COMPARISON OF DIFFERENT DURATIONS OF BOILING AND FREEZING ON THE BONE TRAITS OF POULTRY AND FLEXURE AND CRUSH TESTS IN BONE-BREAKING STRENGTH ANALYSIS IN BROILERS

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https://dx.doi.org/10.4314/gjansci.v14i1.4

ABSTRACT
Four experiments were conducted to investigate the effect of different durations of boiling and freezing on the weight and breaking strength (BS) of bones in Guinea fowls, broilers, and cockerels and compare three-point bending and crushing tests in determining the BS in broilers. In experiment 1 (Guinea fowls), experiment 2 (broilers), and experiment 3 (cockerels), 10 birds were sacrificed in each duration of the trial on d 56 post-hatch. Ten (10) tibiae and femur were boiled in experiments 1, 2, and 3 for 0, 5, 10, or 15 min. Other sets of 10 tibiae and femur were frozen for 7 days for 0, 12, 18, or 24 hrs. In experiment 4, 3-point bending and crushing tests were compared in testing the tibiae and femur BS at 28 and 56 d in broilers. In experiment 1, the tibial weight was reduced ($P < 0.05$) following 15 min of boiling. Increasing the boiling time beyond 5 min led to a reduction ($P < 0.05$) in femur weight (% body weight) in Guinea fowls. The femur weight (% body weight) and BS of Guinea fowls were only reduced ($P < 0.05$) when it was not frozen. In experiment 3, boiling at 5 min increased ($P < 0.05$) the tibial BS. In experiment 4, the tibial BS of broilers was higher ($P < 0.05$) under the crushing method at d 28 while femur BS was unchanged by either of the methods. The BS of both the tibia and femur were similar at d 56 regardless of the technique used. The duration of boiling or freezing as a preconditioning of bone might make a difference in BS in poultry. Also, the crushing test might serve as a potential alternative to the bending test in assessing the BS of bone in broilers. However, further studies are needed to buttress the current findings.

Keywords: *bone weight; bone-breaking strength; crushing test; poultry; three-point fixture*

INTRODUCTION
Bone development and health are important welfare concerns in the poultry industry. Recent advances in the genetic selection and nutrition of modern chickens have necessitated the need to pay more attention to bone health (Rath *et al*., 2000; Kapell *et al*., 2017; Santos *et al*., 2022). This is because genetic selection in broilers has led to rapid growth that outpaces bone development, resulting in metabolic bone diseases (Shim *et al*., 2002; Stratmann and Toscano, 2004; Pedersen *et al*., 2015). Thus, heavier birds are more likely to suffer from leg deformities and lameness. Therefore, the assessment of bone traits has become an important parameter in most recent studies involving especially chickens. The tibia and the femur have been used in assessing bone strength (BS) in most chicken studies (Chew *et al*., 2019; Nguyen *et al*., 2021).

Excision of the tibia and femur to measure traits such as weight and BS is laborious and timeconsuming, especially in older birds. The tendons and ligaments are difficult to remove in older birds thus increasing the time of de-fleshing. Boiling the leg in water for some time reduces the task of de-fleshing (Cifuentes-Alcobendas and Domínguez-Rodrigo, 2019). Additionally, in a practical chicken trial where the testing facility used for determining BS is not readily accessible, the bones are preconditioned by freezing for a period. It is not known if the duration of freezer storage (and thawing), due to an unstable electricity supply, could impact the results of BS. Some work on the effect of boiling and freezing has been done on other animals (Goh *et al*., 1989; Linde and Sørensen, 1993; Kuninori *et al*., 2009; Kaye *et al*., 2012). However, there is a paucity of information on the effect of boiling and freezing on the bones of different poultry.

