Research Article

Changes in Leukocyte Indices of Holstein Cows Under Prolonged Heat Stress Conditions

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This study assessed the effects of prolonged heat stress (HS) on leukocyte indices in Holstein cows. Blood samples from 18 multiparous Holstein cows were analyzed: a hyperthermia group (HYP, n = 8) exposed to THI ≥ 72 and a control group (CON, n = 10) under thermal comfort. Integral leukocyte indices, including the Nuclear Shift Index (NSI), Neutrophil-to-Monocyte Ratio (NMR), and Lymphocyte-Granulocyte Index (LGI), were calculated using validated clinical methodologies. Results revealed a 2.2-fold increase in band neutrophils (p=0.0035) and a 78.7% elevation in the NSI (p=0.0246) in response to HS. In contrast, lymphocyte and monocyte counts decreased by 23.7% (p=0.0404) and 42.1% (p=0.0183), respectively, accompanied by significant declines in adaptive capacity indices such as the LGI and the Index of Adaptation by Garkavi (IAG). These findings highlight the physiological trade-offs in immune responses under HS, emphasizing the utility of leukocyte indices as biomarkers for assessing thermal stress impacts. Developing strategies to mitigate HS-induced effects is crucial for enhancing the welfare and productivity of dairy cows.

Introduction

Heat stress (HS) challenges dairy farming by affecting cow health, metabolism, and immunity, reducing productivity and welfare[1]. Elevated environmental temperatures, particularly during prolonged heat waves, induce systemic stress responses that involve dynamic changes in leukocyte populations and immunometabolic indices[2][3]. Despite the growing use of non-invasive heat stress assessments, blood parameters remain reliable biomarkers of physiological status under hyperthermia[4][5].

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Prolonged heat stress in dairy cows is typically defined as THI ≥ 72 for at least three to five consecutive days, leading to systemic physiological and metabolic shifts[6]. However, the long-term effects of chronic exposure remain less understood. In this study, prolonged HS is considered as continuous exposure to THI ≥ 72 for at least 45 consecutive days, including multiple heatwave episodes, with the most recent lasting five days before blood sampling.

Leukocytes play a pivotal role in the immune response to heat stress by modulating both innate and adaptive immunity. Hyperthermia increases neutrophils, particularly band neutrophils, signaling acute inflammation[7][8]. This neutrophilic response, often accompanied by a left shift in the leukocyte formula, underscores the prioritization of rapid innate immune mechanisms over slower adaptive responses during thermal stress[9]. Additionally, the Nuclear Shift Index (NSI) and the Neutrophil-to-Monocyte Ratio (NMR) are significantly elevated, highlighting systemic inflammatory activation[10].

Conversely, lymphocyte counts tend to decrease during heat stress, reflecting stress-induced lymphopenia. This reduction is attributed to glucocorticoid-mediated redistribution of lymphocytes from circulation to peripheral tissues[8]. Changes in leukocyte indices, such as the Lymphocyte-Granulocyte Index (LGI) and the Index of Adaptation by Garkavi (IAG), further demonstrate the shift from adaptive to innate immune dominance[11]. These changes are consistent across studies in various species, emphasizing the conserved nature of stress-induced immunological adjustments[12].

Heat stress also influences the metabolic profile of dairy cows. Elevated non-esterified fatty acids (NEFA) and total proteins indicate enhanced lipolysis and protein catabolism, which provide alternative energy sources during prolonged thermal challenges[13][3]. These metabolic adaptations, while beneficial in the short term, may exacerbate systemic inflammation and oxidative stress[14]. Notably, the reduction in antioxidant defense mechanisms, coupled with increased reactive oxygen species (ROS), suggests a heightened risk of cellular damage under HS[15][16].

The interplay between leukocyte dynamics and metabolic shifts underscores the complexity of the heat stress response. Hyperthermia-induced changes in leukocyte survival and phagocytosis, as observed in both bovine and buffalo leukocytes, highlight the species-specific nuances in immune function during thermal stress[17]. Moreover, transcriptomic analyses have revealed that genes involved in inflammation and thermotolerance are differentially expressed in leukocytes under HS conditions, providing insights into molecular mechanisms underpinning these responses[10].

Leukocyte indices provide a comprehensive assessment of immune function, integrating multiple leukocyte parameters into a single metric. This allows for a more sensitive evaluation of systemic stress responses compared to individual leukocyte counts. Although these indices have been widely used in veterinary diagnostics, their potential in assessing prolonged heat stress remains underexplored.

