KIRKUK UNIVERSITY JOURNAL
FOR AGRICULTURAL SCIENCES

# Polymorphism of the *GnRH1* gene and Its effect on some Physiological parameters of Local chickens

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- Date of research receivd 26 /12 /2022 and accepted 11/1 /2023
- Part of PhD. Dissertation for the first author

## Abstract

The *GnRH1* gene is among the genes that mainly affect reproduction and productive traits in local chickens. The GnRH hormone regulates reproductive activity in poultry by regulating the production of other reproductive hormones, such as LH and FSH, which significantly affect birds' productivity. This study was conducted to detect Genetic polymorphism of the *GnRH1* gene in local chickens using PCR-SSCP sequencing. Samples were collected at ages 2, 4, 6, and 8 months of birds (n=50). The results found homogeneity in all encoded regions except for one. Polymorphism was detected in the Exon-4 1SNP site 147 C>G. The results indicated significant differences ($P \leq 0.05$) for the interaction of sex with the genotype of the LH, as the statistical results show that birds with the genotype CC for both, gender outperformed the birds carrying the genotype CG at the age of 2 and 4 months. The results showed significant differences ($P \leq 0.05$) in the mean genotype within each month in the mean testosterone concentration in males and estrogen in females. Age progression revealed that the homologous genotype CC-carrying birds had an arithmetic advantage over the CG-carrying birds in terms of the average sex hormone concentration. This gene may serve as a marker that is helpful in the marker-assisted selection of local chicken. This study is one of the first to study polymorphisms *GnRH1* in local chickens.

**Key words:** SSCP ,*GnRH1*, Local Chickens**,** LH**,** FSH

Citation: Milad A. Badi; Asaad Y. Ayied; Khalid C. Al-Salhie. "Polymorphism of the GnRH1 gene and Its effect on some Physiological parameters of Local chickens". *Kirkuk University Journal For Agricultural Sciences*, 14, 1, 2023, 196-206. doi: 10.58928/ku23.14116

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## Introduction

*GnRH* (Gonadotropin releasing hormone) was first discovered in mammals due to its effect on stimulating and releasing pituitary gonadotropin-stimulating hormones [1]. This hormone differs from that found in birds, containing two types (*GnRHI* and *GnRHII*, which differ from each other in the chain of amino acids and participate in regulating reproductive activity in chickens [2]. Later the third form, *GnRHIII*, was discovered in songbirds [3]. The reproduction process is subject to a large group of genes and hormones that regulate the functioning of the reproductive system [4]. GnRH is one of the hormones synthesized and secreted by neurons in the brain's hypothalamusAdditionally, it stimulates the pituitary gland to release hormones that activate the reproductive glands[5], such as the follicle stimulating hormone (FSH), which is in charge of numerous reproductive processes, from the stimulation of ovarian follicles in females to the production of sperm in males. The other hormone is Luteinizing hormone (LH), which stimulates ovulation in females and also stimulates the interstitial cells in the testicles to secrete the hormone testosterone [6]. Currently, genetic markers are widely used in poultry farming [7]. Significant progress in molecular genetics provides new tools that can be used to help identify genotypes [8], as the productive performance of birds can be predicted at an early age by using genetic indicators [9].Concluded that the genes *GnRHI* and *GnRHII* are polymorphic and significantly affect body weight, egg production and egg quality traits [10]. gene expression is affected by SNP (single nucleotide polymorphysims), and mutations that occur in the gene have a significant effect either by inhibiting or activating reproductive functions[11], represented by egg production rate and egg quality traits [10] ; [12]. There is also a correlation and effect between the concentration of *GnRH* in blood serum and its gene expression in reproductive activities and some productive traits [13]. The study aims to reveal the presence of genetic polymorphisms of the *GnRH1* gene in local chickens and its effect on some physiological parameters.

## Material and methods

The current study was conducted at a poultry farm in Wasit province, and laboratory tests were completed in the College of Agriculture/ University of Basrah's laboratories from 11/20/2020 to 9/30/2021. Iraqi local chickens were raised from one week until eight months, and blood samples were collected from 50 birds at the age of second months and four months, six months and eight months.

