R. Bras. Zootec., 49:e20180242, 2020
https://doi.org/10.37496/rbz4920180242

Non-ruminants
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

The epiphyseal plate closure phenomena for male and female Japanese quail (*Coturnix coturnix japonica*): histological and biochemical alterations

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ABSTRACT - This study was conducted to determine the effect of sex on ossification processes in quail by determining the exact closure timing of the epiphyseal plate in the proximal region of the femur. This was done by investigating the histological and biochemical parameters affecting the osteogenesis process that takes place following quail hatching to observe if any variation existed between males and females in this regard. For this purpose, blood samples were collected from six male and female specimens via IV catheters every week for the first 42 days that followed hatching. The samples were transferred into serum tubes, and PTH (PTH), triiodothyronine (T3), thyroxine (T4), inorganic phosphorus, calcium, and vitamin D values in the samples, which are known to have an impact on ossification, were analyzed. The specimens from which the blood samples were collected were then euthanized, and histological cut-sections that covered the epiphyseal growth plate were collected, along with the bone sections of the proximal regions of the right femur. Considering decalcification, these histological sections were kept in an ethylene diamine tetra acetic acid (EDTA) solution. Routine histological examinations were then conducted on these sections, after which they were embedded in paraffin. Crossman's modified triple staining method was used to prepare them, and Wilcoxon Signed Ranks Test was used to statistically evaluate whether the inspected biochemical parameters played a role in the ossification process of quail and whether a statistical difference existed between sexes in this regard. The findings of our study revealed that poultry animals also have five zones in the epiphyseal plate as do mammals, and they have calcified cartilage areas. The findings also indicate that, while ossification starts to occur both in male and female quail specimens, calcification occurs more frequently in females. It was determined that the proximal epiphyseal plate regions of the femur close at the end of the sixth week in both sexes of quail, although the calcification and ossification are more advanced in some females compared with males.

Keywords: biochemistry, epiphyseal plate, histochemistry, quail, sex

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Received: November 1, 2018
Accepted: August 25, 2019

How to cite: Karakoç, Z. and İrak, K. 2020. The epiphyseal plate closure phenomena for male and female Japanese quail (*Coturnix coturnix japonica*): histological and biochemical alterations. Revista Brasileira de Zootecnia 49:e20180242. https://doi.org/10.37496/rbz4920180242

1. Introduction

The majority of the mammalian skeleton is formed by a process called endochondral ossification, which occurs when the cartilage excluding the parietal bones gives way to bone tissue. The vertebral column, pelvis, and bones of the extremities form through endochondral ossification. In this kind of osteogenesis, the hyaline cartilage comes into existence first and is ultimately replaced with the bone tissue itself (Ketani and Sağsöz, 2009).

The ossification of long bones starts at the end of the embryonic period. The focus of the first ossification, which occurs to constitute diaphysis, is called the primary ossification center. While it may occur at

various stages, a primary ossification center is present for almost every bone at birth. The focused ossification at the endpoints of the long bones is called the secondary ossification center. The ossification initiated by the secondary ossification center continues by expanding radially from the inner part of the anlage. A cartilage region remains between this ever-growing bone tissue and the diaphysis. This region is called "the cartilage epiphysialis" or "the epiphyseal cartilage". It has significant importance in terms of the longitudinal growth of bones (Atalgin and Çakir, 2006; Serter, 2010).

The endochondral ossification is strictly controlled by the circulation system. There are many elements that play a significant role in the reproduction and differentiation of chondrocytes in the growth plates such as the systemic hormones and signal factors, which are produced locally, protein PTHrP related to the Indian hedgehog (lhh), parathyroid hormone or parathormone (PTH), PTH1R receptor, fibroblast growth factor 18 (FGF-18), and FGFR3 receptor. Vascular endothelial growth factor (VEGF), connective tissue growth factor (CTGF), bone morphogenetic proteins (BMP), and Wnt proteins, as well as transcription factors Sox9 and Runx2/Cbfa1 are also known to influence the ossification process (Zhang et al., 2011; Topaloğlu et al., 2017).

In the extremity bones, the hyaline cartilage gives way to bone tissue with matrix calcification and mineralization in the epiphyseal plate regions by means of "endochondral ossification". Due to this process, the plate region contains three distinct types of chondrocytes: proliferating chondrocytes, mature chondrocytes, and hypertrophic chondrocytes. The region is ossified in the perichondrium and transitions to the periosteum. The growth of the bone length comes to a permanent halt when the entire epiphyseal plate becomes ossified (Özer, 2014; Topaloğlu et al., 2017).

The epiphyseal chondrocytes along the ossification area consist of five zones (regions), namely the reserve zone (resting of), proliferative zone (proliferation zone), hypertrophic cartilage zone (maturation zone), calcified cartilage zone (calcification zone), and ossification epiphysis-diaphysis zone (Özer, 2014; Topaloğlu et al., 2017).

