STUDIES ON HEAT SHOCK PROTEIN GENE POLYMORPHISM IN SAHIWAL AND CROSSBRED COWS

By

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON HEAT SHOCK PROTEIN GENE POLYMORPHISM IN SAHIWAL AND CROSSBRED COWS"** submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** of P. V. Narsimha Rao Telangana Veterinary University, Rajendranagar, Hyderabad is a record of the bonafide research work carried out by **Mrs. J. SAI PRASANNA (I. D. No. RVD/16-11)** under my guidance and supervision.

No part of the thesis has been submitted for any other degree or diploma or has been published. The published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged.

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LIST OF ABBREVIATIONS

=	-	Equals
%	-	Per cent
±	-	Plus or Minus
>	-	Greater than
<	-	Less than
°C	-	Degree Celsius
µg	-	Microgram
µl	-	Microlitre
APS	-	Ammonium persulfate
AFC	-	Age at First Calving
AFS	-	Age at First Service
bp	-	Base pair
CI	-	Calving Interval
Db	-	Dry bulb temperature
DMRT	-	Duncan's Multiple Range Test
DNA	-	Deoxyribo Nucleic Acid
dNTP	-	Deoxyribo Nucleotide Triphosphate
DP	-	Dry Period
DDW	-	Double Distilled Water
EDTA	-	Ethylene Diamino Tetraacetic Acid
g	-	Gram
GP	-	Gestation period
HF	-	Holstein Friesian
HSP	-	Heat Shock Protein
HTC	-	Heat Tolerance Coefficient
Kbp	-	Kilo-base pair
LL	-	Lactation Length
KDa	-	Kilo Dalton
mA	-	Milliampere
mg	-	Milligram
Mgcl2	-	Magnesium chloride

min	-	Minute
ml	-	Millilitre
mM	-	Milli Molar
NFW	-	Nuclease Free Water
ng	-	Nanogram
OD	-	Optical Density
P	-	Probability
PAGE	-	Poly Acrylamide Gel Electrophoresis
PCR	-	Polymerase Chain Reaction
pm	-	Pico moles
RBC	-	Red Blood Cells
rpm	-	Revolutions per minute
RR	-	Respiration Rate
RT	-	Rectal Temperature
SCC	-	Somatic Cell Count
SDS	-	Sodium Dodecyl Sulphate
sec	-	Seconds
SNP	-	Single Nucleotide Polymorphism
SSCP	-	Single Stranded Conformation Polymorphism
TBE	-	Tris Boric acid EDTA
THI	-	Temperature Humidity Index
TEMED	-	N, N,N',N'-Tetra Methyl Ethylene Diamine
TLMY	-	Total Lactation Milk Yield
TNZ	-	Thermo Neutral Zone
V	-	Volts
w/v	-	Weight by Volume
WBC	-	White Blood Cells
Wb	-	Wet bulb temperature

DECLARATION

I, **Mrs. J. SAI PRASANNA,** hereby declare that the thesis entitled **"STUDIES ON HEAT SHOCK PROTEIN GENE POLYMORPHISM IN SAHIWAL AND CROSSBRED COWS"** submitted to P. V. Narsimha Rao Telangana Veterinary University for the Degree of **DOCTOR OF PHILOSOPHY** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

Date: 18/6/2021

Place: Hyderabad

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ABSTRACT

The present study was aimed to elucidate genetic polymorphism in different fragments of heat shock protein (*HSP*) genes through Polymerase Chain Reaction and Single-Stranded Conformation Polymorphism (PCR-SSCP) technique and their association with physiological, production and reproduction traits.

Data on physiological, productive and reproductive traits of 50 purebred Sahiwal and 50 crossbred cows were analyzed. Significant influence of genetic group was observed on physiological parameters (average of readings recorded at 8 AM and 2 PM), with crossbreds recording higher mean respiration rate (30.81 ± 0.22 *Vs* 24.16 ± 0.21 breaths/minute), rectal temperature (38.58 ± 0.03 *Vs* 38.29 ± 0.03 °C) and heat tolerance coefficient (2.35 ± 0.02 *Vs* 2.05 ± 0.02) than Sahiwal cows. Season also had a significant influence on the physiological parameters studied. Mean respiration rate was 28.56 ± 0.36, 23.38 ± 0.33 and 20.54 ± 0.38 (number/minute) in Sahiwal and 44.58 ± 0.38, 25.94 ± 0.34 and 21.90 ± 0.37 (number/minute) in crossbreds, during summer, rainy and winter seasons respectively. The corresponding mean rectal temperatures were 38.52 ± 0.03, 38.23 ± 0.03, and 38.13 ± 0.02 °C in Sahiwal and 39.22 ± 0.03, 38.72 ± 0.02, and 37.80 ± 0.03 °C in crossbreds, respectively. Higher heat tolerance coefficient was observed during summer in both the genetic groups.

