Van Vet J, 2023, 34 (2) 133-139

Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Koç Yıldırım E, Kaya M, Dereli Fidan E (2023).** The Effect of Cold Water and Stocking Density on Oxidative Metabolism in Broiler Chickens During Hot Dry Season. *Van Vet J*, 34 (2), 133-139. DOI: https://doi.org/10.36483/vanvetj.1256926

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

The Effect of Cold Water and Stocking Density on Oxidative Metabolism in Broiler Chickens During Hot Dry Season

Ece KOÇ YILDIRIM[1,*] Mehmet KAYA[2] Evrim DERELİ FİDAN[2]

[1] *Department of Physiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, 09000, Aydin, Türkiye*
[2] *Department of Animal Science, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, 09000, Aydin, Türkiye*

Received: 28.02.2023 Accepted: 18.05.2023

ABSTRACT The present experiment was conducted to investigate the effect of drinking water temperature and stocking density (SD) on oxidative metabolism in the heart, liver, *bursa fabricius*, and thymus in broiler chickens raised under heat stress. The experiment comprised of 360 one-day-old Ross 308 male broiler chickens randomly divided to 6 experimental groups with 4 replicates in each group. Experimental treatments included three different SD (low = 12 birds/m^2, medium = 15 birds/m^2 or high = 18 birds/m^2) and two different drinking water temperature (10 ˚C or 31 ˚C) in a 3 x 2 factorial arrangement. At the end of the experiment (42 days of age), two birds per replicate were euthanized for sample collection. The results indicated high SD increased oxidative damage and caused an increase in MDA formation in the heart, liver and thymus. On the other hand, cold water ameliorated the oxidative damage due to the high SD in the thymus. In the study, the statistically non-significant interaction was generally determined between cage stocking density and cold drinking water on the antioxidant system. Besides, while cold water administration increased CAT activity in heart and thymus tissues, decreased GSH activity. In conclusion, drinking water temperature and stocking density are key environmental factors effecting oxidative metabolism when broilers under high temperature conditions; however, more studies are needed in terms of the interactive effects of water temperature and stocking density on antioxidant enzymes under current conditions.

Keywords: *Chickens, Housing, Hot temperature, Oxidative stress, Water.*

ÖZ

Etlik Piliçlerde Sıcak İklimde Soğuk Su ve Yerleşim Sıklığının Oksidatif Metabolizmaya Etkisi

Bu çalışma, sıcaklık stresi altında yetiştirilen etlik piliçlerde, içme suyu sıcaklığı ve yerleşim sıklığının kalp, karaciğer, *bursa fabricius* ve timus dokularında oksidatif metabolizma üzerindeki etkisini araştırmak için yapıldı. Deney, 360 adet bir günlük Ross 308 erkek etlik civcivin, her grupta 4 tekerrür olacak şekilde rastgele 6 deney grubuna bölünmesiyle oluşturuldu. Deneysel uygulamalar, 3 x 2 faktöriyel düzenlemede üç farklı yerleşim sıklığı (düşük = 12 kanatlı/m^2, orta = 15 kanatlı/m^2 veya yüksek = 18 kanatlı/m^2) ve iki farklı içme suyu sıcaklığını (10 ˚C veya 31 ˚C) içerdi. Deney sonunda örnek alımı için her tekrar grubundan 2 tavuk alınarak ötenazi edildi. Sonuçlar, yüksek yerleşim sıklığının oksidatif hasarda ve kalp, karaciğer ve timusta MDA oluşumunda bir artışa neden olduğunu gösterdi. Buna karşın soğuk su, timustaki yüksek yerleşim sıklığı nedeniyle oluşan oksidatif hasarı iyileştirdi. Çalışmada, antioksidan sistem üzerine yerleşim sıklığı ve soğuk içme suyu arasındaki etkileşimler genel olarak önemsiz bulundu. Bunun yanısıra, soğuk su uygulaması kalp ve timus dokularında CAT aktivitesini artırırken GSH aktivitesini azalttı. Sonuç olarak, içme suyu sıcaklığı ve yerleşim sıklığı yüksek sıcaklıkta yetiştirilen etlik piliçlerde oksidatif metabolizmayı etkileyen anahtar çevresel faktörlerdir; ancak su sıcaklığı ve yerleşim sıklığı interaksiyonunun antioksidan enzimler üzerindeki etkilerinin belirlenmesi açısından günümüz koşullarında daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: *Barınak, Oksidatif stres, Su, Tavuk, Yüksek sıcaklık.*

INTRODUCTION

In today's livestock industry, animals are exposed and raised under certain conditions, such as high temperatures, high density of stocking, diseases, inadequate health services, which adversely affect their reproductive performance, health status and well-being. For this reason, researchers are making efforts to improve the response of animals to stress. However, there is limited information on the physiological mechanisms of stress responses in animals exposed to various stress factors (Goo et al. 2019).