Understanding these mechanisms is essential for improving animal welfare and productivity in heat-stressed environments. Therefore, this study evaluates the effects of prolonged heat stress on leukocyte indices, with a particular focus on shifts in innate and adaptive immunity. We hypothesize that extended exposure to heat stress leads to a significant increase in neutrophilic activity and a decline in adaptive immune responses, which can serve as potential biomarkers for thermal stress assessment in dairy cows.

Materials and methods

Experimental Design

Eighteen multiparous lactating Holstein cows in their second or third lactation were randomly assigned to one of two groups using a simple randomization procedure. The randomization was performed by assigning each cow a unique identification number, followed by a computer-generated random sequence to allocate them into either the hyperthermia group (HYP, n = 8) or the control group (CON, n = 10). The cows in both groups were selected to have similar days in milk (DIM) (LSM ± SE: HYP 130.2 ± 3.13 vs. CON 130.5 ± 2.81; $p > 0.05$) and comparable average daily milk yield (HYP 24.8 ± 0.48 kg vs. CON 24.6 ± 0.45 kg; $p > 0.05$), ensuring homogeneity between groups at the start of the experiment. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Commission on Bioethics of the Dnipro State Agrarian and Economic University (protocol No. 5 dated 29 May 2018) and the requirements for humane treatment of animals.

Housing and Feeding Conditions

The study was conducted on a commercial dairy farm located in the Dnipropetrovsk Oblast, Ukraine (48°28′44″ N, 35°36′46″ E). Dairy cows were housed in a naturally ventilated barn using a loose housing system. Sand was utilized as bedding in the cubicles, providing improved hygiene and comfort for the animals.

All cows were fed a corn silage-based total mixed ration (TMR) throughout the year. The diets were balanced for essential nutrients following the National Research Council[18] guidelines. The TMR

composition included high-quality feedstuffs such as barley, oat, and corn grains, alfalfa silage, cereal hay, wheat straw, rapeseed, sunflower, and soybean meals, as well as dried beet pulp and mineral-vitamin supplements. The ingredients were thoroughly mixed in specialized mixers equipped with electronic scales to ensure homogeneity. Feeding frequency and rationing were monitored and controlled using a computerized system.

The barn was equipped with a feeding alley and six water troughs, which were freely accessible to the cows, ensuring constant availability of feed and water. This housing and feeding setup provided optimal conditions to maintain the physiological state and productivity of the animals.

Environmental Conditions

The thermal environment within the barn was monitored using a thermohygrometer (Benetech GM 1360, Shenzhen Jumaoyuan Science and Technology Co., Ltd., Shenzhen, China). Key environmental parameters, such as air temperature and relative humidity, were recorded to calculate the temperature-humidity index (THI), which served as an indicator of heat stress (HS). The THI was determined using Kibler's (1964) formula[19]. Based on previous studies[6], prolonged heat stress in dairy cows is defined as exposure to THI levels exceeding 72 for at least three to five consecutive days. In the present study, cows in the hyperthermia group (HYP) experienced a minimum of 45 consecutive days of THI $\geq$ 72, with recurrent heatwave episodes. The most recent heatwave lasted five days, while a more distant heatwave extended over nine days. These extended periods of heat stress served as the basis for evaluating leukocyte indices under prolonged hyperthermic conditions.

Blood samples from HYP cows were collected on day 5 of a 10-day heatwave, following 45 consecutive days of heat stress. This period included multiple heatwaves, the most recent lasting five days, and a more distant heatwave lasting nine days. At the time points of blood sampling from HYP cows, the minimum THI in the barn was 77.9, with values ranging from 77.9 to 78.6. During the HYP period, cows were exposed to elevated THI conditions for at least 8–10 hours per day, primarily during midday and early afternoon (10:00 AM – 6:00 PM), followed by partial nocturnal cooling. Daily air temperatures during the HYP period reached a maximum of 34°C, accompanied by low relative humidity (26%). Conversely, in October, the control (CON) group was maintained under thermal comfort conditions, with a THI consistently below 68. Blood samples were collected 42 days after the last heat wave (lasting eight days) and 21 days after the last day with heat stress conditions (THI $\geq$ 72). During the control period, maximum daily air temperatures reached 19°C, with relative humidity ranging between 30% and 35%,

corresponding to a THI of 63.1, which is described in more detail in our previous paper[6]. These differences in climatic conditions between the experimental periods provided a basis for evaluating the impact of environmental stress on leukocyte indices and other physiological parameters.