Effect of genetic group was significant on total lactation milk yield and peak yield, with crossbreds outperforming Sahiwal cows. Mean total lactation milk yield, peak yield, and lactation length were 1768.32 ± 109.67 kg, 10.17 ± 0.50 kg and 304.41 ± 13.00 days in Sahiwal and 2983.45 ± 78.32 kg, 14.92 ± 0.36 kg and 324.71 ± 9.29

days in crossbred cows, respectively. Means for reproductive traits, *viz*, gestation period, service period, dry period and calving interval were 277.71 ± 2.24, 182.60 ± 15.51, 167.47 ± 10.68 and 430.23 ± 13.46 days in Sahiwal and 275.34 ± 1.60, 181.09 ± 10.91, 127.01 ± 7.51 and 413.61 ± 9.47 days in crossbred cows, respectively.

Genomic DNA of experimental animals was isolated by phenol-chloroform extraction method and used for PCR after evaluating quality and quantity of DNA. Six fragments, two each of *HSP70, HSP90AA1*, and *HSP90AB1* genes were amplified with specific primers and the amplicons were checked on 1.5% agarose gel with standard DNA ladder.

The amplicons were subjected to PCR-SSCP technique to detect polymorphism. Out of the six fragments studied, two fragments (Fragment II of *HSP70* and Fragment I of *HSP90AB1* gene) revealed a monomorphic pattern, while four fragments revealed polymorphism in both Sahiwal and crossbred cows. The PCR-SSCP of Fragment I of *HSP70* gene revealed genotypes AA and AB in Sahiwal and AA and AC in crossbred cows. The corresponding allele frequencies of A and B in Sahiwal cows were 0.79 and 0.21 and allele frequencies of A and C in crossbred cows were 0.81 and 0.19. The PCR-SSCP of Fragment I of *HSP90AA1* gene yielded two conformational patterns AA and AB corresponding to two allelic variants A and B in both Sahiwal and crossbred cows. The allele frequencies of A and B were 0.78 and 0.22, and 0.84 and 0.16 in Sahiwal and crossbred cows, respectively. The *HSP90AA1* Fragment II yielded two genotypic patterns AA and AB corresponding to two allelic variants with frequencies of 0.85 and 0.15, and 0.81 and 0.19 in Sahiwal and crossbred cows, respectively. Fragment II of *HSP90AB1* gene yielded four SSCP patterns AA, AB, AC, and BC corresponding to three allelic variants A, B, and C whose allelic frequencies were 0.78, 0.14, 0.08, and 0.59, 0.23, 0.18 in Sahiwal, and crossbred cows, respectively. The present investigation indicated that the SSCP technique is a valuable tool for the identification of genetic polymorphism.

PCR-SSCP patterns of different fragments of HSP genes were correlated with the physiological, productive, and reproductive traits in both Sahiwal and crossbred cows. It was observed that *HSP70* fragment I genotype AA had higher peak milk yield in Sahiwal cows while the same genotype had higher total lactation milk yield, lower service period, and calving interval in crossbred cows.

The association analysis of SSCP patterns of the fragment I of *HSP90AA1* gene revealed that genotype AA had higher lactation length in Sahiwal cows, and higher total lactation milk yield and peak yield in crossbred cows.

The influence of *HSP90AA1* fragment II genotypes was non-significant in Sahiwal cows, while in crossbred cows, AB genotype had a longer service period.

The association analysis of SSCP patterns of the fragment II of the *HSP90AB1* gene revealed that cows with genotype BC had a longer calving interval in Sahiwal while BC genotype had higher total lactation milk yield and AA genotype had a lower age at first service in crossbred cows.

In conclusion, the differences found between different SSCP genotypes of *HSP* genes in productive and reproductive traits indicated their possible role in marker-assisted selection. The SSCP patterns obtained in the present study for various fragments of *HSP* genes and their relationship with physiological parameters did not show significant differences among the genotypes indicating that a larger population of cows with a wide genetic base may be needed to elucidate the association of polymorphs.

