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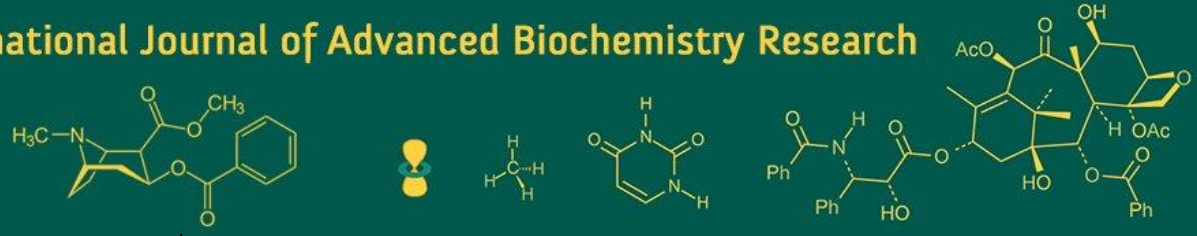

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Thermal stress associated metabolic and biochemical alterations in Landlly crossbred pigs

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Abstract
Pigs are more sensitive to harsh impacts of heat stress among livestock species due to their inherent features such as a smaller number of sweat glands, presence of subcutaneous fat, inefficient panting and small lungs. The present investigation was done at Swine Production Farm, ICAR-Indian Veterinary Research Institute to explore the serum biochemical and metabolic changes in Landlly crossbred pigs amidst of increasing air temperature (AT) and Relative humidity (RH). A total of 24 Landlly crossbred pigs in grower stage with uniform body weight were selected and randomly divided into two as heat stress (control) and heat stress alleviated (treatment) groups. Both groups were housed in same confined shed whereas a sensor-based cooling system was installed in second pen to minimize the heat stress effects. Major micro-climatic variables *viz*, AT, RH and temperature humidity index (THI) was recorded twice daily in both pens. Mean AT, RH and THI around the micro-climate was 32.6±0.15℃, 72.4±0.19% and 80±0.24 respectively. The study was conducted during 4 months (June-September, 2023) and serum biochemical- metabolic parameters such as Total protein (TP), Urea nitrogen (UN), Total cholesterol (TCHO) and Glucose (GLU) were estimated at 21 days interval for all the animals. During each stage of investigation, the treatment group animals provided with cooling assistance outperformed the control group animals. The control group exhibited elevated TP, serum urea nitrogen and TCHO levels ($p<0.001$) during the hot-dry period of the summer season, when high AT were more prevalent than RH. In contrast, glucose levels ($p<0.001$) consistently fell below the optimal range, with a noticeable influence of RH during heat stress in pigs. During the latter months, when relative humidity was higher, glucose levels were lower compared to the initial months. Thus, the biochemical and metabolic alterations during heat stress in pigs can be nurtured as critical indicators to assess the physiological status and animal welfare.

Keywords: Climate change, lipid peroxidation, oxidative stress, thermoregulation, heat stress, pigs

1. Introduction
Various environmental conditions can influence an animal's immune system and productivity, with heat stress emerging as the most critical global factor adversely affecting animal health and performance. The risk of heat stress is rising in tandem with global temperature increases, both in magnitude and duration. Heat stress occurs when an animal's capacity to dissipate the heat produced by its normal metabolic activities is compromised (Gaughan *et al*., 2010) [9]. This typically happens when air temperature levels exceed the thermo-neutral zone (TNZ), the temperature range within which an animal can maintain its normal body temperature without altering metabolic heat production or relying on evaporative heat loss mechanisms. Relative humidity is also crucial in an animal's ability to expel heat and, consequently, in the experience of heat stress. This is because evaporative heat loss is influenced by changes in the ambient water vapor levels. Indeed, heat stress intensifies when both air temperature and humidity levels rise (Dahl *et al*., 2020) [5].
As environmental temperatures rise, the potential for sensible heat exchange diminishes due to the decreasing temperature differential between the animal's body surface and the surrounding environment. Consequently, evaporative heat loss becomes the primary mechanism for regulating body temperature (Milan *et al*., 2016) [14]. Initially, sweating facilitates evaporative heat loss as air temperatures increase, and with more severe heat stress, respiratory heat loss intensifies. As the animal's heat load escalates in its effort to maintain a normal core temperature, its physiological systems are impacted.