Bone-breaking strength is a common trait that researchers have measured in most poultry research (Lee *et al*., 2012; Zanu *et al*., 2020a; Nguyen *et al*., 2021; Wang *et al*., 2021). In most of these studies, the measurement of the bone-breaking strength used the flexural test under 3-point loading conditions in which a 3-point fixture is employed to evaluate the strength of bone with a universal testing machine (UTM). In the procedure, the bone is placed on two supporting beams, and a compression plate is moved slowly downwards to touch and break the bone parallel to the two beams (Zeng *et al*., 2014). The peak force at which the object breaks estimates its strength. However, the challenge with this method is that because the UTMs are not originally designed for testing the strength of the bones of chickens the 3-point fixture is mostly not fit to use and requires the fabrication of a more appropriate bending bench. This poses a challenge to the researcher. It is not known if an alternative and an easier technique called the crushing test (Liu *et al*., 2017; Zhang *et al*., 2022) which does not require the two supports needed in the 3-point bending technique could play a similar role in bone testing in poultry research. The crushing strength method determines the crushing strength of objects which is the ultimate withstanding ability of the object up to the point it gets permanently crushed under the influence of an applied force.

Therefore, it was hypothesized in this study that different durations of boiling or freezing as a preconditioning treatment of bones would not have any difference in the weight and BS of bones in Guinea fowls, broilers, and cockerels. The second hypothesis was that bone determination with either the 3-point bending or crushing strength tests would give similar outcomes and thus offer poultry researchers an alternative technique for testing the BS of the bone of broilers.

MATERIALS AND METHODS

This study comprised 4 experiments involving local male Guinea fowls (exp 1), Cobb 500 male broilers (exp 2), and cockerels (exp 3). In exp 4, a 3-point fixture bending was compared to the crushing method in the testing of BS of the tibia and femur of broilers at d 28 and d 56. In each of the exp (1-3), the birds were reared together under the same management conditions. A diet was formulated using the CFC4 feed formulating tool (Table 1). They were fed *ad libitum* throughout the trial.

Table 1: Ingredient and feed composition of the diet (%, as-is basis)

Ingredient	Starter diet	Grower-finisher diet
Maize	58.0	60.0
Wheat bran	12.5	18.0
Soybean meal	16.0	11.0
Fishmeal	11.0	9.0
Premix	0.50	0.50
Oyster shell	0.50	0.50
Salt	0.50	0.50
Dicalcium phosphate	1.00	0.50
	100	100
Calculated nutrient composition		
Protein %	23.0	18.0
Energy, K cal/g	2900	3210
Ca	0.90	0.80
Available P	0.45	0.40

Vitamin A, 8,000,000 IU; Vitamin B1, 1300 mg, Vitamin B2, 2500 mg, Vitamin D3, 3000 IU; Vitamin E, 10, 000 IU; Vitamin K3, 1,500 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6 mg, Nicotinic Acid, 5,000 mg, Pantothenic Acid, 4000 mg; Choline Chloride, 8000 mg; Copper, 2,500 mg; Cobalt, 700 mg; Iron, 4,500 mg; Zinc, 55, 000 mg; Methionine, 50,000 mg; Lysine, 200,000 mg; Selenium (1%), 1,300 mg; Iodine, 2,000 mg; Manganese, 60, 000 mg; Antioxidant, 625 mg

Data collection in experiments 1-3

In each exp (1-3), 80 birds (10 birds in each duration of boiling or freezing) of average body weight were sacrificed by rapid cervical head dislocation on d 56 post-hatch. In each sampling, the right tibiae and femur were excised from each bird. For the determination of BS, the tibiae and femur were defleshed of muscle and tissue with a sharp knife and immediately boiled or frozen. In the boiling method, the 10 tibiae and femur each were boiled at 100°C for either 0, 5, 10, or 15 min. In the freezing method, the 10 tibiae and femur were put in zip-lock bags and frozen for 7 days at -20°C for either 0 hrs (no freezing), 12 hrs (and 8 hrs of thawing), 18 hrs (and 4 hrs of thawing), or 24 hrs (no thawing). After boiling or freezing, the bones were air-dried for 5 days to reduce moisture that might hasten decaying of the bones before the bone breaking was carried out. The weight was taken with a New Classic digital balance (Mettler Toledo, Greifenasee, Switzerland). The tibia and femur were then subjected to testing on a universal testing machine (Inspekt table50-1, Hegewald & Peschke, Meß-Germany) set up with a 50 KN load cell and 3-point bending test fixture (Plate 1) at a test speed of 10 mm per minute. The machine was run by a BenQ computer (24-inch IPS monitor) with Blue Hill 3 software. The force was applied at the midpoint of each tibia and femur with a 4 cm span between the 2 fixed supports. Supports of the fixture were adjusted for the tibia and femur to accommodate the length of the bones.

