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Effect of molting programs and *Humulus lupulus* plant on tibia characteristics and expression of TRPV6 gene in different molting periods of laying hens

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ABSTRACT. The aim of this study was to determine the effects of molting programs and *Humulus* (*Humulus lupulus*) plant on tibia characteristics and expression of TRPV6 gene in Pre, during and post-molting layers. Experiment 1, Hy-line W-36 (75-76 weeks, n= 272) laying hens 20 hens slaughtered before molting at end of 76 week for checking status. Experiment 2, A total of 252 Hy-line W-36 were managed in 7 treatments, 6 replicates and 6 birds in each. Treatments including: (A) feed withdrawal (FW), (B) basal diet + 2% Portulaca oleracea plant powder, (C) basal diet + 30% sunflower seed hulls + 2% Portulaca oleracea powder, (D) basal diet + 2% white button mushroom waste, (E) basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, (F) basal diet + 30% sunflower seed hulls and (G) basal diet (Control treatment). Experiment 3, A total hens of treatments in experiment 2 after slaughter of 20 hens maintained for experiment 3, then each treatment diet was modulated in to two treatments (with and without *Humulus*). In this step, this 224 Hy-Line W-36 laying hens were designed by 14 treatments, 4 replicates and 4 hens in each in a 7×2 factorial arrangement with completely randomized design (CRD). The highest tibia weight and bone cortical thickness were observed in the treatment contain *Portulaca Oleracea* powder ($p < 0.05$). A higher tibia weight, bone cortical thickness, ash and density were indicated by *Humulus* plant in post-molting periods ($p < 0.05$). The bone cortical thickness and ash were increased by basal diet + 30% sunflower seed hulls + 2% Portulaca Oleracea powder and basal diet + 30% sunflower seed hulls treatments at post molting periods ($p < 0.05$). During the post-molting period, there was no significant effect on maximum strain and elastic modulus by *Humulus* and molting methods ($p > 0.05$). Also, no significant differences were found in expression of TRPV6 mRNA in the jejunum, kidney and egg shell gland (ESG) during molting and post-molting periods ($p > 0.05$). In conclude, improve tibia characteristics during molting and post-molting periods by *Portulaca oleracea* powder and white button mushroom waste. Moreover, *Humulus* plant improved tibia status without affecting TRPV6 mRNA expression.

Keywords: Gene expression; laying hen; medicinal herbs; molting; tibia characteristics.

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Introduction

The replacement pullets with egg producers molting programs could lead to benefit induced status to extend egg production into multiple egg laying cycles is one of the most expensive costs in the commercial egg industry (Bell, 2003). Induced molting enhanced mineralization of the medullary tibia and recovery during the post-molting period (Mazzuco & Hester, 2005). The typical induced molting methods used in the egg industry such as feed withdrawal (Park et al., 2004), dietary imbalances of certain minerals such as Cu, Zn (Stevenson & Jackson, 1984), Na, Cl (Harms, 1991), or high fiber diets (Landers et al., 2008). Almost all previous reports indicated that induced molting improved egg production and mineralization of the medullary tibia (Abdelqader et al., 2013). *Portulaca* (*Purslane oleracea*) is one of the most herbal medicines in the world, which is a rich source of flavonoids including quercetin, kaempferol, myricetin, apigenin, luteolin, genistein, and genistin (Zhang et al., 2007; Zhu et al., 2010). Recent studies have suggested that higher flavonoids intake is associated with increased bone mineral content, and reduced markers of bone resorption (Messina et al., 2004; Sahin et al., 2007). Mushrooms have long been appreciated as an important source of bioactive compounds of medicinal value, which is rich in ergosterol, which is the pre-cursor of vitamin D2 (Jasinghe et al., 2006). The decrease in shell quality and mineralization of the medullary tibia in the old hen

has been attributed to a decline in vitamin D metabolism and expression through the formation of cholecalciferol through calcium binding protein (calbindin) (Berry & Brake, 1991) or intestinal uptake of Ca^{2+} (Yosefi et al., 2003). The evidence of their research has suggested three-step process for epithelial Ca^{2+} absorption: first the calcium comprising the passive entry of Ca^{2+} into enterocytes via the transient receptor potential vanilloid channel type 5 or 6 (TRPV5, TRPV6); the cytosolic transfer of Ca^{2+} bound to the protein calbindin D28K and the extrusion of Ca^{2+} across the basolateral membrane via Ca^{2+} ATPase (PMCA 1b) (Yang et al., 2011). The TRPV6 protein is expressed in epithelial tissues such as the intestine, kidney, bone, skin, placenta and this ion channel have been significant effects in Ca2+ absorption (Bianco et al., 2007). Little information is available in the scientific literature on the effects of a non-fasting molting regimens on hen skeletal health, also rarely information, however, exists regarding change of expression of TRPV6 mRNA gene in the jejunum, kidney and egg shell gland (ESG) at pre-, during and post-molting of layers. Therefore, the objective of the current study was to determine the effect of an induced molting using non withdrawal of feed and *Humulus* (*Humulus lupulus*) plant on the bone mineralization and expression of TRPV6 mRNA gene at pre, during and post-molting hens.

Material and methods

The experiment was located at the Faculty of Agricultural in, Bu Ali Sina University, Hamedan, Iran. All hens were weighted and arranged into the replicates cages as similar body weights.

Experiment 1

A total, 272 old Hy-line W36 (75-weeks) laying hens were housed at cage during 2 weeks for monitoring tibia characteristic and expression of TRPV6 mRNA status before molting. During this time, the birds were fed a complete layer ration ad-libitum and at the end of the feeding period (2 weeks), the 20 hens were slaughtered. Oviduct, small intestine, kidney and tibia were rapidly removed and storage in ice pack.

