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ORIGINAL ARTICLE

HABITUATION MITIGATES ADVERSE EFFECTS OF HEAT STRESS IN BROILER CHICKEN DURING THE HOT-DRY SEASON

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published in July 14, 2020 (https://doi.org/10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Heat stress is a significant challenge in broiler chicken production, particularly in tropical and subtropical regions. This study evaluates the effectiveness of habituation in mitigating adverse effects of heat stress in broiler chickens during the hot-dry season. Sixty-day-old male broiler chicks (Arbor Acres) were randomly divided into control (n = 30) and habituated groups (n = 30). The habituated group was subjected to 4 days of habituation training from days 17 to 20. The two groups were subjected to tonic immobility (TI) test on day 21. Heterophil-to-lymphocyte (H:L) ratio, serum heat-shock protein 70 (Hsp70) expression, and erythrocyte osmotic fragility were assessed after the TI test. The results indicated that the thermal environment during the study period was characterised by high dry-bulb temperatures and high temperature-humidity index (THI), indicating significant thermal challenge on the broiler chickens. Habituated chickens showed significantly reduced TI durations compared to control. The H:L ratio and serum Hsp70 concentrations were markedly decreased ($P < 0.05$) in habituated chickens relative to controls. There was decreased ($P < 0.05$) erythrocyte membrane fragility in the habituated group, with reduced haemolysis compared to the control group. In conclusion, these findings show that the adverse effects of heat stress during the hot-dry season was mitigated in the habituated broiler chickens, as evidenced by reduced TI durations, a lower H:L ratio, decreased serum Hsp70 concentrations, and enhanced erythrocyte membrane stability. The study highlights the importance of habituation as a behavioural strategy in reducing sensitivity to heat stress in broiler chickens.

Keywords: erythrocyte osmotic fragility; habituation; heat-shock proteins 70; heterophil-lymphocyte ratio; tonic immobility

INTRODUCTION

Habituation in broiler chickens involves adaptation to repeated exposure to stimuli [22]. While initial exposure to a stressor typically elicits strong behavioural and physiological responses, subsequent exposures often result in a diminished response, indicating habituation [28]. Unlike associative learning, which involves linking two or more stimuli, habituation is a non-associative learning process that enables organisms to filter out irrelevant or repetitive information, thereby optimising energy for productive purposes [10, 24]. It plays a crucial adaptive role by mitigating the negative effects of chronic stress [17, 18]. Previous studies have explored the use of habituation to reduce adverse stress responses in poultry. For example, Jones [19] highlighted the importance of habituation to reduce fear and stress responses in poultry when exposed to humans, suggesting that regular, positive human interaction can lower stress-induced behaviours. Additionally, habituation through regular handling has been shown to improve the behavioural responses of laying hens, resulting in a decrease in stress-related behaviours [5]. However, a significant gap in the literature exists, particularly regarding the influence of habituation on poultry during thermal environmental stress conditions. In the Northern Guinea Savannah zone of Nigeria, the seasonal environmental temperature fluctuations during the hot-dry season pose significant challenges for broiler chickens [3]. High ambient temperature and relative humidity (RH) during the season are important thermal environment factors that cause heat stress, which is a major issue in broiler production in tropical and subtropical regions.

Neurologically, habituation reduces the activation of the hypothalamic-pituitary-adrenal (HPA) axis, a key component of the body's stress response system. The dampening effect of habituation protects the body against the adverse effects of prolonged stress [23]. Studies have shown that habituation mechanisms in animals help modulate stress responses, preventing the unnecessary depletion of resources and enabling adaptive responses to genuine threats [17]. Habituation to repetitive handling in chickens can lead to reduced physiological stress, enhancing overall welfare and resilience [20, 28]. Stress response in broiler chickens can be effectively measured via fear-related behavioural tests such as tonic immobility (TI) tests [30], and the TI test has been modified to study habituation in broiler chickens [24]. Studies have shown that broiler chicken exhibiting reduced TI durations indicates enhance welfare [13]. Thus, this study aimed to investigate the mitigating effects of habituation in broiler chickens during environmental heat stress conditions such as the hot-dry season.

