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ANALYSIS OF SELECTED POLYMORPHISMS FOUND WITHIN GENES INVOLVED IN BONE METABOLISM SIGNALING PATHWAYS WITH MORPHOMETRIC PARAMETERS OF BONE TISSUE IN BROILERS

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ABSTRACT

The constant improvement of genetic tools, mainly due to the influence of genetic selection, leads to a permanent increase in the growth rate of commercial broiler chickens. The result is animals that reach market weight in a relatively short time. However, this often affects the health of the limbs, when such rapid growth is associated with skeletal disorders, when ideal bone development does not occur due to the rapid increase in body weight. While the time it takes to gain slaughter, weight has steadily decreased over the past few years, the time it takes for healthy bone development remains more or less the same. Therefore, studies carried out from the point of view of a better understanding
of a relatively little-explored area such as the genetic architecture of bones, can bring insights that can be used in poultry breeding. One of the relatively important signaling pathways for bone metabolism is the RANK/RANKL/OPG and Wnt pathways. Our study is focused on the search for polymorphisms within selected genes (*TNFRSF11A* and *WISP1*) of these pathways that might show associations with the traits we are monitoring. Testing was done on 43 broilers, namely fast-growing Ross 308, Cobb 500, and slow-growing Hubbard M22BxJA87A broilers. A total of thirteen polymorphisms were found, three of which were synonymous and five in an intron. Among the found polymorphisms, no one showed associations with the traits we were monitoring.

Keywords: polymorphism, bone, bone metabolism, gene, signaling pathway

INTRODUCTION

More and more studies point to the importance of genetics in the difference in bone characteristics of modern broiler lines. This is not the only important factor, other such factors include, for example, the significant influence of nutrition, breeding management, and environmental factors. Above all, breeding for very fast growth and therefore the extreme physiology of broilers negatively affects the development of the skeleton and brings various problems with the limbs, which can lead to fractures. This lower bone quality is caused by low calcification and high porosity, when bone development is not adapted to the rate of body weight increase **(Pang *et al*., 2021; Sanchez-Rodriguez *et al*., 2019; González-Cerón *et al*., 2015)**. The importance of studying bone abnormalities does not need to be emphasized, even from the point of view that potential problems may not be immediately visible at first **(Ventura & Silva, 2019)**. Various genetic changes can underlie many diseases, including those related to bone metabolism. Whether it is mutations within one gene, digenic, or possibly more complex polygenic, various structural variations in the form of single nucleotide polymorphisms (SNPs), as well as in larger sections. Above all, SNPs underlie differences between individuals and play an important role in the variability of disease susceptibility **(Hannan *et al*., 2019; Koromani *et al*., 2019)**. A key element in the process of bone remodeling is balanced resorption and formation, with local regulation critical to their balance. This is corrected by two very important pathways, RANK/RANKL/OPG and Wnt, which ensure the transmission of systemic and locally produced signals **(Kenkre & Bassett; 2018)**.

The RANK/RANKL/OPG signaling pathway is essential for bone metabolism, particularly in the regulation of osteoclastogenesis and osteoclast activity. This pathway's precise modulation is crucial for maintaining bone homeostasis and preventing bone metabolic disorders such as osteoporosis **(De Leon-Oliva *et al*., 2023; Kim *et al*. 2020)**.