The osteoblast membranes contain PTH receptors, which trigger the secretion of the osteoclast-stimulating hormone when stimulated by PTH. Osteoblasts also secrete a high level of hormone during the active bone formation process and cause an increase in blood level of alkaline phosphatase (ALP) enzyme. The ALP blood concentration is an important indicator of the ossification process status (Oznurlu et al., 2016). The PTH enables osteoclasts to provide acid hydrolases to the matrix by increasing the lysosome synthesis in the osteoclast. This also initiates bone resorption and generates many invaginations by increasing the membrane convolutions. The Ca and phosphate (PO_4) ions are released as a result of increasing bone destruction, and their amounts in the blood are increased. This increase halts the PTH secretion. The non-secretion of PTH stimulates and activates the osteoblasts, and bone formation is stimulated (Atalgin and Çakir, 2006; Özer, 2014).

The studies concerning bone tissues mostly focus on bone tissue damage and osteoporosis (Kürtül et al., 2009; Bagi et al., 2011). The osteoporosis of proximal femur, lumbar vertebra, and bone properties of the mandibula takes place as pathophysiology for humans, and is frequently assessed in preclinical research (Bagi et al., 2011).

Bone tissue studies in poultry animals are performed to investigate the skeletal mutations, determine embryonic development conditions in laboratory conditions, describe endochondral ossification of various bones in embryonic period, and reveal the teratogenic results of new medicines (Pourlis et al., 1998; Simsa and Monsonego Ornan, 2007; Kürtül et al., 2009; Bakır and Kocamiş, 2011; Pourlis and Antonopoulos, 2014). In the literature survey performed for the present study, we found no studies regarding sex-based changes in biochemical parameters for the histology of epiphyseal plate closure process in quail in the post-hatching period.

This study was conducted to determine the effects of sex on ossification process following quail hatching by determining the exact closure timing of the epiphyseal plate in the proximal region of femur, using the histological and biochemical parameters that are known to influence the osteogenesis, and observe if any variation exists between males and females in this regard.

2. Material and Methods

The study was carried out in the Siirt province, and the research on the animals was conducted with the approval of the institutional committee on animal handling (2016/10).

A total of 84 animals was used in the study, of which 42 were males and 42 females. The animals were kept in cages at 35 °C temperature and 30% relative humidity conditions, whereas the temperature was reduced to 30 °C in the second week. Artificial fluorescent lighting and natural sunlight were used in the cages with a 24-h lighting program. Quail were fed 5% sugar-added water for 4-6 h following hatching, and with broiler, starter feed until the third week. Starting in the third week, both male and female quail were fed a fattening feed. The same feed was given to both groups. The nutritional composition of starter and fattening feed given to quail were prepared according to Tufan and Bolacalı, 2017 (Table 1). All quails were given the same quality feeds and water *ad libitum* during the study.

Table 1 - Ingredient composition and analyzed content of nutrients of diets used in the trial

	Diet	
	Starter	Grower
Ingredient (g kg^{-1})		
Yellow corn	450.0	527.8
Wheat	83.1	90.0
Vegetable oil	30.0	10.0
Soybean meal (48% CP)	300.0	270.0
Fish meal (64% CP)	35.0	-
Sunflower meal (32% CP)	80.0	70.0
Limestone	9.5	12.5
Vitamin and mineral premix[1]	2.5	2.5
Salt	3.5	3.5
DCP	4.1	13.5
Antioxidant	0.8	-
DL-methionine	-	0.2
L-threonine	1.5	-
Nutritional content (g kg^{-1})		
Dry matter	900.5	898.3
Metabolic energy (kcal kg^{-1})[2]	3,005	2,905
Crude protein	239.0	200.0
Crude fat	46.0	28.0
Crude fiber	44.2	44.5
Crude ash	56.2	61.0
Calcium	8.2	9.2
P	3.7	3.8
Na	2.0	1.8
Cl	2.8	2.6
Methionine + cysteine	8.5	7.1
Lysine	13.0	10.3
Threonine	10.6	7.6
Tryptophan	3.1	2.7

CP - crude protein.
[1] Supplied the following per kilogram of diet: 13,000 IU vitamin A; 3,500 IU vitamin D3; 100 mg vitamin E; 3 mg vitamin K3; 3 mg vitamin B1; 8 mg vitamin B2; 6 mg vitamin B6; 30 mg vitamin B12; 30 mg niacin; 8 mg calcium-D-panthotenate; 2 mg folic acid; 70 mg vitamin C; 70 mg D-biotin; 200 mg choline chloride; 2 mg canthaxanthin; 0.75 mg apo carotenoic acid esther; 120 mg Mn; 100 mg Zn; 90 mg Fe; 16 mg Cu; 1.5 mg I; 0.75 mg Co; 0.30 mg Se.
[2] Calculated according to NRC (1977) table values.