MATERIALS AND METHODS

Experimental site and location

The experiment was performed in the poultry house of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria (110 10' N, 070 38' E), located in the Northern Guinea Savannah Zone of Nigeria. The study was carried out from March to April, during the hot-dry season [3].

Experimental flock and management

A total of sixty-day-old male chicks (Arbor Acres) were used for the experiment. They were raised on a deep litter system and properly identified via tags. They were fed a standard diet from day 1 to day 21 (Table 1). The broiler chickens were given access to water and feed *ad libitum*. Biosecurity measures were followed throughout the experiment.

Table 1. Nutrient contents of the broiler chickens feed

Nutrient contents	Amount
Metabolisable energy (kcal.kg^{-1})	2800
Proximate analysis* (%):	
i) Dry matter	97.50
ii) Crude protein	24.74
iii) Crude fibre	4.61
iv) Oil	3.40
v) Ash	4.50
vi) Nitrogen-free extract	62.75

* Analysed in the biochemical laboratory, Department of Animal Science, Ahmadu Bello University, Zaria, Nigeria

Thermal microenvironmental parameters

The dry- and wet-bulb temperatures of the micro-environment of the poultry pen were measured three times daily using a dry- and wet-bulb thermometer (Aura Labtech, India) at 07:00 h, 13:00 h, and 19:00 h (GMT+1) from days 8 to 28. From the data, the RH was calculated each day using an online relative humidity calculator (www.1728.org/relhum.htm). The temperature-humidity index (THI) on each day was determined using the following formula as described by Tao and Xin [29]:

$$THI = 0.85 \times DBT + 0.15 \times WBT$$

(where DBT = dry-bulb temperature and WBT = wet-bulb temperature).

Experimental design and groupings

Sixty-day-old broiler chicks were divided into two groups of 30 chickens. Using simple random sampling, the birds were assigned to control and habituated groups. Each broiler chicken in the habituated group was subjected to 4-days of habituation training from day 17 to 20 as described by Nash and Gullap [24]. Briefly, the habituation training consists of manually restraining a bird on dorsal recumbency for 15 seconds in a U-shaped cradle covered with cloth by trained personnel. The duration of each resulting immobility episode was terminated after about 15 seconds by gentle prodding. Each bird was given five inductions per day, with a 15-second intertrial interval. On day 21, all broiler chickens in both groups were subjected to TI test. The habituation training and tonic immobility test were conducted once on each of the designated days and commenced at 07:00 h (GMT+1). On day 21, a total of 2 mL of blood per bird was collected from 7 broiler chickens per group via the wing vein, after the TI test, into anticoagulant (K_2EDTA) and anticoagulant-free sample bottles. The blood samples containing anticoagulant were taken immediately to the laboratory for the determination of heterophil and lymphocyte count, and erythrocyte osmotic fragility (EOF). The anticoagulant-free sample bottles containing blood were allowed to clot for 10–20 minutes at room temperature and were subsequently centrifuged at 2000 ×g for 20 minutes to obtain the serum. The separated serum was frozen until the determination of Hsp70 expression.

Tonic immobility test

The TI test was carried out as described by Ogundeji and Ayo [25].

Determination of heterophil-lymphocyte ratio

Heterophil and lymphocyte counts were determined using conventional staining techniques [8]. From the data, H:L ratios were obtained.

Determination of serum heat shock protein 70 expression

The serum level of Hsp70 was determined using a commercially available ELISA kit (Wuhan Fine Biotech Co., Ltd., Wuhan, China) as described by the manufacturer's protocol.

Determination of erythrocyte osmotic fragility

The EOF was determined according to the method described by Faulkner and King [12] on day 21. Briefly, 0.02 ml of blood collected from each bird in each group were added to tubes, containing increasing concentrations (0, 0.1, 0.3, 0.5, 0.7, and 0.9%) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4. The tubes were gently mixed and incubated at room temperature (25–26 °C) for 30 minutes. The contents in each tube were then mixed and centrifuged at 400 ×g for 10 minutes, after which the supernatant was decanted. The optical density of the supernatant was determined spectrophotometrically at 540 nm. The degree of haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0 % NaCl) as 100 %. The percentage haemolysis was calculated using the following formula:

$$\text{Percentage haemolysis (\%)} = \frac{\text{Optical density of test solution} \times 100}{\text{Optical density of standard solution}}$$

Data analyses

The data obtained were expressed as mean ± standard error of the mean (mean ± SEM). The TI duration was analysed using the Mann-Whitney test. The H:L ratio, Hsp70, and EOF were analysed using the Students' t-test. Values of $P < 0.05$ were considered significant. The analyses were performed using GraphPad 8.02 for Windows (San Diego, CA, USA).