Experiment 2

In experiment 2, two hundred fifty-two (252) Hy-Line W36 laying hens (77-weeks of age) were management in completely randomize design (CRD) and arranged into 7 dietary treatments, 6 replicates and 6 birds in each. The diets were iso-energetic and iso-nitrogenous. Treatments including: (A) feed withdrawal (FW), (B) basal diet + 2% *Portulaca oleracea* powder, (C) basal diet + 30% sunflower seed hulls + 2% *Portulaca oleracea* powder, (D) basal diet + 2% white button mushroom waste, (E) basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, (F) basal diet + 30% sunflower seed hulls and (G) basal diet as control treatment (Table 1). During molting, the following characteristics were evaluated in obtain slaughter 20 hens in this period of experiment: tibia characteristics, biomechanical properties, tibia mineral content and plasma phosphorous calcium and expression of TRPV6 mRNA in the jejunum and kidney were considered. In this respective, diet and water were allowed ad libitum and hens were placed on an artificial lighting program of 8L: 16 D during the 30 D molting period. The body weight and feather condition (checked in the head, neck, back, breast, leg, belly, wing and tail) of all the hens were recorded on days 3, 6, 9, 12, 15, 18, 21, 24 and 27.

Table 1. Ingredients and nutritional composition of the experimental diets.

Ingredients (%)	Pre-Molting	Molting							Post-Molting	
		1	2	3	4	5	6	7	With *Humulus*	Without *Humulus*
Corn	58.31	0	56.44	81.34	56.44	81.34	58.1	83	57.22	57.5
SBM	25.14	0	9.044	13.034	9.044	13.034	9.31	13.3	26.37	26.5
SSH	0	0	30	0	30	0	30	0	0	0
POP	0	0	2	2	0	0	0	0	0	0
WBMW	0	0	0	0	2	2	0	0	0	0
Humulus	0	0	0	0	0	0	0	0	0.5	0
Soybean oil	3.22	0	0	0	0	0	0	0	2.96	2.98
DCP	1.92	0	1.496	2.156	1.496	2.156	1.54	2.2	2.55	2.57
Oster shell	10.78	0	0.949	1.369	0.949	1.369	0.95	1.37	9.72	9.77
NaCl	0.27	0	0.034	0.049	0.034	0.049	0.035	0.05	0.3	0.3
Sodium Bicarbonate	0.17	0	0.03	0.03	0.03	0.03	0.03	0.03	0.2	0.2
Mineral premix[1]	0.025	0	0.017	0.0245	0.017	0.0245	0.017	0.025	0.025	0.025
Vitamin premix[2]	0.025	0	0.017	0.0245	0.017	0.0245	0.017	0.025	0.025	0.025
Methionine	0.14	0	0	0	0	0	0	0	0.129	0.13

Calculated analysis %										
AMEn (Mcal kg^{-1})	2778	0	2220	2220	2220	2220	2220	2220	2800	2800
CP	15.86	0	8.90	8.90	8.90	8.90	8.90	8.90	15.86	15.86
Ca	5	0	2	2	2	2	2	2	4.35	4.35
AP	0.41	0	0.38	0.38	0.38	0.38	0.38	0.38	0.54	0.54
Na	0.19	0	0.03	0.03	0.03	0.03	0.03	0.03	0.19	0.19
Cl	0.19	0	0.03	0.03	0.03	0.03	0.03	0.03	0.19	0.19
D-CAB	225	0	127	127	127	127	127	127	211.79	211.79
SID Lysine	0.81	0	0.4	0.4	0.4	0.4	0.4	0.4	0.799	0.799
SID Methionine	0.44	0	0.17	0.17	0.17	0.17	0.17	0.17	0.367	0.367
SID Methionine +cystine	0.73	0	0.38	0.38	0.38	0.38	0.38	0.38	0.600	0.600
SID Threonine	0.67	0	0.2	0.2	0.2	0.2	0.2	0.2	0.550	0.550
SID Arginine	0.85	0	0.43	0.43	0.43	0.43	0.43	0.43	1.030	1.030
SID Valine	0.79	0	0.28	0.28	0.28	0.28	0.28	0.28	0.703	0.703
Determined analysis										
DM	94.35	0	94.32	92.88	94.77	93.49	94.51	93.65	93.41	93.81
Total ash	5.84	0	5.16	6.35	5.63	5.83	5.71	6.56	6.44	6.72
CP (N × 6.25)	15.67	0	7.79	7.82	8.08	8.21	7.95	7.88	15.94	15.50
CF	8.36	0	68.04	17.32	66.91	14.09	57.88	9.55	10.21	10.86
Ca	5.32	0	2.23	2.29	2.21	2.19	2.22	2.31	4.61	4.52
P	0.64	0	0.40	0.38	0.39	0.41	0.40	0.39	0.65	0.63

SBM: Soy bean meal, SSH: Sunflower seed hulls, POP: *Portulaca Oleracea* powder, WBMW: white button mushroom waste, DCP: Di-calcium phosphate, DM: dry matter, CP: crude protein, Ca: calcium, AP: Avail.phosphorus, CF: Crude fiber, [1] Vitamin premix supplied per kg of diet: vitamin A: 7.2 g; vitamin D_3: 7 g; vitamin E: 14.4 g; vitamin K_3: 1.6 g; vitamin B_1: 0.72 g; vitamin B_2: 3.3 g; vitamin B_3 (Calcium pan-thotenate): 12.16 g; vitamin B_5 (Niacin): 12 g; vitamin B_6: 6.2 mg; vitamin B_{12}: 0.6 g; Biotin: 0.2 g; and Cholin chloride: 440 mg. [2] Mineral premix supplied per kg of diet: Manganese: 64 mg; Iron: 100 mg; Zinc: 44 mg; Copper: 16 mg; Iodine: 0.64 mg and Selenium: 8 mg.

