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Original Article

Comparative study of productive and reproductive parameters of Holstein Friesian cows in different agroecological zones under subtropical conditions of Pakistan

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Abstract

The current study aimed to evaluate the impact of three different agroecological zones on the productive and reproductive performance of Holstein-Friesian (HF) cows, along with their comparative analysis of physiological and molecular markers under subtropical conditions of Pakistan. The productive (milk yield and composition) and reproductive (service per conception and calving interval) performance of genetically identical (n=210) HF cows placed across three agroecological zones: irrigated lowlands (Okara), wet (Abbottabad), and dry (Queta) highlands were analyzed. Additionally, heat stress markers (superoxide dismutase, glutathione peroxidase, serum cortisol, bovine heat shock protein 70 (HSP-70), and blood glucose) from five cows in each location (n=15 in total) were also investigated in early and late summers using commercial ELISA and calorimetric kits. Furthermore, the mRNA levels of heat shock protein genes *HSPA8* and *HSP90AB1* were also quantified in three agroecological zones through qRT-PCR. The results revealed that cows raised in the wet highlands of Abbottabad exhibited significantly higher ($P<0.05$) milk production (daily and total lactational yield), and the lowest service period, calving interval and number of services per conception as compared to the cows from the other two agroecological zones. Furthermore, there was a significant effect ($P<0.05$) of agroecological zones on the heat stress indicators (SOD, GPX, cortisol, blood glucose, and HSP70) profiles and expression of heat shock protein genes in HF cows. The dairy cows from Abbottabad (Wet highlands) showed a significantly lower profile of heat stress indicators as compared to the cows from the other two agroecological zones. It can be inferred that HF cows function better in moderate agroecological zones of subtropical countries.

Keywords: Heat stress indicators, Subtropics, Holstein-Friesian cows, Agroecological zone

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Introduction

Pakistan is a subtropical country broadly divided into ten agroecological zones based on precipitation, climate, topography, and soil characteristics (Ghafar et al., 2020; Baig et al., 2021). Pakistan's agrarian economy is primarily defined by cultivating crops and rearing livestock, engaging 45% of the nation's workforce and constituting 21% of the Gross Domestic Product (GDP). Milk, often referred to as "white gold," is Pakistan's essential agricultural commodity, with 8 million families actively involved in the livestock industry, generating 30-40% of their income from this sector (Aftab and Ali, 2023).

Although local dairy breeds of Pakistan are well adapted to subtropical environmental conditions, however, suboptimal milk production from these dairy breeds necessitates the import of high milk-producing exotic dairy cows (Mahmood et al., 2021). The average milk yield of native dairy breeds of Pakistan ranges from 10 to 14 liters per day which is 3-4 times less than developed countries. Among the imported dairy breeds, the Holstein-Friesian (HF) cows have taken the lead (Khan, 2022). The HF cow breed in Pakistan has become increasingly important in recent years (Tahir et al., 2019). The country's dairy industry has experienced significant growth, and the breed's high milk production makes it particularly well-suited for commercial dairy farming. However, HF cows require increased housing management to combat heat stress (Mahmood et al., 2021). HF cows originally belong to temperate climatic zone, where conditions are generally mild and conducive to their health and productivity. In subtropical conditions of Pakistan, HF cows may face challenges due to higher temperature and humidity which can affect their comfort, health and production.

Several physiological and environmental factors can significantly influence the productive potential of these dairy cows in tropical and sub-tropical environments (Ribeiro et al., 2020; Mattone et al., 2022). Heat stress arises when the environmental temperature exceeds the ideal range for dairy cows' comfort. The conventional comfort zone for dairy cows spans from 5°C to 25°C, and temperatures beyond 25°C trigger heat stress among dairy cows (Mylostyvyi et al., 2019). In subtropical regions, cows are exposed to prolonged periods of high temperatures and humidity, resulting in a decline in milk production, reduced feed intake, and lower fertility rates (Recce et al., 2021; Rodríguez et al., 2022). Heat stress can also increase the incidence of metabolic disorders, such as ketosis and mastitis (Fukushima et al., 2020; Becker et al., 2020), further decreasing the cow's productivity. Heat stress management is crucial in tropical and subtropical dairy production. Provision of shade and ventilation/ cooling facilities, ensuring access to clean fresh drinking water, changing feeding schedules, and monitoring cow health are all critical measures for maintaining cow health and production in excessive heat and humidity (Habimana et al., 2023).

Despite more than three decades since the introduction of Holstein Friesian (HF) cows, dairy farmers in Pakistan are still battling to obtain maximum milk production from HF cows, and Pakistan's dairy industry has not achieved self-sufficiency in milk production (Abbas et al., 2022; Khan et al., 2013). This situation likely arises from multiple factors, including heat stress and a subtropical environment. High temperatures, low rainfall, and water scarcity characterize Pakistan's arid and semi-arid regions can negatively affect dairy cows' productive and reproductive performance, including HF cows (Carvajal et al., 2021; Yue et al., 2023). In addition, the physiological effects of heat stress can also lead to increased health problems in dairy cows (Becker et al., 2020).

Milk composition is also affected by heat stress in dairy cows (Carvajal et al., 2021; Wang et al., 2020). High temperatures can reduce conception rates, increase embryonic mortality, and irregular estrus cycles (Ali et al., 2017; Sammad et al., 2020). Heat stress also impairs spermatogenesis and conception rates, thereby extending calving intervals and diminishing the number of lactations per cow (Roth, 2020; Li et al., 2022).