RESULTS

Thermal micro-environment parameters during the study period

The thermal environment parameters inside the broiler chickens' house during the study period are presented in Table 2. The DBT and THI range from 24.14 ± 0.66 °C to 38.29 ± 0.97 °C and 23.83 ± 0.66 °C to 37.02 ± 0.98 °C, respectively. RH fluctuated between 55.71 ± 5.57 % and 23.83 ± 0.66 %. The overall average daily DBT, RH, and THI during the study period were 31.38 ± 0.61 °C, 64.29 ± 2.84 %, and 30.50 ± 0.61 °C, respectively.

Table 2. Thermal environment parameters inside the broiler chickens house during the study period

Parameter	Hour of the Day			
	7:00 h	13:00 h	19:00 h	Average Daily
DBT (°C)	24.14 ± 0.66 (19 – 28)	38.29 ± 0.97 (32 – 43)	31.71 ± 0.90 (27 – 38)	31.38 ± 0.61 (27.33 – 33.67)
RH (%)	82.97 ± 1.84 (68.50 – 92.00)	54.50 ± 3.51 (35.30 – 93.80)	55.71 ± 5.57 (16.70 – 79.90)	64.29 ± 2.84 (43.20 – 78.90)
THI	23.83 ± 0.66 (18.85 – 27.70)	37.02 ± 0.98 (31.10 – 41.65)	30.64 ± 0.80 (25.95 – 35.75)	30.50 ± 0.61 (26.68 – 32.97)

Values in parentheses are the minimum and maximum. DBT: Dry-bulb temperature; RH: Relative humidity; THI: Temperature-humidity index; *n* = 14 days.

Tonic immobility duration of the broiler chickens

The control group exhibited a significantly (P < 0.05) longer TI duration of 81.42 ± 12.80 seconds compared to the habituated group, which had a TI duration of 40.61 ± 4.56 seconds (Fig. 1).

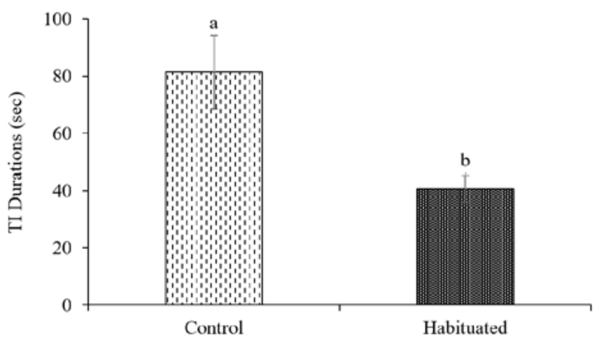

Fig. 1. Effects of habituation on tonic immobility (TI) duration in broiler chickens during the study period

Heterophil-to-lymphocyte (H:L) ratio in the broiler chickens

A significant decrease (P < 0.05) in the heterophil-to-lymphocyte (H:L) ratio was recorded in the control group compared with the habituated group (Table 3).

Table 3. Effects of habituation on haematological responses in broiler chickens during the study period

Parameters	Control	Habituated
Heterophil (× 10^3 µL)	1.36 ± 0.11	0.97 ± 0.20
Lymphocyte (× 10^3 µL)	6.36 ± 0.41	6.23 ± 1.03
H:L Ratio	0.22 ± 0.03[a]	0.14 ± 0.01[b]

[ab] Means for the same parameter having different superscript letters across the row are significantly (P < 0.05) different; H/L: heterophil lymphocytes ratio.

