Association of polymorphisms of vasoactive intestinal peptide and its receptor with reproductive traits of turkey hens

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Abstract

The aim of this study was to identify variations of vasoactive intestinal peptide *(VIP)* and VIP receptor-1 *(VIPR-1)* genes that might be associated with turkey reproductive traits. One hundred twenty turkey hens were recorded for age at first egg (AFE), first egg weight (FEW), egg number (EN), total egg weight (TEW), laying period (LP), and broodiness. The DNA was isolated from blood samples and subjected to PCR amplification of the meleagrine *VIP* and *VIPR-1* genes. The SNPs were detected by single-strand conformation polymorphism and the variant DNA fragments were sequenced. One mutation in 3'-UTR of VIP (G5846A) and two SNPs in intron 2 of *VIPR-1 (*C17687T and A17690T) were found, all of them novel. The associations of the three detected SNPs with the reproductive traits of turkeys were evaluated. The detected polymorphisms were used for marker-trait association analyses. The results of association analysis showed that G5846A on 3' UTR of VIP has a significant association with LP, EN, TEW, and AFE. The G allele of G5846A was the favourable SNP allele for LP, EN, and TEW traits. The AA genotype of A17690T on intron 2 of *VIPR-1* was significantly associated with higher LP, EN, and TEW. AGAA haplotype showed association with higher EN and TEW. These results suggest that the SNPs in 3'-UTR of *VIP* and intron 2 of *VIPR-1* genes may influence egg production traits in turkey hens.

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Introduction

Reproduction traits have been of primary interest to breeders in the poultry industry. Many environmental factors such as photoperiod and nutritional diet (Liu, 2004; Lewis & Gous, 2006) can affect reproduction performance, and genetic factors also influence these traits (Miazi *et al.*, 2012; Nikbin *et al.*, 2018; Zhu *et al.*, 2018). It has been shown that immunization against vasoactive intestinal peptide (VIP) inhibits reproduction via neuronal and hormonal pathways (Son *et al.*, 2016; Vistoropsky *et al.*, 2016). The effects of *VIP* and its receptor (*VIPR-1*) genes on the reproduction traits of poultry have recently been a focus of interest (Xu *et al.*, 2011; Ngu *et al.*, 2015; Pu *et al.*, 2016).

VIP is a 28-amino acid polypeptide that belongs to a family of regulating peptides such as secretin (Sheward *et al.*, 1995). VIP is a prolactin (PRL)-releasing factor that can promote the secretion of PRL (El Halawani *et al.*, 1990; Kansaku *et al.*, 2016). VIP antibody injection inhibits broodiness and reduces serum PRL in incubating hens (Sharp *et al.*, 1998). Active immunization against VIP in turkey hens terminates broodiness and increases egg production (El Halawani *et al.*, 2000). The *VIP* gene is located on chromosome 2 of turkey and has 6 exons (NCBI ref no: NC_015012.2). The association of *VIP* and *VIPR-1* polymorphisms with egg production of chicken has been reviewed (Xu *et al.*, 2011; Pu *et al.*, 2016).

VIP induces its effects by interacting with its specific receptor, namely intestinal peptide receptor-1 (VIPR-1), which is located on the membrane surface of the anterior pituitary cells in birds (El Halawani *et al.*, 1990). VIPR-1 is a glycoprotein, and belongs to the class II subfamily of the 7-transmembrane G-protein-coupled receptors superfamily (Gaudin *et al.*, 1998). Xu *et al.* (2011) reported that VIP and its receptor affect

reproduction traits of poultry via influencing gonadotropin releasing hormone (GnRH) secretion. It has been proved that the *VIPR-1* gene influences the endocrine mechanism of broodiness and, hence, involves the regulation of broodiness in avian species. (Kansaku *et al.*, 2001; Chaiseha *et al.*, 2004). Although the expression of the *VIPR-1* gene occurs in both the hypothalamus and the pituitary, only the variation of its mRNA expression in the pituitary has been associated with reproductive traits (Chaiseha *et al.*, 2004). You *et al.* (2001) stated that variation in the expression of *VIPR-1* is associated with changes in the reproductive traits of egg-laying quail. Although several research projects have studied the effects of *VIP* and *VIPR-1* expressions at transcriptional level (Chaiseha *et al.*, 2004; Kansaku *et al.*, 2001; You *et al.*, 2001) and translational level (El Halawani *et al.*, 2000; Kang *et al.*, 2002) on reproductive traits of avians, studies on the influences of *VIP* and *VIPR-1* DNA variations on reproductive traits of turkey hens are scarce. Indigenous turkeys of Iran have relatively low reproductive performance. To develop an indigenous turkey industry, the reproductive traits of the turkeys must be improved. The objective of this study is therefore to investigate the polymorphism of turkeys' *VIP* and *VIPR-1* genes and their association with reproductive traits, including AFE, EN, EW, LP and broodiness behaviour of the native turkey of northwest Iran.