CHAPTER I
INTRODUCTION

Ever increasing human population is exerting tremendous pressure on natural resources including livestock (Herrero and Thornton, 2013). Changing global environment is also posing a major threat to the sustainability of livestock production systems (Naqvi and Sejian, 2011; Polsky and Keyserlingk, 2017 and Rojas-Downing *et al.,* 2017). Global warming impairs production and reproduction performance, metabolic health and immune competence of livestock. Negative impact of global warming on total milk production for India has been estimated to be around 3.2 million tons by 2020 and more than 15 million tons by 2050 (Upadhyay *et al.,* 2008).

Selection and breeding methods are the only tools available for animal breeders to meet these challenges. Though crossbreeding was proved to be the fastest way of increasing milk production, adaptability of crossbreds was poor as reflected by lower breeding efficiency. Further, crossbreds are found to be highly susceptible to diseases and less tolerant to heat stress (Singh, 2016).

Holstein Friesian is extensively used for crossbreeding in India (Wakchaure *et al.,* 2015). Several crossbred populations with Friesian inheritance ranging from 35.93% to 93.75% were investigated and performance evaluated. It was observed that crossbreds with 62.5% of Friesian inheritance were found to be superior to all other crosses (Bhaduria and Katpatal, 2003, Lakshmi *et al.,* 2010).

Sahiwal is among the best milch breeds of the Indian sub-continent (Nivsarkar *et al.,* 2013), with an average lactation milk yield of 1880 kg (Verma *et al.,* 2016). They are highly tolerant of tropical diseases and are known for their adaptability to heat stress. However, their number is fast dwindling due to indiscriminate crossbreeding and is resulting in the loss of unique heat stress genes due to genetic recombination (Singh, 2016). Therefore, there is an urgent need to characterize these unique genes in Sahiwal and other indigenous cattle breeds.

Current strategies focussing on managemental manipulations to mitigate the effects of heat stress are partially successful and are only short term measures (Berman, 2005). Feed and housing modifications with focus on genetic manipulations could be a sustainable long term strategy to reduce the effects of heat stress (Boonkum *et al.,* 2011; Scholtz *et al*., 2013).

Cellular tolerance to heat stress is mediated by heat shock proteins (*HSP*s). Among different families of these proteins, *HSP70* and *HSP90* are especially considered to be related to the development of temperature tolerance (Hue *et al*., 2013). Unraveling polymorphism in heat shock protein genes could be a step towards the identification of genetic markers for selecting heat-tolerant cattle. Although differences in thermotolerance at physiological and cellular level are documented (Collier *et al.,* 2006; Chaiyabutr *et al.,* 2008; Wilson and Crandall, 2010 and Dalcin *et al.,* 2016) in both *Bos indicus* and *Bos taurus* cattle, information on polymorphism of *HSP* genes in Sahiwal cattle and Holstein Friesian crossbreds is scarce. There are few reports from India regarding the association of *HSP70* and *HSP90* gene polymorphism with heat tolerance in Tharparkar cattle (Bhat *et al.,* 2016), Deoni cattle (Kerekoppa *et al*., 2015), Jersey crossbred cows (Sailo *et al.,* 2015b), and from abroad in Holstein cow (Li *et al.,* 2011). Therefore, the present investigation was undertaken with the following objectives.

1. To study the polymorphism in different fragments of *HSP* genes in Sahiwal and crossbred cows through PCR-SSCP.
2. To study the physiological parameters of Sahiwal and crossbred cows and their association with polymorphism of *HSP* genes.
3. To study the association of polymorphism of *HSP* genes with the production and reproduction traits of Sahiwal and crossbred cows.

CHAPTER II
REVIEW OF LITERATURE

Growing demand for improving milk production and rising temperatures due to global warming has increased the thermal load on dairy animals. When the heat load of an animal is greater than its capacity to lose heat, a part of the metabolizable energy that is used for production must be diverted to maintain thermal balance. This causes decreased milk production and reduced reproductive performance in dairy animals (Rivington *et al*., 2009).

An attempt has been made here under, to review the impact of heat stress on production and reproduction traits along with other relevant aspects as per the objectives of the present study.

