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Original article

Study of selected genes of Wnt signaling pathway in relation to the parameters in the bone tissue of the laying hens

Michala Steinerova [a], Cenek Horecky [a], Eliska Horecka [a], Ales Knoll [a], Sarka Nedomova [b], Petr Slama [a], Ales Pavlik [a,*]

[a] Department of Animal Morphology, Physiology and Genetics, Mendel University in Brno, Faculty of AgriSciences, Zemědělská 1/1665, 613 00 Brno, Czech Republic
[b] Department of Food Technology, Mendel University in Brno, Faculty of AgriSciences, Zemědělská 1/1665, 613 00 Brno, Czech Republic

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ABSTRACT

The Wnt signaling pathway plays a critical role in almost all aspects of skeletal development and homeostasis. Many studies suggest the importance of this signaling pathway in connection with bone metabolism through many skeletal disorders caused by mutations in Wnt signaling genes. The knowledge gained through targeting this pathway is of great value for skeletal health and diseases, for example of increased bone mass in the case of osteoporosis. Our objective was to focus on the detection of single nucleotide polymorphisms and investigate the associations between possible polymorphisms in selected genes that are part of those signaling pathways and parameters of bones in hens of ISA Brown hybrids (bone breaking strength, length, width, and bone mass). Different regions of the *GPR177, ESR1* and *RUNX2* genes were studied, using PCR and sequencing, in a total of forty-eight samples for each marker. Thirteen polymorphisms have been discovered in selected regions of studied genes, whereas these polymorphisms were only within the *GPR177* gene. Eight of these polymorphisms were synonymous and five were in the intron. The tested regions of the *ESR1* and *RUNX2* genes were monomorphic. The only statistically significant difference was found within the *GPR177* gene (exon 2) and the bone length parameter, in the c.443 + 86G > A polymorphism. However, this polymorphism was found in the intron, and no other one was found within the selected regions to show associations with the observed bone parameters.

1. Introduction

Various cellular processes, such as cellular proliferation, differentiation, body axis patterning, are controlled by cellular and tissue functions in which the Wnt signaling pathway is involved, and this signaling is also required in bone for proper development (Bullock and Robling, 2017). Bone growth is stimulated by WNT ligands, indicating a very important role for Wnt signaling pathway in the bone healing process (Houschyar et al., 2018).

The skeletal health is constantly maintained by the renewal of the skeleton, which occurs in a very energy-intensive process of bone remodeling. This process, in which old bone is replaced and localized bone defects are repaired, makes bone a dynamic organ with unique mechanical properties. Due to the complex arrangement of bone characteristics and possible continuous modifications, the bones are mainly strong, rigid, but also elastic and resistant to deformation. In addition to aging, many other factors can contribute to bone weakness, including estrogen deficiency (Hardy and Fernandez-Patron, 2020; Osterhoff et al., 2016).

The Wnt signaling pathway plays an important role in a wide range of biological processes. It includes a large family of secreted glycoproteins that control processes such as cell proliferation, differentiation, apoptosis, migration, and polarity in a variety of cell types, with significant involvement in embryonic development (including skeletal patterning) (Manolagas, 2014). Wnt signals are transmitted by at least two intracellular pathways, where the so-called canonical Wnt pathway (β-catenin-dependent) is best studied. The second, β-catenin-independent, is the non-canonical pathway, which is further divided into Wnt/planar cell polarity (PCP) and the Wnt/Ca2 + pathway. Many human diseases, which can include many types of cancer, but also other degenerative diseases and developmental defects are associated with misregulation of

* Corresponding author.
E-mail address: ales.pavlik@mendelu.cz (A. Pavlik).
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Wnt signaling (Azbazdar et al., 2021). It has been found that gene mutations that are part of Wnt signaling are associated with many skeletal diseases, in which bone strength is affected. Such diseases include, for example, osteoporosis (Lu and Han, 2018). This chronic skeletal disease leads to impaired bone quality, especially in strength and high susceptibility to fracture. Osteoporosis is characterized by a loss of bone mass and a change or even a defect in its structure, with consequent increased fragility. Pain, frequent fractures, and physical limitations make osteoporosis a disease that causes considerable suffering (Mäkitie et al., 2019; Zhu et al., 2021), which is a problem not only in humans but also in animals. For example, in caged laying hens, the fragility of their bones is a serious problem (Steinerova et al., 2019a, 2019b, 2020), whereas in commercial flocks, fractures occur in more than 30% of all laying hens during their lifetime. The disease is multifactorial, with environmental, nutritional, and genetic components (Horecka et al., 2015, 2018).