### Blood samples

Blood samples of five males and five females were collected from birds from the wing vein at 2, 4, 6 and 8 months using a 1 ml medical syringe in tubes free of anticoagulant to estimate some biochemical traits. The serum was separated from the other blood components using a centrifuge for 15 minutes at a speed of 3000 rpm. After that, the serum was preserved by freezing until the required examinations were performed.

### FSH, LH, Testosterone and Estrogen

According to the FSH and LH hormone concentration, using the ready-made measuring kit prepared by the American company Monobind Inc, using the ELISA technique to perform the examination, and following the leaflet attached to the (Kit).

**Genomic DNA isolation**

One drop of peripheral blood was collected from each female and male local chick's wing vein. Genomic DNA was extracted from all 50 birds using a manual salting-out procedure [13]. The quantity and quality were described in birds [8]. The genomic DNA integrity was visualized by direct electrophoresis on ethidium bromide pre-stained 0.08% agarose evaluated using a nanodrop spectrophotometric method (Biodrop, μLITE, UK).

**Polymerase chain reaction**

Three PCR fragments were designed using NCBI Primer-BLAST software [14] . The primary designing approach for these three primer pairs was to cover all three coding regions of the GnRH1 gene so that each PCR amplicon would represent one specified exon. The lyophilized oligonucleotides were purchased from Bioneer (Daejeon, South Korea). The PCR amplification reactions were performed using a Bioneer PCR premix . The optimum amplification conditions for the three designed amplicons were empirically determined using a gradient PCR, as in table 1. Standard PCR experiments were performed on a PCR thermocycler (USA). The amplification protocol was initiated by one cycle of denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 45 s, and elongation at 74°C for 45 s, and was concluded with a final extension at 72°C for 5 min. PCR amplicons were verified by electrophoresis on 1.5% agarose gel.

**Single-strand conformation polymorphism (SSCP)**

SSCP experiments were performed using a rapid high voltage approach [15] [16] with several modifications. Briefly, each PCR amplicon was treated with an equal volume of SSCP denaturing-loading buffer (95% formamide , 0.05% xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA, pH 8). After denaturation for 7 min, 2 μl of PCR amplicons were immediately placed on ice and frozen for about 10 min. Subsequently, 2 μl of samples were loaded on neutral polyacrylamide gel (0.1 mm thickness, 10 cm length, and 10 cm width). Electrophoresis conditions were optimized according to the details described in table 2. Gels were fixed and stained [17].

**DNA sequencing**

Each detected genotype on the SSCP gel was subsequently exposed to sequencing reaction from both termini according to instructions of Macrogen laboratories . (Macrogen, Geumchen, Seoul, Korea). The referring database of the GnRH1 nucleic acid sequences was retrieved (https:// www. ncbi. nlm. nih. gov). The sequenced SSCP genotypes were visualized and annotated by BioEdit ver 7.1. (DNASTAR, Madison). The amino acid reading frames of the SSCP variants were determined. Each detected variant was visualized using SnapGene Viewer ver. 4.0.4(http:// www. snapg ene. com).

**Genetic diversity analysis**

The assessment of the genetic polymorphism of the *GnRH1* gene variants was performed by calculating allele and genotype frequencies, observed heterozygosity (Ho), expected heterozygosity (He), and the effective number of alleles (Ne) were performed using PopGen32 software, v. 1.31 [18]. Chai-squared test ($\chi 2$) was also calculated to verify the possible

deviation from Hardy–Weinberg Equilibrium (HWE) expectations for the distribution of genotypes. The polymorphism information content (PIC) was computed using the following formula [19].

$$PIC = 1 - = \sum_{i=1}^{m} Pi^2 - \sum_{i=1}^{m-1} \sum_{j=i+1}^{m} 2Pi^2 Pj^2$$

where *pi* and *pj* are the frequencies of the *i*th and *j*th allele, respectively, and m is the number of alleles.