Blood samples were collected from six male and six female specimens via IV catheters every week for 50 days following hatching into serum tubes. Then, they were transported to the laboratories and stored following cold-chain procedures. Values of PTH, T3, T4, IP, Ca, and vitamin D, which are known to influence the ossification process, were then evaluated.

After the seventh week, the subjects were euthanized. Histological cut-sections covering the epiphyseal growth plate and bone sections of the proximal region of the right femurs of the quail were collected. These cut-sections were kept in EDTA solution for decalcification purposes. The routine histological inspections of sections were conducted with a 70% alcohol series following the elution procedure and embedded in paraffin afterwards.

Five-micrometer thick serial sections were cut from paraffin blocks using a Leica RM 2125 Rotary Microtome. Crossman's modified triple staining method was applied to sections after the deparaffinization and rehydration procedures. Following the staining procedure, the preparations were observed with a Nikon-Eclipse 400 digital camera attached to a research microscope. Histopathology evaluations were performed by investigating the proliferative chondrocytes, mature chondrocytes, and density of calcification regions during the ossification process of the epiphyseal plate region. Chondrocytes and calcification zones were assessed using blinded preparations and an independent assessor (Z.K.). Furthermore, the widths of epiphyseal regions of male and female quail were measured (Figure 1). The one-way ANOVA and post hoc (Bonferroni) tests were used to compare the epiphyseal plate widths. Data were presented in the tables as median ± standard deviation. $P<0.05$ was accepted as statistically significant.

A normality test was performed to determine the appropriate analytical method for the study. This test revealed that the data did not display a normal distribution. One of the nonparametric tests, Wilcoxon Signed Ranks Test (SPSS software version 20.0, Chicago, IL, USA), was used to compare the repeated groups. $P<0.05$ was accepted as statistically significant.

$z = (Ws-n(n+1)/4)/\sqrt{(n(n+1)(2n+1)/24)}$ using the Wilcoxon Signed Ranks Test (Demirutku et al., 2005), in which, n = the number of pairs of observations in the sample, and W = Sum of the R+ ranks.

Simple linear regression analysis was used to evaluate the weekly results for biochemical parameters. A P-value of ≤0.05 was interpreted as different.

$$Y = \alpha + \beta X,$$

in which Y represents the dependent variable, X is the independent variable, and α and β are two constraints which are known as regression coefficients.

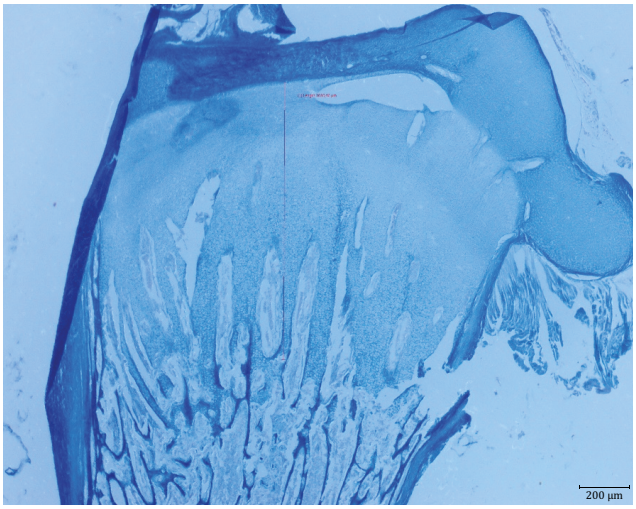


Figure 1 - Measurement site in epiphyseal plate region (Crossmann's triple 200 µm).

3. Results

Comparing the first week results of male and female quail (Table 2), it was determined that the ALP activity (P<0.05), IP, and T4 levels (P<0.01), and Ca, T3, vitamin D, and PTH levels (P<0.001) are different between the sexes in a statistically significant manner. Based on the findings of the second week, it was determined that the difference between the sexes in terms of PTH level was also statistically significant (P<0.01). Parathormone and ALP values were found to be significantly higher in females compared with males (P<0.001), while IP and Ca levels were higher in females compared with males in the third week of the study (P<0.05). Another observation was that the IP level was highest in the fourth week in male quail. T4 levels, on the other hand, were higher in female quail in the fifth week compared with males, whereas they were lower in the first and seventh weeks. A statistically significant difference was also detected between sexes in terms of PTH levels (P<0.05) and ALP activity (P<0.01). When the sixth-week findings were evaluated, it was determined that the IP (P<0.01), PTH, and Ca levels (P<0.05) were higher in females compared with males, and the T3 level was higher in males (P<0.01). The T3 levels of females were noticeably lower compared with previous weeks. When the findings of the final week of the study were examined, it was found that the Ca value was higher in females (P<0.001), while T4 and vitamin D levels (P<0.01) and ALP activity were higher in males (P<0.01).

