locus genotype Ser52Ser/Ser52Ser (ANOVA,  $F = 5.61$ ,  $P < 0.01$ ; **Figure 2**).

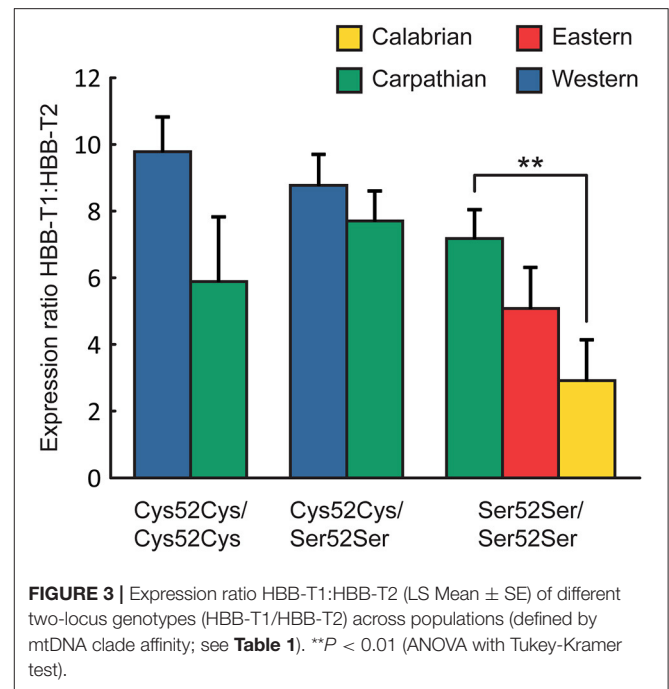
Further analysis revealed that the two-locus genotypes did not, however, show significantly different expression ratios between populations (defined by mtDNA clades), with the exception of genotype Ser52Ser/Ser52Ser, which was associated with a significantly low ratio in the Calabrian population (2.9) vs. the Carpathian population (**Figure 3**).

## DISCUSSION

### Regulatory Variation in the HBB Genes

Although our RT-qPCR analysis revealed broader variation in the HBB-T1:HBB-T2 ratio than previously reported from RNA-seq of three individuals (Kotlík et al., 2014), the data for 47 voles demonstrated that the HBB-T1 is the major adult-expressed gene for  $\beta$ -globin in the bank vole, accounting for an average of 85% of total  $\beta$ -globin mRNA. This agrees with previous findings (Kotlík et al., 2014) from protein electrophoresis analyses that showed that the major electrophoretic Hb polymorphism (Hb S/Hb F) (Hall, 1979) across 145 bank voles was explained by the HBB-T1 genotype, supporting that the relative transcript abundance of HBB-T1 and HBB-T2 reflects the relative abundance of Hb tetramers that incorporate the translated  $\beta$ -globin subunits of those two genes.

The  $\beta$ -globin gene cluster of mammals contains a set of developmentally regulated genes that are arranged in their temporal order of expression, with the 5' end of the cluster



delimited by the embryonic HBE gene and the later expressed (adult) HBD and HBB genes at the 3' end of the cluster (Hoffmann et al., 2008; Gaudry et al., 2014). While in some mammals, products of HBD or HBB/HBD fusion genes combine with  $\alpha$ -globin chains to produce up to 100% of adult Hb (Gaudry et al., 2014), one or more copies of HBB are the only functional adult  $\beta$ -globin genes in rodents (Hoffmann et al., 2008; Kotlík et al., 2014). Surprisingly little is, however, known about the relative transcript abundance of the HBB duplicates. Our findings of higher expression of HBB-T1 relative to HBB-T2 are in agreement with those from  $\beta$ -globin genes of the house mouse (*Mus musculus*), where the assessment of relative transcript abundance demonstrated about a 4-fold higher expression of the upstream gene-duplicate compared to the downstream paralog (Weich et al., 1988; Runck et al., 2009), and the Hb isoform encoded by HBB-T1 accounts for  $\sim 80\%$  of total Hb (Whitney, 1977).

We found no significant difference in HBB-T1:HBB-T2 ratio between the two-locus genotypes Cys52Cys/Cys52Cys and Cys52Cys/Ser52Ser, and thus, there is no evidence that a change in the HBB-T1:HBB-T2 ratio by itself causes variation in Hb Cys content in the bank vole. However, we found allelic differences in the HBB-T1 expression, where there was an approximately two-fold difference in mean expression level between genotype Cys52Cys and genotype Ser52Ser. Although the difference was not statistically significant when normalized to *Ppia*, genotype Cys52Cys was also associated with a significantly elevated HBB-T1:HBB-T2 ratio, which does not depend on normalization. This also means that the variation in HBB-T1 expression level is likely not explained by higher rates of Hb synthesis (i.e., upregulation of all globin genes) in some individuals, for example due to age or physiological conditions (Stankiewicz et al., 2014).

