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A survey on the effect of plasma vitamin C on white blood constituents under heat stress condition for dairy cows

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1 ABSTRACT

The objective of this study was to investigate the effect of seasonal change in thermal environment on plasma vitamin C (VC) concentration, white blood constituents and their relationship in heat stress conditions, in dairy cows under Mediterranean climate conditions of north-west of Tunisia. Two experiments were carried in two different periods: spring P_1 (from 1st February to 15th March) and summer P_2 (from 1st to 30 August), using 48 Holstein cows. Cows were classified according to level milk production either high or low productive cows (HPC, LPC). Mean Temperature Humidity Index THI values were 65.62 ±1.32 and 83.27 ±1.90 in P_1 and P_2, respectively. Leucocytes cell counts and VC concentrations were affected by test period (*P*<0.001). However, Decrease in plasma VC concentration in P_2 was positively correlated to lymphocytes and neutrophils (*P*<0.01) and negatively correlated to eosinophils (*P*<0.05) and monocytes (*P*<0.01) in HPC and LPC .The coefficient of determination R^2value suggested that a large part of the variation leucocytes percentages could therefore be attributed to decrease in plasma VC in heat stress. However, the decrease of VC concentration during summer compared to spring could be considered as an evidence of the suppression of cows' immune system under heat stress.

2 INTRODUCTION

In dairy cows, several empirical observations suggest that stressful environmental conditions alter host resistance. A recent study on dairy cows (Rejeb (a) *et al*., 2016) indicates that a large reduction in plasma VC concentration was reported in dairy cows stressed by high environmental conditions and the immune function is affected by the plasma VC level. Therefore, Kelly (1980) suggested that heat stress increases the susceptibility of many animal species to infectious diseases. In the other hand, Koubkova *et al.* (2002) described a significant rise in hemoglobin concentration and hematocrite value and found that the proportional distribution of white blood cells changes at high temperatures. It has been documented that a rising relative humidity at an elevated temperature exerted marked by the increased of physiological variable of heterophiles/lymphocytes ratio in especially layer chickens (Joachim *et al.,* 2010). Minka and Ayo (2008) found that stressed layers supplemented with VC, showed a significant increase in eosinophiles, but a non-difference in the numbers of basophiles and monocytes. So heterophiles/lymphocytes ratio has been used as a reliable indicator of stress in birds (Altan *et al.,* 2000). However, Broucek *et al.* (1984) found a decrease in neutrophiles and eosinophiles and an increase in lymphocytes and monocytes at high temperature. Furthermore, VC increases neutrophiles protection against oxidative stress induced by free radicals associated with the oxidative burst (Wolf, 1993). Goetzl *et al.* (1974) reported that the VC stimulates interferon production, which protects leucocytes responses and could be related to variation of white blood cells types at hot environmental conditions. Several studies have been carried out to asses and confirmed, that high producing cows have more metabolic activity and produce more body heat than low producers; thus, a higher milk yield may increase heat stress if the cause of stress is not mitigated (Ben Younes (a) *et al.*, 2011; Najar *et al.*, 2011) . Therefore, level milk yielding could be related to heat stress, it becomes important to know the immune response to heat stress and their relation with level milk production in dairy cows. Moreover, investigate the relationship between plasma VC concentration and leucocytes cells in high and low yielding dairy cows under significant climate changes in a Mediterranean climate in North-West of Tunisia.

3 MATERIELS AND METHODS

3.1 Animals, feeding and management: This study was carried out at BADROUNA dairy farm, Bousalem Jandouba (north-west of Tunisia) which is located 36°.6' latitude north and 8°.9' longitude west. The experiment was conducted in two different periods. Spring (P_1:15 March-15 April; mean daily THI value 63.46 ±1.62, no heat stress) and summer (P_2: 15 July-15 August; mean daily THI value 83 ±0.5, stress conditions). Sixteen multiparious and primipares lactating Holstein Friesian dairy cows (Table 1) were used. They were housed in a covered tie stall barn with straw bedding. Iron sheets covered the stalls.