This involves a reduction in heat generation and an increase in heat loss (Dahl *et al*., 2020) [5]. When animals are exposed to environmental conditions outside their TNZ, the priority of nutrient utilization shifts to maintaining euthermia, leading to decreased production efficiency and productivity. Thus, productivity declines during the hot summer months, affecting not only tropical regions but also temperate countries (Renaudeau *et al*., 2012) [19].

Climate change affects livestock both directly and indirectly (Bernabucci *et al*., 2010) [3]. Animal's exhibit altered physiological and behavioral responses to heat stress, including increased respiration rate (RR), panting, elevated body temperature, and changes in feeding and rumination patterns, favoring more standing, drinking, and seeking shade. Climate change significantly impacts the sustainability (Das *et al*., 2016) [6] and viability (Gaughan *et al*., 2010) [9] of animal production systems globally. The health, welfare, and productivity of livestock can all be negatively impacted by climate change, which can also indirectly affect the quality and quantity of animal feed. Animals adjust their physiological and behavioral responses as a crucial aspect of thermoregulation in hot thermal conditions to maintain their internal body temperatures within a safe range. However, prolonged or intense exposure to heat beyond thermoregulatory capacity can lead to elevated internal body temperatures, triggering various heat stress reactions in animals (Islam *et al*., 2021) [12]. Thermal stress significantly impacts metabolic and serum biochemical activities in animals (Sejian *et al*., 2014) [21]. High air temperatures can cause an increase in the activity of certain metabolic enzymes, including alanine aminotransferase (ALT), which has been consistently observed in pigs during heat stress episodes.

Estimating serum biochemical parameters is an essential tool for thoroughly understanding and managing the effects of heat stress on livestock animals. These parameters offer valuable insights into the physiological changes that occur in response to heat stress, as well as nutritional and dehydration status, and organ function. This information is crucial for developing better management and mitigation strategies. Additionally, these parameters serve as direct indicators of an animal's health and metabolic state. The purpose of this investigation is to unveil the dynamic alterations in serum biochemical profile in pigs amidst of heat stress condition.

2. Materials and methods

2.1. Experimental population

The data for the current study was derived from a herd of Landlly (75% Landrace × 25% Ghurrah) crossbred pigs, housed at the Swine Production Farm (SPF), Livestock Production and Management section at ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Uttar Pradesh, India. The Landlly crossbred variety was developed under the All India Coordinated Research Project (AICRP) on pigs at the ICAR-IVRI. This study involved selecting 24 pigs (18 males and 6 females) in the grower stage, approximately 45-60 days old. These pigs were evenly divided into two groups: Control (first group) and Treatment (second group), with each group containing 12 animals (9 males and 3 females). Careful attention was paid to ensure that the average body weight in both groups was nearly identical, being 29.99±1.39 kg for the control group and 30.45±1.74 kg for the treatment group. Throughout the study period, the animals were provided with a uniform diet consisting of crushed maize (48%), soybean meal (20%), wheat bran (30%), salt (0.5%), mineral mixture (MM) (1.5%), and high-quality green fodder, meeting their specific nutritional requirements for the grower stage, which include 18% crude protein and 3170 kcal/kg of digestible energy per animal. Fresh drinking water was made available ad libitum in both pens at all times.

2.2. Experimental design

The study was carried out over a four-month period from June to September 2023. The first two months (June and July) experienced a hot-dry micro-climate, while the subsequent two months (August and September) were characterized by a hot-humid condition. During the investigation, the average air temperature (AT) was 32.6±0.15 ℃, relative humidity (RH) was 72.4±0.19%, and the temperature-humidity index (THI) was 80±0.24. The control group was housed under standard farm conditions, whereas the treatment group was housed in an area equipped with a sensor-based evaporative cooling system to mitigate heat stress. Both control and treatment group pens were located within the same shed, with precautions taken to prevent the microclimates of the pens from interacting. Animals were introduced to the pens 7 days prior to the start of the trial to ensure proper acclimatization. Space allocation for each animal followed BIS recommendations, providing 0.9-1.2m² per animal for both covered and open areas.