Materials and Methods

In this research, 120 turkey hens were used from the turkey research station of north-western Iran. Genomic DNA was extracted from the whole blood of the turkeys according to a modified protocol of Bailes *et al.* (2007). Reproductive traits, including egg number (EN), age at first egg (AFE), first egg weight (FEW), total egg weight (TEW), egg weight (EW), laying period (LP), and broodiness behaviour, were recorded from the turkey hens. All of the hens were kept in individual cages. Duration of laying was recorded as laying period. All laid eggs were counted and weighed to provide EN and TEW records, respectively. The hens that showed broodiness behaviour after a few layings were recorded as broody hens. The duration of the recording was from 10 March to 15 October. All recording and blood sample collections were performed in accordance with the protocol approved by the Animal Welfare Committee of University of Mohaghegh Ardabili.

Two pairs of primers were designed by Primer3 v. 0.4.0 software (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) based on the sequence of *VIP* (GenBank NC_015012.2.1) and *VIPR* (GenBank XM_019616272.1) genes. The designed primers F: 5′- tgaaggacacctagcaaactc -3′ and R: 5′- acttcacacgttctcttgctc -3′ were used to amplify exon 6 and 3'-UTR of the *VIP* gene (*VIPe6*). To amplify exon 2 and a part of intron 2 of the *VIPR* gene (*VIPRe2*), the forward and reverse primers were designed as F: 5′- tcccccaggttgcagaag -3′ and R: 5′- tgcagaatcgtggagatgtg -3′. The targeted fragments were 433 and 316 bp for *VIPe6* and *VIPRe2*, respectively. Polymerase chain reaction (PCR) was performed in a reaction volume of 25 µL containing 50–100 ng of the genomic DNA, 12.5 µL PCR Mastermix (Cinnagen, Iran), 1 µL of 10 µM of each primer and 2 µL 10 mM dNTPs, and 8.1 µL ddH$_2$O. The PCR program that was used was 5 min pre-denaturation at 95 °C; 32 amplification cycles of 95 °C for 30 seconds; 59.5 °C for 30 seconds; 72 °C for 1 min; and final extension at 72 °C for 10 min. The PCR products were examined by electrophoresis on 1.5% agarose gel stained by GelRed® nucleic acid gel stain (Biotium Inc, Hayward, CA).

To identify polymorphisms for each gene, the single-strand conformation polymorphism (SSCP) method was performed. The PCR products were electrophoresed on 8% polyacrylamide gel and the bands were visualized using the silver staining method. The PCR products of patterns that were obtained for each locus were sequenced commercially (Macrogen, South Korea).

BioEdit software (Hall, 1999) was used to align the sequences of patterns for each locus to find polymorphisms. PopGen32 software was used (Yeh *et al.*, 2000) to identify genotype and allele frequencies of the candidate genes and Hardy-Weinberg equilibrium (HWE., The association of polymorphisms or haplotypes with the traits was analysed using SAS GLM procedure (SAS Institute Inc., Cary, NC, USA). Multiple comparisons of Tukey's test were performed for means separation. The χ2 distribution test was performed for broodiness trait for each biallelic locus.

Results

The descriptive statistics of the recorded traits of the turkey hens are shown in Table 1.