Experiment

After induce molting, total remaining hens (224 hens) in treatments experiment 2 divided into two treatments issues with and without *Humulus* plant. Therefore experiment 3 was includes 14 treatments, 4 replicates and 4 layers in each with 7×2 factorial arrangement in completely randomized design (CRD). Basal diets were formulated according to Hy-Line W36 recommend in 2015 for pre, during and post molting period, as shown in Table 2. In obtained slaughter 20 hens during this period, tibia characteristics and the expression of TRPV6 mRNA in the jejunum, kidney and ESG were evaluated.

Table 2. Proximate analysis of white button mushroom waste, sunflower seed hull, *Humulus* and *Portulaca oleracea* powder (%).

	Sunflower seed hull	*Portulaca Oleracea* powder	White button mushroom waste	*Humulus*
DM	95.22	82.54	78.32	94.11
Total Ash	2.18	25.83	14.75	15.36
EE	2.74	3.15	2.63	6.79
CP (N ×6.25)	1.21	10.30	4.32	7.82
CF	48.88	9.86	13.24	14.61
Ca	0.74	3.14	2.25	0.94
P	0.23	2.06	1.75	1.00
ADF	14.91	41.72	44.50	30.61
NDF	9.28	23.33	39.36	22.14

DM: dry matter, EE: Ether Extract, CP: crude protein, CF: Crude fiber Ca: calcium, P: phosphorus, ADF: acid detergent fiber and NDF: neutral detergent fiber.

Tibia properties

For the bone measurements, two birds from each treatment were selected at random, weighed and euthanized in this particular case. At the end of every period, hens were euthanized, and the right tibias were cleaned of attached tissue (All samples were kept on ice at the time of sample collection) and stored in plastic bags at -20°C. Tibia length, diameter, ash and weight were measured. Tibia volume was calculated assuming that the specific gravity of water is 1.0 g cm^{-3} at room temperature (Zhang & Coon, 1997). Bone density was obtained by dividing the tibia weight to volume.

Mechanical properties

The mechanical properties of samples measured by means of the axial test device (BT1-FR0.5TH.D14, Zwick, Ulm, Germany) equipped with a force gauge (X force Hp nominal Force: 500N Capacity) and 3-point bending system. Mechanical properties including maximum force, toughness and elasticity modulus were calculated from the foece-deformation curve. The sample is placed on two supporting pins a set distance

apart. The distance between supporting pins is 62 mm and the speed of test is 0.5 mm min.$^{-1}$: when displacement is enforced, the response in force is measured. Force/ displacement curves are recorded using the Test expert II software (from Zwick). The fracture work, which is the work done to failure the sample is calculated as the area under the force, deformation curve. The elasticity modulus was calculated as the slope of the liner portion of stress-strain curve between 25 and 75% of ultimate failure.

Bone ash measurement

Following bone density and bone breaking strength measurements, tibia samples were dried in an oven at 100ºC for 24h and weighed. The bones were then ashed at 600°C overnight, cooled in a desiccator, and weighed. The percentage ash was calculated by dividing the ash weight of each bone by tibia dry matter (DM).

Quantitative RT-PCR

Transient receptor potential vanilloid channel type 6 (TRPV6) mRNA gene expression in the kidney, jejunum and ESG segments were quantified and normalized to β-actin (Sugiyama et al., 2007) expression using an ABI-prism 7300 Sequence Detection System. The primers for TRPV6 (forward primer: 5′-TGGAACGGACTAAGTCAGAAGTTG-3′; reverse primer: 5′ CGTTATGGCTGGGATGTTGTT-3′, 141 bp, GenBank accession no. XM416530, 49-197 bp) and for β-actin (forward primer: 5′-TGCGTGACATCAAGG AGAAG-3′ and reverse primer: 5′-TGCCAGGTACATTGTGGTA-3′, 300 bp, GenBank accession no. L08165, 694 993 bp) (Yang et al., 2011) were designed by Prime Premier 5.0 and synthesised by Invitrogen Biotechnology Ltd. (Shanghai, China). Aliquots of the cDNA samples were used for RT-PCR in a final volume of 20 µ L, containing 10 µ L SYBR Premix Ex Taq (2×), 0.4 µ L ROX Reference Dye (50×) and 0.8 µ M of each forward and reverse primers. The PCR protocols included an initial denaturation (30 s at 95ºC), and a two-step amplification program (5 s at 95ºC, 31 s at 62ºC, repeated 40 times). The method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time RT-PCR data (Livak & Schmittgen, 2001).

Statistical analysis

The obtained data were submitted to analysis of variance using the General Linear Model (GLM) procedure of SAS statistical package Statistical Analytical Systems (SAS Institute, 2012). Means were compared with the test of Duncan at 0.05 probability level.