For serum estimation, blood samples were aseptically collected in serum vacutainers on the first day (0^{th} day) of the study and subsequently every 21 days until the study concluded. After collection, the samples were left at room temperature for 20-30 minutes to allow for coagulation. Serum was then separated from 5 ml of blood by centrifuging the samples at 3000 rpm for 15 minutes and subsequently stored at -40 °C. All serum samples were later analyzed using commercial kits based on standard methods employing the chem5X-Erba Mannheim analyzer. The biochemical parameters measured during the study included the following:

2.2.1. Serum total Protein (gm/dl)

Serum total protein levels were measured using a commercial kit from Erba, Transasia Bio-medicals LTD, HP, employing the Biuret method. In this method, proteins' peptide bonds react with copper ions in an alkaline solution, producing a blue-violet complex. The color intensity of this complex is directly proportional to the protein concentration in the sample. This is quantitatively determined by measuring the absorbance at 546 nm through colorimetric techniques.

2.2.2. Serum urea nitrogen (mg/dl)

Serum urea nitrogen levels were measured using a commercial kit from Erba, Transasia Bio-medicals LTD, HP, utilizing the GLDH-Urease method. In this process, urea in an alkaline solution reacts enzymatically with urease, releasing ammonia. This ammonia then reacts with alpha-ketoglutarate in the presence of the glutamate dehydrogenase enzyme, producing glutamate. The decrease in absorbance at 340 nm is directly proportional to the urea concentration in the serum sample.

2.2.3. Serum glucose (mg/dl)
Serum glucose levels were measured using a commercial kit from Erba, Transasia Bio-medicals LTD, HP, following Trinder's method. In this method, glucose in the sample is oxidized by glucose oxidase, producing gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminoantipyrine and phenol in the presence of peroxidase, forming a colored quinoneimine complex. The intensity of the resulting pink color is directly proportional to the glucose concentration in the sample, and it is measured photometrically at 505 nm.

2.2.4. Serum total cholesterol (mg/dl)
Serum total cholesterol levels were determined using a commercial kit from Erba, Transasia Bio-medicals LTD, HP, which employs the dynamic extended stability CHOD-PAP method. In this process, cholesterol esters in the sample are hydrolyzed by cholesterol esterase, releasing cholesterol and fatty acids. The free cholesterol then reacts with oxygen to generate hydrogen peroxide. This hydrogen peroxide subsequently reacts with phenol and 4-aminoantipyrine to produce Quinoneimine. The concentration of cholesterol in the sample is directly proportional to the absorbance of Quinoneimine at 505 nm, measured through colorimetric techniques.

3. Results and Discussion
In this investigation, we assessed the important biochemical parameters related to metabolic changes in the body to explore the effects of thermal stress on pigs. The significant findings are as follows:

a) Total protein (TP) (gm/dl)
A significantly higher total protein (TP) concentration was observed in animals experiencing greater heat stress compared to those in the treatment group at all stages. Despite fluctuations in relative humidity (RH) and air temperature, the TP levels in the treatment group consistently remained within the normal range for pigs. In contrast, the control group exhibited elevated TP levels during the hot-dry period of the summer season, when high air temperatures were more prevalent than RH. Although significant changes were not observed within the control group, the variation across different periods was more pronounced compared to the treatment group. During the hot-dry period (June-July), TP levels in the control group exceeded the upper limit of the required range, whereas they approached the borderline during the more humid parts of the season. The clear difference in TP concentration between the two groups indicates the effectiveness of the newly developed cooling system in maintaining normal body functions. The mean TP values for the control and treatment groups were 5.84±0.07 and 4.49±0.04 gm/dl respectively (p<0.001). The variation in TP levels at fortnightly intervals for both the treatment and control groups is illustrated in Figure 1.