Table 1 Descriptive statistics of reproductive traits of turkey hens

Traits	Mean	se	Min	Max
Laying period (day)	136.09	4.94	38	201
Egg number	50.81	2.19	13	90
First egg weight (gr)	72.46	0.73	61.4	86.1
Total egg weight (gr)	3940.05	180.66	1000.7	6982.2
Age at first egg (day)	246.94	0.86	240	281
Egg weight (gr)	76.8	0.51	66.8	86.5

The identified SNPs of *VIPe6* and *VIPRe2* are shown in Figure 1. A substitution of G with A was detected in *VIPe6* at position 5846 bp (G5846A), which occurred in 3'-UTR region of the *VIP* gene.
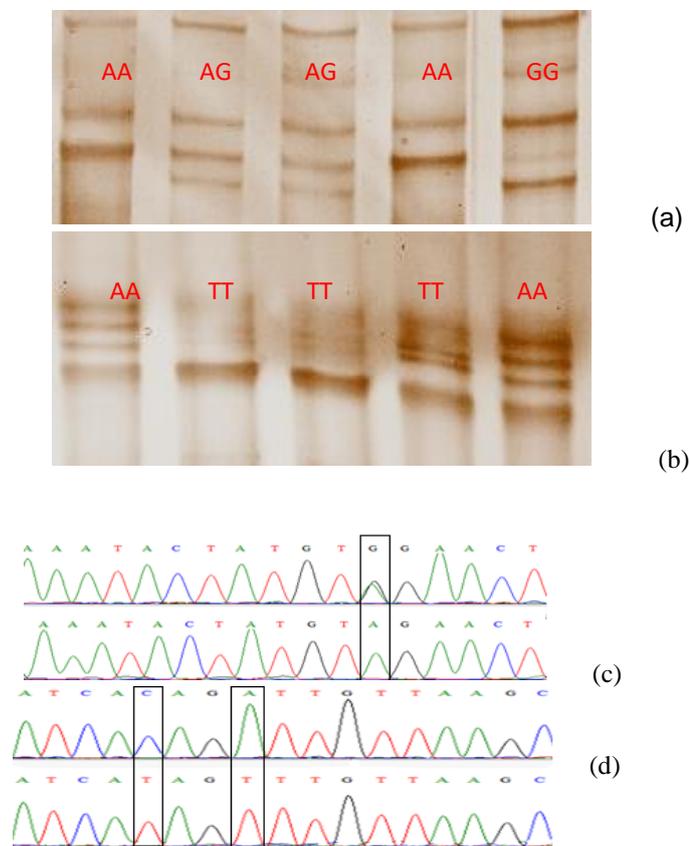
Figure 1 Polyacrylamide gel image and genotypes of (a) VIPe6, and (b) VIPRe2, and sequence electropherograms showing SNP sites (c): G5846A for *VIPe6*, and (d): C17687T and A17690T for *VIPRe2*

A transition in position of 17687 bp (C17687T) and a transversion in position of 17690 bp (A17690T) were detected in intron 2 of *VIPR* gene. Both sequences were submitted to NCBI GenBank. Accession numbers for *VIPRe2* were MG770236-MG770237 and for *VIPe6* were MG770238-MG770239. Allele and genotype frequencies of *VIPe6* and *VIPRe2* loci are presented in Table 2. Three genotypes were observed for *VIPe6*, and two genotypes were observed for *VIPRe2*. The highest genotype frequency was detected for AA of *VIPe6* (0.85), while GG of *VIPe6* was the lowest (0.04). Observed heterozygosity of *VIPe6* was 0.11, and no heterozygous genotype was observed for *VIPRe2*. The effective number of alleles per loci were 1.2532 and 1.4751 for *VIPe6* and *VIPRe2*, respectively. The results of chi-square test revealed significant deviations from Hardy-Weinberg equilibrium (HWE) for both loci (Table 2).

Table 2 Genotype and allele frequencies, effective number of allele and Hardy-Weinberg equilibrium test for the polymorphic loci in the turkey hens.

Locus	n_a	n_e	Allele frequency		Genotype frequency			Expected heterozygosity	Nei	HWE Test
			A	G	AA	AG	GG			
VIPe6	2	1.25	0.905	0.095	0.85	0.11	0.04	0.17	0.17	**
			A	T	AA	AT	TT			
VIPRe2	2	1.48	0.202	0.798	0.20	0.00	0.80	0.32	0.32	**

n_a: number of alleles; n_e: effective number of alleles, HWE: Hardy-Weinberg equilibrium
**significant difference from equilibrium

The results of association analysis are presented in Table 3. The statistical analysis revealed that G5846A on the 3'-UTR region of *VIP* gene has associations with EN, TEW, and AFE (P <0.05). No significant association of G5846A with LP, FEW, or EW was found. The GG genotype of *VIPe6* was associated with higher LP and AFE (P <0.05). However, AG was a favourable genotype for EN and TEW traits compared with those of the AA genotype, while no significant difference was observed between the AG and GG genotypes for EN and TEW.

