

# Response of tibia bone morphology, biomechanics and minerals to organic hawthorn (*Crataegus tanacetifolia*) fruit vinegar application in broiler chickens exposed to cyclic heat stress

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**ABSTRACT.** This study aimed to determine the effects of adding different doses of organic hawthorn fruit vinegar (HFV) to the drinking water of male broilers (Ross 308) reared under cyclic heat stress (CHS) on tibia bone morphology, biomechanics, and minerals. Broilers were distributed into six groups based on a 2 × 3 factorial design, testing two ambient temperatures (24 and 35°C CHS for 8 h/day starting from day 21) and three HFV levels (0, 2, and 4 ml/L). Cyclic heat stress had no effect on the morphological and biomechanical properties of the tibia bone, except for a decrease in cortex thickness ( $P < 0.05$ ). However, CHS significantly decreased the bone Ca, P, Mg, Cu, Zn, Mn, and K levels, while increasing the Fe levels ( $P < 0.01$ ). The addition of 2 and 4 ml/L HFV had no effect on the morphological and biomechanical properties of the tibia bone ( $P > 0.05$ ), except for an increase in bone diameter with 2 ml/L HFV ( $P < 0.05$ ). HFV additions were not sufficient to increase the Ca level decreased by CHS, and the tibia bone Ca level of birds under CHS decreased further with HFV additions ( $P < 0.01$ ). Conversely, 2 ml/L HFV increased Mg levels, and both 2 and 4 ml/L HFV increased Cu, Fe, Zn, and Mn levels, whereas P levels decreased ( $P < 0.01$ ). K level decreased with 2 ml/L HFV and increased with 4 ml/L HFV supplementation. In conclusion, HFV supplementation has positive effects on bone mineralization in broilers exposed to HS and can be used up to 4 ml/L to mitigate the negative effects of heat stress.

**Keywords:** broilers, hawthorn fruit vinegar, cyclic heat stress, tibia bone

## INTRODUCTION

Heat stress causes various behavioral, physiological, and neuroendocrine changes that threaten the poultry industry worldwide and negatively affect the health and performance of poultry (Bayraktar *et al.*, 2021; Kaya, 2023; Bayraktar *et al.*, 2023). Broiler farming causes skeletal diseases in chickens, especially in the leg bones, owing to rapid weight gain and delayed skeletal development (Santos *et al.*, 2022). Prolonged stress disrupts and triggers cortisol rhythm, causing many metabolic problems and diseases (Bayraktar & Bayraktar, 2019). Heat stress also causes bone loss in broilers (Hosseini-Vashan *et al.*, 2016) and laying hens (Koelkebeck *et al.*, 1993) as a result of decreased calcium consumption and absorption. Maintaining and improving leg health in poultry is an important prerequisite for optimum production performance and welfare of broiler chickens (Tekce *et al.*, 2020). Therefore, it is imperative to prevent leg diseases and improve the health of broiler legs (Dixon, 2020).

Vinegar is an important element that can be effective against the negative effects caused by HS in broiler chickens, which are more sensitive to leg problems compared to other poultry species. Hawthorn vinegar is a type of vinegar obtained by fermenting hawthorn fruit using various methods and turning it into vinegar. Hawthorn vinegar contains bioactive substances such as gallic acid,

catechin, epicatechin, chlorogenic acid, caffeic acid, coumaric acid, and ferulic acid (Budak *et al.*, 2014), and has antioxidant, antimicrobial, hypolipidemic, and intestinal Ca absorption-enhancing effects (Kishi *et al.*, 1999; Chou *et al.*, 2015). This study aimed to investigate the effects of different doses (2 and 4 ml/L water) of organic HFV added to the drinking water of broiler chickens subjected to CHS on tibia bone morphology, biomechanics, and tibia minerals.

## MATERIALS AND METHODS

### Animals and experimental design

A total of 300 one-day-old male commercial broiler chicks (Ross 308) were utilized in this 42-day study. After a 7-day acclimation period, the chicks were randomly placed in two separate poultry coops according to a 2 × 3 experimental design. This design included two groups based on ambient temperature (thermoneutral (TN) at 24°C and heat stress (HS) at 35°C) and three groups based on HFV levels (0, 2 and 4 ml/L water) added to the drinking water. The study was conducted with five replicates, each containing 10 broilers. Broilers were reared in two poultry coops with similar characteristics, featuring the same number and position of hanging feeders and drink-

ers, automatic heaters, wood shavings litter, and 121 × 110 × 108 cm floor compartments.