✉ *Corresponding author: vetece04@hotmail.com.tr

One of the biggest problems in livestock farming in many countries is heat stress. Among livestock animals, especially poultry are the most susceptible to heat stress. Poultry lack the inhibition of body heat production due to the fact that their bodies are almost entirely covered with feathers and they have limited sweat glands. In poultry, which are frequently exposed to heat stress, first of all, feed consumption decreases. This leads to loss of body weight and rapid depletion of fat reserves (Quinteiro-Filho et al. 2010). Hormonal balance, immune system and blood values as well as feed consumption are negatively affected by heat stress (Aengwanich, 2007). It induces oxidative stress and causes respiratory alkalosis (Teeter et al. 1985; Altan et al. 2003; Lin et al. 2006). Heat stress can cause an increase in lipid peroxidation products and protein carbonyls in plasma and tissues, whereas the severity and duration of heat stress change the activity of antioxidant enzymes (Akbarian et al. 2016). Thus, the performance and health of the animals are adversely affected.

Stocking density can also be a critical stress factor in intensive poultry farming, as high stocking density is highly associated with problems in poultry health, performance and welfare. Possible causes of these problems are reduced access to feed and water, abnormal behavior, inadequate air and poor soil quality. (Esteyez 2007). In addition, high stocking density may result in increased temperature in the micro-environment surrounding the broilers and reduced heat loss from the body, resulting in moderate heat stress (Cengiz et al. 2015). High stocking density causes increase in heterophile to lymphocyte ratio, blood stress hormones, and oxidative stress but decrease in immune response (Mustafa et al. 2010; Najafi et al. 2015; Astaneh et al. 2018; Nasr et al. 2021). Therefore, high stocking density may induce some pathological events similar to heat stress (Goo et al. 2019).

In order to diminish the possible negative effects of heat stress, some practices such as changing the feed content, feed restriction, intermittent feeding and lighting programs are recommended. Apart from these, giving cold water can be an important strategy. Although water is not a nutritional element on its own, it is very necessary to evaluate the feed taken and to keep the body temperature of the animal constant (Park et al. 2014). It is observed that broilers under heat stress cannot regulate their body temperature when their water consumption decreases, but it is reported that cooling the drinking water positively affects the animals' ability to cope with heat stress (Bruno et al. 2011). In addition, there are studies suggesting that cold water given to poultry animals exposed to heat stress positively affects the development and performance of the animals (Park et al. 2014; Farghly et al. 2018).

In the present study, it was aimed to specify the effects of cold drinking water and different stocking density on oxidative metabolism of selected organs in broiler chickens raised under high temperature.

MATERIAL AND METHODS

Birds, Experimental Design and Management

The present study was approved by Aydin Adnan Menderes University Animal Ethical Committee (ADÜ-HADYEK Approval no: 64583101/2020/065).

The experiment comprised of 360 one-day-old Ross 308 male broiler chickens randomly divided to 6 experimental groups with 4 replicates in each group as a totally random design with 3 x 2 factorial arrangement of the stocking density (SD) [low = 12 birds/m^2 (LSD), medium = 15 birds/m^2 (MSD) or high = 18 birds/m^2 (HSD)] and the drinking water temperature (10 ˚C or 31 ˚C). The birds were housed in coops with a floor area of 1 m^2, excluding the feeder and water areas, and 5-7 cm in height, homogeneously laid with wood shavings. A 23L:1D lighting program was applied up to 7 days and 18L:6D thereafter until day 42. The temperature was sustained at 32°C until day 7 followed by a reduction of 3°C per week until day 21 and a temperature of 24–26°C was sustained afterwards.

Starter feed containing 3000 kcal/kg metabolic energy (ME) and 23% crude protein (CP) in the age period of 0-10 days, grower feed containing 3100 kcal/kg ME and 21.5% CP between days 11-24, and finisher feed containing 3200 kcal/kg ME and 19.5% CP between days 25- 42 was given *ad libitum*.Free access of birds to feed and water was being certain throughout the experiment. The experiment's duration was 42 days.