Concerning biochemical parameters of male quail (Table 3), the IP value changes are significant for the weeks 1-2, 1-5, 1-6, 2-5, 2-6, 3-5, 3-7, and 5-7; Ca value changes are significant for weeks 1-7, 2-5, 2-6, 3-7, and 5-6; ALP value changes are significant for weeks 1-2, 1-5, 1-6, 2-3, 2-5, 4-7, 5-6, and 6-7; T4 value changes are significant for weeks 1-2, 1-6, 1-7, 2-6, 2-7, 3-4, 3-5, and 4-5; T3 value changes are significant for weeks 1-2, 2-5, 2-6, 4-5, 4-6, 4-7, 5-6, 5-7, and 6-7; vitamin D value changes are significant for weeks 2-4, 2-5, 2-6, 2-7, 3-5, 4-6, 4-7, 5-6, 5-7, and 6-7; and PTH value changes are significant for weeks 1-3, 1-4, 1-6, 3-4, 3-6, 4-6, and 5-7.

Table 2 - Weekly averages of some biochemical parameters of female and male quail

Parameter	Sex	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
IP	Male	3.60±0.12	7.03±0.26	8.23±0.15	9.40±0.21	7.47±0.47	5.43±0.50	6.23±0.32
	Famale	4.73±0.09	7.30±0.25	9.73±0.47	7.70±0.50	7.57±0.22	8.40±0.35	6.33±0.20
	P-value	0.001**	0.502^{ns}	0.037*	0.035*	0.856^{ns}	0.008**	0.804^{ns}
Ca	Male	5.65±0.58	7.55±0.51	6.89±0.34	7.98±0.28	8.20±0.24	7.77±0.20	8.90±0.24
	Female	9.35±0.06	7.10±0.48	8.46±0.28	8.18±0.28	8.00±0.17	9.77±0.57	16.11±0.51
	P-value	0.000***	0.560^{ns}	0.024*	0.635^{ns}	0.531^{ns}	0.030*	0.000***
T4	Male	0.42±0.01	0.23±0.04	0.48±0.08	0.36±0.01	0.48±0.08	0.46±0.07	1.00±0.05
	Female	0.34±0.01	0.24±0.05	0.47±0.03	0.42±0.08	1.73±0.26	0.34±0.03	0.60±0.03
	P-value	0.002**	0.917^{ns}	0.941^{ns}	0.504^{ns}	0.010*	0.201^{ns}	0.003**
T3	Male	8.90±0.02	5.80±0.25	5.98±0.13	6.07±0.19	4.54±0.33	4.89±0.12	4.67±0.29
	Female	6.39±0.02	6.37±0.95	5.80±0.33	6.23±0.32	3.97±0.39	2.76±0.29	4.79±0.35
	P-value	0.000***	0.597^{ns}	0.653^{ns}	0.690^{ns}	0.330^{ns}	0.002**	0.811^{ns}
VitD	Male	6.79±0.02	14.01±2.70	6.10±0.88	22.22±1.12	20.91±1.31	14.89±1.40	27.49±1.37
	Female	4.07±0.03	18.24±1.83	7.51±0.40	23.49±2.28	22.41±0.47	21.74±2.17	12.85±1.82
	P-value	0.000***	0.264^{ns}	0.220^{ns}	0.643^{ns}	0.343^{ns}	0.057^{ns}	0.003**
PTH	Male	15.50±0.06	5.90±0.72	10.31±0.25	4.56±0.49	3.34±0.35	2.42±0.34	4.27±0.14
	Female	13.17±0.07	16.93±2.00	6.53±0.20	3.25±0.58	1.42±0.34	6.67±1.28	2.90±0.62
	P-value	0.000***	0.007**	0.000***	0.162^{ns}	0.017*	0.033*	0.098^{ns}
ALP	Male	893.33±6.64	2389.33±117.79	2229.67±44.25	1295.67±118.94	1114.33±21.76	978.67±30.33	776.33±19.06
	Female	933.00±7.94	2274.33±47.89	1790.67±69.90	1431.00±87.31	1309.33±25.76	937.33±37.22	571.67±52.70
	P-value	0.019*	0.417^{ns}	0.006**	0.411^{ns}	0.004**	0.438^{ns}	0.022*

IP - inorganic phosphorus; T4 - thyroxine; T3 - triiodothyronine; VitD - vitamin D; PTH - parathormone; ALP - alkaline phosphatase enzyme; ns - non-significant (P>0.05).
* P<0.05.
** P<0.01.
*** P<0.001.