### Feed

HFV, which was used as an additive in this study, was obtained from a commercial company and added to the drinking water of the chicks. No additives were added to the feed. Broiler starter feed was used between days 0-10, grower feed between days 11-24, and finisher feed between days 25-42. The amount of vinegar that broiler chicks reared in TN conditions would consume with drinking water each day (for 42 days) was calculated and added to their drinking water, except for the control groups. Consequently, the same amount of HFV was consumed by the chicks under both TN and HS conditions. For this purpose, after the animals were deprived of water for 1 h, 100 ml of water with the calculated amount of HFV was given to the treatment groups, and 100 ml of water without additives was given to the control groups. After the consumption of the given water, the experiment was continued with fresh water. Throughout the experiment, feed and drinking water without additives were consumed *ad libitum*.

### Birds heat, lighting and humidity

The temperature of the experimental coops was initially maintained at 33°C for the first 7 days, then gradually reduced to 24°C until day 21. For the thermoneutral (TN) environmental conditions, the coop temperature was maintained at approximately 24 °C for 24 h between days 21 and 42, and the relative humidity was maintained between 50-60%. For the heat stress (HS) applied coop, the temperature was raised to 35°C for 8 h daily between

9:00 am and 5:00 pm, starting from day 21 (Rocchi *et al.*, 2022; Zhang *et al.*, 2021). For the remaining 16 h (5:00 pm to 9:00 am), the temperature in the HS-applied coop was maintained at approximately 24°C. The temperature of the HS-applied coop was increased to approximately 35°C for 8 h between 9:00 am and 5:00 pm starting from day 21, and was kept at approximately 24°C for 16 h between 5:00 pm and 9:00 am (Zhang *et al.*, 2021). The relative humidity of this coop varied between 60% and 70% from day 21 to the end of the study. The temperature and humidity were monitored at four points in each room using a temperature-humidity recording system. A 24-hour fluorescent lighting program was applied with an average light intensity of 40 lux/m<sup>2</sup> during the study.

### Analytical Procedures

**Analyzes of hawthorn fruit vinegar.** The total phenolic compound quantities were determined using a Folin-Ciocalteu reagent and the method described by Singleton *et al.* (1999), with slight modifications (Gülçin *et al.*, 2002). The total phenolic content of HFV was determined to be 1551.29 mg GAE/L. The 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity was determined using the method described by Köksal *et al.* (2009). The ABTS scavenging activity of HFV was 11.075 (IC<sub>50</sub>, µg/ml). The phenolic component and organic acid profiles of HFV were determined using high-performance liquid chromatography (HPLC) (Coklar & Akbulut, 2017). The phenolic compound and organic acid profiles are presented in Table 1.

**Table 1.**

Phenolic compound and organic acid profiles of hawthorn fruit vinegar (Kaya, 2023).

Phenolics			Organic acids		
Parameters	Value		Parameters	Value	
	mg/L	%		mg/L	%
Gallic acid	104.12	10.6	Formic acid	1873.18	11.8
Catechin	18.24	1.9	Lactic acid	1314.94	8.2
Gentisic acid	308.44	31.6	Acetic acid	10776.21	67.7
Rutin	123.93	12.6	Fumaric acid	214.83	1.3
Ferulic acid	78.38	8.0	Succinic acid	1745.22	11.0
Naringin	24.57	2.5	Total	15924.38	100
Neohesperidin	83.71	8.6			
Coumarin	25.60	2.6			
Resveratrol	36.14	3.7			
Quercetin	54.77	5.6			
t-Cinnamic acid	82.76	8.5			
Hesperidin	29.77	3.1			
Flavone	6.85	0.7			
Total	977.28	100			

**Determination of Tibia Bone Morphological and Biomechanical Properties.** On the 42nd day of the study, 60 fasted birds (10 from each group, two from each replicate) were selected and slaughtered by cervical dislocation. Their right tibia bones were collected. One right tibia from each replicate was used to determine the bone mineral concentration, while the other was used to measure bone morphology and mechanical properties. The tibia bones were cleaned of meat and fat, bagged, and stored in a deep freezer at  $-82^{\circ}\text{C}$  until analysis. Prior to measurements, bone samples were thawed for 6 h at room temperature in an air-conditioned room. All flesh and proximal cartilage were removed from the bones.