Determination of Indicators

Blood samples were collected by puncturing the jugular vein and directly filling 2-ml EDTA Vacutainer® tubes (Aichele Medico AG, Basel, Switzerland). The blood analysis included the determination of the total leukocyte count (WBC) and the leukocyte formula. The total leukocyte count was measured using an automatic hematology analyzer Sysmex XS-1000i (Sysmex Corporation, Japan). The leukocyte formula was determined by microscopic examination of blood smears stained using the Romanowsky-Giemsa method. A total of 200 cells were counted per smear, including band and segmented neutrophils, lymphocytes, monocytes, eosinophils, and basophils. To assess systemic immune responses and adaptive capacities, integral leukocyte indices were calculated using standard clinical methodologies[20]. These indices included the Nuclear Shift Index (NSI), which evaluates the ratio of band neutrophils to segmented neutrophils, the Leukocyte Index (LI) reflecting general leukocyte activity in systemic inflammation, and the Leukocyte Index of Intoxication by Kalf-Kaliph (LII), which serves as a marker of endogenous intoxication. Additionally, the Lymphocyte-Granulocyte Index (LGI) was used to determine the immune balance between lymphocytes and granulocytes, while the Index of Neutrophil to Lymphocyte Ratio (NLR) was applied as a systemic inflammation marker. Other indices included the Index of Lymphocyte to Eosinophil Ratio (LER), an indicator of allergic sensitivity, the Index of Neutrophil to Monocyte Ratio (NMR) to assess innate immune activation, and the Index of Lymphocyte to Monocyte Ratio (LMR), which provides insight into adaptive immunity. Furthermore, the Index of Allergenization (IA) was evaluated as a marker of potential allergic responses, the Index of Adaptation by Garkavi (IAG) was used to assess adaptation to environmental stressors, and the Index of Immune Reactivity (IIR) served as a general measure of immune functionality and resilience.

Statistical analysis

Initially, the data were presented as mean values (Mean) and the standard error of the mean (SE). To determine whether the data followed a normal distribution, the Shapiro-Wilk test was performed for all variables. Since most indicators did not meet normality criteria ($p < 0.05$ in multiple cases), non-parametric statistical methods were employed for further analysis.

Significant differences between groups were determined using the Mann–Whitney U test, which was selected as a non-parametric alternative to the T-test for comparing mean ranks between independent samples. This approach was chosen because it ensures robust statistical inference in cases where data deviate from normality and sample sizes are relatively small. The probability level of $p < 0.05$ was considered statistically significant.

Statistical processing was performed using Statistica 12 (StatSoft, Inc., Tulsa, OK, USA).

Results

Changes in Leukocytes

Under hyperthermia group (HYP), the total leukocyte count (WBC) increased by 17.49% compared to the control group (CON), though this difference was not statistically significant (Table 1). The increase in WBC is likely associated with the activation of neutrophils, which play a critical role in the immune system's response to stress. Notably, there was a pronounced increase in band neutrophils, which was 2.2 times higher in HYP compared to CON ($p = 0.0035$). This significant mobilization of immature neutrophils reflects an enhanced inflammatory response to thermal stress. The NSI, which evaluates the ratio of band to segmented neutrophils, also increased by 78.7% in HYP ($p = 0.0246$). This indicates a heightened neutrophil activation and a shift in the immune response towards rapid inflammatory activity, with band neutrophils acting as the first line of defense against external stressors.

Indicator	Experimental groups		p-value
	HYP	CON	
White blood cells, cells/μL	11.77±4.013	10.02±1.518	0.2144
Types of leukocytes, %			
Band	4.58±2.503	2.08±1.443	0.0035
Segmented	55.83±10.894	45.42±10.378	0.0525
Monocytes	2.75±1.422	4.75±2.261	0.0183
Lymphocytes	34.42±9.995	45.17±11.158	0.0404
Eosinophils	4.50±2.664	3.87±1.959	0.7905
Integral leukocyte indices			
Nuclear shift index	0.084±0.049	0.047±0.035	0.0246
Leukocyte index	0.24±0.154	0.16±0.185	0.3701
Leukocyte index of intoxication by Kalf-Kaliph	0.23±0.158	0.15±0.075	0.4689
Lymphocyte-granulocyte index	0.61±0.250	1.05±0.02	0.0528
Index of neutrophil to lymphocyte ratio	2.09±1.328	1.16±0.498	0.0529
Index of lymphocyte to eosinophil ratio	11,96±8.479	13.57±6.716	0.5612
Index of neutrophil to monocyte ratio	26.36±10.661	12.06±5.157	0.0035
Index of lymphocyte to monocyte ratio	14.23±5.822	13.04±9.902	0.2851
Index of allergization	0.11±0.089	0.09±0.036	0.9537
Index of adaptation by Garkavi	0.66±0.270	1.15±0.526	0.0139
Index of immune reactivity	15.19±6.334	13.85±10.537	0.2919