Relatively newly identified proteins include the GPR 177 protein (G protein-coupled receptor 177), which accompanies Wnt ligands after secretion. It is also known as Wintless (Wls) (Zhong et al., 2012). Through activation by different extracellular signals, the G protein-coupled receptors superfamily provides a number of biological processes, while it is hugely structurally and functionally diverse. Some human studies point to several mutations which disrupt bone development or metabolism (Luo et al., 2019). GPR177 may be necessary for several important steps in osteogenesis, mainly due to its crucial role in Wnt signaling. Conditional deletion of the gene, which leads to serious damages of the craniofacial and body skeletons, is used in mice to evaluate its functionality. Based on the defects in the expansion of skeletal precursors, it is clear, that GPR177-mediated signaling cannot be replaced (Maruyama et al., 2013). Important regulators of skeletal growth and maintenance include estrogens with the estrogen receptor alpha being the major mediator of these estrogenic results on bone (Börjesson et al., 2013). Functional estrogen receptor and Wnt /-catenin signaling are important for bone response to mechanical signals (Liedert et al., 2020).

Runx2, which is necessary for osteoblast development and bone formation, and belongs under the Runt family of gene domains, is also called CBfa1. By regulating the expression of selected proteins, including osteocalcin, it aids in the maturation and function of osteoblasts (Haxaire et al., 2016; Komori, 2019).

The purpose of this study was to find single nucleotide polymorphisms in selected genes that are involved in the Wnt signaling pathway and to identify the relationship of the found polymorphisms to selected bone mechanical parameters in laying hens. This signaling pathway has been selected for its important role in bone metabolism, which was highlighted in several studies. However, the results in the form of polymorphisms, which would show associations with the monitored parameters, can help in understanding this issue and be significant in the fact that protein production and bone formation are affected.

2. Material and methods

The tested laying hens of ISA Brown hybrids, which were kept according to 74/99 / EC Council Directive in enriched cages, were slaughtered by decapitation, during which their blood was taken. This collected blood was stabilized with heparin and used for further analyzes. These laying hens were fed with balanced feed in the amount of 116 to 170 g of feed per hen per day and light conditions were provided according to the technological instructions for breeding the given hybrid. Laying hens were put into production at the 16th week of age, their laying period begins from the 18th week according to ISA Brown cage guide (https://www.isa-poultry.com/documents/597/ISA_Brown_CS_cage_English_guide.pdf). At the age of 26 weeks, at the peak of production, hens were slaughtered. DNA was isolated using a DNA Lego kit (Top-Bio, Prague, Czech Republic). A set of 48 laying hens was tested and bone parameters were determined on the femur. Femur was cleaned and the dimensions of the bones, as well as their width and length, were determined using a Vernier caliper, and the same measurement procedures were followed for all bones. In the case of length, bone was measured at the longest distance between the end of the distal and proximal epiphysis and the its width as the greatest distance between the *facies caudalis* and *facies cranialis* at the fracture point. In the event of bone strength, a three-point bending test was used using the TIRATEST 27,025 testing machine (TIRA Maschinenbau GmbH, Schalkau, Germany).