The tibia length, weight, and volume parameters were determined before the bones were fractured. Bone length and the distance between the distal and proximal ends were measured using a digital caliper with an accuracy of 0.001 mm. Bone weight was measured using a digital balance with a sensitivity of 0.001 g, and the bone volume was measured using a beaker. The Robusticity Index (RI) was calculated according to Reisenfeld (1972), and the Seedor Index (SI) was calculated according to Seedor *et al.* (1991). The bone diameter and cortex thickness were measured using digital calipers from two points on the central axis of the fractured tibia to determine the mechanical properties.

Bone mechanical properties were determined using a BESMAK testing device (model BMT-100S; BESMAK, Ankara, Türkiye) and Test Works 4 software package (version Litest X; BESMAK, Ankara, Türkiye). This was performed via a three-point bending test (ASAE Standard S459, 2001) using a load-deflection curve. The crosshead speed was 5 mm/min. A double shear block apparatus was used to perform shear tests on the tibia. A 15 mm (0.59 inch) section in the middle of the diaphysis was subjected to shear force. The fracture energy of the bones was calculated using these data along with the shear force-deformation diagram data (Wilson & Ruzsler, 1996). These tests allowed for the evaluation of the ultimate shear force and shear stress for each bone. These biomechanical traits of bones were defined by Wilson and Ruzsler (1996). The fractured bones, whose biomechanical properties were determined, were dried in an oven at  $105^{\circ}\text{C}$  for 24 h and then weighed on a 0.001 g digital balance to determine the tibia dry weight. The dry matter content of tibia bone was determined using the standard calculation method based on the dry to wet weight ratio. The bones were burned in a muffle furnace at  $600^{\circ}\text{C}$  for 4.5 h, and the ash weight was determined by weighing on a digital scale with a sensitivity of 0.001 g (Sultan *et al.*, 2018).

**Determination of tibia mineral content.** The tibia bones were thawed one day before the analysis. Flesh, fat, and marrow were removed, and the bones were then dried at  $100^{\circ}\text{C}$  for 24 h and ground to a size that would pass through a 0.5 mm sieve. Seven ml of nitric acid ( $\text{HNO}_3$ ) and 1 ml of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) were added to 0.5 g

of tibia bone sample and calcined in two different stages (first stage; 15 min at  $200^{\circ}\text{C}$ , TFM120 W/jar, second stage; 15 min at  $200^{\circ}\text{C}$ , TFM120 W/jar) in a microwave wet calciner (Milestone, START D, Italy) withstanding 45 bar pressure. The bone mineral content of the tibia was then determined using an atomic emission spectrophotometer (ICP MS, Agilent Technologies 7700 series, Japan) in samples made up to 25 ml with distilled water (Mertens, 2005).

**Statistical analysis.** SPSS 23.0 was used to analyze the data obtained from the experiments using the  $2 \times 3$  factorial statistical analysis model (general linear model). Duncan's multiple comparison test was used to compare the effects of the additive doses, and the independent samples t-test was used to compare the effects of heat stress.

## RESULTS

The effects of different levels of Hawthorn Fruit Vinegar (HFV) supplementation in the drinking water of Cyclic Heat Stress (CHS)-treated broilers are presented in Table 2 for tibia dry weight (DW), dry matter (DM), tibia ash weight (AW), and tibia ash percentage (AP). The morphological characteristics are detailed in Table 3, and the biomechanical characteristics are presented in Table 4. Overall, neither CHS nor HFV supplementation significantly affected the tibia bone weight, ash values, or most morphological and biomechanical properties ( $P > 0.05$ ). The only exceptions were that 2 ml/L HFV supplementation improved tibia bone diameter, and CHS reduced tibia bone cortex thickness ( $P < 0.05$ ). Furthermore, no significant interaction between temperature (T) and HFV was observed for these parameters ( $P > 0.05$ ).