Table 1. Impact of prolonged heat stress on leukocyte blood profiles in Holstein cows

Note. The table presents the values of indicators and the significance of differences between the HYP and CON groups (p-value), determined using the Mann–Whitney U test.

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Linking Leukocyte Changes with Indices

The dominance of neutrophils under thermal stress is further highlighted by the doubling of the NMR in HYP ($p = 0.0035$). This reflects a significant reduction in monocyte levels, which decreased by 42.1% in HYP ($p = 0.0183$). The shift away from monocyte activity under HS reallocates the immune system's resources towards the faster and more immediate neutrophil-driven immune pathway. Similarly, lymphocyte levels decreased by 23.7% in HYP compared to CON ($p = 0.0404$), which is typical of stress responses that favour neutrophilic over lymphocytic activity. The LGI decreased by 41.9% in HYP ($p = 0.0528$), indicating a trend towards a marked shift from lymphocytic to granulocytic dominance. Although this result did not reach statistical significance, it suggests that the immune system may prioritize faster and more effective responses during thermal stress.

The LI and the LII did not exhibit statistically significant differences between HYP and CON, indicating that compensatory mechanisms within leukocyte subpopulations might mitigate the overall impact on these indices. However, the IAG, which assesses the organism's adaptive capacity, showed a substantial reduction of 1.3 times (75%) in HYP compared to CON ($p = 0.0139$). This decrease underscores the organism's diminished ability to adapt to prolonged high-temperature exposure, highlighting the strain imposed on its physiological and immune systems. In addition, the IIR was virtually unchanged. This indicates an overall decline in the immune system's capacity to respond to stressors under hyperthermic conditions.

Collectively, these findings reveal a clear pattern: while neutrophilic activity increased significantly to provide a rapid response to thermal stress, other immune components, such as lymphocytes and monocytes, were suppressed.

Significant dispersion of indices in the HYP group underscores the importance of assessing not only mean values but also the range of variation as an indicator of individual variability. The high disparity may be attributed to differences in cows' heat tolerance, their adaptive capacity, and the mobilisation of neutrophil reserves under stress conditions. For instance, the Nuclear Shift Index (NSI) ranged from 0.03 to 0.2, indicating substantial variation in the activation of neutrophils during heat stress. Similarly, the Leukocyte Index (LI) exhibited a broad range (0.09 to 0.63), reflecting heightened immune activity in response to prolonged thermal stress. The Lymphocyte-Granulocyte Index (LGI) fluctuated between 0.2 and 0.92, suggesting varying degrees of lymphocytic suppression and granulocytic dominance among individuals. The most pronounced variability was observed in the Index of Neutrophil to Monocyte Ratio

(NMR), which ranged from 8 to 41, highlighting individual differences in innate immune activation under hyperthermic conditions. This substantial variation in leukocyte indices suggests that heat stress does not elicit a uniform immune response but rather triggers heterogeneous physiological adaptations influenced by genetic predisposition, prior exposure to heat stress, and individual resilience.

Discussion

Heat stress (HS) alters leukocyte profiles, reflecting adaptive shifts between innate and acquired immunity. During HS, a substantial increase in neutrophil counts, particularly band neutrophils, was observed. This aligns with findings by Koch et al.[8], who reported upregulated pro-inflammatory markers, such as TNF-α and IFN-γ, and heightened neutrophil activity under heat stress. The observed increase in the NSI by 78% and the twofold rise in the NMR highlight the dominance of neutrophilic responses in acute stress conditions. This response is likely an adaptive mechanism aimed at mitigating systemic inflammatory challenges triggered by prolonged hyperthermia.

HS-induced lymphopenia and monocyte reduction align with Joo et al.[7], who linked these changes to cortisol-driven immune suppression, favouring neutrophilic activation over adaptive responses. These changes were further reflected in the LGI, which decreased by 41.9%, emphasizing the shift towards granulocytic dominance. Similarly, the 42.6% reduction in the IAG suggests compromised adaptive capacity under thermal stress.