2.1 HEAT STRESS

Stress is defined as an external event or condition that stresses a biological system (Collier *et al.,* 2017). It is a reflex reaction, revealed by the animal's inability to cope with its environment, which leads to many adverse consequences, ranging from discomfort to death (Manuja *et al*., 2012). Livestock is subjected to various forms of stress, including physical, chemical, nutritional, psychological, and thermal. Among all, heat stress is the most detrimental, affecting livestock production (Rivington *et al*., 2009).

Heat stress is any combination of environmental factors such as air temperature, relative humidity, air movement, and solar radiation that causes the effective temperature of the environment to be higher than the thermoneutral zone of the animal (Herbut *et al.,* 2018). It is the sum of external forces acting on an animal that causes a rise in body temperature and evokes a physiological response (Dikmen and Hansen, 2009).

Heat stress negatively influences production and reproduction in dairy cattle (Hyder *et al.* 2017; Polsky and Keyserlingk, 2017; Lees *et al*., 2019 and Sammad *et al.,* 2020). Though dairy cattle have several adaptive mechanisms to cope with heat stress, these mechanisms only help the animals survive the stress by compromising their production and reproduction performance. Heat stress in cattle reduces feed intake, growth, milk production and reproductive potential; increases susceptibility to

diseases and in extreme cases causes death resulting in substantial revenue loss to farmers (Sejian *et al*., 2012; Noordhuizen and Bonnefoy, 2015 and Herbut *et al*., 2019).

2.1.1 Temperature-Humidity Index (THI)

Temperature-humidity index (THI) is a single measure depicting combined effects of air temperature and relative humidity (Du Preez, 2000). This index was developed as a weather safety index to control and decrease heat stress-related losses in livestock (Habeeb *et al*., 2018). The environmental temperature alone does not provide complete information on the level of heat stress on animals and thus, temperature combined with relative humidity accurately measures the heat stress intensity. It was initially developed by Thom (1959) and later adapted by Kibler (1964) for dairy cows and is commonly used to evaluate the effect of heat stress on the livestock worldwide (Aharoni *et al*., 2003). THI is calculated from dry and wet bulb air temperatures for a particular day according to the formula given by National Research Council (NRC 1971).

$$THI = 0.72 (Wb + Db) + 40.6$$

Where, Wb is wet-bulb and Db is dry-bulb temperatures in °C

THI values are categorized into mild, moderate, and severe stress levels for cattle by the Livestock Conservation Institute (Armstrong, 1994). The upper limit of THI at which cattle maintain stable body temperature was between 72 and 76 (Ravagnolo *et al*., 2000a). Livestock is comfortable at THI from 65-72, under mild stress from 72-78, and severe stress above 80. High thermal stress (THI > 78) requires greater efforts to dissipate heat (Upadhyay *et al*., 2008).

The THI map was developed, based on hundred different locations of India by Upadhyay *et al*. (2008), according to which THI values in coastal regions go beyond the comfortable conditions during most of the year, affecting the productivity of dairy cattle. THI was generally higher than 73 from March to September in most areas (Northern and Southern India), which caused discomfort to animals. In northern areas, THI from March to September ranged from 70 to 75. In hilly areas, the THI levels were generally low (70) and animals remained comfortable throughout the year.

It was observed that the milk yield of crossbred cows in India (e.g., Karan Fries, Karan Swiss, and other Holstein and Jersey crosses) were negatively correlated with the temperature-humidity index (Mandal *et al*., 2002). Lactating dairy cows experienced heat stress when THI rose above 72, with severe heat stress occurring when THI exceeded 88 (Thatcher *et al*., 2010). The relationship between the temperature-humidity index and heat stress levels in dairy cattle is shown in Table 2. 1. The THI up to 72 had no adverse effect, while severe effects were found on milk production and reproduction for THI ranging from 90 to 98 and resulted in death beyond this level (Habeeb *et al.,* 2018).

Table 2.1: Temperature-Humidity Index and its relationship with heat stress effects in dairy cattle

THI	Stress level	Effects
<72	None	No adverse effect produced in dairy cows
72 to 79	Mild	Dairy cows adjust by seeking shade, increasing respiration, and dilation of blood vessels. There is minimum effect on milk production
80 to 89	Moderate	The respiration rate and body temperature increase. Feed intake decreases, water consumption increases. Milk production starts to decline. Reproductive performance is also decreased.
90 to 98	Severe	Cows become very uncomfortable. There is rapid respiration (panting) and excessive saliva production. Milk production and reproduction decrease markedly
>98	Danger	Potential cow death occurs

(Source: Habeeb *et al.,* 2018)

2.1.2 Thermo neutral zone (TNZ)

The Thermo neutral zone (TNZ) is defined as the range of ambient temperature within which thermoregulation is achieved only by control of sensitive heat loss, without regulatory changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 2001). An animal is assumed to be in its TNZ when it is within a temperature range that needs the least thermoregulatory effort and temperature regulation is achieved by non-evaporative physical processes alone (Hillman, 2009).