The AA genotype of *VIPRe2* was associated with greater LP, EN, and TEW (P <0.05). No significant association of A17690T with AFE, FEW, and EW was found. The sequencing results showed four haplotypes for the turkeys. The haplotypes' association results are presented in Table 3. The haplotype AAAG was favourably associated with EN and TEW. The AAGG turkeys showed later puberty and had higher AFE compared to the other haplotypes (P <0.05). The results of chi-square analysis revealed that none of the polymorphisms on *VIPe6* and *VIPRe2* was associated with the broodiness of the turkeys.

Table 3 Association of *VIPe6* and *VIPRe2* genes and the haplotypes with egg production traits of indigenous turkey hens

	Genotype	Laying period	Egg number	First egg weight	Total egg weight	Age at first egg	Egg weight
VIPe6	AA	133.61[b] ± 4.13	48.55[b] ± 1.81	72.3 ± 0.59	3786.25[b] ± 150.24	246.28[b] ± 0.67	76.67 ± 0.4
	AG	149.91[b] ± 10.92	65.55[a] ± 4.93	72.13 ± 1.57	5121.15[a] ± 397.5	245.82[b] ± 1.77	77.9 ± 1.07
	GG	155.75[a] ± 18.11	61.50[ab] ± 8.18	71.55 ± 2.6	4602.5[ab] ± 659.18	261.75[a] ± 2.92	74.88 ± 2.6
	P-value	0.2151	0.0032	0.9588	0.0061	0.0001	0.32
VIPRe2	TT	131.92[b] ± 3.59	47.79[b] ± 1.66	72.65 ± 0.55	3687.0[b] ± 132.12	246.58 ± 0.68	76.78 ± 0.37
	AA	151.79[a] ± 7.85	62.26[a] ± 3.27	72.80 ± 1.21	4819.59[a] ± 289.15	248.89 ± 1.49	77.24 ± 0.8
	P-value	0.023	0.0001	0.9077	0.0005	0.16	0.596
Haplotype	TTAA	132.7 ± 4.46	48.22[b] ± 2.02	72.19 ± 0.62	3744.5[b] ± 162.1	246.34[b] ± 0.72	76.83 ± 0.4
	AAAA	145.5 ± 18.39	54.00[ab] ± 8.34	72.92 ± 2.52	4207.4[b] ± 668.36	244.5[b] ± 2.98	77.8 ± 1.77
	AAAG	149.9 ± 11.08	65.55[a] ± 5.03	72.13 ± 1.54	5121.1[a] ± 403.04	245.81[b] ± 1.8	77.9 ± 1.07
	AAGG	163.25 ± 18.39	61.5[ab] ± 8.34	71.55 ± 2.56	4602.5[b] ± 668.36	261.75[a] ± 2.98	74.87 ± 1.8
	P-value	0.22	0.01	0.55	0.015	0.0001	0.49

Data are expressed as mean ± SD. Within a row values followed by different letters differ significantly at the 0.05 level by the Tukey test

Discussion

In the present study, both *VIPe6* and *VIPRe2* gene regions showed polymorphisms. This is the first report for the SNP in the 3'-UTR region of avian *VIP*. The number of reports in this region is limited. *VIP/ApoI* (Xu *et al*., 2011), *VIP/HinfI*, and *VIP/VspI* (Vu & Ngu, 2016) have been reported in chickens, but were not

matched with the current findings on 3'-UTR region of *VIP* in turkey. Differences in SNPs between the two species in the same region of the gene might be due to different evolutionary processes with different rates of mutation on the orthologues in the different species. Most of the turkeys had the AA genotype. However, only a low frequency (9%) of G allele was observed. Therefore, the homozygote GG appeared rarely (4%) in this study.

In *VIPRe2*, both detected SNPs (C17687T and A17690T) were novel and have never been reported in turkeys. Only two haplotypes were detected for *VIPRe2* in the turkeys. Since no similar work on this gene has been found, the frequencies of the alleles at these loci were not available for comparison. However, Zhou *et al.* (2008) stated that there are seven variation sites in the region from exon 6 to exon 11 in chickens. Two more SNPs were reported by Pu *et al.* (2016) in exon 4 (G373T) and exon 6 (A313G) of laying quail. Chicken *VIPR1/TaiI, VIPR1/HahI*, and *VIPR1/TaqI* polymorphisms (Vu & Ngu, 2016; Xu *et al.*, 2011) did not match with SNPs of *VIPRe2* of the present study either.