Regarding biochemical parameters of female quail (Table 4), the IP value changes are significant for the weeks 1-2, 1-5, 1-7, 2-5, 2-7, 3-5, 4-6, and 5-7; Ca value changes are significant for weeks 1-2, 1-3, 1-6, 2-3, 2-6, 3-6, and 4-7; ALP value changes are significant for weeks 1-6,1-7, 2-3, 2-4, 3-4, 4-6, and 6-7;

Table 3 - Weekly regression analysis results of biochemical parameters of male quail

Weekly change of parameters		P-value					
		2nd week	3rd week	4th week	5th week	6th week	7th week
IP	1st week	0.000	0.212	0.595	0.009	0.001	0.264
	2nd week		0.166	0.682	0.005	0.003	0.211
	3rd week			0.202	0.027	0.439	0.000
	4th week				0.822	0.321	0.158
	5th week					0.053	0.042
	6th week						0.515
Ca	1st week	0.060	0.061	0.698	0.392	0.071	0.000
	2nd week		0.627	0.389	0.017	0.000	0.132
	3rd week			0.079	0.690	0.668	0.022
	4th week				0.059	0.357	0.488
	5th week					0.013	0.585
	6th week						0.150
ALP	1st week	0.005	0.054	0.349	0.000	0.006	0.138
	2nd week		0.003	0.783	0.016	0.080	0.440
	3rd week			0.779	0.104	0.271	0.836
	4th week				0.233	0.083	0.002
	5th week					0.001	0.076
	6th week						0.015
T4	1st week	0.001	0.907	0.408	0.366	0.011	0.001
	2nd week		0.722	0.178	0.153	0.000	0.017
	3rd week			0.021	0.028	0.502	0.592
	4th week				0.000	0.086	0.689
	5th week					0.070	0.636
	6th week						0.054
T3	1st week	0.002	0.769	0.313	0.079	0.158	0.235
	2nd week		0.852	0.120	0.014	0.044	0.079
	3rd week			0.069	0.287	0.167	0.106
	4th week				0.005	0.000	0.000
	5th week					0.000	0.002
	6th week						0.000
VitD	1st week	0.664	0.396	0.331	0.545	0.711	0.637
	2nd week		0.066	0.002	0.031	0.000	0.000
	3rd week			0.225	0.000	0.055	0.074
	4th week				0.138	0.003	0.002
	5th week					0.024	0.035
	6th week						0.000
PTH	1st week	0.085	0.000	0.000	0.754	0.002	0.880
	2nd week		0.114	0.129	0.284	0.261	0.108
	3rd week			0.000	0.667	0.001	0.971
	4th week				0.630	0.000	0.988
	5th week					0.402	0.001
	6th week						0.719

IP - inorganic phosphorus; T4 - thyroxine; T3 - triiodothyronine; VitD - vitamin D; PTH - parathormone; ALP - alkaline phosphatase enzyme.

Table 4 - Weekly regression analysis results of biochemical parameters of female quail

Weekly change of parameters		P-value					
		2nd week	3rd week	4th week	5th week	6th week	7th week
IP	1st week	0.001	0.269	0.141	0.016	0.527	0.000
	2nd week		0.114	0.313	0.001	0.829	0.004
	3rd week			0.625	0.028	0.190	0.375
	4th week				0.601	0.010	0.085
	5th week					0.803	0.036
	6th week						0.398
Ca	1st week	0.000	0.000	0.769	0.215	0.000	0.489
	2nd week		0.001	0.571	0.344	0.001	0.330
	3rd week			0.917	0.145	0.000	0.620
	4th week				0.119	0.919	0.001
	5th week					0.144	0.267
	6th week						0.621
ALP	1st week	0.393	0.367	0.103	0.620	0.002	0.000
	2nd week		0.000	0.006	0.080	0.151	0.259
	3rd week			0.005	0.090	0.136	0.238
	4th week				0.339	0.018	0.050
	5th week					0.975	0.811
	6th week						0.000
T4	1st week	0.020	0.000	0.019	0.000	0.668	0.037
	2nd week		0.007	0.000	0.012	0.156	0.000
	3rd week			0.007	0.000	0.516	0.016
	4th week				0.012	0.161	0.000
	5th week					0.591	0.025
	6th week						0.107
T3	1st week	0.016	0.413	0.253	0.527	0.000	0.146
	2nd week		0.951	0.025	0.810	0.038	0.607
	3rd week			0.284	0.000	0.288	0.003
	4th week				0.204	0.370	0.638
	5th week					0.384	0.009
	6th week						0.083
VitD	1st week	0.004	0.022	0.269	0.003	0.000	0.060
	2nd week		0.000	0.064	0.055	0.001	0.258
	3rd week			0.021	0.130	0.007	0.431
	4th week				0.619	0.168	0.873
	5th week					0.013	0.005
	6th week						0.116
PTH	1st week	0.005	0.659	0.040	0.000	0.214	0.426
	2nd week		0.267	0.001	0.007	0.573	0.133
	3rd week			0.099	0.706	0.167	0.000
	4th week				0.049	0.925	0.035
	5th week					0.188	0.466
	6th week						0.318

IP - inorganic phosphorus; T4 - thyroxine; T3 - triiodothyronine; VitD - vitamin D; PTH - parathormone; ALP - alkaline phosphatase enzyme.