Table 1: Description of cows used in experiment during the two periods P_1 and P_2

	P_1		P_2	
Animals	HPC†	LPC‡	HPC	LPC
Cows (number)	15	15	15	15
Age (months)	70 (±3)	74.5 (±1)	69.25 (±4)	73 (±2.5)
Body weight(kg)[a]	519.25 (±13)	509 (±19)	521 (±15)	519.9 (±20)
Days In Milk (days)	173 (±19.5)	172 (±16)	172.5 (±18.5)	171 (±20.5)
Number of lactation	3 (±1)	3.5 (±1)	2.5 (±1)	3 (±1)
Milk yield (kg/d)	33.11 (±1.8)	22.55 (±1.02)	28.5 (±1.92)	19.32 (±1.30)

† HPC: High producing cows;
‡ LPC: Low producing cows;
(a) Estimated according to Heinrichs *et al.*, (1992), BW = 4.134 × (HG – 318.51);
( ): Standard deviation;

The diets were typical of those in the region with forage ratio of 63%, 57% in P_1 and P_2 respectively, on dry matter (DM) basis. The concentrate (8 kg/cow/day) was fed in four equal meals daily. Food and water were available *ad libitum*. Ingredients and chemical composition of diets fed to animals during the experiment are reported in Table 2. Diets were defined including ingredients commonly used in North-west of Tunisia. Crude protein and neutral detergent fiber fibre content in diets ranged from 13.6 to 15.8% and 39.8 to 42.8% DM, respectively.

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Table2: Ingredients and chemical composition of the total mixed ration diet

	Item	P_1	P_2
Feed ingredient (%DM)	Tritical ground green forage	18.50	--
	Bersim green forage	12.20	--
	Alfalfa forage	9.0	13.20
	Oat hay	6.20	8.20
	Corn silage	17.60	--
	Oat silage	--	35.60
	Corn grain grind	--	11.5
	Soybean meal	7.70	9.20
	Barley grain	9	9.9
	Wheat bran	7	8.20
	Vitamins A, D et E	0.8	0.8
	Mineral	2	2.5
	Sodium bicarbonate	--	0.4
	Calcium phosphate	--	0.6
	Chemical composition		
	DM %	29.6	33.4
	CP (% of DM)	15.9	13.6
	Starch (% of DM)	18.7	23.4
	NDF from forages (% of DM)	80.4	76.8
	NDF (% of DM)	42.8	39.8
	NEL (Mcal/kg)	1.49	1.49
	Minerals composition		
	Ca (% of DM)	0.80	0.80
	P (% of DM)	0.50	0.60
	Fer (ppm of DM)	162.80	271.10
	Mg (% of DM)	0.30	0.30
	Cl (% of DM)	0.40	0.50
	K (% of DM)	1.90	2.10
	Na (% of DM)	0.30	0.50

3.2 Measurements, sampling, and laboratory analysis: At each test day ambient temperature, Relative Humidity, Rectal Temperature, Heart rates and Respiratory Rates Ta, RH, RT, HR and RR were recorded. Measures started at 12 p.m. and finished around 3 p.m. Ta and RH were measured using a thermo hygrometer (HI 91610C, Hanna instrument, Portugal). Estimation of THI (Temperature Humidity Index) was performed for each test day using the equation described by Kibler (Kilber, 1964). RT was measured by inserting a veterinary digital thermometer approximately 60 mm into the rectum for 60 s (precision ± 0.01 °C). The HR was determined using a medical stethoscope for one minute (breaths/minute). RR was measured by counting the flank movements of the individual cows for one-minute period of uninterrupted breathing and reported as the number of inspirations per minute (inspirations/minute). At each test day Blood samples was collected from the caudal vein puncture at approximately 1 p.m. into vacuum tubes (10 ml). In each tube, solution of EDTA (anticoagulant substance) was placed before sterilization. The samples were kept in an ice bath for a few hours until centrifugation (3000 tours/mn at 4°C) to recover plasma. Plasma vitamin C concentration (VC) was performed according to the method reported by Roe and Kuether (1942). From each blood samples collected, smears were prepared using wrights-Giesma method (Fisher Scientific Company) for differential leucocytes profile. Total leucocytes number was determined for each smears blood by light microscope.