Acclimatizing imported dairy cattle breeds in subtropical environment is essential for the sustainability of dairy industries of developing countries like Pakistan. Investigating the effects of agroecological/agroclimatic zones and management practices, such as the provision of shades, ventilation, and cooling systems, can help identify strategies to mitigate the negative impact of heat stress / climatic conditions on HF cows (Negrón-Pérez et al., 2019). It is crucial to explore different management strategies to improve the performance of Holstein Friesian cows in arid and irrigated areas of subtropics. It is also crucial to understand how the environment affects these cows' productive and reproductive performance to develop appropriate management strategies that optimize their performance (Manica et al., 2022). Therefore, the current study was executed to evaluate the effects of different agroecological zones on the productive and reproductive performance of HF cows and a comparative analysis of heat stress indicators in these cows under subtropical conditions of Pakistan.

Material and Methods

Duration of study and ethical approval

The duration of this study was one year. Ethical approval for all experiments was obtained from the University of Agriculture Peshawar's Ethical Review Committee in compliance with ARRIVE guidelines (Percie du Sert et al., 2020) vide office note number 7888/L.M.B&G/UoA dated 08/09/2022. No animal was harmed or euthanized during the study.

Location of study and their climatic condition

The study, focusing on the productive and reproductive performance of imported Holstein Friesian (HF) cows, was conducted across three distinct agroecological zones in Pakistan: district Abbottabad (34.1688° N, 73.2215° E), district Okara (30°52'60''N 73°35'60'' E), and District Quetta (30.1798° N, 66.9750° E). The Abbottabad, Okara, and Quetta, are the representatives of three different agroecological zones of Pakistan. Each location exhibits distinct climatic conditions and major dairy farming operations of Pakistan are being managed in comparable agro-ecological conditions. The Abbottabad, situated in the wet mountains zone, experiences summer maximum temperatures around 35°C and winter minimums between 0-4°C. Surrounding Mountain tops are often snow-covered in winter and spring. Rainfall averages 236 mm in summer and 116 mm in winter. Quetta is located in the western dry mountain zone and has a semi-arid highland climate with mild summers and cold winters. Summer temperatures range from 30 to 39°C in the south, while winter minimums vary between 3°C to 7.7°C. Rainfall averages 30-35 mm monthly. In the extreme northwestern area, a sub-humid climate prevails with a 32°C summer maximum temperature and a 2°C winter minimum temperature. Average rainfall is 63-95 mm in winter and 95 mm in summer. Okara is located in the northern irrigated plains and has semi-arid subtropical continental climate. Summers are hot, with an average daily maximum temperature of 39.5°C, while winters are cool, with a daily minimum around 6.2°C. Annual rainfall ranges from 300-500 mm, with summer months (July-September) receiving 75-108 mm and winter months 14-22 mm of rain (Hussain et al., 2023). A graphical illustration of the overall comparison of climatic conditions prevalent in these three agroecological zones has been shown in the figure. 1

Animals and housing

The study involved (n=210) clinically healthy Holstein Friesian (HF) cows, selected from herds located in three distinct agroecological zones of Pakistan: Abbottabad (n_1=70), Okara (n_2=70), and Quetta (n_3=70). The cows imported from a similar genetic source (Australia) were randomly selected from each location. The selection criteria were the successful completion of three lactations and no history of health or reproductive issues.

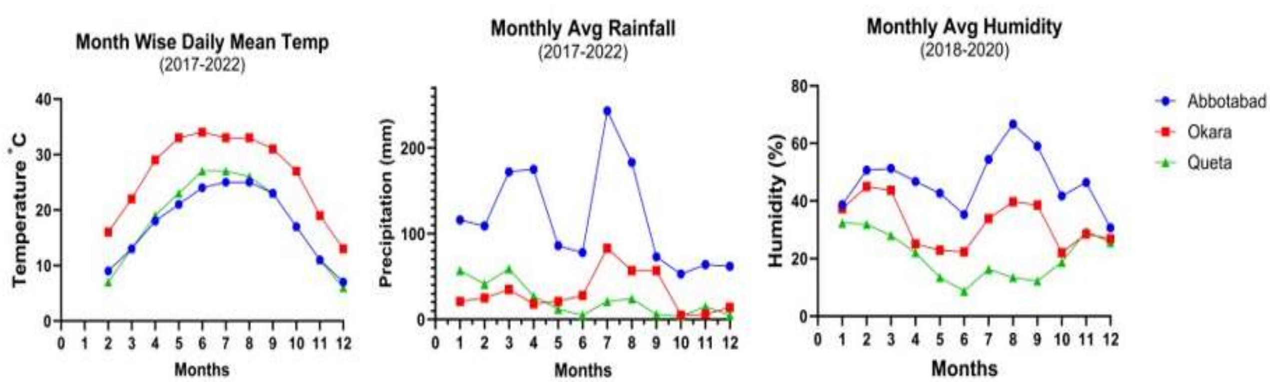

Figure-1. Climatic condition of three different agroecological zones of Pakistan Source: 'NomadSeason' for temperature and rainfall (2017-2022), and 'Weather and Climate' for humidity (2018-2020).