2.1. GPR177, ESR1 and RUNX2 genes testing

Own oligonucleotide primers were used to test selected regions of the genes (Table 1). These primers were designed in OLIGO v4.0 software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA). Using the ABI Veriti 96-Well thermocycler (Life Technologies, Applied Biosystems, Foster City, CA, USA), the thermal profiles of the PCR reactions were determined as follows: 94 °C for 3 min, 30 cycles at 94 °C for 1 min, 53, 55, 57 or 58 °C for 30 s; 72 °C for 1 min, 72 °C for 10 min, then maintained at 4 °C. Annealing temperature of 58 °C was used for GPR177 exon 2; 57 °C for exon 4 and exon 11; 53 °C for ESR1 exon 1, 57 °C for ESR1 exon 4 and 55 °C for RUNX2 exon 6. PCR products were verified by electrophoretic separation on a 2.5% agarose gel (TBE buffer). A GoodView fluorescent dye was used as a visualizer (Ecoli, ltd., Bratislava, Slovak Republic). The separation was performed for 30 min and at 120 V. The conditions for preparing the PCR sequencing template were: 96 °C for 1 min, next 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Purification followed, using a BigDye XTer-

Table 1
GPR177, ESR1, RUNX2 primers.

Gene	Primer sequence	Product size (bp)
GPR177 (exon 2)	**Forward:** 5′-ACTAACGGCAGGGTCAAGGAG-3‘ **Reverse:** 5′-CTCACCCTTCTGCATCATCTCC-3‘	539
GPR177 (exon 4)	**Forward:** 5′-GTTCACTGCTGGGCTCTCGT-3‘ **Reverse:** 5′-AGCGGTCAGAGAGGCACTTC-3‘	443
GPR177 (exon 11)	**Forward:** 5′-GCAGCCCCATTGTAAGCAGTG-3‘ **Reverse:** 5′-CACTGAAGGGCAAACAAGAGCAC-3‘	714
ESR1 (exon 1)	**Forward:** 5′-AATGTGCCTTTCCAGTGCTCA-3‘ **Reverse:** 5′-TGATTCCACAAGATTACGCAGTTC-3‘	749
ESR1 (exon 4)	**Forward:** 5′-AAGCCTCAACAAAGTAAACCAG-3‘ **Reverse:** 5′-AGTGGTCTTGTTGGAAATGC-3‘	775
RUNX2 (exon 6)	**Forward:** 5′-AGTTGTGTTTCATTGTGTTCTCC-3‘ **Reverse:** 5′-TCTTCTTATTAGGGTTTGTCTGC-3‘	382

Legend: *GPR177* – G protein-coupled receptor 177, *ESR1* – estrogen receptor alpha, *RUNX2* – runt-related transcription factor 2.

minator Purification kit (Life Technologies, Applied Biosystems) according to the manufacturer's protocol and sequencing was performed on an ABI PRISM 3500 DNA analyzer (Life Technologies, Applied Biosystems). SeqScape v2.7 (Life Technologies, Applied Biosystems) was used to evaluate the results on the principle of sequence alignments.

2.2. Statistical evaluation

A non-parametric rank-based test, the Kruskal-Wallis test, was used to determine statistically significant differences between the observed indicators, which were as dependent variables and genotype as independent variables, using STATISTICA 12 statistical software (StatSoft Inc., Tulsa, USA). Value of $P < 0.05$ was considered statistically significant.

3. Results

Within the monitored genes, which were studied in forty-eight samples for each marker, a total of 13 polymorphisms were found, with 8 being synonymous and 5 in the intron. These polymorphisms were found only within selected regions of the *GPR177* gene. The *GPR177* gene is in laying hens located on chromosome 8, has a total of 12 exons, and the regions of exons 2, 4, and 11 were examined. The PCR products of *GPR177* exon 2 had size of 539 bp, the *GPR177* products of exon 4 had size of 443 bp and exon 11 had size of 714 bp.

In the case of *GPR177* gene 8 polymorphisms were identified within area of exon 2. Directly in the exon were 4 of them (c.187G > A, c.205G > A, c.226A > G, c.319C > T). All of these polymorphisms were synonymous. The other 4 polymorphisms were found in the intron (c.171-47G > T, c.171-44C > T, c.443 + 71C > T, c443 + 86G > A). The polymorphism c.443 + 86G > A was the only association with the observed parameters, namely bone length (Table 2).

Within the studied region of exon 4 of the *GPR177* gene, only one polymorphism was found (c.676C > T), which was located in the exon, this polymorphism was synonymous (Table 3).