The basal rate of heat production within the TNZ is in equilibrium with the rate of heat loss to the external environment. Below the TNZ is the zone of Lower Critical Temperature (LCT) and above is the zone of Upper Critical Temperature (UCT). The TNZ for livestock ranges from 5 to 25°C (West, 2003).

When the ambient temperature decreases, the animal reaches the LCT, and the rate of metabolic heat production of the resting animal is increased by shivering and/or non-shivering thermogenesis to maintain thermal equilibrium. When the ambient temperature increases, the animal reaches the UCT, and the rate of evaporative heat loss of the resting animal is increased (tachypnea or sweating) to maintain thermal balance (IUPS Thermal Commission, 2001). Thermo neutral zone of animals depends on the age, breed, feed intake, diet composition, previous state of temperature acclimatization, production, and behavior of the animal (Aggarwal and Upadhyay, 2013).

2.1.3 Effect of Heat Stress on Milk Production

There is significant impact of heat stress on milk production due to reduced appetite and decreased feed intake. By decreasing feed intake, cows reduce metabolic heat production to cope with increased ambient temperature. Decreased milk yield under heat stress is caused by associated effects on thermal regulation, energy balance, and endocrine changes (Ominski *et al*., 2002, Usman *et al*., 2013 and Liu *et al.,* 2019).

Ravagnolo and Misztal (2000b) reported a decrease in milk yield by 0.2 kg per unit increase in Thermo Humidity Index (THI) when THI exceeded 72 in Holstein Friesian cows in Georgia. Holstein cows were very susceptible to high ambient temperatures due to their weak thermotolerance as a result of which their milk production declined resulting in substantial economic loss in summer (St-Pieree *et al*., 2003).

Bouraoui *et al*. (2002) reported that reduced feed intake accounts for about 35% of the heat stress-induced decrease in milk synthesis in HF cows. The feed intake dropped within 1 day after the initiation of heat stress, while milk yield decreased after 2 days of heat stress in HF cows (Spiers *et al*., 2004). Berman (2005) reported that

effective environmental heat loads above 35°C activate the stress response systems in lactating HF dairy cows.

Upadhyay *et al*. (2009) reported that a temperature rise of 1.0 or 1.2°C with a minor change in precipitation from March to August in India would affect milk production in cattle and buffaloes. Further it was reported that the estimated annual loss due to heat stress at the all-India level is 1.8 million tones of milk (Rs 2661.62 crore per year), which is 2% of the country's total milk production. The decrease in milk production will be higher in crossbreds (0.63%) followed by buffalo (0.5%) and indigenous cattle (0.4%). Both increase in T max (>4°C above normal) during summer and decline in T min (<3°C than normal) during winter negatively impact milk production of crossbred cattle and buffaloes.

Heat stress not only affects the quantity but also the quality of milk by altering its various components such as fat percentage, Solid non-fat (SNF), protein, casein, and lactose content. Other milk quality parameters like Somatic cell count (SCC) and bacterial counts are also increased during periods of hot humid weather (Summer *et al*., 2018). Nardone *et al*. (2010) reported that higher producing animals that have the highest feed intake are typically the most severely affected. Further heat stress also compromises the immune system in animals making them susceptible to various disease conditions like mastitis.

Gaafar *et al*. (2011) reported that with an increase in THI from 59.82 in the winter to 78.53 in summer, the total lactation milk yield (TLMY), 305 days and daily milk yield reduced by 39.00%, 31.40%, and 29.84%, respectively in HF cows. Total average milk production/cow was significantly ($p<0.05$) higher in spring (42.74 ± 4.98 L) compared to summer (39.60 ± 5.09 L).

Molee *et al*. (2011) reported that Holstein crossed with local breeds in the tropics and subtropics perform better than the purebred Holstein and were also resistant to heat stress.

Rejeb *et al*. (2012) studied heat stress in response to milk yield on 13 Holstein cows and recorded a reduction in milk yield during summer compared

to spring. Reduction in milk production was attributed to changes in metabolism, physiology and feed intake.