In this study, the control group, which lacked any cooling assistance, exhibited elevated serum total protein concentrations, while the treatment group consistently maintained levels within the normal range. This elevation in total protein could be attributed to hemo-concentration resulting from dehydration. Pigs lose significant water through increased respiration and sweating when exposed to high temperatures, leading to a reduction in plasma volume and an increase in the concentration of blood constituents, including proteins. Consequently, serum total protein concentrations appear elevated, even if the absolute amount of protein remains unchanged (Baumgard and Rhoads, 2013) [2]. Additionally, the synthesis of acute phase proteins (APPs) is known to impact serum total protein levels during heat stress. The liver produces these proteins, such as haptoglobin, serum amyloid A, and C-reactive protein, in response to the inflammatory signals triggered by heat stress. The increased synthesis of APPs during heat stress contributes to the elevation in serum total protein levels. Moreover, heat stress can induce increased protein catabolism as the body strives to meet heightened energy demands. This catabolic process involves the breakdown of muscle proteins into amino acids, which are then transported to the liver for gluconeogenesis or other metabolic processes. The redistribution of these amino acids and subsequent increase in plasma protein synthesis contribute to the observed elevation in serum total protein levels (Sejian *et al*., 2018) [20].

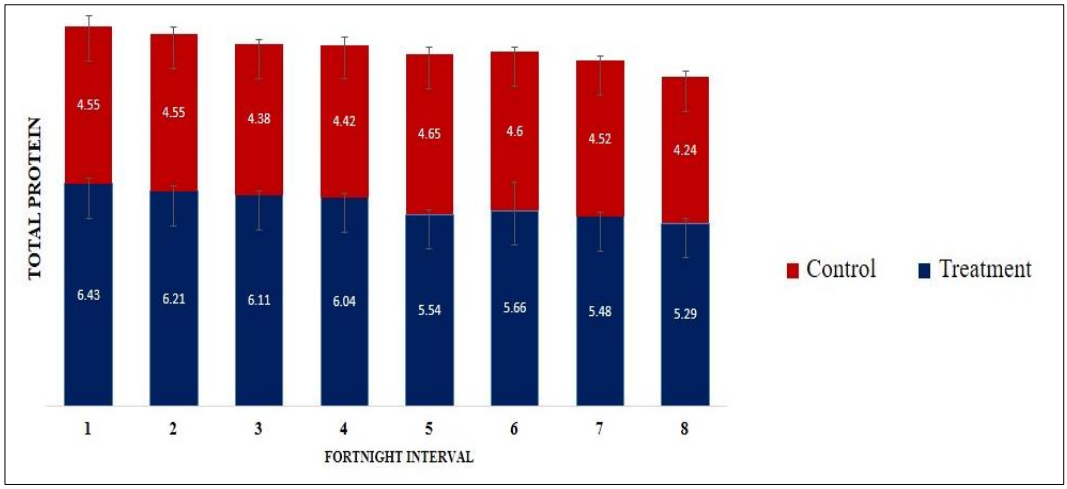

Fig 1: The variation in Total protein level at fortnight intervals for both the treatment and control groups

b) Serum urea nitrogen (mg/dl)
Serum urea nitrogen values, which serve as indicators of dehydration levels, consistently showed higher levels in animals belonging to the control group. While variations were observed in serum urea nitrogen values at different times of the season in the treatment group, these values

consistently remained within the normal range for pigs. In contrast, the control group exhibited more pronounced variation, with significantly elevated levels during the hot-dry period compared to the hot-humid part of the season. Although the treatment group also showed some impact of air temperature on serum urea nitrogen concentration, it was less noticeable. In the control group, serum urea nitrogen concentration exceeded the upper limit during the initial months of heat stress and subsequently normalized as air temperatures decreased. The mean serum urea nitrogen values for the control and treatment groups were 30.73±0.77 and 20.44±0.65 mg/dl respectively (p<0.001). The fluctuation in serum urea nitrogen concentration at fortnightly intervals for both the treatment and control groups is depicted in Figure 2.