A total of 4 polymorphisms were found in the studied region of exon 11. One polymorphism was intronic (c.1427-27G > C) and 3 were in the exon, one being synonymous (c.1462G > A) and 2 non-synonymous (c.1428 T > A, c.1448 T > G) (Table 4).

The *ESR1* gene has 8 exons and is located on chromosome 3. Region of exon 1 and exon 4 were tested. The PCR products of *ESR1* exon 1 had size of 749 bp and products of *ESR1* exon 4 had size of 775 bp.

The *RUNX2* gene is located on chromosome 1 and has 6 exons. The region of exon 6 was studied, and the PCR product had size of 382 bp.

For both the *ESR1* gene and the *RUNX2* gene, all regions of interest were monomorphic.

The range of values for individual parameters of laying hen bones is given above (Table 5). Apart from the above-mentioned polymorphism c.443 + 86G > A of the *GPR177* gene, the effect of other studied polymorphisms on selected bone parameters was not demonstrated.

Table 2
Association of *GPR177* (exon 2) gene polymorphisms with selected bone parameters.

Gene	SNP	Genotype (n)	Bone breaking strength [N]	Length [mm]	Width [mm]	Bone mass [g]
GPR177 (exon 2)	c.171-47G > T	TT (25)	119.97 ± 47.01	84.95 ± 2.75	7.80 ± 0.44	8.44 ± 0.90
		GT (19)	117.12 ± 36.68	86.24 ± 2.04	7.60 ± 0.45	8.62 ± 0.75
		GG (4)	111.45 ± 29.61	87.12 ± 2.03	8.06 ± 0.84	8.52 ± 1.02
	P-value		*0.974*	*0.557*	*0.259*	*0.789*
	c.171-44C > T	TT (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		TC (8)	115.64 ± 29.31	85.43 ± 2.07	7.89 ± 0.46	8.21 ± 0.85
		CC (40)	118.55 ± 42.10	85.83 ± 2.61	7.68 ± 0.55	8.53 ± 0.84
	P-value		*0.891*	*0.432*	*0.383*	*0.494*
	c.187G > A	GG (39)	118.29 ± 42.62	85.64 ± 2.63	7.75 ± 0.55	8.60 ± 0.82
		GA (8)	110.99 ± 41.39	86.53 ± 1.90	7.58 ± 0.73	8.34 ± 0.90
		AA (1)	139.89 ± 0.00	89.42 ± 0.00	7.99 ± 0.00	8.95 ± 0.00
	P-value		*0.616*	*0.302*	*0.446*	*0.614*
	c.205G > A	GG (4)	111.45 ± 29.61	87.12 ± 2.03	8.06 ± 0.84	8.52 ± 1.02
		GA (19)	117.67 ± 35.72	86.19 ± 2.00	7.56 ± 0.46	8.54 ± 0.79
		AA (25)	119.41 ± 45.10	85.28 ± 2.86	7.79 ± 0.52	8.44 ± 0.87
	P-value		*0.974*	*0.557*	*0.259*	*0.789*
	c.226A > G	GG (42)	116.43 ± 41.52	85.69 ± 2.68	7.75 ± 0.55	8.45 ± 0.86
		GA (6)	131.66 ± 29.31	86.50 ± 0.76	7.43 ± 0.38	8.77 ± 0.71
		AA (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	P-value		*0.346*	*0.385*	*0.195*	*0.448*
	c.319C > T	TT (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		TC (10)	114.60 ± 39.88	86.89 ± 2.02	7.63 ± 0.70	8.41 ± 0.87
		CC (38)	118.95 ± 40.73	85.53 ± 2.59	7.73 ± 0.50	8.50 ± 0.84
	P-value		*0.760*	*0.267*	*0.466*	*0.637*
	c.443 + 71C > T	TT (5)	122.62 ± 37.85	87.10 ± 2.33	7.44 ± 0.55	8.65 ± 0.44
		TC (35)	117.99 ± 40.52	85.44 ± 2.59	7.75 ± 0.48	8.47 ± 0.87
		CC (8)	116.41 ± 42.32	86.62 ± 2.01	7.66 ± 0.74	8.49 ± 0.90
	P-value		*0.786*	*0.385*	*0.538*	*0.875*
	c.443 + 86G > A	GG (17)	117.90 ± 33.23	86.91 ± 2.00	7.54 ± 0.58	8.73 ± 0.67
		GA (31)	111.13 ± 33.90	85.64 ± 2.32	7.81 ± 0.51	8.39 ± 0.93
		AA (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	P-value		*0.872*	***0.039***	*0.079*	*0.177*

Legend: n – numbers of individuals of each genotype, analyzed parameters listed in the table are presented as mean ± SD (Standard Deviation).