The RANK/RANKL/OPG signaling axis plays a fundamental role in bone remodeling, a process that involves the coordinated action of bone formation by osteoblasts and bone resorption by osteoclasts. Dysregulation of this pathway can lead to excessive bone resorption, resulting in weakened bones and increased fracture risk **(Khosla & Monroe, 2018)**. The pathway consists of three main components: RANK (Receptor Activator of Nuclear Factor Kappa-B (NF-κB)), where RANK is a receptor expressed on the surface of osteoclast precursors and mature osteoclasts. Activation of RANK by its ligand is crucial for osteoclast differentiation, activation, and survival **(Boyce & Xing, 2008)**. RANKL (Receptor Activator of NF-κB Ligand) is a ligand produced by osteoblasts, osteocytes, and stromal cells. Its binding to RANK triggers a cascade of signaling events that promote the formation and activity of osteoclasts **(Xiong & O'Brien, 2012)**. OPG (Osteoprotegerin) is a soluble decoy receptor produced by osteoblasts that bind to RANKL, preventing it from interacting with RANK. This inhibition is vital for controlling osteoclastogenesis and bone resorption **(Boyce & Xing, 2008)**. When RANKL binds to RANK, it activates several downstream signaling pathways, including NF-κB, MAPK (Mitogen-Activated Protein Kinase), and NFATc1 (Nuclear Factor of Activated T Cells 1), which are essential for osteoclast differentiation and function. These activated osteoclasts then resorb bone by secreting acids and proteolytic enzymes that degrade the bone matrix **(Novack & Teitelbaum, 2008)**. Conversely, OPG acts as a major regulator by binding to RANKL and preventing it from activating RANK, thereby inhibiting osteoclast formation and activity. This balance between RANKL and OPG is crucial for maintaining bone density and strength **(Boyce & Xing, 2008)**. Recent human studies have further elucidated the role of this pathway in bone health. For example, research has shown that increased RANKL expression and decreased OPG levels are associated with bone loss in conditions such as postmenopausal osteoporosis **(Khosla & Monroe, 2018)**. Therapeutic interventions targeting this pathway in humans, such as the use of denosumab, the monoclonal antibody that inhibits RANKL, have shown significant efficacy in reducing bone resorption and fracture risk in osteoporosis patients **(Cummings *et al*., 2009)**.

The WNT signaling pathway is a fundamental regulatory system that controls various cellular processes, including proliferation, differentiation, and gene expression. This pathway is particularly critical in bone metabolism, where it plays a key role in maintaining bone homeostasis, osteoblast differentiation, and inhibiting osteoclastogenesis **(Baron & Kneissel, 2013)**. The Wnt signaling pathway can be divided into two main branches: the canonical (β-catenin dependent) and non-canonical (β-catenin independent) pathways. The canonical pathway is especially crucial for bone metabolism. When Wnt proteins bind to the Frizzled and LRP5/6 co-receptors on the cell surface, this interaction stabilizes and accumulates β-catenin in cytoplasm. The stabilized β-catenin then translocates to

the nucleus, where it acts as a co-activator for TCF/LEF transcription factors. This results in the transcription of target genes essential for the proliferation and differentiation of osteoblasts, the cells responsible for bone formation **(Liu *et al*., 2022; Krishnan *et al*., 2006)**.

The RANK/RANKL/OPG and Wnt signaling pathways are integral to bone metabolism and regulate the delicate balance between bone resorption and formation. Understanding the components and mechanisms of these pathways in conjunction with the study of SNP in genes involved in these pathways provides fundamental insight into bone health and offers therapeutic potential for the treatment of bone-related diseases.

MATERIAL AND METHODS

Tested animals

Broilers were tested in a total number of 43 animals, namely the breeding program Ross 308, Cobb 500, i.e. fast-growing broilers and slow-growing broilers Hubbard M22BxJA87A. Experiments were carried out following the guidelines of The Committee of Experts for Ensuring the Welfare of Animals of the Mendel University in Brno (Reference number of accreditation 57199/2020-MZE-18134) and those corresponding to the purposes specified in § 15, §16, and §7 of the Law no. 246/92 Coll. as amended. The animals were exposed to defined conditions and were housed in the experimental barn of the Mendel University in Brno, while the housing was in accordance with Council Directive 2007/43/EC on minimum rules for the protection of chickens reared for meat production. The fattening of the tested broilers was carried out according to the principles stated in the technological manual for a specific hybrid combination when a commercial feed mixture labeled BR1 was given for the first ten days, a commercial feed mixture BR2 from the tenth day, and a commercial feed mixture BR3 from the twenty-eighth day. Feed was given ad libitum. Broilers were killed by decapitation at the age of 35 days.

Genotyping

Genotyping itself was preceded by DNA isolation. This was isolated using a commercially available DNA Lego Kit (Top-Bio, Prague, Czech Republic) when 100 µl of blood was used. Methods such as PCR and sequencing have been used to determine genotypes. The PCR reaction was prepared in a volume of 10 µl, of which 0.2 µl was DNA. For each specifically tested region of a particular gene, specific oligonucleotide primers were designed (Table 1) using Oligo v4.0 software (Molecular Biology Insights, Inc., Colorado Spring, CO, USA).