Comparative studies across species reveal similar stress-induced patterns. For instance, Radsikhovskii et al.[21] observed substantial increases in neutrophil-dominated indices under thermal stress in laying hens, highlighting the conserved nature of such responses. Additionally, Minias[22] emphasized the utility of heterophil-to-lymphocyte (H/L) ratios as indicators of physiological stress in birds, findings that parallel the observed leukocyte profile changes in heat-stressed dairy cows. This cross-species similarity suggests that heat stress-induced shifts in leukocyte populations follow a fundamental immunological response, prioritizing innate immunity over adaptive mechanisms to cope with thermal challenges.

Blond et al.[16] demonstrated that prolonged heat stress correlates with increased pro-inflammatory markers and reduced adaptive immunity, findings that align with the significant reductions in the LMR and LGI in our study. These results reinforce the concept of immune system prioritization under stress, favouring mechanisms that address immediate threats at the expense of adaptive and long-term immune functions.

The association between leukocyte indices and conventional biomarkers of HS, such as cortisol, glucose levels, and oxidative stress markers, underscores their diagnostic potential. Previous studies have demonstrated that HS elevates cortisol concentrations, leading to immunosuppressive effects that manifest as lymphopenia and increased neutrophilic activity[23]. Furthermore, metabolic stress responses, including increased glucose utilization and altered insulin sensitivity, correlate with shifts in leukocyte profiles, particularly the Neutrophil-to-Lymphocyte Ratio (NLR) and Nuclear Shift Index (NSI). Elevated oxidative stress levels, as demonstrated by Mylostyva et al.[24], further corroborate the role of leukocyte indices in assessing systemic stress responses. Increased ROS production under HS conditions may impair leukocyte function, exacerbating immune shifts towards innate immunity and reducing adaptive responses.

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Elevated systemic inflammation during HS often coincides with metabolic shifts, including increased lipolysis and protein catabolism, as reported in studies on Dazu black goats[13]. These metabolic changes provide the energy necessary to sustain the immune response, explaining the observed neutrophil proliferation and lymphocytic reductions.

The methodology for calculating leukocyte indices is well-documented in Lomako and Shylo[25]; however, we believe that further modifications are necessary to enhance their applicability for assessing immune system shifts under prolonged heat stress conditions. Our recent findings indicate that specific leukocyte indices, particularly NSI and LGI, may serve as more precise biomarkers for thermal stress-induced alterations in immune function. Future studies should refine these indices by incorporating additional immunophysiological markers relevant to hyperthermia-induced inflammatory responses.

Overall, heat stress induces a profound systemic shift in leukocyte profiles and indices, prioritizing rapid neutrophil-driven responses while suppressing adaptive immunity. These changes, while necessary for immediate survival, may compromise long-term immune competence, emphasizing the need for management strategies that mitigate the adverse effects of hyperthermia in dairy cows.

A limitation of this study is the relatively small sample size, which may restrict the generalizability of the findings to larger populations. Additionally, variations in individual heat tolerance among cows could have influenced the immune response and leukocyte dynamics observed in this study. While all cows were kept under similar management conditions, slight differences in barn microclimates, feed intake variability, and genetic predisposition to thermotolerance may have contributed to the observed immune responses. Furthermore, other potential confounding factors, such as prior exposure to heat stress in earlier lactations and underlying health conditions, could not be entirely ruled out. These factors should be considered in future studies to refine our understanding of leukocyte index variations under prolonged heat stress conditions.

Future research involving a greater number of animals is necessary to validate these results and further explore the observed relationships under varying environmental and management conditions.

Conclusions

Prolonged heat stress in Holstein cows alters immune function, promoting innate immune dominance while suppressing adaptive responses. Leukocyte indices serve as key biomarkers for stress evaluation, highlighting the need for mitigation strategies. To minimize the negative impact of heat stress on the immune system of dairy cows, it is recommended to implement environmental cooling strategies such as increased ventilation, misting systems, and shade structures. Additionally, nutritional adjustments, including the supplementation of antioxidants and electrolytes, may enhance adaptive capacity under heat stress conditions.

This study focused exclusively on Holstein cows, which are known for their high milk yield but also increased sensitivity to thermal stress. While the observed leukocyte shifts provide valuable insights, breed-specific differences in thermotolerance should be considered. Future studies should investigate whether similar immune responses occur in heat-adapted breeds, such as Jersey or indigenous cattle, to refine stress adaptation strategies.

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