Sample Collection and Determination of Oxidative Metabolism

At the end of the experiment a total of 48 birds, two birds per replicate, were slaughtered and the heart, liver, *bursa fabricius* and thymus tissue samples were collected to determine the oxidative metabolism. Tissue samples were first diluted 10 times with cold 150 mM PBS (pH 7.4) and homogenized for 1-2 minutes at 2,000 rpm with a tissue homogenizer (IKA WERKE Yellowline OST Basic S2 Analog Overhead Stirrer, Athy, Ireland). Homogenates were centrifuged at 12000 rpm for 10 minutes at +4^{o}C. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) activities were established in the supernatants obtained after centrifugation. The determination of MDA was made as per the method reported by Ohkawa et al. (1979). In this method, a pink colored pigment was formed when thiobarbituric acid and MDA react in acidic pH and hot environment, and this color was measured at 532 nm and the results were given as nm/mg protein. GSH was determined as described by Tietze (1969). In this procedure, 5,5'-dithiobis,2-nitrobenzoic acid (DTNB) is reacted to yield a product measured at 412 nm within 4 minutes, and the results are expressed as mg/g protein. CAT activity was measured in supernatants according to the method determined by Luck (1965). In this method, the conversion of substrate H_2O_2 to H_2O was observed spectrophotometrically at 240 nm at 20-second intervals and the decrease in absorbance was measured. Enzyme activity was given using k/mg protein. SOD activity was specified as per the method of Sun et al. (1998). In this method, superoxide radicals form formazone dye in the presence of nitro blue tetrazolium. This color intensity was measured spectrophotometrically at 560 nm. The percent inhibition was calculated depending on SOD activity and the results were expressed as U/mg protein.

Statistical Analysis

The data were statistically analyzed using the SPSS software package (version 22.0 SPSS Inc., Chicago, IL, USA). Levene's test was used to confirm the homogeneity of variances. The oxidative stress data (MDA, SOD, CAT, and GSH) were subjected to ANOVA using the General Linear Model (GLM) procedure with cold water and stocking density as the main effects along with their interactions included in the following model:

$x_{ijk} = \mu + M_i + D_j + (MD)_{ij} + e_{ijk}$,

Where, x_{ijk} = analyzed measurement, μ = Overall mean, M_i = cold water (10 ˚C or 31 ˚C), D_j= effect of stocking density (12, 15, and 18 birds/m^2), $(MD)_{ij}$= effect of interaction,

e_{ijk}= residual random error. In analyzes GLM was designed to reveal the effect of cold water and stocking density on oxidative stress parameters. The partial effects of cold water and stocking density for each factor were analyzed with Least Squares Means Test and multiple comparisons were performed with a Duncan Test.

RESULTS

High SD (18 birds/m²) in broilers significantly increased MDA levels of the heart, liver, and thymus compared to low SD (12 birds/m²) (p=0.029) (Table 1). The interaction between SD and drinking water temperature was also significant in regard to thymus MDA level (p=0.026) (Table 1).Cold water led to a reduction in SOD activity in *bursa fabricius*. (p=0.003) (Table 2).

In addition, cold water applied to broilers increased CAT activity in heart and thymus tissues (p=0.040 and p=0.000, respectively), while reduced GSH activity in both tissue (p=0.002 and p=0.045, respectively) compared to normal water administration (Table 3 and 4).

The interaction between SD and drinking water temperature caused a significant reduction only in cardiac GSH activity (p=0.048) (Table 4).

In addition to the antioxidant enzyme activities in the selected tissues of drinking water, the stocking density affected only liver GSH activity. High SD significantly decreased liver GSH activity compared to low SD (p=0.039) (Table 4).

Table 1: Effect of drinking water temperature (WT) and stocking density (SD) on MDA activity of the selected organs in broilers (nmol/mg protein).