Dikman *et al*. (2014) observed that cows in hot humid climatic regimes show decreased milk yield and feed intake because of their continuous exposure to high humidity and high air temperature.

Cowley *et al*. (2015) reported that HF cows subjected to heat stress reduced their ingestion and produced less milk when compared with cows raised in normal climate. Pragna *et al*. (2017) observed that apart from decreased feed intake, there is decrease in nutrient absorption, alteration in rumen function, and hormonal imbalance which contributes to reduced milk production during heat stress.

2.1.4 Effect of Heat Stress on Reproduction

Heat stress has a significant adverse impact on the reproductive performance of cattle in both sexes. In females, heat stress hampers the growth of oocytes by altering the secretion of progesterone, luteinizing hormone, and follicle-stimulating hormones during the estrous cycle (Ronchi *et al*., 2001).

Heat stress was responsible for impaired ovarian function and increased embryonic mortality (Samal, 2013 and Liu *et al.,* 2019); delayed fetal growth and increased fetal loss (Wolfenson *et al*., 2000; Be´nyei *et al*., 2001; Hansen, 2007, Polsky and Keyserlingk, 2017) and poor estrus expression and endometrial responses (Wolfenson and Roth, 2019) in dairy cows. The conception rate of lactating cows during summer declined by 20 to 27% (Lucy, 2002 and Chebel *et al*., 2004) and there was a considerable increase in non-return rate to the first service in HF breed of cattle of United States (Al-Katanani *et al*., 1999; Ravagnolo and Misztal, 2000a).

Upadhyay *et al*. (2009) reported decreased intensity and length of the oestrus period, decreased growth, size, and development of ovarian follicles, decreased conception rate, decreased fetal growth and calf size, increased embryonic deaths, increased number of AI per conception and increased incidence of silent heat. In bulls, semen concentration, number of sperms, and motile spermatozoa per ejaculation were lower in summer than in winter and spring (Nichi *et al*., 2006).

2.2 PHYSIOLOGICAL PARAMETERS

Physiological parameters such as respiration rate and body temperature mainly determine the adaptability of animals. (Costa *et al.,* 2015). An animal's ability to endure the rigors of climatic stress under warm conditions has been physiologically assessed by changes in body temperature, respiration rate, and pulse rate (Dalcin *et al*., 2016).

Beatty *et al*. (2006) reported that *Bos taurus* cattle experience significant physiological modifications when exposed to prolonged and continuous periods of elevated heat and humidity. These changes persist for a few days even after the heat stress conditions have settled. *Bos indicus* also experience similar but less marked physiological changes as they are better able to regulate their body temperature in response to heat stress than *Bos taurus* breeds (Dalcin *et al*., 2016).

During genetic adaptation, *Bos indicus* cattle have acquired thermotolerant genes (Hansen, 2004), and thus have a higher degree of thermotolerance compared to *Bos taurus* animals. When exposed to specific heat stress conditions, the genetic adaptation enables *Bos indicus* cattle to have lower respiration rates and rectal temperatures than *Bos taurus* animals (Gaughan *et al*., 2000; Pereira *et al.,* 2014). The mean values for physiological parameters of Sahiwal and HF crossbred cows as reported by various authors in the literature are summarized in Table 2.2. Significant breed difference was observed; *Bos taurus* cattle had higher respiration rate, rectal temperature and heat tolerance coefficient values than *Bos indicus* cattle at all ambient temperatures from 18 to 41°C (Gaughan *et al*., 2010) indicating that crossbred cattle were more prone to heat stress.

2.2.1 Respiration Rate (RR)

Respiration rate (RR) refers to the number of breaths per minute. The normal respiration rate in adult cattle varies from 24 to 36 breaths per minute but may have a greater range, between 12 and 36 breaths/min (Kumar *et al.,* 2018). Published literature revealed that the mean respiration rate ranged from 12.78 ± 1.64 (Das*,* 2014) to 35.54 ± 0.18 (Sengar *et al.,* 2018) in Sahiwal cows and from 13.62 ± 1.52 (Varma *et al.,* 2015) to 49.22 ± 2.23 (Das*,* 2014) in crossbred cows.