Results

Average tibia characteristics and tibia biomechanical properties of the laying hens fed by the layer diet at the pre-molting period (75-76 weeks) have shown in Table 3. Also, in the Table 4 show status tibia, mineral plasma content and the expression of TRPV6 mRNA in the jejunum, kidney and egg shell gland (ESG) in the pre-molting period. The results related to the tibia characteristics are shown in Table 5. No significant differences were observed in body weight and tibia length at the molting period ($p > 0.05$). The highest tibia weight was observed in the treatment C ($p < 0.05$). Bone cortical thickness, tibia ash and density were increased by the treatments D and E as compared with other treatments. The effect of molting programs on tibia biomechanical properties at the molting period are presented in Table 6. Maximum strain decreased by the treatments G and A, as compared with other treatments within the molting period ($p < 0.05$). As can be seen, no statistically significant differences ($p > 0.05$) were found in the all treatments in ultimate force and elastic modulus. The Breaking strength of tibia were elevation in hens fed by treatment B ($p < 0.05$). The effect of molting programs on tibia mineral content and plasma phosphorous and calcium at the molting period can be seen in Table 7. The greater tibia calcium was found by treatment C but greater tibia phosphorous was shown by treatments C and F in molting period ($p < 0.05$). The plasma phosphorous was considerably lower in treatment A as compared with other treatments within the molting period ($p < 0.05$). The highest level of plasma calcium was indicated in the treatments B and G, in molting period ($p < 0.05$). The results have shown no significant effect (main and interaction effects) by treatments on BW and tibia length ($p > 0.05$) Table 8. A higher tibia weight, bone cortical thickness, ash and density were observed by the main effects of *Humulus* plant in comparison without *Humulus* plant treatment ($p < 0.05$). Tibia weight and tibia weight/body weight index were not affected by molting programs in post molting periods ($p > 0.05$). Significantly increased tibia weight and tibia weight/body weight index were shown by treatment contain *Humulus* plant in comparison to other treatments at the post-molting period ($p < 0.05$). The highest bone cortical thickness and ash observed in W-A, W-C and W-F treatments in comparison to other treatments. Bone density was increased by W-D treatment in post molting periods ($p < 0.05$).

Table 3. Average tibia characteristics and tibia biomechanical properties of the laying hens fed by the layer diet at the pre-molting period (75-76 weeks).

	BW (g)	Tibia length (mm)	Tibia weight (g)	Bone cortical thickness (mm)	Bone ash (%)	Bone density (g cm^{-3})	Bending stress (N mm^{-2})	Maximum strain (mm)	Ultimate force (N)	Breaking strength (kg g^{-})	Elastic modulus (N mm^{-2})
Pre-molting	1762.71	111.48	7.81	7.05	0.20	0.19	90.92	2.85	132.15	4.55	12078.27

BW: body weight.

Table 4. Average tibia and mineral plasma content and the expression of TRPV6 mRNA in the jejunum, kidney and egg shell gland of the laying hens fed by the layer diet at the pre-molting period (75-76 weeks).

	Tibia Ca/Tibia Weight (%)	Tibia P/Tibia Weight (%)	phosphorus (mg dL)	calcium (mg dL)	kidney	jejunum	Egg shell gland
Pre- molting	18.64	8.87	6.59	18.21	0.75	0.84	2.81

Table 5. The effect of molting programs on tibia characteristics at the molting period.

Treat	BW (g)	Tibia length (mm)	Tibia weight (g)	Bone cortical thickness (mm)	Bone ash (%)	Bone density (g cm^{-3})
A	1290.830	113.450	0.569^{cd}	6.406^{b}	0.192^{c}	0.154^{c}
B	1317.500	116.010	0.576^{bcd}	6.548^{b}	0.192^{c}	0.179^{bc}
C	1347.830	113.610	0.614^{a}	6.643^{ab}	0.207^{ab}	0.207^{ab}
D	1351.010	114.680	0.590^{abc}	6.945^{a}	0.198^{bc}	0.183^{bc}
E	1326.500	113.670	0.566^{cd}	6.946^{a}	0.211^{a}	0.225^{a}
F	1356.670	114.230	0.558^{d}	6.708^{ab}	0.197^{bc}	0.180^{bc}
G	1359.830	113.930	0.601^{ab}	6.613^{ab}	0.201^{abc}	0.181^{bc}
SEM	20.752	0.836	0.009	0.107	0.004	0.012
P-value	0.1150	0.1723	0.0042	0.0084	0.0068	0.0094

$^{a-d}$ Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls and G: basal diet.

Table 6. The effect of molting programs on tibia biomechanical properties at the molting period.

Treat	Bending stress (N mm^{-2})	Maximum strain (mm)	Ultimate force (N)	Breaking strength (kg g^{-1})	Elastic modulus (N mm^{-2})
A	76.66^{d}	2.14	113.13	3.63^{c}	10614.99
B	81.80^{c}	2.61	111.16	4.53^{a}	10681.28
C	89.68^{ab}	2.27	108.83	4.10^{b}	10651.58
D	87.01^{b}	2.46	110.01	4.17^{ab}	10669.12
E	88.71^{ab}	2.45	108.85	4.11^{ab}	10657.16
F	86.36^{bc}	2.54	111.40	4.21^{ab}	10676.24
G	92.15^{a}	2.16	108.33	4.02^{b}	10644.84
SEM	1.6468	0.1346	2.3680	0.1344	46.4392
P-value	<.0001	0.1022	0.7740	0.0038	0.9620

$^{a-c}$ Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls and G: basal diet.

Table 7. The effect of molting programs on tibia mineral content and plasma phosphorous and calcium at the molting period.

Treat	Tibia Ca/Tibia Weight (%)	Tibia P/Tibia Weight (%)	phosphorus (mg dL^{-1})	calcium (mg dL^{-1})
A	16.86^{c}	7.72^{c}	5.61^{b}	11.79^{b}
B	17.37^{bc}	7.97^{bc}	6.34^{a}	16.17^{a}
C	18.23^{a}	8.48^{a}	6.66^{a}	13.48^{ab}
D	17.46^{b}	8.13^{b}	6.47^{a}	14.30^{ab}
E	17.58^{b}	8.15^{b}	6.52^{a}	14.22^{ab}
F	17.93^{ab}	8.46^{a}	6.63^{a}	13.71^{ab}
G	17.44^{b}	8.05^{b}	6.41^{a}	15.26^{a}
SEM	0.1881	0.0938	0.2139	0.9574
P-value	0.0005	<.0001	0.0258	0.0412

$^{a-c}$ Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls and G: basal diet.

Table 8. The effect of molting programs on tibia characteristics at the post-molting period.