Factors	MDA			
	Heart	**Liver**	***Bursa fabricius***	**Thymus**
Water temperature				
Normal	43.97	19.52	8.68	28.08
Cold	43.15	19.33	8.69	26.45
SEM[1]	2.55	0.75	0.27	1.30
Stocking density				
12 birds/m²	38.65[b]	17.42[b]	9.09	23.95[b]
15 birds/m²	41.58[ab]	19.23[ab]	8.34	28.55[ab]
18 birds/m²	50.46[a]	21.63[a]	8.63	29.31[a]
SEM[2]	4.42	1.30	0.48	2.26
WTx SD Interactions				
Normal-12 birds/m²	47.19	20.24	9.40	25.74[ab]
Normal-15 birds/m²	35.44	20.80	7.89	27.09[ab]
Normal-18 birds/m²	49.29	17.52	8.75	31.43[a]
Cold-12 birds/m²	35.98	18.22	8.78	31.36[a]
Cold-15 birds/m²	41.85	22.45	8.80	27.18[ab]
Cold-18 birds/m²	51.63	17.33	8.51	20.80[b]
SEM[3]	1.80	0.53	0.19	0.92
Significance of main effects			**p value**	
Water temperature	0.821	0.862	0.972	0.381
Stocking density	**0.029**	**0.009**	0.306	**0.047**
WTX SD Interaction	0.126	0.379	0.265	**0.026**

[a, b]: Means with different superscript letters in the same column differ (p<0.05), [1, 2]: Standard error of the mean, [3]: Standard error of the mean for interaction effect.

Table 2: Effect of drinking water temperature (WT) and stocking density (SD) on SOD activity of the selected organs in broilers (nmol/mg protein).

Factors	SOD			
	Heart	**Liver**	***Bursa fabricius***	**Thymus**
Water temperature				
Normal	4.38	1.47	1.86[a]	3.05
Cold	3.82	1.64	1.66[b]	2.96
SEM[1]	0.20	0.07	0.04	0.13
Stocking density				
12 birds/m^2	4.06	1.61	1.85	3.27
15 birds/m^2	3.86	1.53	1.74	3.02
18 birds/m^2	4.38	1.52	1.69	2.73
SEM[2]	0.36	0.13	0.07	0.24
WTx SD Interactions				
Normal-12 birds/m^2	4.36	1.56	1.99	3.26
Normal-15 birds/m^2	3.94	1.41	1.84	3.09
Normal-18 birds/m^2	4.83	1.43	1.75	2.80
Cold-12 birds/m^2	3.76	1.67	1.71	3.27
Cold-15 birds/m^2	3.78	1.65	1.63	2.95
Cold-18 birds/m^2	3.92	1.60	1.64	2.67
SEM[3]	0.14	0.05	0.03	0.09
Significance of main effects			**p-value**	
Water temperature	0.065	0.124	**0.003**	0.657
Stocking density	0.360	0.733	0.132	0.097
WTX SD Interaction	0.577	0.879	0.549	0.939

[a, b]: Means with different superscript letters in the same column differ ($p<0.05$), [1, 2]: Standard error of the mean, [3]: Standard error of the mean for interaction effect.

Table 3: Effect of drinking water temperature (WT) and stocking density (SD) on CAT activity of the selected organs in broilers (k/mg protein).

Factors	CAT			
	Heart	**Liver**	***Bursa fabricius***	**Thymus**
Water temperature				
Normal	0.47[b]	2.29	0.11	0.23[b]
Cold	0.71[a]	1.70	0.14	0.60[a]
SEM[1]	0.08	0.21	0.01	0.06
Stocking density				
12 birds/m^2	0.61	2.36	0.11	0.47
15 birds/m^2	0.68	1.71	0.12	0.30
18 birds/m^2	0.48	1.92	0.13	0.47
SEM[2]	0.14	0.37	0.01	0.11
WTx SD Interactions				
Normal-12 birds/m^2	0.62	2.59	0.10	0.19
Normal-15 birds/m^2	0.55	2.14	0.11	0.27
Normal-18 birds/m^2	0.25	2.12	0.12	0.22
Cold-12 birds/m^2	0.61	2.12	0.12	0.75
Cold-15 birds/m^2	0.82	1.27	0.14	0.33
Cold-18 birds/m^2	0.72	1.72	0.15	0.72
SEM[3]	0.05	0.15	0.00	0.04
Significance of main effects			**p-value**	
Water temperature	**0.040**	0.060	0.073	**0.000**
Stocking density	0.364	0.213	0.388	0.218
WTX SD Interaction	0.242	0.789	0.952	0.057

[a, b]: Means with different superscript letters in the same column differ ($p<0.05$), [1, 2]: Standard error of the mean, [3]: Standard error of the mean for interaction effect.

Table 4: Effect of drinking water temperature (WT) and stocking density (SD) on GSH activity of the selected organs in broilers (mg/g protein).