Serum heat shock protein 70 concentration in broiler chickens

The results showed a significant (P < 0.05) decrease in the serum heat-shock protein 70 (Hsp70) concentration in the habituated group of broiler chickens compared with the control group (Fig. 2). The habituated group presented an Hsp70 concentration of 5.90 ± 0.27 $ng.mg^{-1}$, whereas the control group presented a higher concentration of 6.66 ± 0.15 $ng.mg^{-1}$.

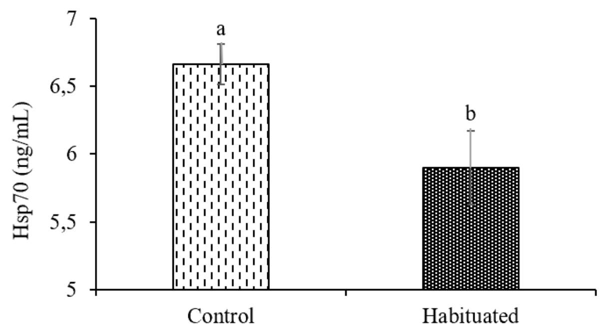

Fig. 2. Effects of habituation on serum heat-shock protein 70 concentration in broiler chickens during the study period

Erythrocyte osmotic fragility in broiler chickens

Erythrocyte osmotic fragility in broiler chickens was assessed during the study period (Fig. 3). At a NaCl concentration of 0.7 %, the percentage haemolysis recorded in the habituated group was significantly lower (P < 0.05), measuring 4.15 ± 0.42 %, compared to the control group, which presented a higher haemolysis value of 7.65 ± 0.64 %. No significant differences in haemolysis were detected across the experimental groups at NaCl concentrations of 0.9 %, 0.5 %, 0.3 %, and 0.1 %.

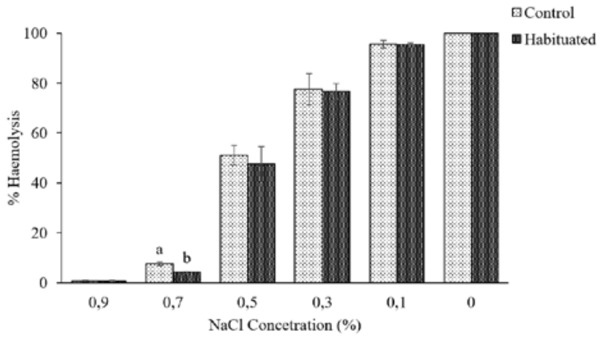

Fig. 3. Effects of habituation on erythrocytes osmotic fragility in broiler chickens during the study period

DISCUSSION

The results of this study highlight the impact of the Northern Guinea Savannah's thermal microenvironment on broiler chickens during the hot-dry season, revealing that the birds were exposed to significant heat stress. The fluctuations in DBT ranging from 24.14 ± 0.66 °C to 38.29 ± 0.97 °C, along with RH levels between 54.50 ± 5.57 % and 82.97 ± 1.84 %, indicate the presence of both mild and extreme heat conditions that impede the chicken's ability to thermoregulate. This aligns with previous studies [31], which reported that broilers in similar climates experience considerable physiological stress, diverting energy from growth to thermoregulation. The study's findings on the THI, with values ranging from 23.83 ± 0.66 °C to 37.02 ± 0.98 °C, suggest that broilers were subjected to moderate to severe heat stress, particularly as THI values above 30 °C are considered critical for poultry [21, 29]. These results emphasise the necessity for effective heat mitigation strategies in poultry management.

The result of the present study indicates that habituated broiler chickens exhibit reduced TI duration when subjected to TI test. This is in agreement with the report by Nash and Gullap [24], who demonstrated that habituation reduces the TI response in broiler chickens. The TI test is a fear-realated behavioural test capable of activating the stress response mechanism in chickens [13]. Several studies have shown a link between stress and fear responses in poultry. For example, Hemsworth et al. [17] reported that handling stress impacts fear responses and physiological stress indicators in broiler chickens. Sulimova et al. [27] further highlighted that chronic stress influences physiological responses and emphasised the physiological impact of fear. DeHaas et al. [9] illustrated these associations in commercial laying hens, revealing that heightened fear responses correspond with physiological stress indicators. Thus, this finding of the present study suggests that the habituation may be reducing the physiological burden on the animal stress mechanism. Since the neurobiology of fear and stress response is superimposed, the damping effects of habituation in the broiler chickens may cause a decrease in stress response during the hot-dry season.