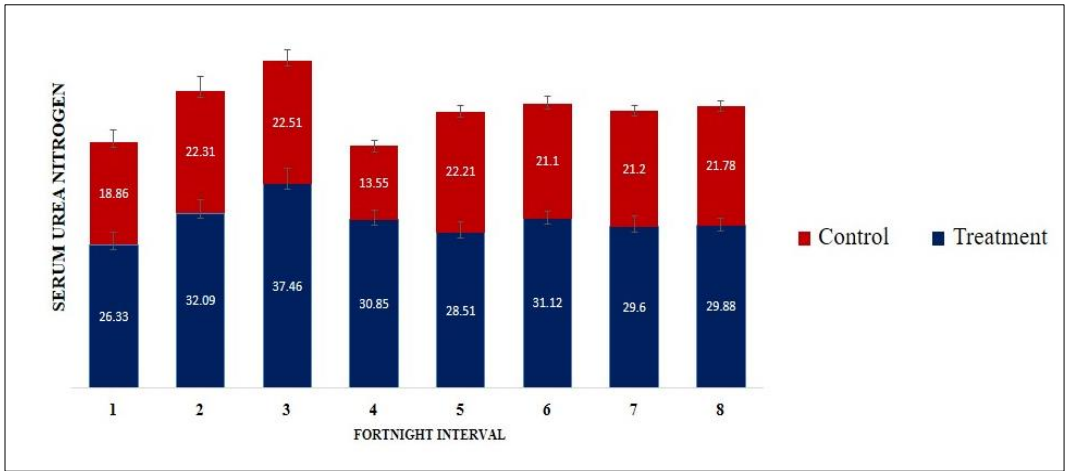

Fig 2: The variation in serum urea nitrogen level at fortnight intervals for both the treatment and control groups

The serum urea nitrogen levels were observed to be high in the control group during the heat stress period, especially when air temperatures were elevated. This increase in serum urea nitrogen is attributed to metabolic adjustments in response to high environmental temperatures. Pigs alter their metabolic pathways during heat stress, leading to the catabolism of muscle protein to provide energy substrates (Huang *et al*., 2024) [10]. This metabolic shift results in elevated serum urea nitrogen levels, which is further supported by metabolomic profiling studies showing changes in amino acid metabolism and markers of protein catabolism during heat stress episodes. Dehydration can also concentrate blood constituents, including urea, as pigs mobilize body reserves to maintain water and electrolyte balance, increasing protein breakdown and urea production. Heat stress triggers an inflammatory response that releases cytokines and acute-phase proteins, leading to increased muscle protein breakdown and amino acid catabolism, contributing to elevated urea nitrogen levels. Additionally, exposure to high temperatures increases oxidative stress, damaging proteins and cellular components, leading to their degradation and removal, further elevating serum urea nitrogen levels (Huau *et al*., 2024) [11]. Impaired kidney function and reduced glomerular filtration rate (GFR) during heat stress can also decrease urea clearance, leading to its accumulation in the blood. Furthermore, elevated cortisol levels during heat stress enhance gluconeogenesis and proteolysis, increasing urea production. Researches has explored various nutritional strategies to mitigate the effects of heat stress, including dietary supplementation with amino acids like glutamine and arginine, which can modulate protein metabolism and reduce urea nitrogen accumulation (Garcia *et al*., 2019) [8].

c) Total cholesterol (mg/dl)

Throughout the heat stress period, animals in the control group consistently exhibited significantly higher levels of total cholesterol, regardless of fluctuations in relative humidity (RH) and air temperature (AT). The mean serum total cholesterol concentration for the control and treatment groups was 150.22±1.23 and 125.44±1.78 mg/dl respectively (p<0.001). In the treatment group, total cholesterol levels consistently remained within the normal range recommended for pigs. While no significant changes were observed in the results, cholesterol levels tended to be higher during periods of elevated air temperature. However, as air temperature decreased and relative humidity gradually increased, circulating cholesterol levels began to decline. Initially, in control animals, cholesterol values approached the upper limit of the required range but later normalized. This suggests a transient increase in total cholesterol levels in circulation during heat stress. The fluctuation in serum total cholesterol concentration at fortnightly intervals for both the treatment and control groups is depicted in Figure 3.

Heat stress exerts an independent influence on lipid metabolism, as evidenced by the increased serum levels of total cholesterol, low-density lipoprotein-cholesterol, and triglycerides (TG) observed in this investigation, consistent with findings reported by Fang *et al*. (2020) [7]. In the control group, the estimated total cholesterol levels were elevated, nearing the upper limit of the required range for pigs. Heat stress primarily impacts lipid metabolism in pigs by reducing lipolytic capacity while increasing fat deposition and triglyceride storage, regardless of any heat-induced changes in feed intake. This alteration in lipid metabolism may be attributed to enhanced lipid deposition due to altered insulin sensitivity, along with reduced lipolysis activity in adipose tissue, which serves as an adaptive mechanism to limit heat production in heat-stressed animals (Qu & Ajuwon, 2018) [18].