Table 5 presents the effects of different HFV levels on the tibia bone mineral content of broilers exposed to CHS. CHS exposure significantly decreased tibia bone levels of Ca, P, Mg, Cu, Zn, Mn, and K ( $P < 0.01$ ) while increasing Fe levels ( $P < 0.01$ ). With the addition of 2 and 4 ml/L HFV to the drinking water, tibia bone Ca and P levels decreased ( $P < 0.01$ ), but Cu, Fe, Zn, and Mn levels increased ( $P < 0.01$ ). Tibia bone K levels showed a mixed response, decreasing with 2 ml/L HFV supplementation and increasing with 4 ml/L HFV ( $P < 0.01$ ). Conversely, Mg levels increased specifically with 2 ml/L HFV ( $P < 0.01$ ). No significant interaction between T and HFV was observed for bone P content ( $P > 0.05$ ). However, a significant interaction between T and HFV was observed for bone Ca, Mg, Cu, Fe, Zn, Mn, and K parameters ( $P < 0.01$ ).

## DISCUSSION

### Morphological properties of the tibia bone

Except for the fact that 2 ml/L HFV added to the drinking water of stressed chickens improved tibia bone diameter ( $P < 0.05$ ), stress and HFV supplementation had no effect on tibia bone weight, ash content (Table 2), and morpho-

**Table 2.**

Effect of hawthorn (*Crataegus tanacetifolia*) fruit vinegar supplementation in drinking water on tibia bone weight and ash of broiler reared under cyclic heat stress

		HFV (ml/L water)	DW (g)	DM (%)	AW (g)	AP (%)
T	TN	0	8.33	49.1	3.34	40.3
		2	8.20	49.5	3.21	39.0
		4	8.02	48.5	3.27	40.6
	HS	0	7.49	48.4	2.96	39.5
		2	8.21	48.7	3.24	39.4
		4	7.66	51.0	3.00	39.2
SEM			0.15	0.35	0.08	0.4
T	TN		8.18	49.0	3.28	40.0
	HS		7.78	49.4	3.07	39.3
HFV	0		7.91	48.8	3.15	39.9
	2		8.20	49.1	3.23	39.2
	4		7.84	50.0	3.14	39.9
<i>P</i> -values						
T			0.21	0.61	0.20	0.50
HFV			0.60	0.51	0.89	0.76
T*HFV			0.55	0.10	0.55	0.73

HFV, hawthorn fruit vinegar; T, temperature; TN, thermo-neutral; HS, heat stress; SEM, standard error of the mean; DW, dry weight; DM, dry matter; AW, ash weight; AP, ash percentages

**Table 3.**

Effect of hawthorn (*Crataegus tanacetifolia*) fruit vinegar supplementation in drinking water on the morphological properties of the tibia bone of broiler reared under cyclic heat stress

		HFV (ml/L water)	Length (mm)	Wet weight (g)	Volume (cm <sup>3</sup> )	RI (cm/g)	SI (g/cm)	Diameter (mm)
T	TN	0	103.2	16.97	13.06	4.02	1.65	9.44 <sup>ab</sup>
		2	103.1	16.59	12.76	4.05	1.61	9.63 <sup>ab</sup>
		4	105.1	16.59	12.57	4.13	1.57	9.24 <sup>ab</sup>
	HS	0	102.2	15.52	12.30	4.10	1.52	9.03 <sup>b</sup>
		2	102.6	16.82	13.52	4.01	1.64	9.79 <sup>a</sup>
		4	102.4	14.97	12.54	4.16	1.46	9.49 <sup>ab</sup>
SEM			0.6	0.33	0.24	0.02	0.03	0.09
T	TN		103.8	16.71	12.80	4.07	1.61	9.44
	HS		102.4	15.77	12.79	4.09	1.54	9.43
HFV	0		102.7	16.24	12.68	4.06	1.58	9.23 <sup>b</sup>
	2		102.8	16.71	13.14	4.03	1.62	9.71 <sup>a</sup>
	4		103.8	15.78	12.55	4.14	1.52	9.37 <sup>ab</sup>
T			0.27	0.16	0.99	0.61	0.20	0.98
HFV			0.75	0.52	0.60	0.14	0.30	0.05
T*HFV			0.76	0.45	0.46	0.53	0.44	0.20

HFV, hawthorn fruit vinegar; T, temperature; TN, thermo-neutral; HS, heat stress; SEM, standard error of the mean; RI, Robusticity index; SI, Seedor index.