These cows, aged 7 to 8 years, were housed in open sheds constructed according to each region's climatic conditions. In Abbottabad and Quetta, measures were in place to mitigate harsh winter effects, while in Okara, sprinklers were installed alongside fans available in all locations to manage temperature and comfort. Open night paddocks were provided across all sites for optimal animal welfare. The cows were fed seasonal green fodder, maize silage, wheat straw, and a precisely designed concentrate, which was complemented by unlimited access to clean, fresh drinking water. The nutritional routine was adjusted to meet the National Research Council's requirements (Council, 2001), delivering consistent and balanced dietary consumption throughout three diverse agroecological zones. The specific quantities of concentrate ration and green fodder or silage were consistent for all cows, independent of location. Furthermore, the feeding plan was created to disperse the feed over numerous smaller meals throughout the day, maximizing digestion and nutritional absorption. During the study, breeding was managed by expert veterinarians through artificial insemination, ensuring high-quality semen and adherence to best insemination practices (López-Gatius, 2022).

Data collection (productive and reproductive performance)

A detailed retrospective analysis technique was used to collect productive and reproductive performance data from three commercial dairy farms located in Abbottabad, Okara, and Quetta. The data encompassed comprehensive records of lactational yield, milk composition, reproductive performance, cow health status, and management practices. Key parameters like milk production per lactation, milk quality (fat and protein contents), reproductive metrics (number of services per conception, service period, gestation period, dry period and calving interval), and environmental factors, particularly the temperature, humidity, and rainfall, were methodically extracted. This approach also included assessing the cows' health records and detailed feeding regimes.

Blood sampling

Blood samples were aseptically collected during two critical periods i.e. early and late summer to capture variations in physiological stress responses correlated with seasonal climatic changes. The collected blood samples were stored in EDTA vacutainers. No animal was harmed or euthanized during this procedure (Hussain et al., 2018).

Measurement of heat stress indicators

Blood samples were obtained from five cows in each of the three locations (n =15) to analyze heat stress markers. The heat stress markers studied included superoxide dismutase (SOD), glutathione peroxidase (GPX), cortisol, heat shock protein 70 (HSP70), and blood glucose. The blood samples were processed and analyzed using respective ELISA kits based on sandwich ELISA. The tests were performed using superoxide dismutase (SOD, Catalog # Y-889701, Lot # 20247688), glutathione peroxidase (GPX, Catalog # Y-889421, Lot # 20240677), cortisol (Catalog # SH300179, Lot # 20240202), and heat shock protein 70 “HSP70” (Catalog # Y-889760, Lot # 20246032) ELISA kits manufactured by Zokeyo Biotechnology Co., Ltd. Briefly speaking, all reagents were warmed to room temperature before use. The wash solution was diluted 1:20 with distilled water. The wells were assigned to the standards (50 µL) and samples (10 µL of sample + 40 µL of diluent). Each well received 100 µL of HRP-conjugated detection antibody. The plate was then covered and incubated for 60 minutes. After incubation, the wells were washed many times to eliminate unattached materials. Substrates A and B (50 µL) were added to each well and incubated for 15 minutes at 37°C in a dark place. After the second incubation, 50 µL of stop solution was added to each well till the color changed from blue to yellow. The absorbance was measured at 450 nm with a microplate reader within 15 minutes after adding the stop solution. The standard curve was used to determine the concentration of the target marker in the samples.

Blood glucose levels were measured using the Arena BioScien calorimetric kit (BS.1/GL04.125.0500), and analysis was performed by an automatic chemistry analyzer (BK-200, Biobase China). All measurements were specifically conducted during the early (May to June) and late (August to September) summer season across the three different agroecological zones of Pakistan.

RNA extraction, cDNA synthesis and qRT-PCR analysis of heat stress genes (*HSPA8* and *HSP90AB1*)

For comparative analysis of differential gene expression in imported HF cows under three different agroecological zones of Pakistan, three blood samples from each location (n=9 in total) were collected. These samples were drawn from cows in their third lactation and within the 3rd to 4th month after calving. Aseptic technique was adhered for blood collection, utilizing EDTA vacutainers for storage and further processing. RNA was extracted through trizol method (Uyen et al., 2022). The integrity and concentration of the extracted RNA were evaluated using a Nanodrop plate (Skanit RE 4.1, Thermoscientific). Subsequent conversion of RNA to cDNA was facilitated using the Vivantis cDNA synthesis kit (cDSK01-050), and cDNA was stored at -20 °C for future analyses (Raza et al., 2022).

Gene expression quantification was conducted through real-time polymerase chain reaction (RT-PCR), utilizing specifically designed primers (Khan et al., 2020). Primer-3 software was used for designing the primers for the target genes. The primer characteristics, including length and optimal conditions for gene amplification, were initially determined computationally, followed by empirical validation through gradient PCR to optimize the primer conditions. Gradient PCR was performed on Galaxy XP Thermal Cycler (BIOER, PRC). For *HSP90AB1* gene, the optimum annealing temperature was 57.4°C with 1:10 dilution of cDNA (figure 2b), whereas for the *HSPA8* gene, the annealing temperature was set at 62.4°C with 1:100 dilution of cDNA (figure 2c). Moreover, the specific sequences of the primers utilized in this study are listed in Table 1. To calculate relative expression, *GAPDH* was used as housekeeping gene (Fang et al., 2021) and the annealing temperature for *GAPDH* was set at 54.3°C with 1:10 dilution of cDNA (Figure 2a).