In this research, the polymorphic loci were not in HWE. This may have happened because of factors such as selection, mutation, and/or migration, which influence allele and genotype frequencies through generations (Falconer & Mackay, 1996). Since the population was under selection, immigration of new males, and deviation from HWE were predictable.

The current research is the first attempt to investigate the relationship of the *VIP* and *VIPR-1* loci polymorphism with reproductive traits in turkey. Replacing the allele A with the allele G of *VIPe6* was a synonymous mutation. Substituting A with G significantly increased EN and TEW in AG hens. The GG genotype also showed longer LP. These results showed that the G allele was the favourable allele for LP, EN, and TEW traits. However, it was also associated with higher AFE in the turkey hens. Although the mutation of 3'-UTR has no effect on the amino acid sequence of the related protein, it may alter the appropriate expression of the *VIP* gene. It was revealed that SNP in 3'-UTR can be associated with phenotype changes via altering expression of the gene responsible for the phenotype (Yue *et al.*, 2017; Zhang *et al.*, 2016). Many studies have proved that SNPs in 3'-UTR may regulate expression of their genes (Reamon-Buettner *et al.*, 2007; Kataoka *et al.*, 2016; Zuo *et al.*, 2014). Changes in the 3'-UTR sequence are significantly associated with mRNA stability (Kamiyama *et al.*, 2007) and could influence the rates of localization, translation, and mRNA degradation (Reamon-Buettner *et al.*, 2007). Recent studies revealed that miRNA reacts with 3'-UTR of mRNA, which may negatively regulate the gene transcripts and, consequently, alter the related protein synthesis (Dumortier & Obberghen, 2012; Egervari *et al.*, 2016). Additionally, it may influence adenylation of 3'-UTR and/or cleavage, which accordingly affects gene expression (Liu *et al.*, 2010; Skeeles *et al.*, 2013). The importance of the SNPs in 3'-UTR and its effect on the regulation of the gene expression cannot be ignored. Therefore, the substitution of G5846A might influence the *VIP* gene expression through this way, and thus the reproductive traits of the turkey hens. Zhou *et al.* (2010) reported a significant association between a polymorphism of intron 2 of the chicken *VIP* and egg numbers from 90 to 300 days old, and AFE.

Prolactin gene expression and its plasma concentrations are influenced by VIP levels in the anterior pituitary gland (Chaiseha *et al.*, 2004; Kansaku *et al.*, 2001). Since prolactin affects egg production and serum oestrogen level (Zhang *et al.*, 2007), its influence of VIP level on egg production traits can be explained. Meek *et al.* (2016) reported that VIP increased the level of pepsinogen secretion from the stomach by affecting the parasympathetic nerves, which resulted in improved digestion and indirectly increased the energy, protein, and minerals that are required for egg production. This can also affect the total weight of the egg produced during a period by maintaining the chicken's physiological condition (Bu *et al.*, 2015; Van Emous et al., 2015). On the other hand, the VIP gene stimulates growth hormone releasing hormone (GHRH) secretion by stimulating the secretion of ghrelin. The secretion of GHRH causes the release of growth hormone (Osterstock *et al.*, 2010). Consequently, growth hormone increases follicles and egg production in poultry (Pitts *et al.*, 1996).

The effect of VIP on GnRH via the VIP receptors in species of both mammals and poultry was reported (Christian & Moenter, 2008), such that VIP and its receptor, which affect GnRH, can reduce AFE in poultry (Xu *et al.*, 2011). It may explain how G5846A is responsible as an effective SNP on LP, EN, TEW, and AFT traits.

Although this SNP has never previously been reported, similar to the current result, other polymorphisms of *VIP* showed significant influence on EN of chickens. Zhou *et al.* (2010) revealed that the VIP/*VspI* SNP associated with EN from 90 to 300 days of age and that *VIP/HinfI* influenced broodiness in chickens. Xu *et al.* (2011) also reported three polymorphisms, *VIP/ApoI, VIPR1/TaqI*, and *VIPR1/HhaI*, associated with egg number at 300 days old.