Also, the absence of differences in the expression ratio of each genotype between geographical populations indicates that the observed variation likely cannot be attributed to population-specific effects independent of HBB genotype, such as neutral divergence due to genetic drift (Staubach et al., 2010; Bryk et al., 2013). Rather, our findings are consistent with either an increase of expression of the Cys52 allele or decrease of expression of the Ser52 allele, or both. Such a pattern can be explained most reasonably by linkage disequilibrium between the HBB-T1 polymorphism and a regulatory genetic variant that influences HBB-T1 expression (Morley et al., 2004; Dimas et al., 2008). The basis for this regulatory polymorphism is presently unknown, but it can possibly be a SNP in a regulatory region, such as a promoter or enhancer, which is physically linked to HBB-T1 (*cis*-acting) and binds a *trans*-acting factor to regulate mRNA abundance (Stuve and Myers, 1990; Signor and Nuzhdin, 2018).

Interestingly, a similar pattern of allelic differences in gene expression seems to be present in the low-expression HBB-T2 paralog (Figure 1), although not statistically significant, which indicates the same *cis*-acting polymorphism may be found close to HBB-T2, possibly as a result of gene conversion with HBB-T1.

Balanced synthesis of  $\alpha$  and  $\beta$  chains is necessary for the normal development and function of RBCs and sole upregulation or downregulation of a HBB gene would likely be deleterious, as it would distort the chain stoichiometry (Chaisue et al., 2007). Therefore, some type of compensatory regulatory mechanism most likely exists (Glassberg et al., 2019), whereby the HBB-T1 change leads to a compensatory adjustment of the expression of HBB-T2 (*cis*-acting) or of both genes (*trans*-acting) (Signor and Nuzhdin, 2018).

### Possible Evolutionary Consequences

If, as our results indicate, the relative abundance in bank vole RBCs of the Hb tetramers that incorporate  $\beta$ -globin chains that differ in Cys content (Ser52 vs. Cys52 allele) is modulated by *cis*-regulatory genetic variation, it may have important evolutionary implications. Similar to reactive cysteine-containing Hbs of other rodents (Rossi et al., 1998; Storz et al., 2012), bank vole isoform incorporating the  $\beta$ -globin chain encoded by the Cys52 allele (Hb F) significantly increases the resistance of RBCs to oxidative stress (Kotlík et al., 2014). This resistant phenotype is explained by Hb F taking part in the RBC protein redox system involved in ROS detoxification via the highly-reactive thiol (–SH) group of Cys52, together with the glutathione (GSH) redox system (Rossi et al., 1998; Kotlík et al., 2014; Petersen et al., 2017). Because of the very high concentration of Hb in RBCs, regulatory variation differentially affecting the contribution of the  $\beta$ -globin alleles that differ in Cys content to Hb could provide an effective mechanism for modulating the antioxidant capacity of RBCs (Rossi et al., 1998; Storz et al., 2012; Petersen et al., 2017). A high proportion of redox-active Hb is likely beneficial under conditions conducive to oxidative stress (Rossi et al., 1998; Storz et al., 2012; Kotlík et al., 2014). However, Cys is a precursor for a variety of vital biomolecules, including GSH, and its potential limiting role is considered the source of physiological trade-offs in various organisms (Romero-Haro and

Alonso-Alvarez, 2015; Galván, 2018). In the bank vole, there is likely a trade-off between the use of Cys for Hb synthesis and its use for synthesis of other biomolecules (including GSH), with the outcome of this trade-off possibly being linked to the levels of environmental oxidative stress (Strážnická et al., 2018). Therefore, it has been proposed that the allelic as well as interlocus variation in Hb Cys content provides a means of adjusting the RBC thiol level within populations (Strážnická et al., 2018), maintaining it high enough to provide protection against oxidative damage in RBCs, but without critically reducing the Cys availability for other biosynthetic processes (Beutler et al., 1998; Strážnická et al., 2018). Thus, although they require further empirical evidence, the results of the present study suggest the intriguing possibility that gene regulatory polymorphism may contribute to adaptation of the RBC phenotype in the bank vole by helping to fine-tune the intracellular redox-active thiol levels in response to selection. Therefore, we posit that the  $\beta$ -globin system of the bank vole can serve as a novel model system to study the interaction of gene-regulatory variation with a well-defined protein-coding variation controlling an adaptive physiological trait.

### DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

### AUTHOR CONTRIBUTIONS

VD, LN, SM, and MH carried out the experiments. VD and LN analyzed data. VD and PK wrote the manuscript. PK conceived and supervised the study. All authors commented on the manuscript.

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### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2019.00514/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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