Table 3
Association of *GPR177* (exon 4) gene polymorphisms with selected bone parameters.

Gene	SNP	Genotype (n)	Bone breaking strength[N]	Length [mm]	Width [mm]	Bone mass [g]
GPR177(exon 4)	c.676C > T	TT (0)	0.00 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		TC (34)	116.23 ± 42.30	86.08 ± 2.98	7.77 ± 0.51	8.67 ± 1.14
		CC (14)	109.87 ± 18.11	85.94 ± 3.16	7.80 ± 0.58	8.62 ± 0.92
	P-value		*0.929*	*0.813*	*0.883*	*0.953*

Legend: n – numbers of individuals of each genotype, analyzed parameters listed in the table are presented as mean ± SD (Standard Deviation)

Table 4
Association of *GPR177* (exon 11) gene polymorphisms with selected bone parameters.

Gene	SNP	Genotype (n)	Bone breaking strength [N]	Length [mm]	Width [mm]	Bone mass [g]
GPR177 (exon 11)	c.1427-27G > C	GG (12)	106.21 ± 29.83	86.79 ± 2.74	7.92 ± 0.66	9.16 ± 1.40
		GC (34)	123.42 ± 43.71	85.58 ± 3.15	7.71 ± 0.48	8.54 ± 0.90
		CC (2)	102.01 ± 16.32	87.10 ± 2.57	7.60 ± 0.56	7.79 ± 0.47
	P-value		*0.770*	*0.658*	*0.700*	*0.328*
	c.1462G > A	GG (8)	108.27 ± 31.25	86.42 ± 2.17	7.77 ± 0.57	8.89 ± 1.42
		GA (34)	124.59 ± 44.26	85.40 ± 3.20	7.75 ± 0.48	8.57 ± 1.04
		AA (6)	109.68 ± 21.80	87.31 ± 2.40	7.49 ± 0.44	8.64 ± 0.41
	P-value		*0.997*	*0.546*	*0.660*	*0.873*
	c.1428 T > A	TT (47)	120.09 ± 40.85	85.80 ± 3.04	7.72 ± 0.50	8.63 ± 1.07
		TG (1)	69.58 ± 0.00	89.92 ± 0.00	9.07 ± 0.00	9.96 ± 0.00
		GG (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	P-value		*0.129*	*0.171*	*0.091*	*0.197*
	c.1448 T > G	TT (47)	120.09 ± 40.85	85.80 ± 3.04	7.72 ± 0.50	8.63 ± 1.07
		TA (1)	69.58 ± 0.00	89.92 ± 0.00	9.07 ± 0.00	9.96 ± 0.00
		AA (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	P-value		*0.129*	*0.171*	*0.091*	*0.197*

Legend: n – numbers of individuals of each genotype, analyzed parameters listed in the table are presented as mean ± SD (Standard Deviation).

Table 5
Maximum and minimum values of individual monitored parameters of laying hens.

Bone breaking strength [N]	Length [mm]	Width [mm]	Bone mass [g]
39.74–271.67	78.74–90.44	6.66–9.07	7.29–11.97

Legend: N – newton, mm – millimeter, g – gram.

4. Discussion

In this study, only one single nucleotide polymorphism (SNP) was found, specifically c.443 + 86G > A in the *GPR177* gene, which showed significantly association with bone length. It was found among the *GG* × *GA* genotypes, indicating a longer bone length for the *G* allele than for the *A* allele. The mere presumption, unsupported by any study, is that increasing bone mass, in the form of their greater length, may be at the expense of their strength, from which it could be concluded that they will be more easily prone to fractures. However, it should be noted that this is a polymorphism found in the non-coding region, the intron. No other similar study was found that would address the studied genes in laying hens, as well as in other animal species, in relation to bone parameters.