Lipids play a crucial role in regulating various biological processes involved in the response to heat stress (Péter *et al*., 2021) [17]. Cholesterol, a vital lipid component, plays a unique role among mammalian lipids. Heat stress induces fluidization of cellular membranes, which is essential for

heat sensing and signaling to maintain cellular function (Leach *et al*., 2014) [13]. Cholesterol, as a major component of cell membranes, regulates the fluidity of the lipid bilayer and contributes to heat adaptation by modulating heat shock protein signaling (Balogh *et al*., 2013) [1].

Therefore, investigating the effect of heat stress on cholesterol metabolism is crucial for understanding the role of different lipids in heat stress adaptation. Studies by Pearce *et al*. (2013) [16] have indicated that heat-stressed pigs tend to exhibit increased circulating serum cholesterol levels.

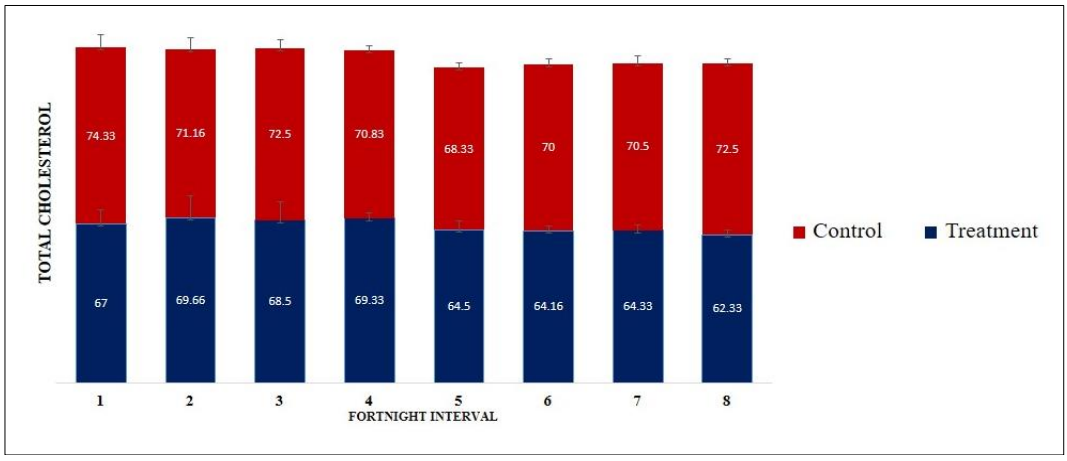

Fig 3: The variation in Total cholesterol level at fortnight intervals for both the treatment and control groups

Elevated temperatures alter the lipid composition and architecture of cell membranes, leading to increased membrane fluidity. This change could be counteracted by elevating cholesterol levels, as observed in our study. Indeed, cholesterol levels in the membrane rise with increasing body temperature to stabilize membranes (Crockett, 1998) [4]. Additionally, cholesterol may regulate the survival and sensitivity of mammalian cells to high temperatures by modulating physical membrane properties, thus serving as a primary defense mechanism through the up-regulation of heat shock protein expression (Nagy *et al*., 2007) [15]. This elevation in serum cholesterol levels observed in our study may have served as a primary defense mechanism against cellular damage and adverse effects during the early stages of heat exposure in the control group.

d) Serum glucose (mg/dl)

The mean serum glucose level in the treatment group was notably higher than that in the control group, indicating more severe heat stress experienced by animals housed in standard pens without a cooling system. The mean serum glucose levels in the control and treatment groups were 66.22±0.82 mg/dl and 71.39±0.53 mg/dl respectively (p<0.001). Although serum glucose levels in the treatment group consistently remained within the normal range, they tended to be close to the lower limit of the required range for pigs. There was no significant fluctuation in glucose levels corresponding to shifts in air temperature (AT) and relative humidity (RH) in this group, indicating that the cooling system helped mitigate the effects of heat stress to a considerable extent. However, in the control group, glucose levels consistently fell below the optimal range, with a noticeable influence of RH. During the latter months, when relative humidity was higher, glucose levels were lower compared to the initial months. The variation in serum glucose levels at fortnightly intervals for both the treatment and control groups is illustrated in Figure 4.