Table 1 *TNFRSF11A* and *WISP1* primers

Gene/exon	Primer sequence	Product size
TNFRSF11A (exon 1)	**Forward:** 5'- AATAGGATGCCAGAAGTTTGTGAC -3'	619 bp
	Reverse: 5'- TCCTACGTACGTGCCTGAAGAC -3'	
WISP 1 (exon 5)	**Forward:** 5'- CACACCTCTAGATCCGGACTGTAG -3'	729 bp
	Reverse: 5'- CTGGCTGCTCTTAATGGATGG -3'	

Legend: TNFRSF11A – Tumor necrosis factor ligand superfamily member 11a, *WISP1* – Wnt1-inducible signaling pathway protein-1.

The annealing temperature was optimized, setting it at 55 °C for the tested regions. The PCR reaction conditions were as follows: Initial denaturation took place for 3 min at 94 °C, followed by denaturation (94 °C/60 s), annealing (55 °C/30 s), and elongation (72 °C/60 s), and this in a total of 30 cycles. Final elongation was at 72°C for 10 min, followed by cooling at 4°C, using an ABI Veriti 96-Well automated thermal cycler (Life Technologies, Applied Biosystems, Foster City, CA, USA).

PCR products were verified by horizontal agarose gel electrophoresis, using a 2.5% gel with GoodView visualization dye (Ecoli, ltd., Bratislava, Slovak Republic). The electrophoretic separation time was 30 minutes, at a voltage of 120 V. This was followed by the preparation of the PCR sequencing mixture in a volume of 10 µl, the conditions of which were as follows: Initial denaturation lasted 60 seconds at a temperature of 96 °C, followed by denaturation (96 °C/10 s), annealing (50 °C/5 s), and elongation (60 °C/4 min), in a total of 25 cycles and cooling to 4 °C. The BigDye Xterminator purification kit (Life Technologies, Applied Biosystems) was used for purification when the supernatant was subsequently removed from each sample and transferred to a sequencing plate. An ABI PRISM 3500 DNA genetic analyzer (Life Technologies, Applied Biosystem, Foster City, CA, USA) was utilized for these analyses, followed by The SeqScape v2.7 program (Life Technologies, Applied Biosystem, Foster City, CA, USA). This program was necessary for detection, analysis of mutations, i.e. identification of individual alleles and genotypes, by comparison with the initial sequences taken from the Genebank database (http://www.ncbi.nlm.nih.gov/genbank/). These genotypes are listed in Tables 2 and 3.

Analysis of monitored bone parameters

All analyses of bone parameters were performed on the femur. These parameters included bone strength, weight and length, determination of internal, external diameter, and thickness of compact bone tissue. Testing was done by separating the right thigh of the animals, extracting the femur, and removing all the muscles. Analysis of bone strength was determined on a universal testing machine TIRATEST 27025 (TIRA Maschinenbau GmbH, Schlkau, Germany) by a three-point bending test. Bone dimensions were assessed using a caliper, where bone length was determined as the longest distance between the ends of the distal and proximal epiphysis of the femur, and width as the greatest distance between *facies cranialis* and *facies caudalis*.

Statistical data processing

Due to the small number of tested animals, the non-parametric Kruskal-Wallis test was chosen. This is not only due to the size of the tested group but also due to the nature of the data, which are not normally distributed. Even from the point of view of normality, this test appears to be a better choice than the classical one-way analysis of variance (ANOVA). The evaluation was performed with the statistical software STATISTICA 12 (StatSoft Inc., Tulsa, USA), where bone parameters were monitored as the dependent variable and the genotype was the independent variable. The overall level of statistical significance was defined as $P < 0.05$.

RESULTS AND DISCUSSION

A total of 13 polymorphisms were found in the studied genes *TNFRSF11A* and *WISP1* within the tested set of 43 broiler individuals of hybrid combinations Ross 308, Cobb 500 (fast-growing), and Hubbard M22BxJA87A (slow-growing). Specifically, exon 1 was tested for the *TNFRSF11A* gene and exon 5 for the *WISP1* gene. In poultry, the *TNFRSF11A* gene is located on chromosome 2 and has 10 exons. A total of 8 polymorphisms were found in the studied region of this gene (c.11G>T, c.31G>A, c.37C>G, c.57C>G, c.90T>C, c.94C>G, c.96G>A, c.220+47C>T), when three of these polymorphisms were synonymous (c.57C>G, c.90T>C, c.96G>A), in which there is no change in the coded amino acid sequence. One of the polymorphisms (c.220+47C) was found in an intron. The remaining polymorphisms were located in the exon (c.11G>T, c.31G>A, c.37C>G, c.94C>G), and none of them showed conclusive associations with the traits we monitored (Table 2).