Treat	BW (g)	Tibia length (mm)	Tibia weight (g)	Bone cortical thickness (mm)	Bone ash (%)	Bone density (g cm^{-3})
Without *Humulus*	1754.340	1754.340	8.014^{b}	0.458^{b}	7.453^{b}	0.215^{a}
with *Humulus*	1770.770	1770.770	8.642^{a}	0.516^{a}	8.044^{a}	0.295^{a}
SEM	17.465	0.100	0.035	0.004	0.043	0.006
Molting						
A	1774.500	112.636	8.3102	0.489	7.752^{ab}	0.253^{ab}
B	1744.700	112.682	8.285	0.494	7.733^{ab}	0.277^{a}
C	1732.200	112.960	8.381	0.493	7.942^{a}	0.250^{ab}
D	1821.400	112.413	8.325	0.478	7.730^{ab}	0.251^{ab}
E	1749.000	113.011	8.323	0.480	7.553^{b}	0.233^{b}
F	1797.000	112.660	8.364	0.482	7.790^{ab}	0.271^{a}
G	1719.100	112.927	8.330	0.492	7.721^{ab}	0.249^{ab}
SEM	32.675	0.188	0.065	0.007	0.080	0.011
Humulus ×Molting						
W-A	1761.200	112.701	8.000^{b}	0.455^{b}	7.381^{ed}	0.203^{ef}
N-A	1787.800	112.561	8.620^{a}	0.523^{a}	8.114^{a}	0.304^{a}
W-B	1759.200	112.723	8.123^{b}	0.463^{b}	7.385^{ed}	0.251^{bcd}
N-B	1730.200	112.644	8.444^{a}	0.525^{a}	8.070^{ab}	0.304^{a}
W-C	1729.400	112.980	8.101^{b}	0.438^{b}	7.700^{cde}	0.231^{cde}
N-C	1735.000	112.940	8.660^{a}	0.518^{a}	8.181^{a}	0.270^{abc}
W-D	1830.200	112.423	7.986^{b}	0.475^{ab}	7.434^{ed}	0.212def
N-D	1812.600	112.400	8.663^{a}	0.519^{a}	8.033^{abc}	0.290^{ab}
W-E	1734.800	113.123	7.924^{b}	0.456^{b}	7.354^{e}	0.179^{f}
N-E	1763.200	112.884	8.723^{a}	0.482^{ab}	7.745^{bcd}	0.288^{ab}
W-F	1757.400	112.884	8.025^{b}	0.458^{b}	7.450^{ed}	0.232^{cde}
N-F	1836.600	112.442	8.705^{a}	0.490^{ab}	8.145^{a}	0.309^{a}
W-G	1708.200	112.842	7.966^{b}	0.466^{b}	7.440^{ed}	0.199^{ef}
N-G	1730.000	113.00	8.703^{a}	0.519^{a}	8.014^{abc}	0.300^{a}
SEM	46.209	0.266	0.091	0.010	0.113	0.015
P-value						
Humulus	0.5087	0.4242	<.0001	<.0001	<.0001	<.0001
Molting	0.2882	0.2652	0.9535	0.5399	0.0576	0.0434
Humulus ×Molting	0.9369	0.9564	0.2055	0.6217	0.7407	0.1807
Treat	0.7029	0.6835	<.0001	<.0001	<.0001	<.0001

$^{a-f}$ Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, EW: egg weight, FCR: feed conversion ratio, EP: egg production. A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls, G: basal diet; W-A and N-A molting by FW and fed with and without *Humulus* respectively, W-B and N-B: molting by PoP and fed with and without *Humulus* respectively, W-C and N-C: molting by sunflower seed hulls + PoP and fed with and without *Humulus* respectively, W-D and N-D: molting by WBMW and fed with and without *Humulus* respectively, W-E and N-E: molting by sunflower seed hulls +WBMW and fed with and without *Humulus* respectively, W-F and N-F: molting by sunflower seed hulls and fed with and without *Humulus* respectively and W-G and N-G: molting by the basal diet and fed with and without *Humulus*, respectively.

There was an increased in the bending stress and breaking strength in the birds fed by *Humulus* plant in the post-molting ($p < 0.05$); but no significant respond was found for bending stress by using the molting program (Table 9). During the post-molting period, there was no significant by *Humulus* plant and molting methods on maximum strain ($p > 0.05$). Inclusion of *Humulus* plant had no significant effect on ultimate force, but it was also observed to be highest in the treatments E, as compared with other treatments ($p < 0.05$). No significant differences in the elastic modulus were observed by using *Humulus* plant, in the post-molting period. However, there were significantly increased by treatment G in this respect ($p < 0.05$). Tibia calcium and phosphorous were increased by *Humulus* plant in the post-molting ($p < 0.05$) Table 10. Similar results were pointed out in plasma calcium and phosphorous by *Humulus* plant in laying hens which have shown the highest plasma calcium and phosphorous by the treatments W-E, W-A and W-G respectively ($p < 0.05$). The results have shown that no significant effect on the tibia calcium and phosphorous and plasma calcium by molting methods ($p > 0.05$), but plasma phosphorous was increased by treatment E, at the post-molting period. The results have shown that treatments had no significant effect on expression of TRPV6 mRNA in the jejunum and kidney during molting periods ($p > 0.05$) Tables 11 and 12. In addition no significant effect were observed between *Humulus* plant and molting methods by the expression of TRPV6 mRNA in the jejunum, kidney and ESG in the post-molting period ($p > 0.05$).

Table 9. The effect of molting programs on tibia biomechanical properties at the post-molting period.