<sup>a-b</sup>, Values within a column with different superscripts differ significantly ( $P < 0.05$ )

logical characteristics (Table 3) ( $P > 0.05$ ). Similar to the results obtained in the present experiment, previous studies on tibiae of 42-day-old broilers showed that increasing environmental rearing temperature had no effect on bone length, weight and RI parameters compared to the group reared at neutral thermal temperature (Zhang *et al.*, 2021). On the other hand, it has been reported that bone ash decreases with increasing environmental temperature during the growth of broilers (Zhang *et al.*, 2021; Rocchi *et al.*, 2022). However, Jankowski *et al.* (2015) reported that the stress caused by high housing density and high temperature in turkeys increased the weight, length, and volume of the tibia bone on day 28, while these parameters decreased on day 126. Consistent with the present study, their findings indicated that stress did not affect the tibia ash ratio at any time period.

As demonstrated in Table 4, the addition of 2 ml/L and 4 ml/L HFV to the drinking water of broilers raised under CHS conditions did not result in any significant alterations ( $P > 0.05$ ) in the biomechanical properties of the tibia bone. The results of the present study conducted under the CHS are consistent with those of studies examining the effects of acidic feed additives on the bone biomechanical properties of animals reared under TN environmental conditions. Similar to the present study, it has been reported that the addition of organic acid mixture to broiler diets does not affect tibia bone shear force (Smulikowska *et al.*, 2010), bone cortex thickness, shear force, and bone stress parameters (Świątkiewicz & Arczewska-Włosek, 2012); the addition of fermentation product (Han *et al.*, 2015) and citric acid (Soliman & Al-Youssef, 2020) does not affect tibia bone shear force; and the addition of organic acid mixture to laying hen diets does not affect tibia bone cortex thickness, shear force and bone stress parameters (Świątkiewicz *et al.*, 2010). However, the reports that different acidifier additions to broiler diets do not affect tibia bone diameter (Akyurek *et al.*, 2011; Abdelaziz, 2015; Han *et al.*, 2015; Soliman and Al-Youssef, 2020) and that bamboo vinegar addition increases tibia bone shear stress (Fu *et al.*, 2013) differ from the results of the current study. Moreover, according to Koelkebeck *et al.* (1993), the shear force of the tibia bone increases with the addition of carbonate to the drinking water of old laying hens reared under CHS conditions. Abdelaziz (2015), who reported that the addition of formic acid to broiler diets reduced tibial shear force, emphasized that bone stability and fracture strength are generally determined by the degree of mineralization of the bone matrix. Low intestinal pH caused by short-chain fatty acids, such as formic, propionic, and acetic acids, added to broiler diets may improve bone mineralization and quality by increasing the solubility and absorption of Ca and P in the small intestine (Hossain & Nargis, 2016). Mineral metabolism disorders in broilers that are encouraged to grow rapidly in intensive rearing systems generally lead to bone abnormalities and lameness, which have negative consequences on both the

economic profitability of production and animal welfare (Świątkiewicz & Arczewska-Włosek, 2012).

### Tibia Bone Mineral Contents

The feed intake, water consumption, and growth performance data for the broilers in this study were presented in detail in a previously published article by Kaya (2023) within the scope of the same experiment. Briefly, that study reported that cyclic heat stress negatively affected growth performance ( $P < 0.05$ ), but HFV supplementation in drinking water had no significant effect on these parameters ( $P > 0.05$ ). Mineral balance is one of the important problems in poultry nutrition. This study determined that CHS significantly decreased tibia bone levels of Ca, P, Mg, Cu, Zn, Mn, and K, while increasing Fe levels ( $P < 0.01$ ) (Table 5). High ambient temperature increases serum corticosterone levels (Kaya, 2023) and negatively affects bone mass (Yan *et al.*, 2019). In addition, oxidative stress caused by high environmental temperature contributes to skeletal damage. In broilers exposed to HS, the decrease in the amount and absorption of Ca taken into the body due to decreased feed consumption causes bone loss (Hosseini-Vashan *et al.*, 2016).