**Statistical analysis**

The data were analyzed using a statistical package (SPSS version 26.0 2019), where the analysis of variance was used for two factors, the gender of the birds (males and females) and genotype (two genotypes), and the means were compared using the least significant difference.

Table 1. The oligonucleotide primer sets designed for the amplification of the *GnRH1* in local chickens.

| Set | Targeted locus | Primer sequences (5′-3′) | Length (bp) | Tm C° |
|---|---|---|---|---|
| 1 | Exon-2- F<br>Exon-2-R | CTCCAGAAGCTTTCCCATGATTC<br>ATCATAGCAAAAACGAGAGCTGC | 271 | 63 |
| 2 | Exon-3- F<br>Exon-3-R | AACCTGGGGAGATTTGCCTT<br>AGTGATGCAAAGCTGGGAGT | 337 | 63 |
| 3 | Exon-4 F<br>Exon-4-R | CAGAGAGATCACGTCGGGTCA<br>ATTCTGGGTTTGTTGATGGTGTTG | 224 | 56.9 |

Table 2. SSCP electrophoresis conditions of the amplicons of the *GnRH1* gene in local chickens. All PCR amplicons were electrophoresed on 0.1 mm thickness of 10 × 10 (L × W) cm diameters polyacrylamide gels.

| Set | Amplicons | Gel concentration | Running time | Running voltage | Running amperage | Running temperature |
|---|---|---|---|---|---|---|
| 1 | Exon-2 | 8% | 180min | 200v | 100mA | 20 |
| 2 | Exon-3 | 8% | 210min | 200v | 100mA | 18 |
| 3 | Exon-4 | 8% | 180min | 200v | 100Ma | 20 |

**Results**

The results of sequencing amplification of GnRH gene 224 bp site show heterozygous. The discovery of silent mutant at147 (C>G). The results of the genetic sequencing of the exon-2, and exon-3, with an amplification size of 271 bp and 337 bp, confirmed the homologous genotype recorded by PCR-SSCP results without any detected SNP. Figure (1) shows the presence of two patterns.CC and CG The frequency of CC genotype (76%), and CG frequency (24%) were shown in table (3). Polymorphism of the GnRH1 gene genotypes for the fourth exon of the analyzed site and the test of Hardy-Weinberg equilibrium. Observed heterozygosity Ho is 0.25, He 0.21875, $\chi$2 0.563, PIC 0.02.

Table 3. Genotype and allele frequencies and genetic diversity variables for 147= SNP locus of the *GnRH1* exon4 gene in local chickens.

| Genotype frequencies | | Allele frequencies | | Ho | He | Ne | $\chi 2$ | PIC |
|---|---|---|---|---|---|---|---|---|
| CC | CG | C | G | | | | | |
| 0.76 n=38 | 0.24 n=12 | 0.875 | 0.125 | 0.24 | 0.21875 | 0.3889 | 0.563 | 0.02 |

* number of samples, $\chi 2$ chi-square, Ho observed heterozygosity, He expected heterozygosity, Ne effective allele number, PIC polymorphism information content. All Chi-square tests have one degree of freedom and are within the significance level ($P \leq 0.05$).