A17690T and C17687T SNPs in *VIPRe2* have not previously been reported. Based on the current results, the two SNPs were close together. However, the authors found only one haplotype. Therefore, they regarded them as one allele (A17690T) in statistical analysis. Different haplotypes from the two SNPs may

be identified in larger populations. The A allele was a favourable allele for LP, EN, and TEW. The allele was located in intron 2. Since the SNP was detected in the intron region, it would not cause any change in the sequence of the protein. However, the SNPs in the intron region may affect transcription of the gene (Berulava & Horsthemke, 2010) and consequently influence the level of VIPR. Olivier (2004) suggested that intronic SNP may affect the level of expression of the gene. There are many reports about significant association between intron regions and phenotypes (Anh *et al.*, 2015; Berkowicz *et al.*, 2012; Luna-Nevarez *et al.*, 2011). Other reports also indicated that any change in the intronic region was effective on the splicing process, and consequently influenced the gene expression level (Komar, 2007). Several research articles have been published about the potential effects of splicing on the trait phenotype (Faustino & Cooper, 2003; Ho *et al.*, 2011; Nissim-Rafinia & Kerem, 2002). Therefore, the SNP in intron 2 may change the expression level of the *VIPR-1* gene and then influence the phenotypes.

VIPR-1 is distributed widely in the gastrointestinal tract, genitourinary system, and other smooth muscle and secreting glands. By binding to the G protein, VIPR-1 activates adenylyl cyclase and increases cAMP (Ishihara *et al.*, 1992). Therefore, VIPR-1 indirectly affects the activity of digestive gland cells and consequently increases the digestive enzymes, improves feed efficiency, and thus increases egg production in turkeys. The effect of VIP on gonadotropin-releasing hormone via VIPR-1 (Christian & Moenter, 2008) can influence levels of gonadotropins and, as a result, the reproductive performance of turkeys. The expression of the VIPR-1 gene showed a positive correlation with broodiness in chickens and turkeys, which affects egg production directly (You *et al.,* 2001). Several studies have shown association between VIPR-1 gene polymorphisms and reproductive traits of avian species. VIPR-1 was introduced as an MAS marker for decreasing the rate of broodiness and improving egg production (Zhou *et al.*, 2008). Chaiseha *et al.* (2004) revealed that the VIPR-1 gene plays a role in regulating broodiness in birds via influence on the endocrine system. Association between polymorphisms of *VIPR-1* and egg number of quail (Pu *et al.*, 2016) and chickens (Ngu *et al.*, 2015) has also been reported. Similarly Zhou *et al.* (2008) showed that *VIPR-1* polymorphisms affected incubation behaviour and age at first egg significantly. Similar to the present study, Xu *et al.* (2011) did not find any association between the polymorphisms of *VIPR-1* (VIPR-1/*HahI* and VIPR-1/*TaqI*) and age at first egg in Ga Noi chickens. The statistical results revealed that the haplotype AAAG was favourably associated with EN and TEW.

VIPe6 and *VIPRe2* polymorphisms did not show association with broodiness of turkeys. These results were in contrast with some reports on chickens and quail. This may occur because of the differences in SNPs and species between the present study and previous research. Although Zhou *et al.* (2010) demonstrated that C338T SNP in intron 2 of VIP was associated with incubation behaviour, the AGG indel in 5'-UTR did not affect broodiness in chickens. Investigation of the *VIPR-1* gene of chickens revealed that C+598T in intron 2 is associated with frequency of broodiness, but *VIPR-1* was found to have no effect on broodiness frequency (Zhou *et al.*, 2008).

Conclusion

Three novel SNPs were found (one SNP in 3'-UTR of *VIP* and two SNPs in intron 2 of *VIPR-1*). The results revealed that these SNPs in meleagrine *VIP* and *VIPR-1* were associated with egg production traits, egg number, total egg weight, and laying period. The results can be used for selection and breeding programmes, to improve the reproductive performance of turkey hens. The effects of the SNPs on the expression level of the *VIP* and *VIPR*-1 genes require further investigation.

Authors' Contributions
SN planed the research, supervised its conduct and wrote the manuscript. GE-Z carried out recording and sampling. LH carried out the lab experiments and prepared the data. SN and NH-E supervised preparation of materials and data analysis. All authors have read and approved the manuscript.

Conflict of interest declaration
The authors declare that they have no conflict of interest.

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