In general, there are not many studies that deal with SNP analyzes in animals, and there are minimal ones that focus on poultry. A relatively large study of polymorphisms in laying hens was published by Raymond et al. (2018). In this study, they performed a whole-genome association study to identify loci associated with bone strength. They tested a population-pure line of laying hens in a quantity of 752 animals. After quality control, an association study, a total of 52 SNPs were found among the several chromosomes tested, where the most significant associations were on chromosome 8. These polymorphisms showed significant associations with the tibia breaking strength. Among the many candidate genes, the authors mentioning the *BRD2* gene, which plays a major role in normal bone physiology. Another comprehensive study that mapped genes that would have a demonstrable effect on bone quality within chromosome 1 was published by Guo et al. (2017). They found a total of 4 genes in this region that met these conditions (*SERPINE3, RANK, POSTN* and *INTS6*), with nine SNPs. A total of 1534 animals were tested.

Among other authors who studied polymorphisms in relation to bone parameters in poultry were Johnsson et al. (2015) and Fornari et al. (2012). Study that deal with our selected genes, but in the field of human medicine, was performed by Zhong et al. (2012). They analyzed SNPs in the genomic region (1p31.3), encoding the GPR177 protein, in individuals of Asian and European descent, finding significant links to bone mineral density. A total of eleven polymorphisms from WNT pathway genes (*LRP4; LRP5; GPR177*) were analyzed by Kumar et al. (2011) for association with bone mineral density in two cohorts of Swedish women. A significant interaction was observed across all of these genes, with it being associated in the *GPR177* gene for both bone mineral density and rate of bone loss. Pineda et al. (2010) conducted an association study on 776 women to elucidate whether polymorphisms in the *RUNX2* gene may be associated with changes in bone density. In testing two polymorphisms in the promoter region and the exon 2 region, they found an association with the observed bone parameters. A similar study within the *RUNX2* gene was performed by Lee et al. (2009). There are several studies on candidate genes and SNPs that are associated with osteoporosis in humans (Liu et al., 2017; Ye et al., 2018; Zhang et al., 2021).

Within the monitored gene regions, most of the detected SNPs were synonymous. This means that there is no amino acid change. Others have been found in introns, i.e. non-coding regions of genes. Given the position of the introns, it may seem that the SNPs found in them may not have such a corresponding value. Disruption of mutations that would lead to exon skipping and activation of cryptic junctions could lead to the intron being included in an alterna-

tive form of the protein, as the mRNA would undergo alternative splicing. This would have implications for protein expression and genotype abnormalities. Mutations that could cause this phenomenon are deep in the intron sequences and affect exonic and intron enhancers or splice suppressors. Therefore, although these splice anomalies do not occur frequently, SNPs in non-coding regions may also play an important role in identifying specific associations (Cai et al., 2015; Cooper, 2010; Seo et al., 2013).

5. Conclusion

Bone fractures in laying hens can be partially solved by proper nutrition and housing, but genetic selection can provide a more permanent solution. Therefore, it is very important to gain insight into the genetic architecture to genetically improve bone strength. Analysis of the effect of polymorphisms can help to understand the effect of these polymorphisms on gene function and the overall health of the individual. This information can help identify new, especially useful markers of SNPs, for selection purposes and help to discover new knowledge that would be beneficial, not only, in the breeding of laying hens, but also in terms of deepening the facts about control of genes over bone metabolism, possibly by clarification the distribution and quantification of the monitored proteins in the bone tissue. As part of the deepening of knowledge about osteoporosis, this study identified polymorphisms in regions of genes that have not yet been tested for bone strength in laying hens within the femur. The results of this study have not yet been published by other authors and its novelty lies mainly in the use of this information by producers of final hybrids of laying hens in the breeding process, but of course for further studies of bone cell metabolism in relation to fractures in avian species kept for egg production. However, these results can also be used in areas other than animal breeding. This study should be further expanded to identify polymorphisms in the genes that the authors cite as part of bone metabolism.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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