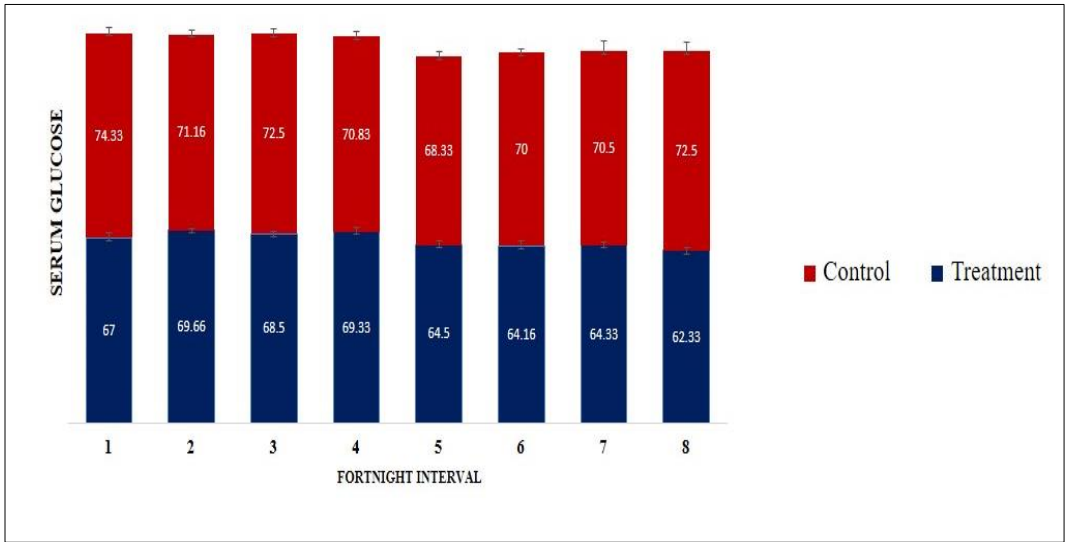

Fig 4: The variation in serum glucose level at fortnight intervals for both the treatment and control groups

Lastly, serum glucose levels were significantly lower in the control group compared to the treatment group, particularly during periods of high air temperature. Heat stress stimulates cortisol release, promoting gluconeogenesis and glycogenolysis, initially increasing glucose levels but eventually depleting glucose reserves through muscle protein breakdown for energy (Baumgard and Rhoads, 2013) [2]. Heat-induced insulin resistance further impairs glucose uptake by tissues, reducing serum glucose levels despite its presence in the bloodstream. Increased respiratory rate during heat stress also increases glucose demand for energy, leading to its decreased serum levels. Redistribution of blood flow away from internal organs, reduced dry matter intake, and oxidative stress during heat stress further contribute to the decline in serum glucose levels (Sejian *et al*., 2018) [20].

4. Conclusion

As the climate niche occupied by domesticated livestock becomes hotter and wetter, the risks of heat stress will become more widespread, making adaptation essential. During heat stress in pigs, an increased level of total protein (TP), total cholesterol (TCHO), and urea nitrogen is evident, with a transient decline occurring as the air temperature decreases. Relative humidity (RH) appears to have a negligible impact on these alterations. However, glucose levels are estimated to be lower during heat stress in pigs, with RH having a noticeable influence. The major factors contributing to the changes in serum total protein, urea nitrogen, glucose, and total cholesterol include hemo-concentration, a high rate of protein catabolism, the generation of oxidative free radicals, reduced lipolytic capacity, augmented cortisol release, and heat-induced insulin resistance. Evaluating these metabolic variations can assist in the early detection of stress-related events, as blood-level reactions to heat stress may occur before noticeable behavioral or physiological alterations.

5. CRediT authorship contribution statement

Argana Ajay, Sakshi Vaishnav, Tapendra Saini, Babita Mishra & Ajoy Das: Conceptualization, Investigation, Methodology, Validation, Statistical analysis, Writing – original draft, Writing – review & editing. Ayon Tarafdar & Anuj Chauhan: Conceptualization, Funding acquisition, Methodology, Writing – review & editing, Validation.

6. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. Data availability

Data will be made available on request

8. Acknowledgement

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