Factors	GSH			
	Heart	**Liver**	***Bursa fabricius***	**Thymus**
Water temperature				
Normal	18.42[a]	16.99	31.20	7.35[a]
Cold	12.13[b]	15.67	30.20	5.50[b]
SEM[1]	1.36	0.62	1.59	0.63
Stocking density				
12 birds/m^2	15.83	17.81[a]	32.03	7.60
15 birds/m^2	14.75	16.26[ab]	29.82	5.14
18 birds/m^2	15.25	14.93[b]	30.24	6.53
SEM[2]	2.36	1.08	2.76	1.09
WTx SD Interactions				
Normal-12 birds/m^2	16.69[ab]	18.67	31.63	8.98
Normal-15 birds/m^2	16.73[ab]	14.75	28.83	4.77
Normal-18 birds/m^2	21.83[a]	17.54	32.93	8.29
Cold-12 birds/m^2	14.96[abc]	16.95	32.24	6.21
Cold-15 birds/m^2	12.77[bc]	15.10	30.80	5.52
Cold-18 birds/m^2	8.67[c]	14.97	27.55	4.77
SEM[3]	0.96	0.44	1.12	0.44
Significance of main effects	**p-value**			
Water temperature	**0.002**	0.147	0.659	**0.045**
Stocking density	0.901	**0.039**	0.698	0.092
WTX SD Interaction	**0.048**	0.393	0.383	0.127

[a, b, c]: Means with different superscript letters in the same column differ ($p<0.05$), [1, 2]: Standard error of the mean, [3]: Standard error of the mean for interaction effect.

DISCUSSION AND CONCLUSION

Heat stress and stocking density induce oxidative stress and damage the immune system and antioxidant system. Lipid peroxidation is a stress indicator of the autocatalytic mechanism that causes oxidative degradation of cellular membranes. MDA is the main final product of lipid peroxidation but a high production of MDA has been reported as an indicator of oxidative stress (Dalle-Donne et al. 2006). High SD (18 birds/m^2) in broilers significantly increased MDA levels of the heart, liver, and thymus. A similar result was reported by Simsek et al. (2009) and it was stated that crowding increases oxidative damage and causes an increase in MDA formation. Cold water application decreased the thymus MDA level in animals raised in high stocking density. Farghly et al. (2018) found that cold water application in Muscovy ducklings decreased serum MDA levels similar to this study. Cold water administration can positively affect performance by causing feed consumption and daily body weight gain in chickens raised under heat stress (Beker and Teeter 1994), as well as may reduce oxidative stress due to high stocking density.

The effect of reactive oxygen species (ROS) is kept in balance by non-enzymatic and enzymatic antioxidants. SOD and CAT are enzymatic, while GSH is part of the non-enzymatic antioxidant system. Hydrogen peroxide (H_2O_2) is the product of the reaction catalyzed by SOD which is the first line of defense of the antioxidant system, the substrate of both CAT and glutathione peroxidase GPx (Irato and Santovito 2021). GSH plays a role as a cofactor for GPx and reacts directly with ROS by its sulfhydryl groups (Michelli et al. 2016). In this study, cold water administration to broilers reared under high ambient temperature increased cardiac and thymus CAT activity, while decreasing GSH activity. In addition, cold water led to a reduction in SOD activity in *bursa fabricius*. Cold water application did not ameliorate the decreased cardiac GSH

activity due to high stocking density. In contrast to our study, Farghly et al. (2018) suggested that cold water increased serum total antioxidant capacity in Muscovy ducklings. Although there is no study on the effect of cold water on different antioxidant enzymes in different tissues in birds raised under heat stress, our results are thought to confirm those of Fadillioglu et al. (2002) that antioxidant enzymes can have complementary roles for each other for the tissue injuries. Finally, in addition to these effects of water temperature, Nasr et al. (2021) reported similar findings that the SD had an effect only on liver GSH activity, and high SD decreased liver GSH activity.

As a result, high stocking density in broilers raised under high ambient temperature increased oxidative stress in heart, liver and thymus tissues. Cold water application was not effective in reducing oxidative stress in tissues, except thymus. The effect of cold water on the antioxidant system is controversial. While it increased CAT activity in heart and thymus tissues, decreased GSH activity. The stocking density and their interactions did not have a significant effect on the antioxidant system.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENT

We gratefully thank Dr. Cemre KOC for correcting the English grammar mistakes of the manuscript.

AUTHOR CONTRIBUTIONS

Idea / Concept: EDF, MK
Supervision / Consultancy: EDF
Data Collection and / or Processing: MK, EKY
Analysis and / or Interpretation: EKY
Writing the Article: EKY
Critical Review: MK

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