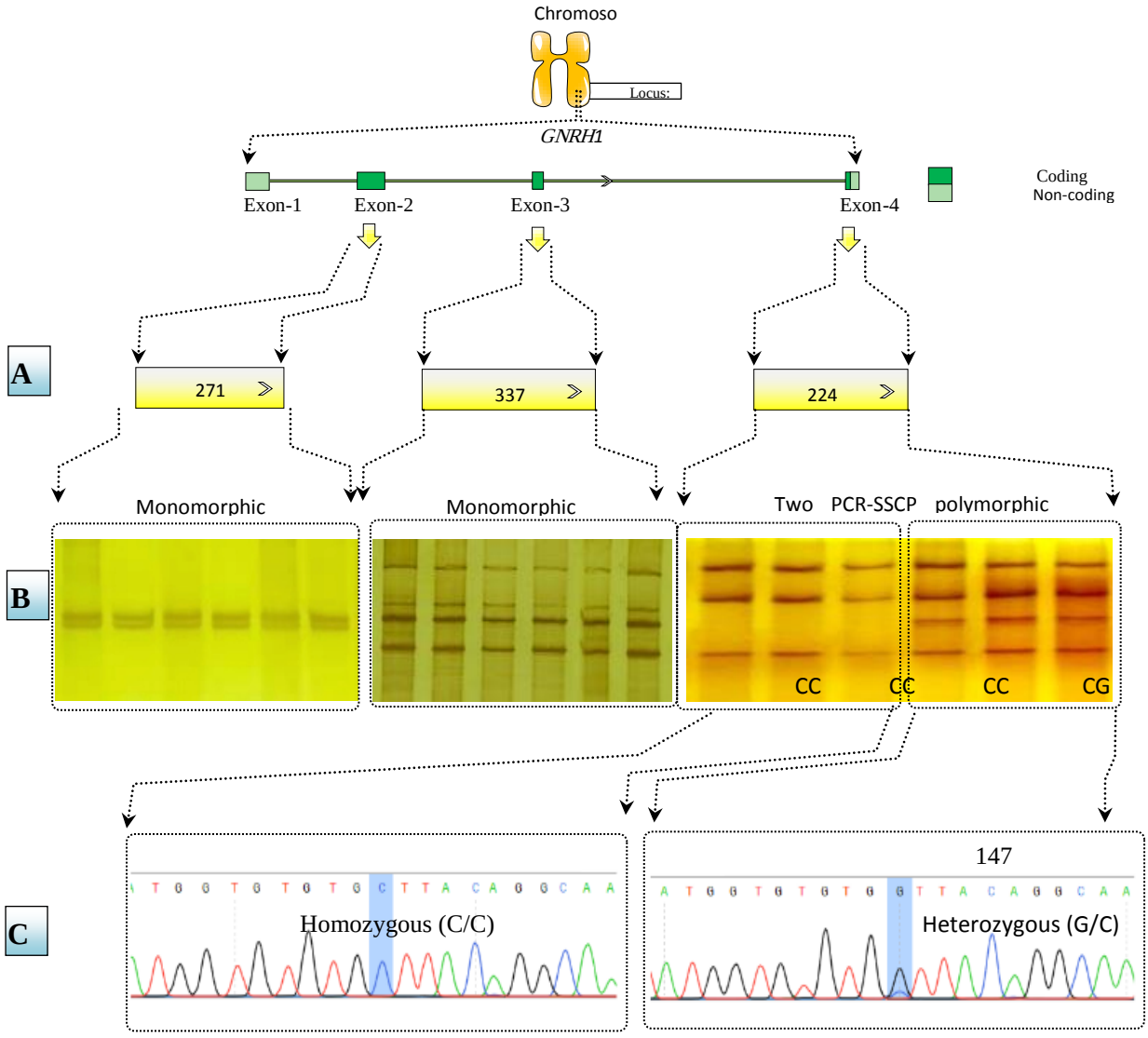


Figure 1. A schematic diagram for the *GnRH1* gene-based PCR-SSCP-sequencing strategy in local chickens. A PCR design of three PCR-specific primers pairs for the amplification of 271 bp, 337bp, and 271 bp in exon 2, exon 3, and exon4, respectively. B Post-PCR genotyping using SSCP technique, in which only exon 4 showed two different genotypes. C Sequencing reactions interpretation of the detected genotypes, in which only one SNP was detected in the exon 4.