Unfortunately, the tibia and femur of the cockerels for exp 3 were unavailable for analysis for the freezing tests due to a technical failure.

Experiment 4

Eighty (80) Cobb 500 female broilers were used in this exp. In each case of BS analysis at d 28 and d 56 using the 3-point bending or crushing test, 10 tibiae, and 10 femurs were used. The inner and outer diameters of the bone were determined. The average tibial length and diameter at d 28 were 70 mm and 6.3 mm respectively. The average femur length and diameter were 5.5 mm and 6.3 mm respectively. In the crushing test method, a 50 mm x 50 mm x 150 mm Eucalyptus wood block was used as the support on a crushing plate (Plate 2). The crushing load was applied from the loading head (load cell of 50 KN) at a rate of 10 mm/min using the UTM (Inspekt table50-1, Hegewald & Peschke, Meß-Germany). The load at which the bone was completely crushed was recorded and the crushing strength was determined.

Statistical analyses

Data for experiments 1, 2, and 3 were analyzed using the General Linear Models (GLM) procedure in Minitab 21.1. Tukey's mean separation test was used to make comparisons between treatment means. In experiment 4, the data means were compared using an independent *t*-test. All statistical analyses were considered significantly different at $p < 0.05$.

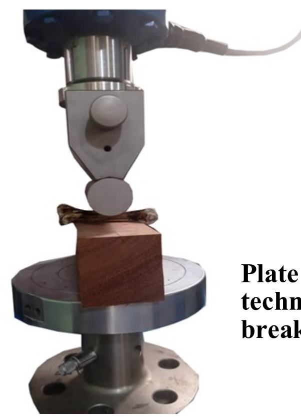

Plate 1: Crushing technique of bone breaking

Plate 2. Three-point bending technique of bone breaking

RESULTS

Experiment 1

In exp 1, the tibial weight (% body weight) of the Guinea fowls at d 56 was not influenced (P > 0.05) at 0, 5, or 10 min of boiling (Table 2). However, at 15 min of boiling the tibial weight decreased (p < 0.05). The tibial weight (% body weight) was unchanged at either 10 or 15 min of boiling (P > 0.05). The BS (Table 2) was not influenced (P > 0.05) by the various durations of boiling in the Guinea fowls at d 56. Again, in exp 1, increasing the duration of boiling beyond 5 min reduced (P < 0.05) the femur weight (% body weight) of the Guinea fowls (Table 2) indicating that the weight of the femur following boiling at either 10 or 15 min was lower (P < 0.05) than boiling at 5 min. The BS (Table 2) of the Guinea fowls was not influenced (P > 0.05) by the different durations of boiling in exp 1.

The different hours of freezing did not have any effect (P > 0.05) on tibial weight (% body weight; Table 3) and BS (Table 3) of Guinea

Table 2: The effects of different times of boiling on tibial and femur of weight and breaking strength of Guinea fowls, d 56 of experiment 1.

Treatments	Tibia		Femur	
	Weight, (%)	BS, (N)	Weight, (%)	BS, (N)
0 mins	0.399[a]	147	0.358[a]	190
5 mi	0.397[a]	154	0.342[ab]	200
10 mins	0.368[ab]	159	0.317b[c]	186
15 mins	0.353[b]	154	0.303[c]	184
SEM	0.005	4.150	0.005	5.230
P-value	0.001	0.801	0.001	0.729

Means that do not share a letter are significantly different (P < 0.05);
Treatments consisted of different boiling times of 0, 5, 10, or 15 mins.;
BS, Breaking Strength;
SEM, pooled standard error of means.