As Table 2 shows, not all data has been published. These were cases where the distribution of genotypes was the same for different polymorphisms or monomorphic sets. Specifically, in the case of the Ross 308 hybrid, the genotype distribution for the c.31G>A and c.37C>G polymorphisms was the same as for the aforementioned c.11G>T polymorphism. For the c.94C>G polymorphism, all tested animals had the same genotype. In the case of hybrid Cobb 500, only one allele was present in all polymorphisms in the exon.

The *WISP1* gene is located on chromosome 2 in poultry and has 5 exons. In this study, exon 5 was tested, when a total of 5 polymorphisms were found (c.1103-20C>T, c.1103-7A>G, c.1133A>G, c.1476+32G>C, c.1476+63A>G), of which only one (c.1133A>G) was located in an exon, the others were in an intron (Table 3). No statistically significant difference was found for any of the found polymorphisms.

Table 2 Association of *TNFRSF11A* (exon 1) gene polymorphisms with selected bone parameters in hybrids

Gene	SNP	Genotype (n)	Bone breaking strength [N]	Length [mm]	Width [mm]	Bone weight [g]
TNFRSF11A (exon 1)	c.11G>T	*GG* (14)	304.95 ± 67.99	83.92 ± 7.74	9.52 ± 1.23	14.88 ± 4.49
	Ross 308	*GT* (1)	428.57 ± 0.00	86.68 ± 0.00	10.70 ± 0.00	21.65 ± 0.00
	P-value		*0.1111*	*0.8848*	*0.1924*	*0.3106*
	c.11G>T	*GG* (7)	266.99 ± 65.17	77.39 ± 6.69	8.34 ± 1.10	10.80 ± 3.35
	Hubbard	*GT* (7)	284.62 ± 69.45	79.22 ± 7.69	8.34 ± 1.14	10.58 ± 3.42
	P-value		*0.6310*	*0.5218*	*0.8728*	*0.8728*
	c.31G>A	*AA* (5)	267.04 ± 74.48	78.46 7.72	8.59 ± 1.28	11.04 ± 3.69
	Hubbard	*GA* (9)	280.19 ± 63.93	78.23 ± 7.02	8.21 ± 1.02	10.51 ± 3.21
	P-value		*0.8651*	*0.6104*	*0.8651*	*1.0000*
	c.37C>G	*CC* (7)	266.99 ± 65.17	77.39 ± 6.69	8.34 ± 1.10	10.80 ± 3.35
	Hubbard	*CG* (7)	284.62 ± 69.42	79.22 ± 7.69	8.34 ± 1.14	10.58 ± 3.42
	P-value		*0.6310*	*0.5218*	*0.8728*	*0.8728*
	c.94C>G	*GG* (11)	277.59 ± 72.02	78.92 ± 7.71	8.44 ± 1.20	10.76 ± 3.54
	Hubbard	*GC* (3)	266.90 ± 40.59	75.24 ± 2.81	7.84 ± 0.12	10.33 ± 2.47
	P-value		*0.6674*	*0.8299*	*1.0000*	*0.8299*

Legend: *TNFRSF11A* – Tumor necrosis factor ligand superfamily member 11a, *n* - numbers of individuals of each genotype, analyzed parameters listed in the table are presented as mean ± SD (Standard Deviation)

Table 3 Association of *WISP1* (exon 5) gene polymorphisms with selected bone parameters in hybrids