T4 value changes are significant for weeks 1-2, 1-3, 1-4, 1-5, 1-7, 2-3, 2-4, 2-5, 2,7, 3-4, 3-5, 3-7, 4-5, 4-7, and 5-7; T3 value changes are significant for weeks 1-2, 1-6, 2-4, 2-6, 3-5, 3-7, and 5-7; vitamin D value changes are significant for weeks 1-2, 1-3, 1-5, 1-6, 2-3, 2-6, 3-4, 3-6, 5-6, and 5-7; and PTH value changes are significant for weeks 1-2, 1-4, 1-5, 2-4, 2-5, 3-7, 4-5, and 4-7.

In the histological evaluation, it was determined that the epiphyseal plate region of poultry animals consisted of five zones, as in mammalians. Reserve and proliferation zones were detected at the end of the first week for both female and male quail (Figure 2 A-B). These were also observed at the end of the second week (Figure 2 C-D). The epiphyseal plate regions of the specimens consisted of proliferation and maturation zones at the end of the third week (Figure 2 E-F). They were present in the fourth week as well, but the maturation zones were increased in size (Figure 2 G-H). Formation of calcified cartilage zones and ossification processes seem to have started at the end of the fifth week for both sexes (Figure 2 I-J). The calcification was found to be more pronounced in female quail. Finally, the epiphyseal plate region was closed in all specimens, in male and female, at the end of the sixth week, which seems to be their adolescence age (Figure 2 K-L). Measurement of the epiphyseal plate region widths revealed no statistically significant difference for the sexes and between weeks ($P>0.05$; Table 5).