The H:L ratio is a reliable and non-invasive marker for chronic stress [16]. In this study, a significant difference was observed in the H:L ratio between the habituated group and the control group. The H:L ratio in the habituated group was significantly lower compared to the control group, suggesting that habituation helped reduce the physiological stress response of broilers to heat stress. These findings align with previous research where stress mitigation strategies have been shown to reduce the H:L ratio in broilers. For instance, Altan et al. [2] reported that broilers subjected to heat stress exhibited a lower H:L ratio when provided with preconditioning or acclimatisation techniques. Similarly, habituation to stressors, such as human handling or sound exposure, could decrease the H:L ratio in chickens, implying that repeated exposure to mild stressors can desensitize the birds to more severe stressors [7]. The reduction in the H:L ratio observed in the habituated group in the present study indicates that by gradually exposing the birds to controlled fear stimuli, the stress response may have been damped, resulting in lower heterophil counts relative to lymphocytes.

The observed reduction in Hsp70 concentrations in the habituated group provides evidence for a reduced physiological stress response resulting from habituation. Typically, elevated ambient temperatures or stressful conditions trigger an increase in Hsp70 expression as part of the chicken's defence mechanism to maintain cellular homeostasis [6]. However, the reduced Hsp70 levels in the habituated group suggest that habituation might alleviate the need for an intense stress-induced response, thereby diminishing the physiological burden on the birds. This finding aligns with previous research that has explored the stress-mitigating strategies in poultry. For example, Gouda et al. [14] emphasise that heat shock proteins, including Hsp70, are critical components of the chicken's defence system against thermal stress. However, this study's results indicate that habituation – a process by which birds become accustomed to stressors through repeated exposure – can also play a significant role in reducing the expression of these proteins. This could be because the habituated group, through habituation, experienced less fear and anxiety, which in turn mitigated their overall stress response, as evidenced by the lower Hsp70 levels. Additionally, Balakrishnan et al. [4] highlights that Hsp70 serves as a key indicator of how broilers respond to stress at a molecular level. The lower Hsp70 levels in the habituated group suggest that psychological conditioning can influence these molecular pathways, reducing the activation of stress responses in a manner similar to other stress alleviation strategies, such as nutritional supplementation.

The findings of this study highlight a substantial difference in erythrocyte osmotic fragility between the habituated and the control groups, reinforcing the protective effects of habituation during heat stress. The lower percentage of haemolysis in the habituated group, particularly at 0.7 % NaCl concentration, suggests that habituation could play a critical role in enhancing erythrocyte membrane stability. This reduced fragility may be attributed to decreased oxidative stress, which has been identified as a major contributor to erythrocyte membrane damage in heat-stressed broilers [1]. Lower haemolysis values, such as those observed in the habituated group, align with previous studies suggesting that oxidative stress is reduced through interventions that improve erythrocyte resilience [11]. Thus, the observed improvement in erythrocyte membrane integrity in the habituated group may result from reduced heat-induced oxidative damage due to habituation.

CONCLUSION

In conclusion, the findings from this study show that habituation mitigated the adverse effects of heat stress in broiler chickens during the hot-dry season. This is evidenced by reduced TI durations in the habituated broiler chickens, indicating reduced fear and stress levels. The habituated birds exhibited significantly decreased H:L ratio and serum Hsp70 concentrations, suggesting reduced physiological stress; and enhanced erythrocyte osmotic fragility, demonstrating improved resilience to oxidative stress and better membrane stability. The study shows the importance of habituation in reducing sensitivity to heat stress in broiler chickens.

DATA AVAILABILITY STATEMENT

The corresponding author will provide the raw data of this article on reasonable request.

ETHICAL APPROVAL

This study with reference number ABUCAUC/2021/025 was approved by the Ethical Committee on Animal Use and Care of Ahmadu Bello University, Zaria.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

No funding was received for this study.

GENERATIVE AI STATEMENT

The authors declare that no Gen AI was used in the creation of this manuscript.

AUTHORS CONTRIBUTIONS

The authors contributed equally to this study.

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