**The effect of genotypes of the *GnRH1* gene on the sex hormones LH and FSH**

The statistical results show that the birds carrying the homozygous genotype CC for both sexes exceeded (P<0.05) the birds carrying the heterozygous genotype CG at the ages of 2 and 4 months in the level of LH (Table, 4). The mean genotype of the CC genotype (1.68) recorded a significant superiority (P≤ 0.05) over the genotype CG (1.38). The results showed arithmetic differences in the mean concentration of LH hormone between the genotypes at 6 and 8 months. No significant differences were recorded at these ages. (Table 5) indicates that there are significant differences (P≤ 0.05) for the interaction of sex with the genotype in the mean concentration of the hormone that stimulates the growth of follicles FSH, as the statistical results show that the birds carrying the genotype CCoutperformed on the birds carrying the genotype CG at the age of 2 months. The mean genotype of the CC genotype (3.73) recorded a significant superiority (P≤ 0.05) over the CG genotype (3.01). The results showed arithmetic differences that did not reach significance in the mean concentration of FSH hormone between the genotypes at the ages of 4, 6, and 8 months. No significant differences were recorded at these ages. Due to the scarcity of scientific studies that study the link between the polymorphism of the *GnRH1* gene and the concentration of sex hormones in local chickens, to compare our results with them, this study paves the way for understanding the genetic polymorphism of the *GnRH1* gene in local chickens

Table 4. Effect of genotype of the exon-4 in mean hormone LH (IU/L) for both males and females in local chickens (± SD).

| Age (month) | Genotype | Gender | | Genotype |
|---|---|---|---|---|
| | | Males | Females | |
| Month 2 | CC | 1.69 $^{a}$± 0.20 | 1.68 $^{a}$± 0.09 | 1.68 $^{A}$ ±0.15 |
| | CG | 1.51 $^{b}$± 0.00 | 1.31 $^{c}$± 0.03 | 1.38 $^{B}$± 0.11 |
| | Mean Gender | 1.65±0.19 | 1.53±0.20 | 1.59±0.20 |
| Month 4 | CC | 3.87 $^{a}$± 0.16 | 3.62 $^{a}$± 0.15 | 3.76 $^{A}$± 0.19 |
| | CG | 3.63 $^{a}$± 0.00 | 3.24 $^{b}$± 0.02 | 3.37 $^{B}$± 0.22 |
| | Mean Gender | 3.82±0.17 | 3.74±0.23 | 3.64±0.26 |
| Month 6 | CC | 4.95±0.10 | 4.93±0.06 | 4.94±0.08 |
| | CG | 4.98±0.00 | 4.78±0.17 | 4.85±0.16 |
| | Mean Gender | 4.95±0.09 | 4.87±0.12 | 4.91±0.11 |
| Month 8 | CC | 6.17±0.14 | 6.20±0.04 | 6.18±0.10 |
| | CG | 6.29±0.00 | 6.06±0.14 | 6.14±0.16 |
| | Mean Gender | 6.20±0.13 | 6.14±0.10 | 6.17±0.11 |

*Mean with lowercase letters that there are significant differences at the level of (P≤ 0.05) for the interaction between genotype and gender and capital letters for the comparison of the main factors (genotype, gender) within one month.

Table 5. Effect of genotype of the exon-4 in mean hormone FSH (IU/L) for both males and females in local chickens (± SD).

| Age (month) | Genotype | Gender | | Genotype |
|---|---|---|---|---|
| | | Males | Females | |
| Month 2 | CC | 3.95 $^{a}$± 0.27 | 3.38 $^{a\ b}$± 0.52 | 3.73 $^{A}$± 0.47 |
| | CG | 3.05 $^{b}$± 0.00 | 2.99 $^{b}$± 0.21 | 3.01 $^{B}$± 0.15 |
| | Mean Gender | 3.77±0.46 | 3.22±0.44 | 3.50±0.51 |
| Month 4 | CC | 5.49±0.58 | 5.06±0.18 | 5.31±0.48 |
| | CG | 5.76±0.00 | 5.01±0.14 | 5.26±0.44 |
| | Mean Gender | 5.55±0.51 | 5.04±0.15 | 5.29±0.44 |
| Month 6 | CC | 6.01±0.46 | 6.34±0.37 | 6.15±0.43 |
| | CG | 6.90±0.00 | 6.58±0.90 | 6.68±0.66 |
| | Mean Gender | 6.19±0.56 | 6.44±0.54 | 6.31±0.53 |
| Month 8 | CC | 7.23±0.42 | 7.74±0.53 | 7.45±0.51 |
| | CG | 8.97±0.00 | 7.39±0.66 | 7.91±1.02 |
| | Mean Gender | 7.58±0.86 | 7.60±0.53 | 7.59±0.67 |