Treat	BW (g)	Bending stress (N mm^{-2})	Maximum strain (mm)	Ultimate force (N)	Breaking strength (kg g^{-1})	Elastic modulus (N mm^{-2})
without *Humulus*	1754.34	91.80^{b}	2.65	136.06	4.54^{b}	12232.25
with *Humulus*	1770.77	99.14^{a}	2.66	133.64	5.05^{a}	12253.45
SEM	17.465	0.711	0.058	1.176	0.048	23.066
Molting						
A	1774.50	95.61	2.57	137.94^{ab}	4.56^{c}	12015.54^{c}
B	1744.70	92.99	2.56	133.26^{bc}	4.53^{c}	12078.27^{c}
C	1732.20	95.22	2.80	130.88^{c}	5.03^{a}	12262.10^{b}
D	1821.40	94.03	2.58	132.13^{bc}	4.90^{ab}	12308.58^{ab}
E	1749.00	96.15	2.54	140.61^{a}	4.89^{ab}	12295.04^{ab}
F	1797.00	97.66	2.70	133.17^{bc}	4.94^{ab}	12342.38^{ab}
G	1719.10	96.65	2.84	135.98^{abc}	4.69^{bc}	12398.02^{a}
SEM	32.675	1.330	0.109	2.200	0.089	43.153
Humulus ×Molting						
W-A	1761.20	89.77^{d}	2.35^{bc}	142.80^{a}	4.39^{e}	11998.19^{d}
N-A	1787.80	101.45^{a}	2.80^{ab}	133.08^{abc}	4.74^{cde}	12032.89^{cd}
W-B	1759.20	91.50^{cd}	2.52^{abc}	130.75^{c}	4.64^{de}	12061.28^{bcd}
N-B	1730.20	94.48^{bcd}	2.60^{abc}	135.76^{abc}	4.42^{e}	12095.27^{bcd}
W-C	1729.40	94.05^{bcd}	2.65^{abc}	130.77^{c}	4.57^{de}	12287.78^{a}
N-C	1735.00	96.38^{abc}	2.96^{a}	130.99^{c}	5.50^{a}	12236.42^{ab}
W-D	1830.20	89.91^{d}	2.91^{a}	133.12^{abc}	4.39^{e}	12298.39^{a}
N-D	1812.60	98.15^{ab}	2.25^{c}	131.13^{c}	5.41^{a}	12318.76^{a}
W-E	1734.80	91.43^{cd}	2.49^{abc}	141.73^{ab}	4.67^{de}	12219.95^{abc}
N-E	1763.20	100.87^{a}	2.60^{abc}	139.49^{abc}	5.11^{abc}	12370.14^{a}
W-F	1757.40	93.21^{bcd}	2.90^{a}	133.51^{abc}	4.71^{de}	12387.30^{a}
N-F	1836.60	102.12^{a}	2.49^{abc}	132.83^{abc}	5.17^{ab}	12297.46^{a}
W-G	1708.20	92.75^{bcd}	2.75^{abc}	139.73^{abc}	4.43^{e}	12372.85^{a}
N-G	1730.00	100.56^{a}	2.93^{a}	132.22^{bc}	4.96^{bcd}	12423.19^{a}
SEM	46.209	1.880	0.154	3.111	0.126	61.027
P-value						
Humulus	0.5087	<.0001	0.8782	0.1516	<.0001	0.5185
Molting	0.2882	0.226	0.2796	0.0327	0.0005	<.0001
Humulus ×Molting	0.9369	0.146	0.1163	0.2973	0.1235	0.5985
Treat	0.7029	<.0001	0.0239	0.0513	<.0001	<.0001

$^{a-f}$ Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, EW: egg weight, FCR: feed conversion ratio, EP: egg production. A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls, G: basal diet; W-A and N-A molting by FW and fed with and without *Humulus* respectively, W-B and N-B: molting by PoP and fed with and without *Humulus* respectively, W-C and N-C: molting by sunflower seed hulls + PoP and fed with and without *Humulus* respectively, W-D and N-D: molting by WBMW and fed with and without *Humulus* respectively, W-E and N-E: molting by sunflower seed hulls +WBMW and fed with and without *Humulus* respectively, W-F and N-F: molting by sunflower seed hulls and fed with and without *Humulus* respectively and W-G and N-G: molting by the basal diet and fed with and without *Humulus*, respectively.

Table 10. The effect of molting programs on tibia mineral content and plasma phosphorous and calcium at the post-molting period.

Treat	Tibia Ca/Tibia Weight (%)	Tibia P/Tibia Weight (%)	phosphorus (mg dL^{-1})	calcium (mg dL^{-1})
without *Humulus*	19.67^{b}	8.78^{b}	6.90^{b}	18.67^{b}
with *Humulus*	20.34^{a}	9.32^{a}	7.87^{a}	19.51^{a}
SEM	0.072	0.039	0.079	0.063
Molting				
A	19.96^{b}	8.90	7.06^{cd}	19.14
B	19.75^{b}	9.03	6.99^{d}	18.92
C	20.03^{ab}	9.04	7.30^{bcd}	18.95
D	20.04^{ab}	9.05	7.51^{abc}	19.00
E	20.39^{a}	9.09	7.81^{a}	19.26
F	19.74^{b}	9.07	7.64^{ab}	19.08
G	20.14^{ab}	9.19	7.39^{abcd}	19.29
SEM	0.136	0.073	0.148	0.117
Humulus ×Molting				
W-A	19.40^{f}	8.59^{e}	6.74^{ef}	18.61^{df}
N-A	20.52^{ab}	9.21^{abc}	7.37^{cde}	19.68^{a}
W-B	19.32^{f}	8.73^{de}	6.61^{f}	18.51^{ef}
N-B	20.17^{abcde}	9.33^{ab}	7.36^{cde}	19.33^{abc}
W-C	19.63^{ef}	8.87^{de}	6.53^{f}	18.31^{f}