Gene	SNP	Genotype (n)	Bone breaking strength [N]	Length [mm]	Width [mm]	Bone weight [g]
WISP1 (exon 5)	c.1133A>G	*GG* (4)	352.83 ± 101.06	81.04 ± 3.27	9.78 ± 0.71	15.94 ± 3.44
	Ross	*GA* (10)	311.00 ± 58.85	84.07 ± 8.36	9.55 ± 1.35	15.38 ± 4.83
		AA (1)	372.26 ± 0.00	87.06 ± 0.00	9.61 ± 0.00	12.86 ± 0.00
	P-value		*0.4426*	*0.6221*	*0.9580*	*0.6800*
	c.1133A>G	*GG* (7)	411.98 ± 149.89	82.25 ± 5.06	10.43 ± 0.96	15.53 ± 2.82
	Cobb	*GA* (5)	473.54 ± 129.89	84.31 ± 8.15	10.25 ± 1.82	18.01 ± 6.02
		AA (2)	441.96 ± 113.06	82.87 ± 8.52	9.84 ± 1.36	16.69 ± 5.19
	P-value		*0.7761*	*0.9722*	*0.7993*	*0.8960*
	c.1133A>G	*GG* (7)	279.19 ± 58.55	78.22 ± 6.93	8.24 ± 1.18	10.85 ± 3.27
	Hubbard	*GA* (7)	272.42 ± 75.99	78.39± 7.58	8.43 ± 1.05	10.53 ± 3.50
	P-value		*0.6310*	*0.8728*	*0.7488*	*1.0000*

Legend: *WISP1* – Wnt1-inducible signaling pathway protein-1, *n* - numbers of individuals of each genotype, analyzed parameters listed in the table are presented as mean ± SD (Standard Deviation)

This study builds on our previous research, when specifically in the study by **Steinerova *et al.* (2023)** the same genes were tested in another set of broilers. Within the tested regions of the *TNFRSF11A* gene, the same polymorphisms were found, however, unlike this testing, some of them showed conclusive associations, specifically within the Hubbard M22BxJA87A hybrids. In exon 1, it concerned the polymorphisms c.11G>T, c.31G>A, c.37C>G, when, for the monitored parameters of bone strength and their width, individuals with the *GG* genotype demonstrably had less wide and weaker bones than in individuals with the *GT* genotype. Also, no polymorphism was found in the *WISP1* gene that would show conclusive associations. It also follows on from our studies which were carried out in laying hens (**Steinerova *et al.*, 2020**), when no conclusive polymorphism in the exon was found. Differences in results are most likely due to the size of the test group, which is a limitation in these studies. Testing should take place on a larger group of animals and multiple sets to confirm the significance of these polymorphisms. **Jansen *et al.* (2021)** in their study dealt with the identification of polymorphisms and subsequently genes that have a connection with bone stability and are located close to them. Testing was carried out on laying hens in a number of 524 individuals, and the main parameters monitored were bone breaking strength (BBS) and bone mineral density (BMD). They found a total of 240 genes for BBS and 220 for BMD, of which 16 were identified as candidates, 10 were associated with BBS and BMD, 3 with associations with osteoporosis, and 3 were functionally related to the Wnt signaling pathway. Based on these findings, the authors support the assumption that bone strength is determined by multiple genes, each of which has a rather small effect. **Johnsson *et al.* (2023**) performed a genome-wide association study on 860 commercial crossbred laying hens reared in different housing systems. Bone strength, bone mineral density, bone composition, and body weight were analyzed. It was found that individuals who were reared on the floor had stronger bones through higher physical activity, resulting in differences in bone geometry, mineralization, and composition. In the genome-wide study, no significant locus was found for the observed bone traits. Another genome-wide association study was performed by **Li *et al.* (2021)**, which was performed in combination with selection signature analysis in an experimental F2 crossbreed population (offspring of broilers and layers of Chinese origin). A total of 545 individuals were tested, where significant SNPs for bone traits were identified on chromosomes 1, 4, and 27. Also in this study, they identified 21 candidate genes that could play an important role in the regulation of bone growth and development. In a study by **Sallam *et al.* (2023)**, markers of genetic variability for bone composition were sought and their correlations with bone strength were investigated in Rhode Island Red laying hens. **Mignon-Grasteau *et al.* (2016)** detected QTLs in the cross of two chicken lines with different digestion efficiency to investigate the genetic basis of bone and mineral metabolism. They measured tibia size, ash content, phosphorus retention, and plasma concentration at 3 weeks of age and estimated the heritability and genetic correlations of these traits with digestive efficiency. The study identified 35 QTLs associated with various bone traits, with six showing genome-wide significance. Candidate genes for bone regulation have been identified on chromosomes 6, 18, and 26, offering new insights into bone metabolism. Genes involved in bone metabolism have also been studied by **Wang *et al.* (2024)**, **Guo *et al.* (2020), Grupioni *et al.* (2017)**, **Guo *et al.* (2017)**. The last two authors mentioned conducted studies that were very similar, focusing on the RANK/RANKL/OPG signaling pathway genes. Specifically, **Grupioni *et al.* (2017)** focused on testing the *RUNX2* and *TNFSF11* genes for nearly 80 traits related to performance, organs, carcass composition, and those affecting bone integrity. Regarding bone markers, the weight of the chilled femur was collected. The broiler paternal lines were tested, and within each gene and trait, the numbers of individuals evaluated were variable, but for each trait there was always a test group of over 1000 individuals. Genetic associations ($P < 0.05$) were found for the trait of chilled femur weight, as well as selected performance traits and a polymorphism in the *RUNX2* gene (g.124,883*A>G*). Within the *TNFSF11* gene, a SNP (g.14,862*T>C*) was found showing genetic associations ($P < 0.05$) also in