Despite the dearth of research on this subject, studies on the effects of HS on bone minerals have been found to align with the findings of this study. Hosseini-Vashan *et al.* (2016) discovered that the bone Ca and P ratios of broilers exposed to HS, and Yan *et al.* (2019) ascertained that bone mineral content and density of broilers exposed to CHS decreased. In addition to these results, respiratory alkalosis may be a potential physiological mechanism by which CHS negatively impacts bone mineral content. Increased respiratory rate (hyperventilation) is a common adaptation in birds exposed to high temperatures to dissipate heat. This may lead to excessive loss of carbon dioxide (CO<sub>2</sub>) from the blood, leading to a shift in the acid-base balance toward alkalosis. This imbalance may reduce the bioavailability of ionized calcium, which in turn may impair bone mineralization. However, parameters directly indicative of acid-base imbalance, such as blood pH, bicarbonate, or ionized calcium levels, were not measured in the current study. Therefore, the assumption that respiratory alkalosis mediates the mineral losses observed in this trial remains a hypothetical explanation at this time. Future research should evaluate these parameters to better understand this physiological mechanism.

Our experimental results, which show that all levels of HFV supplementation decreased tibia bone Ca and P levels, present a paradoxical finding that warrants further discussion. While organic acids are generally known to enhance mineral absorption, our results suggest that other mechanisms may be involved. It is hypothesized that HFV, particularly at higher concentrations, may contain certain biocompounds that could interfere with calcium and phosphorus homeostasis. Alternatively, an excess of certain minerals, such as potassium, which is often found

**Table 4.**

Effect of hawthorn (*Crataegus tanacetifolia*) fruit vinegar supplementation in drinking water on the biomechanical properties of the tibia bone of broiler reared under cyclic heat stress

		HFV (ml/L water)	Cortex thickness (mm)	Cortex cross-section area (mm <sup>2</sup> )	Shear force (N)	Shear stress (N/mm <sup>2</sup> )	Fracture energy (N.mm)
T	TN	0	1.672 <sup>a</sup>	40.77 <sup>a</sup>	1493	37.07	2404
		2	1.514 <sup>ab</sup>	38.72 <sup>ab</sup>	939	24.47	1013
		4	1.550 <sup>ab</sup>	37.51 <sup>ab</sup>	1006	25.94	1686
	HS	0	1.378 <sup>b</sup>	32.96 <sup>b</sup>	1079	33.39	1480
		2	1.562 <sup>ab</sup>	40.37 <sup>a</sup>	1778	42.94	2605
		4	1.384 <sup>b</sup>	35.14 <sup>ab</sup>	1542	42.85	1667
SEM			0.034	0.92	130	2.93	225
T	TN		1.579	39.00	1146	29.15	1701
	HS		1.441	36.16	1466	39.73	1917
HFV		0	1.525	36.86	1286	35.23	1942
		2	1.538	39.55	1359	33.71	1809
		4	1.467	36.33	1275	34.39	1676
T			0.04	0.10	0.22	0.08	0.63
HFV			0.63	0.26	0.96	0.97	0.89
T*HFV			0.10	0.09	0.13	0.23	0.08

HFV, hawthorn fruit vinegar; T, temperature; TN, thermo-neutral; HS, heat stress; SEM, standard error of the mean  
<sup>a-b</sup>, Values within a column with different superscripts differ significantly ( $P < 0.05$ )

**Table 5.**

Effect of hawthorn (*Crataegus tanacetifolia*) fruit vinegar supplementation in drinking water on tibia bone mineral elements of broiler reared under cyclic heat stress