The real-time PCR (qPCR) for *GAPDH*, *HSPA8* and *HSP90AB1* was performed separately by using Thermofisher's SYBR Green Universal Master mix (Catalog # 4309155). Mic PCR (BioMolecular System) was used to perform RT-PCR were performed on. The cycling conditions were carefully programmed, and each primer pair was run individually. Melting curve analysis was carried out after amplification to verify the specificity of PCR products. Relative expression of each gene was calculated using the double delta C_T method. Two-fold log change of each sample was calculated, which facilitated the analysis of differential gene expression across the studied agroecological zones.

Statistical analysis

The normality of data was checked by the Shapiro-Wilk test. After confirmation of the normality of data, the effects of the agroecological zones on productive (daily milk yield, total lactational yield, fat, protein and lactose percentages), reproductive (number of services per conception, service period, gestational period, dry period and calving interval), heat stress indicators (SOD, GPX, Cortisol, HSP70 and blood glucose) and expression of heat shock protein genes (*HSPA8* and *HSP90AB1*) were analyzed by One-Way ANOVA. The entire statistical analysis was carried out using SPSS 27.0, with a 95% confidence interval.

Table. 1 Sequence of Primers for Heat Shock Protein Genes

Primers	Tm	Sequence	Primers	Sequence	Amplicon
HSP90AB1F	55	AGGAACCCTGATGACATC	HSP90AB1R	GGGGATGAACAGCAGTG	145
HSPA8F	55	GACTTTTTCCAGCTCCTTCT	HSPA8R	GAGGATGAGAAACTTCAGGG	150
GAPDHF	56	CAGCGCCAGTAGAAGCA	GAPDHR	CCACTTTGGCATCGTGG	145

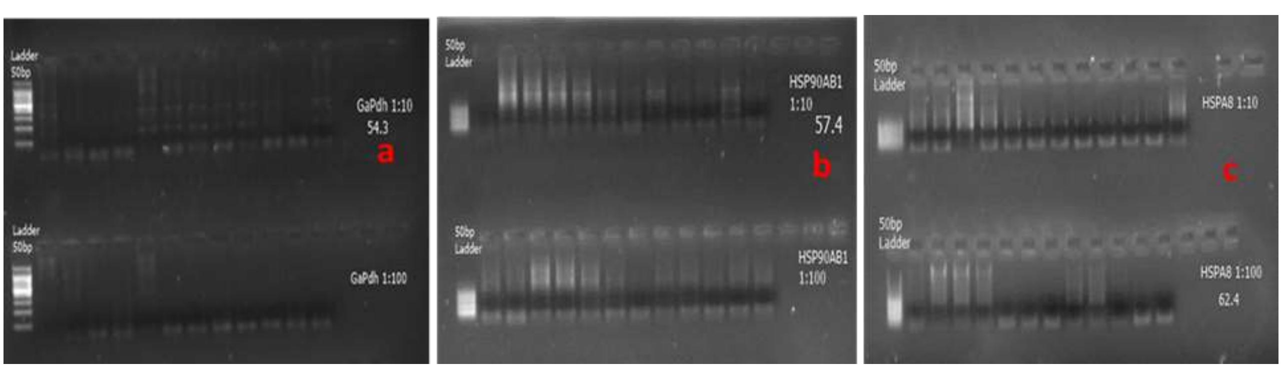

Figure-2. Gel Electrophoresis for *GAPDH*, *HSP90AB1* and *HSPA8* Primer Optimization

Results

Comparative analysis of productive parameters in three different agroecological zones

Milk yield across lactations

In first lactation, Abbottabad exhibited a significantly ($P<0.05$) higher lactational yield (9333.87±1306.49 liters) as compared to Quetta (8711.43±1316.17 liters) and Okara (8846.33±380.13 liters). A similar pattern was followed in the daily milk yield (30.61±4.33 liters) at Abbottabad as compared to the other two groups. This pattern suggests an environmental or management advantage in Abbottabad.

In the second lactation, Abbottabad maintained significantly higher ($P<0.05$) total milk yield (9506.2± 1256.21 liters) and daily milk yield (31.19±4.15 liters), as compared to Quetta and Okara (8858.01 ± 1268.89 liters and 8865.24 ± 367.69 liters, respectively).

During the third lactation, the results showed a notable shift. Although there was no significant difference ($P>0.05$) in total milk yield between Abbottabad (9461.46±1253.18 liters) and Quetta (9170.64±1269.44 liters), but both regions significantly surpassed Okara (8607.04 ± 962.44 liters). However, Abbottabad continued to have the highest mean daily yield (31± 4.08 liters).

Milk composition (fat and protein content) across three lactations

In the first lactation, the fat content (3.92%±0.29) was significantly higher ($P<0.05$) in Abbottabad, followed by Okara and Quetta, suggesting an influence of local environmental conditions on milk composition. However, in terms of protein content, a notable variation was observed across the zones during the first lactation, with Okara cows showing a significantly higher mean (3.42%±0.1) for protein percentage ($P<0.05$). However, the total protein yield in Abbottabad and Okara exhibited comparable levels (Abbottabad=306.74+43.66 Kg; Okara:302.05+15.78 kg), both significantly ($P<0.05$) higher than Quetta. The lactose percentage presented less variation, yet Abbottabad cows still had a marginally higher mean as compared to dairy cows in Quetta ($P<0.05$).

In the second lactation, Abbottabad continued to lead in milk fat composition (3.89 ± 0.33%) compared to Quetta and Okara ($P<0.05$). This pattern of Abbottabad leading in productive parameters was also exhibited in the total protein yield (312.85±41.94 Kg), indicating a consistent performance across the lactations.