3.3 Statistical analyses: In order to determine the effects of period (P_1, P_2) and level of milk production (high, low) on plasma vitamin C concentration and leucocytes percentage, RT, HR, RR we used to the following mixed model:

$$Y_{ij} = \mu + l_i + p_j + (l \times p)_{ij} + e_{ij} \quad (1)$$

Where y_{ij} are the measured values of vitamin C concentration, neutrophils, eosinophils, lymphocytes and monocytes, μ the mean value, p_j fixed effect of period, l_i fixed effect of level milk production, $(l \times p)_{ij}$ interaction level milk production-period and e_{ij} is the residual error. We used the following mixed model to determine the effect of vitamin C concentration and level of milk production (high, low) on neutrophils, eosinophils, lymphocytes and monocytes:

$$Y_{ij} = \mu + c_i + l_j + (c \times l)_{ij} + e_{ij} \quad (2)$$

Where Y_{ij} are the measured values of neutrophils, eosinophils, lymphocytes and monocytes ; μ the mean value, c_i fixed effect of plasma vitamin C concentration, l_j fixed effect of level milk production, $(c \times l)_{ij}$ interaction vitamin C concentration-level of milk production and e_{ij} is the residual error. All analyses were conducted using SPSS (version 17.0) (Aug 23, 2008). Differences were considered significant at $P < 0.05$.

4 RESULTS AND DISCUSSION

4.1 Environmental conditions during the experimental periods and Heat Stress Effect on physical parameters of dairy cows: Mean Ta, RH and calculated THI by the experimental period are shown in Table 3. Average values of environmental variables (Ta, RH and THI) were higher in P_1 than in P_2. Average THI recorded in P_1 was 63.46 (±1.62) and was characterized by a lack of heat stress conditions. As expected, heat stress occurred during P_2 with THI values of 83 (±0.50). This confirms the reported findings by Silanikove (2000) and Gonzalez Pereyra *et al.* (2010), which indicate that high producing cows become heat stressed when the THI index threshold for dairy cattle has been above 78. Kadzere *et al.* (2002) noted that the THI index values of 70 or less are considered comfortable. But, Thatcher *et al.* (2010) reported that lactating cows are considered unstressed when the THI is less than 72.

Table3: Average values of environmental variables (Ta, RH and THI) and physical parameters of dairy cows (RT, HR and RR) at each test period

	Test period		Effect		
	P_1	P_2	P	A	P × A
Ta (°C)	19.14 (2.02) O.n =359	36.95 (2.25) O.n=359	-	-	-
RH (%)	76.20 (0,05) O.n =359	43.05 (0.03) O.n =358	-	-	-
THI	63.46 (1.62)	83 (0.5)	-	-	-
RT (°C)	38.5 (0.07)^a O.n =2155	39.6 (0.08)^b O.n =2160	***	ns	ns
HR (Beat./min)	63 (1.22)^a O.n =1629	77.30 (1.25)^b O.n =1637	***	ns	*
RR (Insp./min)	53.75 (1.22)^a O.n =1620	80.6 (1.21)^b O.n =1637	***	ns	ns

***: $P < 0.001$; *: $P < 00.5$; ns: not significant ($P > 0.05$); Least squares means on the same row with the same letter are not significantly different ($P > 0.05$). Ta: ambient Temperature .

At high environmental temperatures cows attempted to restore their thermal balance. In the present study, heat stress altered (*P* < 0.001) RR (Respiratory rates) and HR (heart rates) (Table 3). RT increased from P_1 (THI = 63.46) with 38.5°C to P_2 (THI = 83) with 39.6°C. Respiratory rates increased from $P_1$with 53.75 Insp. /min to P_2 with 80.6 Insp./min. heart rates increased from P1with 63 Beat/min to P_2 with 77.3 Beat./ min. Such response changes are an adaptive thermal regulatory mechanisms initiated by the cows, in order to maintain heat balance. In summer conditions (P_2) RT, RR and HR raised by 0.33°C, 3.81 Insp./min and 4.7 Beat/min, respectively per increase of THI unit. Similar results were observed by Coppock *et al.* (1982), by Bouraoui *et al.* (2002) and by Ben younes (a) *et al.* (2011).