N-C	20.43^{abc}	9.21^{abc}	8.07^{ab}	19.58^{ab}
W-D	19.79^{def}	8.62^{e}	7.18^{cdef}	18.47^{ef}
N-D	20.29^{abcd}	9.48^{a}	7.83^{abc}	19.52^{ab}
W-E	20.04^{bcde}	8.90^{ce}	7.49^{bcd}	19.11^{bcd}
N-E	20.73^{a}	9.28^{ab}	8.13^{ab}	19.41^{ab}
W-F	19.60^{ef}	8.75^{de}	7.05^{def}	18.82^{cdef}
N-F	19.88^{cdef}	9.40^{a}	8.24^{a}	19.33^{abc}
W-G	19.91^{bcdef}	9.02^{bcd}	6.67^{f}	18.88^{cde}
N-G	20.37^{abcd}	9.37^{a}	8.12^{ab}	19.71^{a}
SEM	0.192	0.104	0.210	0.166
P-value				
Humulus	<.0001	<.0001	<.0001	<.0001
Molting	0.0212	0.2357	0.0020	0.1646
Humulus ×Molting	0.4531	0.1322	0.1098	0.0771
Treat	<.0001	<.0001	<.0001	<.0001

[a-f] Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, EW: egg weight, FCR: feed conversion ratio, EP: egg production. A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls, G: basal diet; W-A and N-A molting by FW and fed with and without *Humulus* respectively, W-B and N-B: molting by PoP and fed with and without *Humulus* respectively, W-C and N-C: molting by sunflower seed hulls + PoP and fed with and without *Humulus* respectively, W-D and N-D: molting by WBMW and fed with and without *Humulus* respectively, W-E and N-E: molting by sunflower seed hulls +WBMW and fed with and without *Humulus* respectively, W-F and N-F: molting by sunflower seed hulls and fed with and without *Humulus* respectively and W-G and N-G: molting by the basal diet and fed with and without *Humulus*, respectively.

Table 11. The effect of treatments on the expression of TRPV6 mRNA in the jejunum and kidney at the molting period.

Treat	kidney	Jejunum
A	0.318	0.430
B	0.366	0.431
C	0.290	0.486
D	0.346	0.450
E	0.315	0.448
F	0.381	0.460
G	0.305	0.381
SEM	0.0351	0.0230
P-value	0.4883	0.0942

[a-c] Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls and G: basal diet.

Table 12. The effect of molting programs on the expression of TRPV6 mRNA in the jejunum, kidney and egg shell gland at the post-molting period.

Treat	kidney	Jejunum	Egg shell gland
without *Humulus*	0.668	0.988	3.952
with *Humulus*	0.666	0.996	4.062
SEM	0.0173	0.0203	0.1797
Molting			
A	0.685	1.049	3.899
B	0.648	0.945	3.905
C	0.674	0.946	4.042
D	0.633	0.957	4.107
E	0.670	1.065	4.228
F	0.691	1.034	3.764
G	0.671	0.949	4.108
SEM	0.0321	0.0382	0.3365
Humulus ×Molting			
W-A	0.678	1.086	3.546
N-A	0.692	1.012	4.252
W-B	0.608	0.902	3.812
N-B	0.688	0.988	3.998
W-C	0.682	0.958	3.678
N-C	0.666	0.934	4.406
W-D	0.644	0.914	4.020
N-D	0.622	1.001	4.194
W-E	0.716	1.106	4.512
N-E	0.624	1.024	3.944
W-F	0.680	1.046	3.826

N-F	0.702	1.022	3.702
W-G	0.672	0.906	4.272
N-G	0.670	0.992	3.942
SEM	0.0458	0.0538	0.4754
P-value			
Humulus	0.9260	0.7893	0.6659
Molting	0.8788	0.0745	0.9677
Humulus ×Molting	0.6935	0.4265	0.7782
Treat	0.9263	0.1807	0.3754

[a-f] Values with different superscripts in the same row are different ($p \leq 0.05$). FI: feed intake, EW: egg weight, FCR: feed conversion ratio, EP: egg production. A: feed withdrawal (FW), B: basal diet + 2% *Portulaca Oleracea* powder (PoP), C: basal diet + 30% sunflower seed hulls + 2% *Portulaca Oleracea* powder, D: basal diet + 2% white button mushroom waste (WBMW), E: basal diet + 30% sunflower seed hulls + 2% white button mushroom waste, F: basal diet + 30% sunflower seed hulls, G: basal diet; W-A and N-A molting by FW and fed with and without *Humulus* respectively, W-B and N-B: molting by PoP and fed with and without *Humulus* respectively, W-C and N-C: molting by sunflower seed hulls + PoP and fed with and without *Humulus* respectively, W-D and N-D: molting by WBMW and fed with and without *Humulus* respectively, W-E and N-E: molting by sunflower seed hulls +WBMW and fed with and without *Humulus* respectively, W-F and N-F: molting by sunflower seed hulls and fed with and without *Humulus* respectively and W-G and N-G: molting by the basal diet and fed with and without *Humulus*, respectively.