* Mean with lowercase letters that there are significant differences at the level of (P≤ 0.05) for the interaction between genotype and gender and capital letters for the comparison of the main factors (genotype, gender) within one month.

**The effect of genotypes of the *GnRH1* gene on testosterone in males and estrogen in females**

The results of Table (6) indicate that there are significant differences (P≤ 0.05) in the mean genotype within one month in the mean concentration of testosterone in males. Age progression and the results showed that the birds carrying the homologous genotype CC showed an arithmetic difference in the mean concentration of testosterone over the birds carrying the genotype CG at the age of 2, 4, and 6 months. The mean genotype did not record a significant difference at eight months. (Table 7) There were statistically significant differences (P≤ 0.05) in the average genotype in one month in the average concentration of estrogen hormone in females, and the statistical results showed that females at the age of 6 months outperformed those at the age of 2 and 4. A homozygous CC genotype showed an arithmetic difference in the mean estrogen concentration of CG-bearing birds at 2, 4, and 6 months of age. The mean genotype did not record a significant difference at 8 months. Due to the scarcity of scientific studies examining the relationship polymorphism of the GnRH1 gene and its effect on the concentration of testosterone and estrogen in local chickens, the study paves the way for understanding the genetic polymorphisms of the GnRH1 gene in local chickens .

Table 6. Effect of genotype of the exon-4 in mean hormone testosterone(ng\ml) for both males in local chickens (± SD).

| Genotype | Age (Month) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| CC | 0.08±0.01 | 1.48±0.06 | 3.37±0.18 | 4.69±0.22 |
| CG | 0.05±0.00 | 1.38±0.00 | 2.93±0.00 | 4.79±0.00 |
| Mean Genotype | $0.07^{C}$± 0.01 | $1.46^{B}$± 0.07 | $3.57^{A}$± 0.39 | 4.71±0.19 |

*Means with capital letters mean that there are significant differences at the level of (P≤ 0.05) to compare the main factors (genotype, gender) within one month.

Table 7. Effect of genotype of the exon-4 in mean hormone estrogen (picogram\ml) in females and local chickens (± SD).

| Genotype | Age (Month) | | | |
|---|---|---|---|---|
| | 2 | 4 | 6 | 8 |
| CC | 27.40±1.81 | 55.48±5.76 | 108.25±8.46 | 155.33±7.02 |
| CG | 23.62±2.07 | 57.31±1.83 | 110.50±9.19 | 153.00±4.24 |
| Mean Genotype | $25.89^{C}$±2.65 | $56.21^{B}$±4.29 | $109.15^{A}$±7.64 | 154.40±5.55 |

*Means with capital letters mean that there are significant differences at the level of (P≤ 0.05) to compare the main factors (genotype, gender) within one month.

## Discussion

There are many reasons for studying this gene and knowing the variation and genotypes in it because the *GnRHI* gene is closely related to the effect on the hypothalamic-pituitary gland [1] , in addition to the essential role in the reproductive and productive performance of birds [20]. affects the work of the reproductive hormones FSH, LH, estrogen, and testosterone, and their levels in the body affect body weight, the development and growth of the reproductive organs in males and females, puberty and sexual maturity [6] . Moreover, sexual behavior[21] and on, the age of production of the first egg, the fertility of the roosters, and, therefore on, the characteristics of hatching and the fertility rate for these reasons, the genetic differences within this gene may have many effects on reproductive and productive performance in chickens . Through the results of the current study, two genotypes were discovered through the technique of genetic polymorphism (SSCP), the discovery of a silent SNP at position 147 in the heterozygous CG genotype, which is C>G. There is no precise mechanism to explain how a silent SNP can affect function as it can alter characteristics [22] [23]. This study is one of the few studies examining genetic polymorphisms in the *GnRH1* gene in local chickens that pave the way to understanding the polymorphism of *GnRH1*.