4.2 The effects of heat stress plasma vitamin C concentration and leucocytes number: Plasma VC concentration (Table 4) was lower (*P* < 0.001) in P_2 (1.58 and 1.65 mg/l in HPC and LPC, respectively) than in P_1 (3.20 and 3.15 mg/l in HPC and LPC, respectively). Number of studies (Padilla *et al.,* 2006; Tanaka *et al.,* 2008) has also found that the concentration in each cow was lower (*P* < 0.001) in the heat-stressing treatment than in the control treatment, which accords with the present findings. As mentioned above, the metabolic demand of VC in dairy cows is influenced by environmental conditions, but heat stress effect did not differ significantly (*P* > 0.05) between HPL and LPL groups. Some other researchers also reported that heat stress decreased plasma vitamin C levels in pigs (Riker *et al.,* 1967) and poultry (Coates, 1984; Sahin *et al.*, 2003). The plasma vitamin C level is affected by the synthesis and consumption of AsA (ascorbic acid). However, the adequacy of VC can be evaluated by analysis of VC or AsA in plasma, serum, leucocytes or urine (McDowell, 1989). AsA is an important water-soluble Antioxidant and can be reversibly oxidized to dehydroascorbic acid (DAsA) (Matsui, 2012). The biosynthetic pathway for AsA starts with the production of UDPglucose from glucose-1-phosphate in the liver (Padilla et al., 2006). Heat stress reduce feed intake in dairy cows (Rejeb (b) *et al.*, 2016; Ben younes (b) *et al.,* 2011; Najar *et al.*, 2011). Glucose is the sole precursor of AsA in animal body , therefore, The reduction in plasma VC in hot environment in the present study is probably attributed to fall in blood glucose, following the decrease in the amount of dry matter intake and hence the amount of carbohydrates during the summer period (P_2) for two groups of cows (HPL, LPL).

Table 4: Heat stress and level milk production in dairy cows (HPL and LPL) effects on VC concentration and Leucocytes percentages.

	P_1		P_2		Effect		
	HPC†	**LPC‡**	**HPC**	**LPC**	**G**	**P**	**G × P**
VC concentration (mg/l)	3.20[a]	3.15[b]	1.58[a]	1.65[b]	ns	***	ns
Lymphocytes (%)	73.4[a]	74.7[c]	64.4[a]	65.1[c]	***	***	***
Eosinophils (%)	1.2[a]	1.2[a]	9.4[a]	9.5[a]	***	***	***
Neutrophils (%)	23.9[a]	23.3[a,b]	20.0[a]	21.0[a]	**	***	**
Monocytes (%)	1.8[a]	1.6[b]	3.9[c]	4.6[b]	***	***	***

† HPC: High producing cows; ‡ LPC: Low producing cows;
***: *P* < 0.001; **: *P* < 0.01; ns: not significant (*P* > 0.05); Least squares means on the same row with the same letter are not significantly different (*P* > 0.05)

Lymphocytes, neutrophils, eosinophils and monocytes levels were significantly affected by heat stress (*P*<0.001). Percentage of lymphocytes and neutrophils in each cow was lower (*P*<0.001) in the heat-stressing treatment than in the control treatment. The lymphocytes depression during summer suggested decrease of the immune system activity. Percentage of eosinophils was higher (*P*<0.001) for heat-stressed cows in HPL and LPL (1.2 vs 9.4 % and 1.2 vs 9.5 % in HPC and LPC, respectively). Percentage of monocytes increased (*P*<0.001) from P_1 (1.8 and 1.6% in

HPC and LPC, respectively) to P_2 (3.9 and 4.6% in HPC, respectively). Koubkova *et al.* (2002) described that the proportional distribution of white blood cells changes at high temperatures. In addition to that, Goetzl *et al.* (1974) suggested that the VC stimulates interferon production, which protects leucocytes responses and could be related to variation of white blood cells types at high ambient temperatures. These results are in accordance with Hartmann *et al.* (1974) and Kamwanja *et al.* (1994) who observed a depression in immune system activity for heat-stressed cows. Furthermore, Broucek *et al.* (1985) found a decrease in the percentage of neutrophils and eosinophils and an increase in the percentage of lymphocytes and monocytes. However, Hartmann *et al.* (1974) found a significant decrease in leucocytes numbers in the alarm phase and leucocytosis in the resistance phase of heat stress. Lee *et al.* (1976) observed that the enhanced ambient temperature evoked leucocytosis in cattle, who noted also an increase of eosinophils count in dairy exposed to high temperature. Lacetera *et al.* (2002) suggested that heat stress did not affect the number of leukocytes. Franci *et al.* (1996) suggested that the parameter changes of lymphocytes in high temperature conditions are treated by the synthesis of heat shock proteins. Both factors, the duration of exposure, and the severity of heat stress, decrease immune activity (Kelley, 1982).