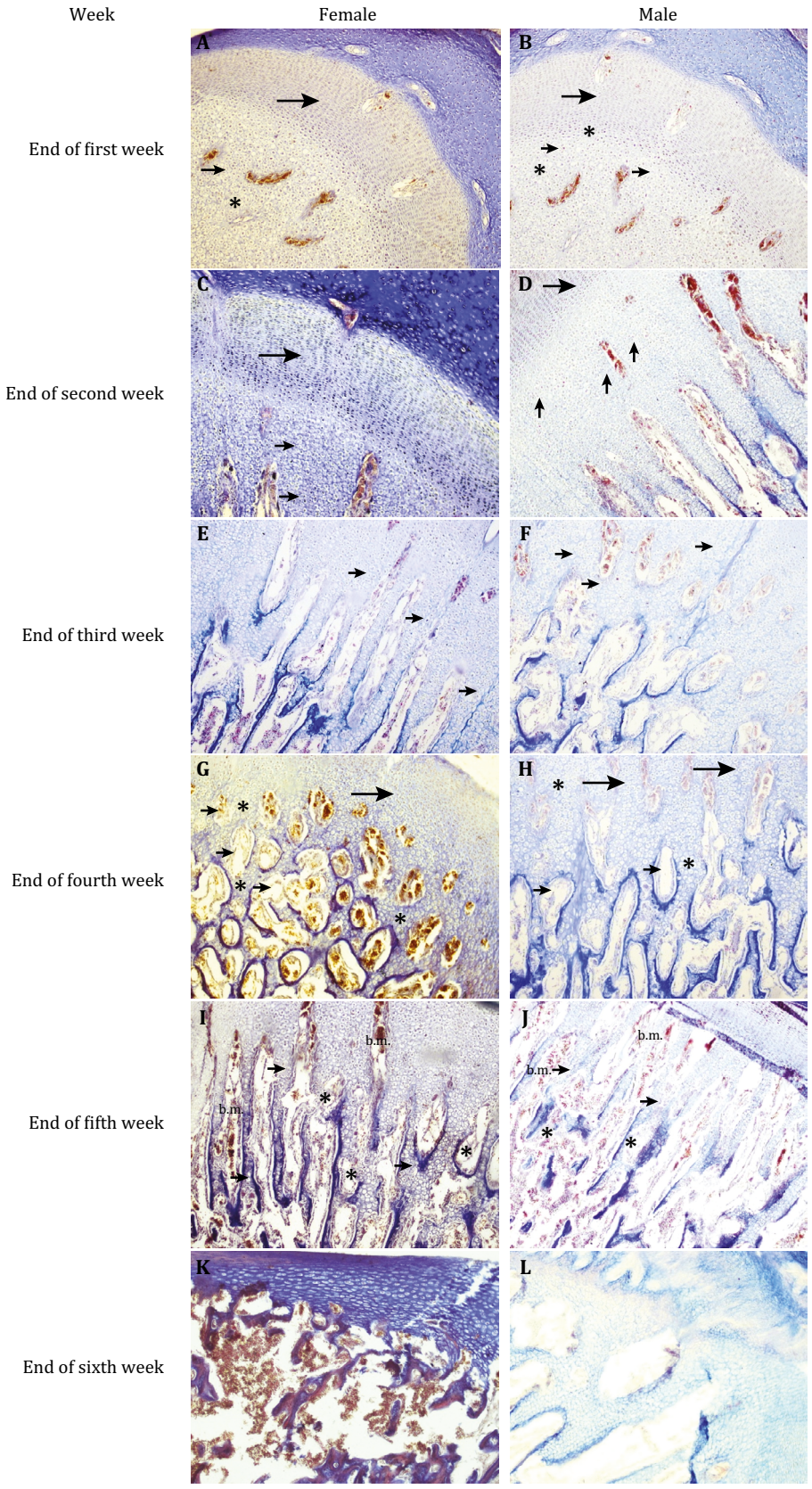
4. Discussion

The PTH, vitamin D, calcium, calcitonin, vitamin A, vitamin C, estrogen, testosterone, certain prostaglandins and cytokines, glucocorticoids, thyroid hormones, insulin, and growth hormone are some of the factors that influence the IGF-I and the acidosis taking place as byproducts of bone metabolism. Serum ALP activity is one of the determining markers in active ossification (Gönenci and Yücel, 2006; Köse and Dinçer, 2015; Oznurlu et al., 2016).

The enzyme ALP secreted by osteoblasts transforms Ca and P ions into $CaPO_4$ ions, and these ions begin to accumulate in the plate zones. This enzyme is continuously secreted by osteoblasts during the process, which continues until the completion of ossification (Özer, 2014; Köse and Dinçer, 2015). Osteoblast membranes contain PHT receptors in the membranes. These secrete osteoclast-stimulant hormones when stimulated by PTH. The ALP level in blood is a significant criterion in terms of the completion of the ossification process. High ALP levels are secreted during active bone formation and its level in blood increases rapidly. The PTH enables osteoclasts to provide acid hydrolases to the matrix by increasing the lysosome synthesis in the osteoclast, initiating resorption, and generating numerous invaginations by increasing membrane convolutions. The Ca and PO_4 ions are released as a result of increasing bone destruction, and their amounts in the blood are increased, halting the PTH secretion. The non-secretion of PTH stimulates and activates the osteoblasts, and bone formation is stimulated (Atalgin and Çakir, 2006; Özer, 2014).

In studies conducted on bones and cartilaginous tissues of male and female animals, the differences observed are attributed to the influence of the estrogen hormone (Gönenci and Yücel, 2006; Ketani and Sağsöz, 2009; Atik and Ciğer, 2012). The difference in Ca level between males and females in weeks 1, 3, 6, and 7 were attributed to the effects of estrogen hormone over Ca metabolism. Weekly regression analysis of Ca levels revealed that the results of the fourth week are similar to results of other weeks, both in males and in females.

In the literature, the T3 hormone is stated to be effective during the period of epiphyseal plate closure to play a significant role in skeletal development, as well as influencing the epiphyseal plates of developing bones and columnar organizations of hypertrophy chondrocytes (Ballock and Reddi, 1994; Serter, 2010; Atik and Ciğer, 2012). While T3 and T4 hormone levels do not display a distinctive increase during the epiphyseal plate closure period, they still have a fluctuating pattern in both male and female quail for seven weeks. Furthermore, a statistically significant difference was observed between the first- and sixth-week T3 hormone levels of male and female quail. The difference between T4 levels of the two sexes, on the other hand, was statistically significant in weeks 5 and 7. Weekly regression analysis showed that the different levels of T4 in female quail in week 6 and T3 levels in week 2 was



A-B: long arrow = normal chondrocyte; short arrow = proliferative chondrocyte; asterisk = proliferative zone. C-D: long arrow = lapped cartilage cells; short arrow = mature chondrocyte. E-F: short arrow = hypertrophic chondrocytes. G-H: long arrow = hypertrophic chondrocyte; short arrow = calcified areas; asterisk = hypertrophic zone. I-J: short arrow = bone cells; asterisk = calcified area; b.m. = bone marrow. K-L: bony tissue in epiphyseal growth plate.
Crossmann's Triple (A-B) X10, (C-L) X20.

Figure 2 - Weekly changes of epiphyseal plaque regions in quail.

