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Acute thermal exposure increases neutrophil lymphocyte ratio of Sahiwal heifers

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Abstract
A study was carried out to find out the effect of temperature humidity index on the neutrophils lymphocyte ratio of Sahiwal heifers in five different periods. The five different temperature conditions maximum ambient temperature/minimum ambient temperature (Tmax/Tmin) were P1: <20 ºC/<10 ºC; P2: >20 ºC/<10 ºC, P3: >25 ºC/<15 ºC; P4: >35 ºC/<20 ºC and P5: >35 ºC/>20 ºC. The study further investigated the effect of acute thermal exposure on neutrophil lymphocyte ratio of Sahiwal heifers. Exposure was done at two intervals *viz.* Exposure I at 40 ºC and 50%RH and Exposure II at 45 ºC and 50% RH. Thermal exposure was done in the climatic chamber for 4 consecutive hours. Neutrophil leukocyte ratio was calculated from differential leukocyte count of blood smears stained with Leishman's stain. Neutrophil lymphocyte ratio of Sahiwal was found to be stable throughout the year in natural climatic conditions. Exposure at at 40 ºC and 50%RH in climatic chamber caused a significant increase in the neutrophil lymphocyte ratio of Sahiwal. The study revealed good thermotolerance of Sahiwal cattle in natural climatic conditions. In response to acute thermal stress Sahiwal heifers showed an increase in neutrophil lymphocyte ratio. The study indicated neutrophil lymphocyte ratio to be a heat stress marker.

Keywords: cold, heat, Sahiwal, neutrophil lymphocyte ratio, stress

1. Introduction
The haematological parameters have been a measure for the detection and diagnosis of various diseases in animals. Factors contributing to variation in haematological parameters include age, sex, stress, diet, body condition, reproductive status, recent activity, hydration, ambient temperature, and altitude [1]. Animals continuously experience some degree of physiological stress due to their exposure to inevitable stressors like extremes of temperature that may be in the form of heat during summer or cold during winter. A study of 2010 on comparison of reference intervals of healthy North American cows from 1957 to 2006 indicated a significant increase in neutrophil count whereas reference intervals for lymphocyte, monocyte, and eosinophil counts as well as hemoglobin concentration had decreased [2].
The neutrophil lymphocyte ratio in adult cattle of different breeds and origins is approximately 1:2 [3-5]. In presence of stress, the leukogram of cattle indicated neutrophilia [4] and lymphopenia [6]. Neutrophil lymphocyte ratio had been indicated to be a marker of acute [7] and chronic [8] stress in laboratory animals, several other types of stress in cattle [9] and long-term stress in pigs [10]. However, there is no report so far on neutrophil lymphocyte ratio of Sahiwal cattle both in natural climatic conditions and in response to thermal exposures.
The present study was carried out to find out the effect of temperature humidity index on the neutrophil lymphocyte ratio of Sahiwal heifers. The study further investigated the effect of acute thermal exposure on neutrophil lymphocyte ratio of Sahiwal heifers.

2. Materials and Methods
2.1 Rearing and maintenance of animals
A total of 30 Sahiwal heifers were selected from the herd maintained at National Dairy Research Institute, Karnal (NDRI), Karnal. The animals were in the age group of 2-2.5 years and the average body weight was 301.3±6.91kg. The animals were fed an ad lib roughage and water as per Kearl standard [11]. Maintenance ration of concentrate mixture (12%CP and 60%TDN) consisting of mustard cake, maize, wheat bran, rice bran, mineral mixture and salt was fed @1kg/animal.

2.2 Study in natural climatic condition:
Five different combinations of maximum ambient temperature (Tmax) and minimum ambient temperature (Tmin) were selected based on the climatograph prepared from the records of past 10 years. The conditions for five different temperature conditions (Tmax/Tmin) were P1: <20 ºC/<10 ºC; P2: >20 ºC/<10 ºC, P3: >25 ºC/<15 ºC; P4: >35 ºC/<20 ºC and P5: >35 ºC/>20 ºC.