Table 3: The effects of different times of freezing on tibial and femur weight and breaking strength of Guinea fowl, d 56 of experiment 1.

Treatments	Tibia		Femur	
	Weight, (%)	BS, (N)	Weight, (%)	BS, (N)
0 hours	0.399	147	0.358[b]	190[b]
12 hours	0.427	156	0.427[ab]	214[ab]
18 hours	0.461	175	0.461[a]	255[a]
24 hours	0.386	160	0.386[ab]	234[ab]
SEM	0.013	6.140	0.013	7.790
P-value	0.161	0.449	0.029	0.017

Means that do not share a letter are significantly different (P < 0.05);
Treatments consisted of different boiling times of 0, 5, 10, or 15 mins.;
BS, Breaking Strength;
SEM, pooled standard error of means.

fowl at 56 d of exp 1. But the weight (% body weight; Table 3) of the femur of the Guinea fowls in exp 1 was decreased ($P < 0.05$) when it was unfrozen (Table 3) and was comparable to 12 and 24 hrs of freezing. The BS of the femur was highest ($P < 0.05$) at 18 hrs of freezing and was comparable to 12 and 24 hrs of freezing (Table 3). The BS was lowest ($P < 0.05$) at 0 hrs of freezing and was similar to 12 and 24 hrs of freezing.

Experiment 2

The different durations of boiling did not affect ($P > 0.05$) the weight (Table 4) and BS (Table 4) of the tibia in broilers at 56 d in exp 2. Boiling at 15 min decreased ($P < 0.05$) femur weight (Table 4) in exp 2 but it was similar to the weight at 5 or 10 min. In exp 2, the different times of freezing did not affect ($P > 0.05$) the weight (Table 5) and BS (Table 5) of either broiler tibia at d 56. Similarly, freezing did not make a difference ($P > 0.05$) in the weight (Table 5) and BS (Table 5) of the broiler femur at 56 d.

Experiment 3

In exp 3, which involved cockerels, the tibial BS was highest ($P < 0.05$) at 5 min of boiling (Table

Table 4: The effects of different times of boiling on tibial weight, length, diameter, and breaking strength of broilers, d 56 of experiment 2.

Treatments	Tibia		Femur	
	Weight, (%)	BS, (N)	Weight, (%)	BS, (N)
0 mins	0.357	307	0.280[a]	342
5 mins	0.342	286	0.273[ab]	293
10 mins	0.339	314	0.262[ab]	298
15 mins	0.320	314	0.242[b]	293
SEM	0.006	8.520	0.004	8.020
P-value	0.144	0.622	0.004	0.082

Means that do not share a letter are significantly different (P < 0.05);
Treatments consisted of different boiling times of 0, 5, 10, or 15 mins.;
BS, Breaking Strength;
SEM, pooled standard error of means.

Table 5: The effects of different times of freezing on tibial and femur weight and breaking strength of broilers, d 56 of experiment 2.

Treatments	Tibia		Femur	
	Weight, (%)	BS, (N)	Weight, (%)	BS, (N)
0 hours	0.357	307	0.280	340
12 hours	0.333	300	0.259	294
18 hours	0.331	283	0.258	281
24 hours	0.320	263	0.262	308
SEM	0.007	9.470	0.006	9.270
P-value	0.359	0.369	0.520	0.104

Means that do not share a letter are significantly different (P < 0.05);
Treatments consisted of different boiling times of 0, 5, 10, or 15 mins.;
BS, Breaking Strength;
SEM, pooled standard error of means.