## Conclusion

A variation was detected in one of the silent SNPs that hurt the production of sex hormones in local chickens. Directing genetic variation in a gene could be a helpful marker in selecting local chickens with the help of genetic markers. Recommend studying several genes responsible for reproductive and productive performance in local chickens to obtain genetic markers that affect reproductive and productive traits that can be used in a genetic improvement program to achieve the economic feasibility of local chicken breeding projects.

**Abbreviations**

GnRH Gonadotropin releasing hormone, FSH (follicle stimulating hormone), LH (luteinizing hormone), DNA (deoxyribonucleic acid), PCR (polymerase chain reaction), SSCP (single-strand conformation polymorphism), NCBI (national center for biotechnology information), bp (base pair), He (expected heterozygosity), Ho (observed heterozygosity), Ne (effective allele number), PIC (polymorphism information content), HWE, (hardy equilibrium), $\chi 2$ (chai-squared test), SNP (single nucleotide polymorphisms).

**Acknowledgements**

This work was partially supported by College of Agriculture, University of Basrah .

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# التشكل الوراثي لجين *GnRH1* و تأثيره في بعض المعايير الفسيولوجية للدجاج المحلي

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**قسم الانتاج الحيواني ،كلية الزراعة ،جامعة البصرة ،البصرة ، العراق**

- **تاريخ استلام البحث 26/12/2022 وتاريخ قبوله 11/1/2023**
- **البحث مستل من رسالة دكتوراه للباحث الاول .**

**الخلاصة**

يعد جين *GnRH1* من بين الجينات التي تؤثر بشكل أساسي على التكاثر والصفات الإنتاجية في الدجاج المحلي. إذ يؤثرعلى هرمون GnRH الذي يتحكم في النشاط التناسلي للدواجن من خلال تنظيم إنتاج الهرمونات التناسلية الأخرى ، مثل LH و FSH ، والتي تؤثر بشكل كبير على إنتاجية الطيور. أجريت هذه الدراسة للكشف عن تعدد المظاهر الوراثية لجين *GnRH1* في الدجاج المحلي باستخدام PCR-SSCP إذ تم جمع العينات في عمر 2 و 4 و 6 و 8 أشهر بعدد 50 طير. بينت النتائج وجود نمط وراثي متماثل في جميع المناطق المشفرة باستثناء منطقة واحدة. تم الكشف عن تعدد المظاهر الوراثية فيها فيExon-4 بسب وجود 1SNPصامتة في الموقع 147 C> G و أشارت النتائج إلى وجود فروق ذات دلالة إحصائية (P≤ 0.05) لتأثيرالجنس مع التركيب الوراثي للهرمون اللوتيني ، إذ أظهرت النتائج أن الطيور التي تحمل النمط الوراثي CC تتفوق على الطيور الحاملة للنمط الوراثي CG في عمر 2 و 4 شهر. و اشارت النتائج وجود فروق معنوية (P≤ 0.05)في متوسط تركيز هرموني التستوستيرون في الذكور والإستروجين في الإناث نستنتج من ذلك ان الطيور التي تحمل التركيب الوراثي المتماثل CC اظهرت فرقا حسابيًا في متوسط تركيز الهرمونات الجنسية مقارنة مع الطيور الحاملة لـ CG . ويمكن اعتماده كواسم وراثي للانتخاب في الدجاج المحلي بمساعدة الواسمات الوراثية الاخرى. على حد علمنا تعد هذه الدراسة واحده من الدراسات النادرة التي تستهدف دراسة تعدد المظاهر الواثية لجين*GnRH1* في الدجاج المحلي.

**الكلمات المفتاحية:** الدجاج المحلي, SSCP , *GnRH1* , FSH, LH,