		HFV (ml/L water)	Ca (ppm)	P (ppm)	Mg (ppm)	Cu (ppm)	Fe (ppm)	Zn (ppm)	Mn (ppm)	K (ppm)
T	TN	0	189780 <sup>a</sup>	72218 <sup>a</sup>	3886 <sup>b</sup>	12.92 <sup>d</sup>	83 <sup>e</sup>	66 <sup>e</sup>	2.11 <sup>c</sup>	3511 <sup>b</sup>
		2	155125 <sup>c</sup>	68192 <sup>ab</sup>	4037 <sup>a</sup>	29.67 <sup>a</sup>	64 <sup>f</sup>	173 <sup>a</sup>	3.63 <sup>b</sup>	2716 <sup>d</sup>
		4	159891 <sup>b</sup>	65544 <sup>ab</sup>	3618 <sup>cd</sup>	27.74 <sup>b</sup>	102 <sup>d</sup>	172 <sup>a</sup>	5.64 <sup>a</sup>	3805 <sup>a</sup>
	HS	0	134374 <sup>f</sup>	64965 <sup>b</sup>	3388 <sup>e</sup>	11.15 <sup>e</sup>	118 <sup>c</sup>	104 <sup>d</sup>	1.77 <sup>c</sup>	2647 <sup>d</sup>
		2	139444 <sup>e</sup>	52646 <sup>c</sup>	3507 <sup>de</sup>	16.04 <sup>c</sup>	155 <sup>a</sup>	117 <sup>c</sup>	3.46 <sup>a</sup>	2874 <sup>c</sup>
		4	149170 <sup>d</sup>	51432 <sup>c</sup>	3704 <sup>c</sup>	15.79 <sup>c</sup>	127 <sup>b</sup>	129 <sup>b</sup>	1.95 <sup>c</sup>	2522 <sup>e</sup>
SEM			3048	1566	41	1.23	5	6	0.24	81
T	TN		168265	68651	3847	23.4	83	137	3.79	3344
	HS		140996	56348	3533	14.3	133	116	2.39	2681
HFV		0	162077 <sup>a</sup>	68591 <sup>a</sup>	3637 <sup>b</sup>	12.0 <sup>c</sup>	101 <sup>c</sup>	85 <sup>b</sup>	1.94 <sup>b</sup>	3079 <sup>b</sup>
		2	147285 <sup>c</sup>	60419 <sup>b</sup>	3772 <sup>a</sup>	22.9 <sup>a</sup>	109 <sup>b</sup>	145 <sup>a</sup>	3.54 <sup>a</sup>	2795 <sup>c</sup>
		4	154530 <sup>b</sup>	58488 <sup>b</sup>	3661 <sup>b</sup>	21.8 <sup>b</sup>	114 <sup>a</sup>	150 <sup>a</sup>	3.79 <sup>a</sup>	3163 <sup>a</sup>
<i>P</i> - values										
T			0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
HFV			0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
T*HFV			0.01	0.16	0.01	0.01	0.01	0.01	0.01	0.01

HFV, hawthorn fruit vinegar; T, temperature; TN, thermo-neutral; HS, heat stress; SEM, standard error of the mean; Ca, Calcium; P, Phosphorus; Mg, magnesium; Cu, Copper; Fe, Iron; Zn, Zinc; Mn, Manganese; K, Potassium.  
<sup>a-f</sup>, Values within a column with different superscripts differ significantly ( $P < 0.05$ ).

in vinegars, could lead to mineral-mineral competition, negatively affecting the absorption and deposition of calcium in the bone. Furthermore, while the increased acidity from HFV may improve the solubility of minerals, it could also alter the intestinal environment in a way that impairs the function of specific mineral transporters. The precise physiological and biochemical mechanisms underlying this observed decrease remain unclear, highlighting the need for future research to investigate these potential interactions more directly.