6). The effect of boiling at 0, 10, and 15 min on the weight (Table 6) was not different (P > 0.05). The femur weight (Table 6), and BS (Table 6) in cockerels were not influenced (P > 0.05) by all the durations of boiling.

As mentioned earlier, the tibia and femur of the cockerels for exp 3 were unavailable for analysis for the freezing tests due to a technical failure.

Experiment 4

Regarding the methods of bone-breaking in exp 4, a higher (P < 0.05) tibial BS of broiler chickens at d 28 was recorded under the crushing method compared to that of the bending technique (Figure 1a). But, the femur BS of broilers was not affected (P > 0.05) at d 28. At 56 d (Figure 1b), the BS of both the tibia and femurs of the broilers were not influenced (P > 0.05) by either the crushing or bending tests.

DISCUSSION

These studies were conducted to investigate the impact of different durations of boiling, freezing, and thawing on the weight and BS of the femur and tibia of Guinea fowls, broilers, and cockerels. Three-point bending and crushing techniques were also compared in the determination of femur and tibial BS in broilers.

The reduction in bone weight following boiling at 15 min at d 28 and 56 in Guinea fowls might be due to damaged cells. This might explain the reduction in the BS of the femur at d 56 in Guinea fowls. In a related study, it was established that heating bones at 150˚C degrades the extracellular organic matrix and increases cell damage (Kaye *et al*., 2012). Boiling leads to dehydration. Dehydration on the mechanical behavior of cortical bone has been reported to decrease the toughness of bone in the collagen phase whereas loss of water associated with the mineral phase decreases both bone strength and toughness (Nyman *et al*., 2006). A similar study in layers indicated that removing the muscle from tibiae without boiling could reduce cell damage, thus influencing BS because the tibia may absorb some water during boiling and result in cell damage (Park *et al*., 2003). Further, it is most probable that boiling increases the loss of fat from the bone, thus, reducing its weight. This observation was attributed to the loss of fat from the bones during boiling. The fact that in the experiment conducted by Orban *et al*. (1993), who investigated the effect of wet or dry pig tibia on BS supports the observation in the present study that not boiling the bone at all increased its strength.

In this study, the weight and BS of the femur were increased following freezing at d 56 in Guinea fowls. The findings of the present study are contrary to those of some studies. How freez-

Table 6: The effects of different times of boiling on tibial and femur of weight, length, diameter, and breaking strength of cockerels, d 56 of experiment 3.

Treatments	Tibia		Femur	
	Weight, (%)	BS, (N)	Weight, (%)	BS, (N)
0 mins	0.411	115^{ab}	0.301	150
5 mins	0.419	150^{a}	0.309	155
10 mins	0.415	90^{b}	0.310	150
15 mins	0.374	95^{b}	0.305	160
SEM	0.008	6.390	0.004	3.750
P-value	0.174	0.001	0.852	0.762

Means that do not share a letter are significantly different (P < 0.05);
Treatments consisted of different boiling times of 0, 5, 10, or 15 mins.;
BS, Breaking Strength;
SEM, pooled standard error of means.