performance traits. In addition, there were suggestive genetic associations ($P < 0.10$) in the abdominal fat index and its yield. The authors also mention the usefulness of these two polymorphisms in SNP-based selection. However, they add that it is also very important to verify these findings in other, especially unrelated, populations before more comprehensive use in the selection process. As for the second study mentioned, this one dealt with laying hens, where **Guo *et al.* (2017)** tested 1534 individuals, performing genome-wide association study of bone quality. They identified a novel locus that was associated with femur bone mineral density (BMD) and was found near *GSG1L*. They mapped that the highest heritability value for bone density was associated with chromosome 1, and the 165-171 Mb region on GGA1 had a significant effect on bone quality. According to the NCBI database, four genes (*RANK*, *SERPINE3*, *INTS6,* and *POSTN*) were identified in this region. The authors also point out the finding of several SNPs (9) within genes known to be associated with bone quality, with three of these genes being associated with osteoporosis in humans. These genes are *ADAMTS*, *SOST* and *RANKL*. They also identified several strong candidate genes or genomic regions associated with bone traits.

The RANK/RANKL/OPG pathway is an essential regulator of the balance between bone formation and resorption. This balance is often disturbed in broilers due to intensive growth and breeding for production parameters. Potential bone problems are often not immediately visible, making them difficult to detect. Molecular and genetic studies can reveal predispositions to these problems before they become apparent at the physical level. Healthy bones reduce mortality and the incidence of defective individuals, which has both economic and ethical benefits. The importance of the RANK/RANKL/OPG pathway is also well known in human medicine, for example, in the treatment of osteoporosis. This approach can be effectively transferred to research in poultry, opening new opportunities for targeted interventions and genetic improvement. Even small sets of animals provide valuable data that can reveal new genetic polymorphisms. These can then be tested in larger and more diverse populations, confirming their importance for bone traits. In our studies, several polymorphisms were found within the above-mentioned signaling pathways. It demonstrates that SNPs associated with bone quality can be identified even in small samples. Unfortunately, previously found polymorphisms that showed associations with selected traits of interest were not confirmed within the different animal test groups, but as mentioned above, we consider our studies to be pilot studies, mainly due to the small number of animals tested, and only on the basis of partial results can larger studies be designed. Future studies should include a larger number of individuals from different breeding lines to confirm the identified polymorphisms and their effect on bone parameters. Other genes and regions associated with the RANK/RANKL/OPG pathway should also be targeted in order to uncover new genetic factors affecting bone metabolism that could help for efficient selection for improved bone structure.

CONCLUSION

Understanding the genetic basis of skeletal health in broilers is of paramount importance to the poultry industry. Genetic studies, particularly those focusing on key signaling pathways such as RANK/RANKL/OPG and Wnt, provide critical insights into the mechanisms governing bone metabolism and strength. These pathways play essential roles in regulating bone remodeling and homeostasis, which are crucial for maintaining skeletal integrity. Identifying and analyzing genetic variations, such as single nucleotide polymorphisms within these pathways, can help develop targeted breeding programs aimed at enhancing bone density and resistance in broilers. This not only leads to healthier poultry but also improves overall production efficiency and animal welfare. Furthermore, advancements in genetic research contribute to reducing mortality rates and minimizing the environmental impact of poultry farming, underscoring the broader benefits of integrating genetic insights into breeding strategies.

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