In the third lactation, Abbottabad again showed the highest fat content (3.87 ± 0.36%), while protein content was highest in Okara (3.42 ± 0.11%). All the results have been summarized in Table 2.

Comparative analysis of reproductive parameter in three different agroecological zones

The reproductive parameters of HF cows across three different agroecological zones in Pakistan were analyzed over three lactation periods. During the first lactation, the number of services per conception was similar across all locations ($P>0.05$). However, the calving interval showed significant differences ($P<0.05$), with Abbottabad cows having a shorter interval (416.19±29.87 days) compared to Quetta (434.74±33.53 days) and Okara (431.09±22.45 days). The service period exhibited a similar trend. However, the gestation days were consistent across all regions ($P>0.05$), indicating uniformity in gestation periods irrespective of agroecological zones.

In the second lactation, the calving interval varied, with Abbottabad cows exhibiting a slightly shorter interval (418.59±30.71 days) as compared to Quetta (440.59±35.43 days) and Okara (429.4±26.77 days), but the differences were statistically significant between Abbottabad and Quetta ($P<0.05$). The service period exhibited a similar trend. However, gestation days remained consistent across all regions ($P>0.05$).

In the third lactation, the pattern of services per conception was similar to the first, with no significant difference across the agroecological zones ($P>0.05$). The service period and calving interval, however, were again shorter in Abbottabad as compared to Quetta and Okara ($P<0.05$). Gestation days were consistent across all regions ($P>0.05$). All the results have been summarized in Table 3.

Table-2. Comparative analysis of productive performance during three lactations in HF cows across three different agroecological zones

Lactation Number	Parameters	Location		
		Abbottabad (n=70)	**Quetta (n=70)**	**Okara (n=70)**
First	**LY (Liter)**	9333.87±1306.49^{a}	8711.43±1316.17^{b}	8846.33±380.13^{b}
	DMY (Liter)	30.61±4.33^{a}	28.56±4.33^{b}	29±1.33^{b}
	Fat (%)	3.92±0.29^{a}	3.67±0.16^{b}	3.73±0.18^{b}
	TF (Kg)	366.26±61.82^{a}	319±48.87^{b}	329.65±18.53^{b}
	Protein (%)	3.29±0.08^{a}	3.18±0.11^{b}	3.42±0.1^{c}
	TP (Kg)	306.74±43.66^{a}	277.02±43.75^{b}	302.05±15.78^{a}
	Lactose (%)	4.58±0.24^{a}	4.47±0.1^{b}	4.53±0.17^{ab}
Second	**LY (Liter)**	9506.2±1256.21^{a}	8858.01±1268.89^{b}	8865.24±367.69^{b}
	DMY (Liter)	31.19±4.15^{a}	29.09±4.19^{b}	29.11±1.26^{b}
	Fat (%)	3.89±0.33^{a}	3.7±0.18^{b}	3.67±0.22^{b}
	TF (Kg)	368.23±50.96^{a}	327.22±46.57^{b}	324.89±23.68^{b}
	Protein (%)	3.29±0.07^{a}	3.2±0.13^{b}	3.41±0.09^{b}
	TP (Kg)	312.85±41.94^{a}	282.18±39.99^{b}	301.9±13.43^{c}
	Lactose (%)	4.65±0.21^{a}	4.49±0.08^{b}	4.48±0.18^{b}
Third	**LY (Liter)**	9461.46±1253.18^{a}	9170.64±1269.44^{a}	8607.04±962.44^{b}
	DMY (Liter)	31±4.08^{a}	30.07±4.21^{ab}	29.41±3.57^{b}
	Fat (%)	3.87±0.36^{a}	3.64±0.16^{b}	3.71±0.18^{b}
	TF (Kg)	366.07±56.08^{a}	334.01±47.83^{b}	318.98±38.39^{b}
	Protein (%)	3.29±0.07^{a}	3.2±0.1^{b}	3.42±0.11^{c}
	TP (Kg)	311.94±41.7^{a}	294.64±42.55^{b}	294.4±35.09^{b}
	Lactose (%)	4.61±0.2^{a}	4.47±0.1^{b}	4.49±0.16^{b}

Note: The comparison is in rows. **LY** (Lactational yield), **DMY** (Daily milk yield), **TF** (Total fat), **TP** (Total protein). The values in the same row not sharing the same superscript are significantly different at ***P <0 .05***

Table-3. comparative analysis of reproductive performance during three lactations in HF cows across three different agroecological zones