2.3 Record of temperature and temperature humidity index
Prevailing temperature in Karnal as recorded at Central Soil Salinity Research Institute (CSSRI), Karnal were obtained. Everyday records of minimum ambient temperature, maximum ambient temperature, dry bulb and wet bulb temperature recorded at I: 0722\ 0830 and II: 1422 h IST were obtained for 1 year. The temperature humidity index (THI) as proposed by National Research Council (12) was calculated by using dry bulb (db) and wet bulb (wb) temperatures in the following formula.

$$THI = 0.72(Tdb + Twb) + 40.6$$

2.4 Thermal exposure in climatic chamber
Sahiwal heifers were kept in a climatic chamber (22'6''x10'10''x8') which was insulated and thermostatically fitted with a heat convector for thermal exposure. Before initiation of thermal exposure animals were kept in the climatic chamber at the prevailing temperature for 4h every day for 10 days to accustom to the chamber environment. Thermal exposure was done at two intervals. Exposure I was done at 40±1 ºC, 50% relative humidity (RH) during P3. Exposure II was done at 45±1 ºC, 50% RH during P4. Thermal exposure was carried out for 4h continuously.
The THI during the thermal exposures was calculated (13) by using ambient temperature (T) and relative humidity (H) in the following formula.

$$THI = 0.08T + RH\frac{T - 14.4}{100} + 46.4$$

2.5 Blood sampling
Blood samples were collected from each of the animals in natural climatic conditions in 5 different periods. Blood samples were also collected from the animals in the thermal exposure at 0h, 1h, 2h, 3h and 4h of thermal exposure. A few drops of blood samples were collected from the jugular vein by using Di-sodium salt of EDTA coated vacutainer tubes with needles.

2.5 Calculation of neutrophil lymphocyte ratio
A small drop of freshly collected blood was placed in the central line of a slide about 1-2 cm, from one end. A spreader slide with a smooth edge was placed at an angle of 45° onto the slide and then moved back to the blood drop. The drop was spread out along the line of contact of the spreader slide. The blood film was dried by waving the slide once in the air. Leishman stain (0.15% in methyl alcohol) was poured enough on the smear to cover it fully and allowed to act for 2 min. Distilled water was added twice the amount of stain and allowed to mix thoroughly by blowing air onto the mixture. Staining was done for 10 min. The smear was washed in the running tap water until the colour stops draining. It was allowed to dry in air by keeping the slide in an inverted position with the broad end of the film up.
Differential leukocyte count (DLC) was done by using oil emersion objective at 100x of an inverted binocular light microscope in a strip running the whole length of the film. The lateral edges of the film were avoided. The film was inspected from head to tail. Approximately more than 200 cells were counted from one slide. DLC was expressed in %. From the result of DLC neutrophil lymphocyte ratio was calculated.

2.6 Statistical Analysis
The data were analyzed by using SPSS 16 version. One way analysis of variance was carried to find out the difference between different periods and between different hours of thermal exposure

3. Results ad Discussion
Average ambient temperatures and THI during different temperature conditions and thermal exposures in the climatic chamber have been presented in Tables 1 and 2 respectively. The neutrophil lymphocyte ratio of Sahiwal heifers in natural climatic conditions and thermal exposure have been presented in Tables 3 and 4 respectively.

3.1 Ambient temperature and THI
The lowest Tmin and lowest average ambient temperature (Tav) were recorded in P1. The highest Tmax and the highest Tav were recorded in P5. Heat stress in dairy cattle starts at THI of 72 which corresponds to 22 ºC at 100% humidity, 25 ºC at 50% humidity, or 28 ºC at 20% humidity [14]. In the present study, the average THI recorded in P4 and P5 were higher than 72 indicating the presence of heat stress. THI >72 was found to cause thermal stress in Sahiwal and Karan-Fries in the form of changes in physiological indices [15, 16]. Experience of thermal stress was also evident in Sahiwal and Karan -Fries cattle during THI >72 by presence of an increase in expression of heat stress markers (HSP70) in Sahiwal and Karan-Fries cattle [16] and antioxidant enzyme genes (Cu-SOD and Mn-SOD) in Sahiwal and Karan-Fries cattle [15].

Table 1: Ambient temperature and temperature humidity index in different periods

Parameters		Periods				
		P1	P2	P3	P4	P5
T	Max	16.70	20.40	30.80	36.00	37.79
	Min	2.50	9.00	13.60	17.80	23.16
	Average	9.60	14.70	22.20	26.90	30.48
T_{db} (ºC)	Max	18.40	19.80	30.50	36.20	35.73
	Min	3.60	10.40	14.90	20.50	27.00
	Average	11.00	15.10	22.70	28.35	31.37
T_{wb} (ºC)	Max	11.40	14.80	20.10	20.70	24.45
	Min	2.80	9.50	14.30	17.40	22.98
	Average	7.10	12.15	17.20	19.05	23.72
THI	Max	62.05	65.51	77.01	81.51	83.93
	Min	45.21	54.98	61.62	67.86	76.58
	Average	53.63	60.25	69.32	74.69	80.26

The THI recorded inside the climatic chamber during thermal Exposure I and II were below the stress level as compared to previous records [14]. However, acute thermal exposure at such THI levels was found to cause thermal stress in Sahiwal and Karan-Fries cattle and the same was detected by presence of higher expression of heat stress marker HSP70 mRNA [17] and HSP70 protein [16].