According to our experimental results, all HFV levels added to the drinking water of broilers reared under CHS conditions decreased tibia bone Ca and P levels but increased Cu, Fe, Zn, and Mn levels ( $P < 0.01$ ). Tibia bone Mg level increased at 2 ml/L HFV level ( $P < 0.01$ ), while K level decreased with 2 ml/L HFV and increased with 4 ml/L HFV (Table 5). As shown in Table 5, HFV supplementation decreased the tibia bone Ca levels in chickens exposed to TN environmental conditions. However, tibia bone Ca levels, which decreased in the HS groups due to the effect of stress, increased in both HFV supplements, especially in the 4 ml/L HFV group; however, this increase was not sufficient to increase the average of the whole experiment. It is thought that the increased intestinal Ca absorption due to the trophic effect and Ca solubility improvement of acetic acid, which constitutes 67.7% of the organic acids in HFV (Kaya, 2023), improves the Ca level that decreases with HS (Kishi *et al.*, 1999; Hossain & Nargis, 2016). In this regard, to prevent bone quality problems, such as weakening of leg bones, developmental disorders, deformation, fracture, and osteoporosis, which are frequently seen in fast-growing broilers, the addition of HFV to the diet may be quite important, especially under high-temperature conditions.

The results of the current study on the effects of heat stress on tibia bone mineral levels in broilers are partially consistent with some previous reports, while showing inconsistencies with others. These differences may be attributed to variations in experimental conditions, such as the active ingredients and levels of additives used, acidity rates, and the species and genotypes of animals. As in the current study, it was determined that the addition of organic acid to the diet containing insufficient P (0.25%) did not affect the tibia bone Ca ratio but decreased the tibia bone P ratio (Akyurek *et al.*, 2011; Soliman & Al-Youssef, 2020); Abdelaziz (2015) reported that the addition of 0.15% and 0.30% formic acid salt to the diet containing insufficient Ca and P did not affect the tibia Ca and P percentage; and Idachaba *et al.*, (2018) reported that the addition of 0.1%, 0.2%, 0.3% and 0.4% organic acid mixture to the diet containing insufficient P (0.40%) increased the tibia bone Ca and P content, especially at the level of 0.3%. Furthermore, the addition of fermentation products at 1, 2, or 4 g/kg of diet was not effective on tibia bone P percentage, but tibia bone Ca percentage increased with 2 g/kg diet addition (Han *et al.*, 2015). Sultan *et al.* (2018) reported that the ad-

dition of 0.5 ml/L organic acid mixture to drinking water decreased the tibia bone Ca level but increased at 1.0, 1.5 or 2.0 ml/L levels, bone P content decreased with 1.0 ml/L organic acid mixture supplementation but increased at 2.0 ml/L level, and emphasized that 2 ml/L organic acid supplementation improved tibial mineralization of Ca and P in broilers. Supplementation of broiler diets with 6% citric acid did not affect Ca requirements and utilization, but affected P requirements and utilization (Boling-Frankenbach *et al.*, 2001). Kishi *et al.* (1999) investigated the effects of 0.4% and 1.6% vinegar addition to ovariectomized rat diets fed a low-Ca diet on Ca absorption, and reported that 1.6% vinegar addition increased Ca absorption and decreased serum parathyroid hormone levels, while 0.4% and 1.6% vinegar addition increased femur Ca content.

## CONCLUSION

The addition of organically produced HFV to the drinking water of broilers exposed to high environmental temperatures did not affect tibia bone morphology and biomechanics, except for increasing bone diameter, decreasing bone Ca and P levels, and increasing Mg, Cu, Fe, Zn, and Mn levels, while it partially improved the Ca level of the tibia bone, especially for the 4 ml/L of HFV group, which decreased with the effect of high temperature. Therefore, we believe that HFV supplementation may have beneficial effects on bone mineral metabolism without negatively impacting the health of broilers under stress. It can also be used at concentrations of up to 4 ml/L to mitigate the undesirable effects of heat stress. However, further research is necessary to draw general conclusions regarding the broader application of HFV in broiler production.

### Conflict of Interest

The author declares that there is no conflict of interest

### Author's contribution

Study conception, data collection, statistical analysis, and writing by H.K.

### Ethics statement

This study was guided pursuant to the approval (dated 18.09.2020 and numbered 2020/13) of the Local Ethics Board for Animal Experiments of Directorate of Veterinary Control Centre Research Institute.

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1. <https://arccjournals.com/journal/indian-journal-of-animal-research/BF-1652>
2. <https://arccjournals.com/journal/indian-journal-of-animal-research/BF-1657>
3. <https://ejournals.epublishing.ekt.gr/index.php/jhvms/article/view/40144>

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