Discussion

At least until now, there is no much study regarding the role of medicinal plants on induced molting. The study has shown that treatments had no influence on tibia length at the molting period. These findings have supported by previous reports that indicated no significantly effect on tibia length by *Portulaca oleracea* powder (0, 1 and 2 percentage) (Jamali et al., 2015). This study has demonstrated considerable positive effects of *Portulaca oleracea* powder on tibia weight and bone cortical thickness. *Portulaca oleracea* is a source of many biologically active compounds, such as β-catotene, alpha-tocopherol, ascorbic acid, polyunsaturated fatty acids (PUFA) and high level of calcium (2%) (Zhang et al., 2007). Tibia ash and density were increased by the treatment G which is agree with other reported by (Kim et al., 2007; 2008). Previous research has shown that feed withdrawal to induce a molting adversely effects on bone mineralization and biochemical properties of bone during molting (Mazzuco & Hester, 2005). Yosefi et al. (2003) also have observed that the tibial ash of fasted hens decreased during an induced molting (feed withdrawal for 8 or 11 day at 67 weeks of age). The results of the current study also have shown reduced bending stress and Breaking strength bone during a fasted molting. However, a non-fasted molt regimen was less deleterious to bone mineralization as compared to a fasted molt. This study describes the tibia biomechanical properties (Maximum strain, Ultimate force and Elastic modulus) were not affected by treatment during molting period. This was consistent with the results obtained by Kwiecieñ and Winiarska-Mieczan (2009), who have observed that increased tibia weight, ash and density by *Humulus* plant in broiler chickens. In more recently, by investigated Effenberger et al. (2005) have shown by the effects of several *Humulus* -derived compounds on cultured osteoblast-like cells and demonstrated that these specific phytoestrogens compounds exerted estrogen-like activities displayed positive effects on bone metabolism.

Recently, the beneficial effects of 8-prenylnaringenin (a prenylflavonoid present in *Humulus*) as a herbal alternative for preserve bone density; also bone biomechanical properties were significantly improved by treatment 8-prenylnaringenin (Sehmisch et al., 2008). The 8-prenylnaringenin as phytoestrogens significantly improves osteoporotic bone quality and mineral density due to have a direct interaction with the estrogen receptors ERα which in turn regulate bone development and maintenance of bone mineral density (Christoffel et al., 2006). It has crucial effects on both trabecular and cortical bone by stimulating target gene transcription through two activation functions (Börjesson et al., 2013). Calcium serum levels is important in molting induction and requirements for eggshell formation along with dietary calcium intake and mobilization from the medullary bone (Landers et al., 2008). It has been demonstrated that mineral content is positively correlated with bone strength, also ash weight was highly correlated with dietary Ca and P levels. This indicating that ash weight could be a reliable predictor of bone mechanical properties (Kim et al., 2007). It has been demonstrated that elastic modulus is positively correlated with calcium content and overall mineral content (Rath et al., 1999). Improving bone mechanical properties can be beneficial to maintain overall bone strength, because bone strength is highly correlated with its structural and material properties (Wilson & Thorp, 1998; Wilson & Ruszler, 1998). Next more study have reported by Sahin et al. (2007), the usage of isoflavones and phytoestrogen as supplements will obviously improve mineral density in bones of Japanese quail. Bone mechanical properties and density of laying hens at the post peak period could be improve by the phytoestrogen (Saberifar et al., 2020). The improvement in tibia mineral content and plasma

phosphorous and calcium at the molting period was probably due to increases in absorption Ca which was increased by supplemental flavonoids in *Portulaca oleracea*. This result is in agreement with the results of previous studies Zhao et al. (2005) have reported that flavonoids supplementation improved eggshell quality and bone mass in laying hens.

The increase of bone cortical thickness, tibia ash and density during molting period by white button mushroom waste supplementation could be due to the increase of ergosterol as pre-cursor of vitamin D2 (Cardwell et al., 2018). It is well known that mushrooms could be used as a supplement of vitamin D2 for insufficiency in this aspect (Jasinghe & Perera, 2005, 2006). It was shown that vitamin D2 from irradiated mushrooms was well absorbed in animals, and has an important positive effect on the bone mineralization process in animal systems (Jasinghe et al., 2006). The Current study also has shown that *Humulus* plant increased tibia calcium and phosphorus content at post-molting period, which was in agreement with Saberifar et al. (2020) who have reported dietary phytoestrogen increased calcium and phosphorus content of tibia laying hens during post peak period. No detectable effects were observed on the expression of gene TRPV6 mRNA in the jejunum and kidney at the molting period. These results have confirmed our previous results with 21-month-old laying hens which suggested that calcium transport gene expression in the laying bird is not age-dependent, although the aged laying hen (19 months old) loses its capability to adapt to changes in Ca demands (Yosefi et al., 2003). There is, however, little information related to the expression patterns of TRPV6 gene in the laying hen during molting and post-molting periods. The major source of calcium is from intestinal dietary Ca absorption, renal Ca reabsorption and skeletal stores (Bar, 2008). The ion channel TRPV6 is likely to function as an epithelial calcium channel in different tissues, such as intestine, kidney, bone and uterus, all of which are characterized by high Ca transport requirements (Hoenderop et al., 2005). There is a significant correlation between TRPV6 gene expression and Ca transport that it is an important rate-limiting entry step in maintaining Ca homeostasis (Bianco et al., 2007). The molting and any other factor that arrests egg markedly reduce the intestinal and ESG TRPV6 gene contents (Bar, 2009). Non-laying or molting birds had a lower calcium transport, but ratios of phosphorus and calcium increased during the post-molting period (Nakada, 1990). Changes of proteins responsible in calcium-regulating, such as transient receptor potential vanilloid channel type 6 (TRPV6), are thought to happen due to the alterations of estrogen levels (Li et al., 2018). The estrogen synthesis is characterized decreased with molting and increasing at post-molting, resulting a change in egg production (Saberifar et al., 2020).

Conclusion

In overall, the results of this study have noted that dramatic improved tibia characteristics and biomechanical properties in the molting and post-molting period by *Portulaca oleracea* powder and white button mushroom waste. Moreover, bone status and tibia mineral content improved by *Humulus* plant in the post-molting period. Finally, the present study has shown that *Humulus* plant and molting program not influence on the expression of gene TRPV6 mRNA in the jejunum, kidney and ESG at the molting and post-molting period, but it was increased as compared with the pre molting period.

Data availability

The data is included in the body of the article. More information will be made available on request from the corresponding author.

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