Parity	Parameters	Location		
		Abbottabad(n=70)	**Quetta (n=70)**	**Okara (n=70)**
First	**SPC (no.)**	2.09±0.28^{a}	2.11±0.4^{a}	2.2±0.5^{a}
	CI (days)	416.19±29.87^{a}	434.74±33.53^{b}	431.09±22.45^{b}
	GP (days)	286.67±9.29^{a}	287.21±8.96^{a}	287.54±9.82^{a}
	SP (days)	129.51±32.73^{a}	147.53±36.63^{b}	143.54±25.95^{b}
	DP (days)	111.19±29.87^{a}	129.74±33.53^{b}	126.09±22.45^{b}
Second	**SPC (no.)**	2.09±0.41^{ab}	2.01±0.47^{a}	2.21±0.45^{b}
	CI (days)	418.59±30.71^{a}	440.59±35.43^{b}	429.4±26.77^{ab}
	GP (days)	287.21±8.78^{a}	285.24±8.77^{a}	288.01±9.55^{a}
	SP (days)	131.37±30.2^{a}	155.34±36.05^{b}	141.39±28.22^{a}
	DP (days)	113.59±30.71^{a}	135.59±35.43^{b}	124.4±26.77
Third	**SPC (no.)**	2.06±0.34^{a}	2.06±0.48^{a}	2.21±0.51^{a}
	CI (days)	413.97±31.51^{a}	438.64±31.11^{b}	430.5±23.05^{b}
	GP (days)	288.36±8.95^{a}	286.91±9.41^{a}	287.19±8.48^{a}
	SP (days)	125.61±34.43^{a}	151.73±33.11^{b}	143.31±24.34^{b}
	DP (days)	108.97±31.51^{a}	133.64±31.11^{b}	125.5±23.05^{b}

Note: The comparison is in rows. **SPC** (services per conception); **CI** (calving interval); **GP** (gestation period); **SP** (service period); **DP** (dry period). The values in the same row not sharing the same superscript are significantly different at ***P < 0.05***

Comparative analysis of biochemical stress markers during early and late summers

The biochemical stress markers of HF cows were compared across Abbottabad, Quetta, and Okara during early and late summer. In early summer, superoxide dismutase (SOD) levels were significantly higher (*P<0.05*) in Quetta (3.89±0.34 ng/ml) and Okara (4.44±0.41 ng/ml) as compared to Abbottabad (2.58±0.43 ng/ml). During late summer, the mean values of SOD increased in all the groups when compared with the corresponding values of early summer. However, the overall trend remained the same. The mean SOD values for Abbottabad, Quetta and Okara were 4.38±0.24, 5.48±0.23 and 6.43±0.25 ng/ml respectively. The differences in SOD values were statistically significant (*P<0.05*) among the three groups in late summer. The results have been summarized in figure 3.

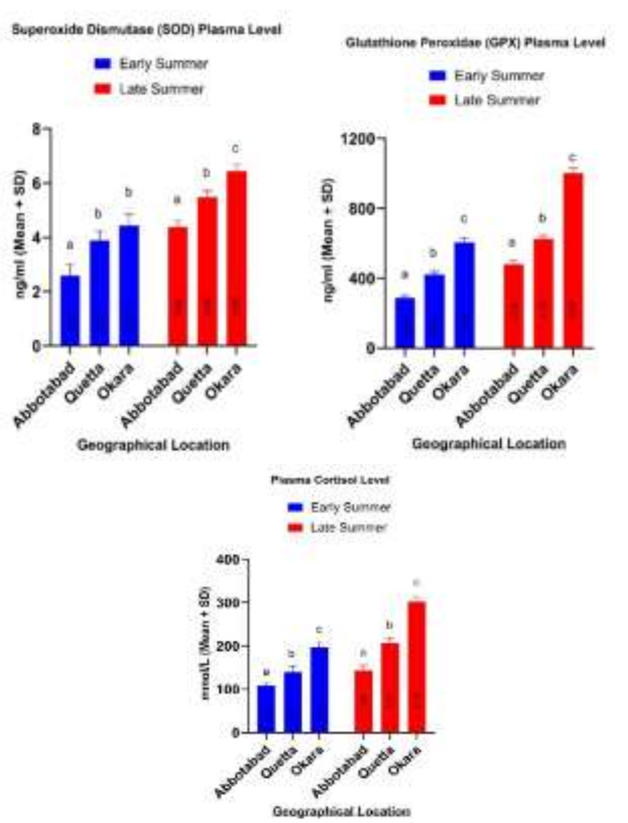

Figure-3. Comparative levels of super oxide dismutase (SOD), glutathione peroxidase (GPX) and plasma cortisol levels during early and late summer across different agroecological zones.

[a-b]Means without common superscript differed (*P<0.05*).

Similarly, glutathione peroxidase (GPX) levels during early summers were significantly different among Abbottabad (286.86±15.13 ng/ml), Quetta (419.63±18.95 ng/ml), and Okara (605.28±27.49 ng/ml) at 95% confidence interval. A similar trend was observed during late summer, although the mean values of GPX were increased among all the groups when compared with the corresponding values of early summer. However, the overall trend remained the same, and the difference in mean GPX values of the three groups were statistically significant (*P<0.05*) in late summers as well. The results have been summarized in figure 3.

The plasma cortisol levels varied significantly among the groups during early summer (*P<0.05*), with Okara exhibiting the highest mean (197.12±9.81 ng/ml), followed by Quetta (138.89±14.94 ng/ml) and Abbottabad (107.86±6.71 ng/ml). During late summer, the mean values of plasma cortisol increased in all the groups when compared with the corresponding values of early summer. This was an indication of possible thermal stress response variation. During late summer, cortisol levels in Abbottabad, Quetta and Okara were 143.06±11.84, 206.51±12.73 and 301.95±10.62 ng/ml, respectively. The mean plasma cortisol values were significantly different among the three groups (*P<0.05*). The results have been summarized in figure 3.