Table 5 - Comparison of epiphyseal plate region widths (µm) of quail

Sex	Week					
	1st week	2nd week	3rd week	4th week	5th week	6th week
Female[a]	1260.13±91.37	1202.25±83.32	1333.40±74.14	1326.15±32.72	1234.27±48.72	1252.50±61.55
Male[b]	1103.22±50.64	1032.86±33.85	1146.06±8.62	1114.06±31.93	1180.51±11.05	1161.73±19.15

a,b - The difference between groups is insignificant (P>0.05).

not statistically significant when compared with other weeks. Similarly, the T4 levels of male quail in week 2 and T3 levels in weeks 2 and 3 were not statistically significant when compared with other weeks. Histological findings showed no irregular structure in chondrocytes, which are organized in the plate region, which can be explained by the effect of the T3 hormone. Moreover, the higher level of T4 in females in the second and fifth weeks give rise to the thought that it could have resulted from the activity of the estrogen hormone on the thyroid gland within the ossification process.

The influence of vitamin D on bone tissue is by regulating the Ca homeostasis and maintaining bone health by aiding absorption and the use of Ca. Moreover, vitamin D and $1,25(OH)_2D_3$ synthesis are closely related to Ca balance, and PTH is regulated by serum Ca and P levels (Özkan and Döneray, 2011; Atik and Ciğer, 2012). The Ca and PO_4 ions are released and transferred into the bloodstream as a result of the increased bone destruction during ossification, and increased activity of these ions in the blood level and their utilization at the ossification zone are directly related to vitamin D (Özkan and Döneray, 2011). In this study, vitamin D, P, and Ca values were highest in the fourth and fifth weeks, and when histological findings were evaluated, it was revealed that the calcification and ossification processes accelerated during this particular period as well. It is logical to assume that the utilization of Ca and P ions, which are needed in this period as explained, was ensured by means of vitamin D.

As part of the study, the PTH levels of both male and female quail were found to be decreasing from the first week, and the difference between the sexes in terms of PTH levels were statistically significant in the first, second, third, fifth, and sixth weeks. The weekly regression analysis results of male and female quail also support these findings. We surmised that this was a result of continuous stimulation of the osteoblasts, and it was influential in ensuring the continuity of bone formation in the epiphyseal plate zone.

In the present study, the ALP activity was determined to be higher in the second and third weeks compared with other weeks for both female and male quail. Evaluation of weekly regression analysis results shows that the ALP activity of male quail had a statistically significant difference between weeks 1-2 and 2-3, whereas the difference was statistically significant between weeks 2-3 for female quail. Yalçın et al. (2007) stated that a high level of ALP activity occurs in the hypertrophic zone (the period when mature chondrocytes are stimulated and activated) and has a lower level in the proliferative zone, which supports our findings. Histological examinations coincide with the biochemical findings that mature chondrocytes are stimulated and activated on days in which ALP activity is also high. The decrease in PTH and the increase in ALP in the fifth week as the calcification increases in females indicate that the osteoblast activity is higher for females than for males. This is further supported by our findings, as the difference between ALP and PTH in the biochemical parameters of the fifth week in females and males was also statistically significant.

In the radiological examinations conducted during the closure process of the epiphyseal plate region of tibiotarsus in male and female quail, Akgül et al. (2017) found that the epiphyseal plate closure process of the entire group was completed in the sixth week, and the closure was completed earlier in 50% of the female quail specimens. In the present study, the comparison of both histological and biochemical parameters revealed that the calcification and ossification phenomena in the epiphyseal plate region were more pronounced in females than in males. Furthermore, the epiphyseal plate region was replaced with bone tissue at the end of the sixth week in both male and female quail.

5. Conclusions

The results indicate that there may be distinct differences between female and male quail regarding the epiphyseal plate closure processes. The faster progression of calcification and ossification in the epiphyseal region observed in female specimens can be attributed to hormonal factors.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Z. Karakoç and K. İrak. Data curation: Z. Karakoç and K. İrak. Formal analysis: Z. Karakoç and K. İrak. Funding acquisition: Z. Karakoç and K. İrak. Investigation: Z. Karakoç and K. İrak. Methodology: Z. Karakoç and K. İrak. Project administration: Z. Karakoç and K. İrak. Resources: Z. Karakoç and K. İrak. Software: Z. Karakoç and K. İrak. Supervision: Z. Karakoç and K. İrak. Validation: Z. Karakoç and K. İrak. Visualization: Z. Karakoç and K. İrak. Writing-original draft: Z. Karakoç and K. İrak. Writing-review and editing: Z. Karakoç and K. İrak.

Acknowledgments

We would like to thank Assist. Prof. Dr. Memiş Bolacali (from the Department of Zootechnics, Veterinary Medicine Faculty, University of Siirt) for his valuable contribution to the statistical analysis of this study.

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