The hyperthermic stress worsens the health condition and affects the behaviour of cows. This study reported that level of milk production has been related to heat stress. However high milk production cows are more sensitive in the term of lymphocytes (P<0.001) and neutrophils (P<0.01) percentages, and less sensitive in the term of monocytes (P<0.001) and eosinophils (P<0.001) percentage, than low milk production cows. The depression of the immune system activity observed in high environmental condition, may have an impact on the occurrence of diseases and an increase in mastitis infections.

4.3 Plasma VC concentration - leucocytes cells relationship: The regressions (figure1, 2, 3 and 4) show that leucocytes number is a function of plasma VC concentration in HPC and LPC. The positive slopes of the regressions lines indicate that the total of lymphocytes (figure 1) and neutrophils (figure 3) decreases as VC concentration decreases in HPC and LPC. The values of the relationships for predictive purposes were relatively high in the regression between lymphocytes and plasma VC concentration (R^2 = 0.82 and 0.86 (P<0.01) in HPC and LPC, respectively) and in the regression between neutrophils and plasma VC concentration (R^2 = 0.86 and 0.89 (P<0.01) in HPC and LPC, respectively). Similarly, our results were in agreement with the findings of Eicher-Pruiett *et al.* (1992) who showed a positive effect of VC supplementation on neutrophils function. Some others researchers (Washko *et al.*, 1995) investigated that the concentration of Lascorbic acid in unstimulated human neutrophils is extremely high and increases approximately 10-fold when the neutrophils is stimulated. Ascorbic acid (VC) is the most important water-soluble antioxidant in mammals. However, the high concentration of ascorbic acid may be needed to protect neutrophils from the oxidants burst. Wang *et al.* (1997) indicated that neutrophils when stimulated take up large quantities of ascorbic acid. The negative slopes of the regressions lines indicate that the total of monocytes (figure 2) and eosinophils (figure 4) increases as VC concentration decreases in HPC and LPC. The values of the relationships for predictive purposes were relatively high in the regression between monocytes and plasma VC concentration (R^2 = 0.81 and 0.88 (P<0.01) in HPC and LPC, respectively) and in the regression between eosinophils and plasma VC concentration (R^2 = 0.84 and 0.72 (P<0.01) in HPC and LPC, respectively). This study results are in accordance with those reported by Minka and Ayo, (2008) who found that layers supplemented with vitamin C and transported by road showed a significant (P <0.05) increase in eosinophils, but a non-significant (P >0.05) difference in the numbers of basophils and monocytes. It has been observed an increase in the values of eosinophils in chickens transported by road (stressed) and administered with vitamins C and E (Joachim *et al.,* 2010). However, hot weather decreased plasma vitamin C caused a greater variation on total number of leucocytes. Essentially all of the decrease in vitamin C was caused by a decrease in ascorbic acid concentrations in heat stress conditions, thereby increasing the proportion of vitamin C contributed by DAsA. Padilla *et al.* (2006) reported that heat stress suppressed glycogenolysis in dairy cows. The suppression of glycogenolysis may reduce AsA synthesis in heat-stressed cows. There are many articles confirming the glucose decrease during the heat stress (Ronchi *et al.*, 1995; Itoh *et al.*, 1998; Koubkova *et al.*, 2002). Indeed, many mammals, such as ruminants, swine, horses, dogs and cats, can synthesize AsA from glucose in the liver (Matsui, 2012). On the other hand, MacLeod *et al.* (1999) suggested that high producing cows were considered to have low AsA synthesis resulting from the high demand for glucose, especially during the early lactation period. Ketosis is a disease related to the high rate of glucose utilization in the mammary gland and the inability of cows to meet the glucose demand through its supply (Baird, 1982).