Table 2: Ambient temperature and temperature humidity index during thermal exposures

Exposure	Climatic chamber			Environment					
	T (ºC)	RH (%)	THI	Tmax	Tmin	Tav (ºC)	Tdb (ºC)	Twb (ºC)	THI
Exposure I	40±1	50	62.4	30.80	13.60	22.20	22.70	17.20	69.32
Exposure II	45±1	50	65.3	36.00	17.80	26.90	28.35	19.05	74.69

3.2 Neutrophil lymphocyte ratio

During different periods (P1-P5) neutrophil lymphocyte ratio of Sahiwal ranged from 0.36±0.02 to 0.43±0.03 which was well within the normal range [3-5]. There was no significant change in neutrophil lymphocyte ratio of Sahiwal during different periods. In Holando Argentino cows, neutrophil lymphocyte ratio was found to increase in summer [18]. During P4 and P5 of present study, THI as high as 74.69 and 80.26 respectively were recorded in Karnal. However, the present study found no effects of high THI on neutrophil lymphocyte ratio. Maintenance of stable neutrophil lymphocyte ratio throughout the year even during the periods of extremes of temperature in winter and summer was indicative of good thermotolerance of Sahiwal in the prevailing climatic conditions.

Table 3: Neutrophil lymphocyte ratio (mean± SEM) of Sahiwal heifer during different periods

P1	P2	P3	P4	P5
0.36±0.02	0.38±0.02	0.430.03	0.37±0.01	0.41± 0.01

When Sahiwal heifers were subjected to acute thermal stress in climatic chamber, neutrophil lymphocyte ratio was found to change significantly with the time of thermal exposure. During Exposure I, neutrophil lymphocyte ratio was found to increase significantly after 2 h of thermal exposure and thereafter it remained stable till the end of exposure for 4 h. It was notable that, the existing Tmax/Tmin and THI in the environment when the animals were in Exposure I were 30.80 ºC/13.60 ºC and 69.32 respectively. At this time the animals were not under the stress before thermal exposure. On sudden thermal exposure in Exposure I with 40±1 ºC and 50% RH the animals experienced acute thermal stress. Upon exposure to acute thermal stress there was significant change in neutrophil lymphocyte ratio. The increase in neutrophil lymphocyte ratio after thermal exposure was caused by thermal stress. In cattle, several types of stress *viz.* post parturient stress [19], exercise stress [20], abrupt weaning [9] and a sole ulcer [21] were found to increase neutrophil lymphocyte ratio.

On the other hand, during Exposure II, there was a stable neutrophil lymphocyte ratio for 3h of thermal exposure and thereafter it decreased after 4h. It was evident that there was already presence of thermal stress in the environment with the existing Tmax/Tmin and THI of 36.00ºC/17.80 ºC and 74.62 respectively. When the animals were already stressed there was presence of higher ranges of neutrophil lymphocyte ratio prior to thermal exposure. The neutrophil lymphocyte ratio remained high for 3h and thereafter decreased after 4h of exposure. The neutrophil lymphocyte ratio recorded after 4h was however not different significantly from the value recorded prior to thermal exposure.

The present finding indicated that Sahiwal exhibited increase in neutrophil lymphocyte ratio in presence of acute thermal stress. There was no collateral finding available in the literature.

Table 4: Neutrophil lymphocyte ratio (mean± SEM) of Sahiwal heifer during thermal exposure

Exposure	0h	1h	2h	3h	4h
I	0.36^{a} ±0.02	0.36^{a} ±0.01	0.51^{b} ±0.02	0.48^{b} ±0.02	0.49^{b} ±0.01
II	0.48^{ab} ±0.01	0.50^{b} ±0.01	0.52^{b} ±0.01	0.50^{b} ±0.01	0.45^{a} ±0.03

^{a,b} Significant differences between means in the same row.

4. Conclusion

The study found good thermotolerance of Sahiwal cattle in winter as well as summer. Sahiwal heifers maintained a stable neutrophil lymphocyte ratio in natural climatic conditions even in the extremes of cold and hot in winter and summer. Acute thermal exposure caused thermal stress to Sahiwal heifers. Presence of thermal stress was detected by an increase in neutrophil lymphocyte ratio in Sahiwal heifers.

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