The heat shock protein (HSP70) levels varied significantly among three groups during early and late summer (*P<0.05*). In early summer, cows from Okara exhibited the highest mean (195.24±15.35 pg/ml), followed by Quetta (174.11±15.44 pg/ml) and Abbottabad (63.07±8.56 pg/ml). During late summer, the mean values of HSP70 increased in all the groups when compared with the corresponding values of early summer. The mean HSP70 value was significantly different among the groups (*P<0.05*). This was an indication of a possible stress response variation. During late summers, HSP70 levels in Abbottabad Quetta and Okara were 125.61±5.55, 304.19±27.76 and 338.81±28.78 pg/ml respectively. This was an indication of thermal stress response. The results were statistically significant among the three groups as shown in figure 4.

During early summer, the glucose levels were lowest in Okara (4.24±0.74 mmol/L) compared to Abbottabad (5.14±0.38 mmol/L) and Quetta (4.50±0.19 mmol/L) (Figure 8). However, a statistically significant difference (*P<0.05*) existed between Okara and Abbottabad only. During late summer, the difference in blood glucose values was statistically non-significant across all locations ($P>0.05$). However, the mean values of blood glucose were decreased in all the groups when compared with the corresponding values of early summer.

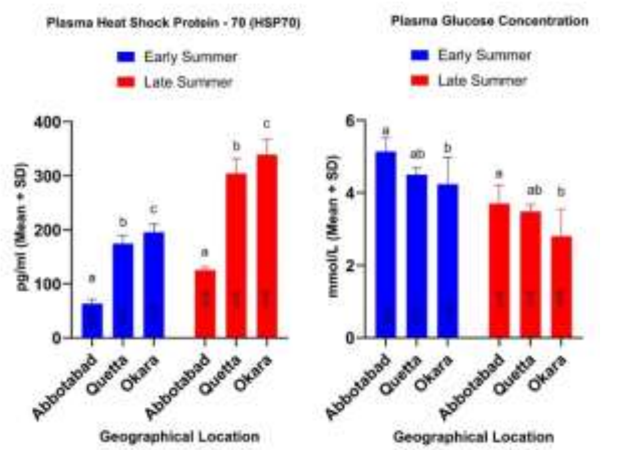

Figure-4. Comparative levels of plasma heat shock protein–70 (HSP70) and plasma glucose during early and late summer across three different agroecological zones.

[a-b]Means without common superscript differed ($P<0.05$).

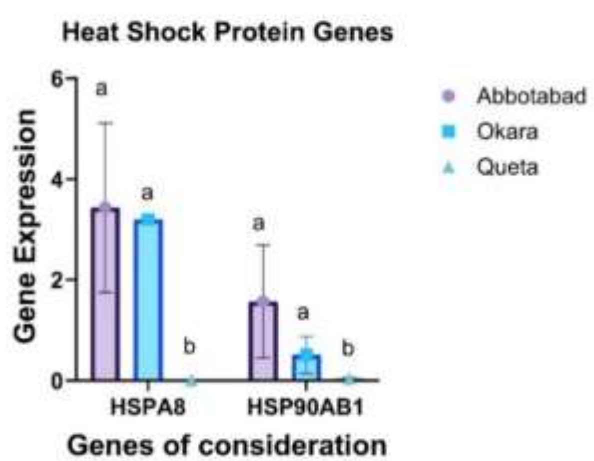

Figure-5. Comparative expression of heat shock protein genes in HF cows across different agroecological zones.

[a-b]Means without common superscript differed ($P<0.05$).

Genes expression of heat shock protein

The expression of heat shock protein (HSP) genes (*HSP90AB1* and *HSPA8*) across three different agroecological zones, i.e., Abbottabad, Okara, and Quetta, revealed significant differences in the expression levels among these zones ($P<0.05$). For *HSP90AB1*, expression was highest in Abbottabad (1.57 ± 1.12), significantly higher than in Okara (0.51 ± 0.37) and Quetta (0.04 ± 0.03), indicating a zone-specific stress response. Similarly, HSPA8 showed increased expression in Abbottabad (3.43 ± 1.68) and Okara (3.20 ± 0.08), with no significant difference between them, but a significantly lower expression in Quetta (0.01 ± 0.00). The results have been summarized in figure 5.

Discussion

The present study was conducted to analyze the productive and reproductive performances, as well as the physiological and genetic responses to heat stress in Holstein Friesian cows in three different agroecological zones under subtropical conditions of Pakistan.

The results of the study suggest that cows from Abbottabad (wet mountains) exhibited significantly higher *($P<0.05$)* total and daily milk yields, along with higher fat content, especially noticeable during the first lactation period. This pattern was consistent across all three lactations, suggesting favorable environmental conditions for milk production in Abbottabad. Conversely, the protein content was notably higher in Okara cows during the first lactation ($P<0.05$), indicating possible regional variations influencing milk quality. These findings are consistent with previous literature that has investigated the productive and reproductive performance of HF cows in different agroecological zones. A study conducted in Ethiopia found that the productive and reproductive performance of HF cows varied depending on the region (Kebede, 2015). Another study conducted in Poland investigated the blood glucose concentration in HF cows at different milk production levels has revealed that lower blood glucose levels were induced by the higher consumption needed to fulfill the energy requirements for milk production (Pawliński et al., 2023). Another study about the effects of different cooling sessions on the physiological and production responses of HF cows found that milk yield was significantly affected by the cooling sessions (Bah et al., 2022). A study in Rwanda about the productive performance of three dairy cows (including HF cows), in different agroecological zones highlighted the impact of environmental changes on milk production (Manzi et al., 2022). Furthermore, research in Zimbabwe revealed that agroecological regions and climatic conditions affect milk production, emphasizing the importance of understanding the environmental factors that impact milk yield (Nyamushamba et al., 2014). Both studies suggested considering the interplay between the genetics of dairy cows and adaptability to environmental conditions to obtain optimum performance from dairy cows.