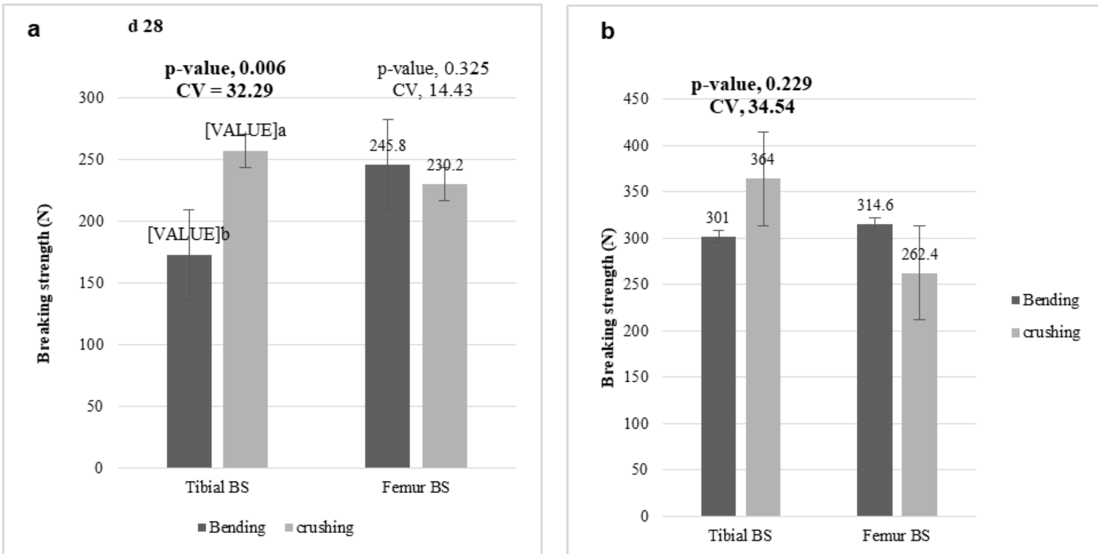

Figure 1: The effects of bending and crushing methods of bone-breaking on the weight and breaking strength of tibial and femur of broilers, d 28 and d 56 of experiment 4.

ing and thawing affect the bone microstructure have been suggested by Boutros *et al*. (2000) to alter the mechanical properties of canine cortical bone. The effect of freezing on bone might be more complicated than just the loss of moisture. On one hand, on freezing, and especially during freeze-thaw cycles, ice crystals form, grow, and converge within and around muscle tissue, bone, and marrow and damage the bone microstructures. A study by Park *et al*. (2003) determined the effects of refrigeration and frozen storage on tibial BS (kg/g) and tibial ash (%) in bones obtained from hens of different ages. It was observed in that study that frozen storage only influenced the bone strength in younger laying hens (72-wk-old) but it could be used for sample storage of bones from older hens (80 or 92-wk-age). When the BS of the tibiae held in refrigerated storage was compared to those of tibiae held in frozen storage, those from 72-week-old hens were stronger than those held in refrigerated storage

On the other hand, preparation of the humeri and tibia by freezing storage before drying and breaking was reported to reduce their breaking in 8-week broilers (Merkley and Wabeck, 1975). Similar findings indicating that freezing degrades the organic components of the extracellular matrix were observed in the work conducted by Kaye *et al*. (2012) which investigated the effects of freezing on the mechanical properties of the bovine femur and human tibia. A decrease in the strength of dry cortical bone tissues from lamb femurs as well as a decrease in fracture resistance has also been associated with the development of microcracks on the bone surface due to freezing in a study by Yin *et al*. (2009).

Except for the differences observed in the bending and crushing techniques in the determination of tibial strength at 28 d, the two techniques largely yielded comparable data indicating that the crushing test might serve as an alternative test to the three-point flexural test. However, the difference observed in the results of the tibial BS at day 28 is not unusual as a similar study that compared two methods showed that the alternative method might be a potential replacer for the golden (older) method though some differences were observed between the two (Kleinheinz and Hernandez, 2016). Additionally, though the BS of the tibia at d 28 in experiment 4 was statistically higher, it makes physiological sense as similar results were observed in broilers at d 29 in previous studies (Zanu *et al*., 2020b; Zanu *et al*., 2021).

CONCLUSION

Though the findings of this study were inconsistent among the three species of poultry, it can be concluded that detailed pre-breakage preconditioning treatments of bones such as duration of freezing or thawing and boiling should be reported in scientific publications as this might make a difference in the results of bone weight and BS. Though the crushing method demonstrated produced results that were comparable to those of the three-point bending technique in analyzing BS in broilers further studies are warranted to fully ascertain this.

ACKNOWLEDGMENT

The authors would like to acknowledge Mr. Haruna Seidu and Mr. Felix Boakye of the Forestry Research Institute of Ghana, Fumesua for the technical assistance in the use of the universal testing machine.

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