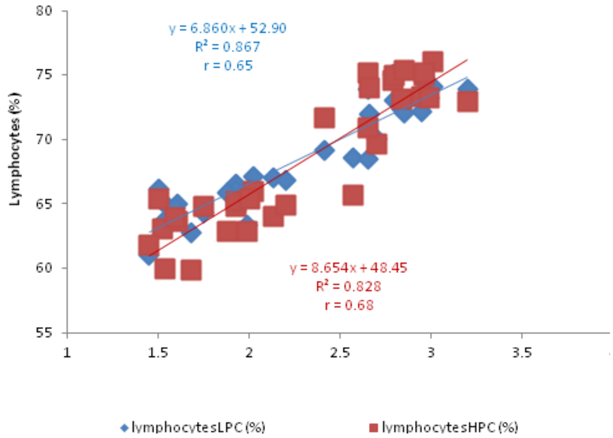

Figure 1: Relationships between lymphocytes and decrease in plasma VC concentration in HPC and LPC; *P*<0.01.

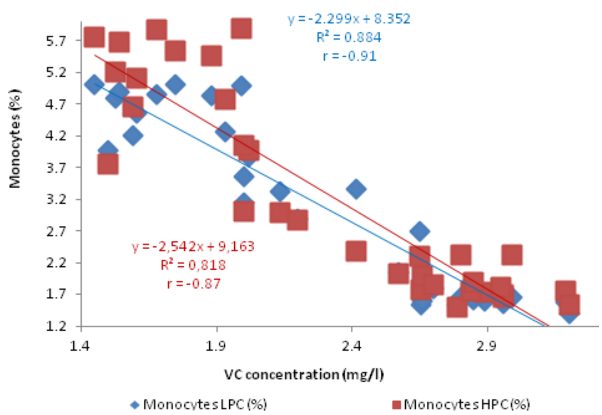

Figure 2: Relationships between monocytes and decrease in plasma VC concentration in HPC and LPC; *P*<0.01.

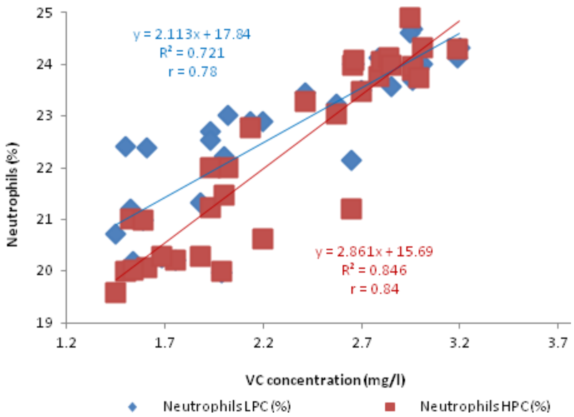

Figure 3: Relationships between neutrophils and decrease in plasma VC concentration in HPC and LPC; *P*<0.01.

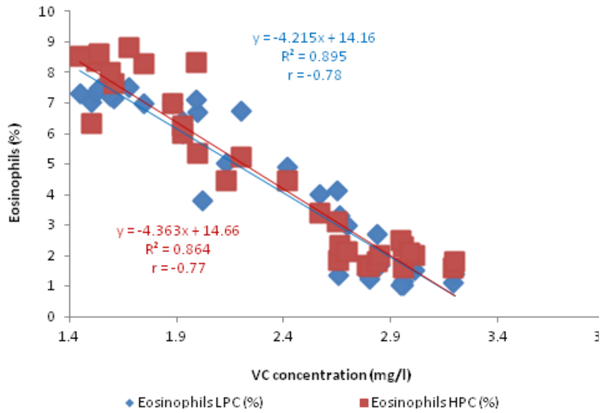

Figure 4: Relationships between eosinophils and decrease in plasma VC concentration in HPC and LPC; *P*<0.05.

5 CONCLUSION

In the present study ,as depicted by the R^2 value a large part of the variation in the percentage of white blood cells profiles could be attributed to the VC concentration decreases. Decreased plasma VC concentration under prolonged exposure at high ambient temperatures and HPC cows had lower effect on eosinophils and monocytes number and higher effect on lymphocytes and neutrophils number compared to LPC. However, white blood cells profile might not be a sensible parameter to heat stress if other stress factors are not controlled. However, management strategies are needed to minimize heat stress and restores the physiological responses to normal values and contain economic losses of dairy farming during summer. In conclusion, dietary supplementation of vitamin C can alleviate the adverse effects of heat stress in dairy cows during the hot weather on Tunisian dairy farms especially during summer months and enhance immune function.

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