In our results of reproductive parameters, there was a uniformity in the number of services per conception across all locations (*P>0.05*), suggesting consistent reproductive efficiency. However, significant differences were observed in service period and calving intervals, with Abbottabad cows having shorter intervals, potentially reflecting superior reproductive management or more favorable environmental conditions for reproduction in this region. These findings are consistent with previous literature that has investigated the reproductive efficiency of HF cows in Pakistan and found that the number of services per conception varied depending on the location (Sattar et al., 2005).

However, in the case of heat stress biochemical markers, a significant difference appears, particularly in Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX) and cortisol levels. Our findings show that SOD, GPX and cortisol levels remained significantly higher across Okara and Quetta facing more climatic stress throughout early and late summer, with an increase in late summer values. This finding is in accordance with prior studies, when SOD levels were found considerably greater in heat-stressed calves, indicating a varied response to thermal stress that may rely on environmental factors or seasonal fluctuations (Mirzad et al., 2018; Abbas et al., 2020). Furthermore, significant changes in Glutathione Peroxidase (GPX) levels across all three agroecological zones, along with an increase in late summer in our study, coincides with the previous literature indicating that GPX normally increases in response to heat stress (Ganaie et al., 2013). The reported low glucose levels in Okara, however, constitute an intriguing oddity, as stress responses are frequently associated with elevated glucose levels for energy (Sammad et al., 2020). This could suggest a unique stress response mechanism or an adaptation specific to this environment. Elevated cortisol levels in cattle in reaction to heat stress have been well established. Another study found higher cortisol levels in nursing ruminants under heat stress, indicating the mitochondrial response to such conditions (Marquez-Acevedo et al., 2023).

Similarly, a rise in cortisol levels under acute heat stress was observed in dairy cattle, emphasizing its importance in thermotolerance selection techniques (Cartwright et al., 2023). Another study found that hair cortisol metabolites in HF cows grew from 6.3 to 24.2 pg/mg with increasing ambient temperatures, demonstrating the long-term impacts of heat stress (Nejad et al., 2019). Correspondingly, our study found significant differences in cortisol and HSP70 levels between seasons and locales (*P<0.05*). In our study, increased levels in Okara during both early and late summer indicate heightened stress response in these environmental conditions. A possible reason for the higher stress response in Okara is higher environmental temperature and humidity during summer season as compared to the other two locations. This concordance with previous literature underlines cortisol's role as a major biomarker for heat stress in cattle.

Several other studies have also reported the impact of heat stress on dairy animals, including cattle and buffaloes. A study on dairy buffaloes in a semi-arid summer found that increasing the frequency and duration of cooling sessions can improve milk yield and the welfare of the animals (Hussain et al., 2023). Another study on Holstein and Jersey cows and heifers on pasture found that heat stress negatively impacts milk production and milk components (de Lima Guimarães Yamada et al., 2023). A study on the effect of seasonal variation in the Temperature Humidity Index (THI) on milk production found that THI is generally used as an indicator of heat stress in dairy animals (Kalyan et al., 2022). A study on the performance of HF cows subjected to different cooling sessions found that the cows in the four cooling sessions group had 0.6% more total solids than those in the group having eleven hours of continuous cooling with sprinklers and the group having two cooling sessions, which could be attributed to both feed intake and physiological response (Bah et al., 2022).

In addition to the biochemical responses, it is essential to consider the environmental differences at our study locations. The variation in cortisol and HSP70 levels across locations, especially the elevated levels in the northern irrigated plains (Okara) may be attributed to distinct environmental stressors present in each agroecological zone. The environmental stress factors include high rise in temperature, humidity and sunlight exposure, all of which are known to affect the physiological well-being of cattle. Relative expression of heat shock protein genes (*HSP90AB1* and *HSPA8*) varied at different agroecological zones in our study. These results align with the general concept of heat shock proteins (HSPs) being responsive to heat stress (Zeng et al., 2023; Habimana et al., 2023; AL-Jaryan et al., 2023). However, our study offers a valuable perspective on geographical variations in HSP gene expression in response to heat stress, complementing the insights gained from the previous studies.

The study's findings on the elevated levels of SOD, GPX, cortisol and HSP70 in Okara during both early and late summer indicate a higher stress response in this region, possibly due to more challenging environmental conditions.

Conclusion

It can be concluded from the current study that wet highlands (Abbottabad) with moderate summers provide the most conducive environment for HF cows under subtropical conditions of Pakistan. This agroecological zone not only enabled superior productive and reproductive performance but physiological stress indicators also remained the lowest. These findings highlight the importance of zone-specific management practices to optimize dairy cattle performance, emphasizing the critical role of environmental adaptation in enhancing dairy productivity and cattle welfare sustainably.

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Contribution of Authors

Rehman MU: Planned the study, performed the experiments, wrote the first draft, performed the statistical analysis, read and approved the final manuscript

Suhail SM & Munir I: Planned and supervised the study, reviewed the literature, conducted data analysis, wrote and edited the manuscript, and approved the final version.

Khan R: Planned and supervised the study, carried out the experiments, reviewed the literature, conducted data analysis, revised the initial draft of the manuscript, and approved the final manuscript.

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