Increased respiration rate is the first reaction observed, when animals are exposed to ambient temperatures above the thermoneutral zone and is the most consistent of all the physiological responses during heat stress (Du Preez, 2000). The significance of this increased respiration rate is that it enables the animal to dissipate the excess body heat by vaporizing more moisture into the expired air (Atkins *et al.,* 2018).

Several authors have reported that the ventilation rate of cattle increases significantly with a rise in ambient temperature especially during summer as compared to other seasons (Das, 2014; Kumar *et al*., 2015a; Sailo *et al*., 2015a and Verma *et al*., 2015). Unlike humans, where heat loss occurs primarily in the form of sweat at high ambient temperatures, the cattle breathe in and breathe out rapidly, so that the evaporative heat loss is increased in an attempt to maintain homeostasis (Hall and Guyton, 2011).

2.2.2 Rectal Temperature (RT)

Rectal temperature (RT) is an ideal indicator for the assessment of heat stress in dairy animals (Dalcin *et al*., 2016). RT is generally considered a useful measure of body temperature and therefore changes in RT reflect changes of a similar magnitude in deep body temperature (Lees *et al.,* 2018). The normal range of rectal temperature is very narrow in most domestic animals. Under normal conditions, the range is from 38.5°C to 39.5°C in calves, from 38.0°C to 39.5°C in heifers, and from 38.0°C to 39.0°C in adult cows (Ma *et al*., 2010).

Published literature revealed that the mean rectal temperature ranged from 37.30 ± 0.09 (Sailo *et al*., 2015a) to 39.05 ± 0.04 (Kumar *et al*., 2017a) in Sahiwal cows and from 37.07 ± 0.22 (Das*,* 2014) to 39.53 ± 0.33 (Deb *et al.,* 2013) in crossbred cows in different seasons.

Table 2.2. Means for physiological traits of Sahiwal and HF crossbreds as reported in the literature

z	Sex	Age	Season	THI	Respiration rate (breaths/ min)	Rectal temperature (°C)	Heat tolerance Coefficient	Author(s)
Sahiwal	F	Adults	Winter	49.7	12.78 ± 1.64	37.30 ± 0.17	1.60 ± 0.01	Das. (2014)
			Spring	64.65	15.09 ± 1.64	38.17 ± 0.17	1.81 ± 0.01	
			Summer	86.44	25.55 ± 1.64	38.79 ± 0.17	2.16 ± 0.03	
	F	Adults	Winter	48.77	14.22 ± 0.43	37.92 ± 0.05	1.66 ± 0.04	Kumar *et al*. (2015a)
			Spring	64.86	18.15 ± 0.43	38.19 ± 0.06	1.79 ± 0.04	
			Summer	92.62	26.26 ± 0.41	38.40 ± 0.06	2.31 ± 0.04	
	F	Adults	Winter	49.7	15.74 ± 0.79	37.30 ± 0.09	1.60 ± 0.02	Sailo *et al*. (2015a)
			Spring	64.5	18.16 ± 0.79	38.18 ± 0.09	1.80 ± 0.02	
			Summer	86.44	29.82 ± 0.79	38.81 ± 0.09	2.16 ± 0.01	
	F	Adults	Winter	48.77	14.03 ± 0.41	37.95 ± 0.05	1.53 ± 0.06	Verma *et al*. (2015)
			Spring	64.86	18.59 ± 0.41	38.23 ± 0.05	1.64 ± 0.06	
			Summer	90.96	26.70 ± 0.42	38.45 ± 0.04	2.20 ± 0.06	
	F	Adults	Summer	86.86	27.50 ± 1.00	39.05 ± 0.04	-	Kumar *et al*. (2017a)
			Winter	60.52	20.52 ± 1.05	37.92 ± 0.04	-	
	F	Adults	Winter	-	32.21 ± 0.18	38.07 ± 0.37	-	Sengar *et al*. (2018)
			Summer	-	35.54 ± 0.18	39.03 ± 0.37	-	
HF crossbreds:								
Frieswal	F	Adults	Summer	-	33.29 ± 0.19	39.53 ± 0.33	-	Deb *et al*. (2013)
Karan Fries	F	Adults	Winter	49.7	17.70 ± 2.23	37.07 ± 0.22	1.70 ± 0.07	Das. (2014)
			Spring	64.65	24.90 ± 2.23	37.98 ± 0.22	1.82 ± 0.07	
			Summer	86.44	49.22 ± 2.23	38.77 ± 0.22	2.35 ± 0.07	
	F	Adults	Winter	48.77	13.62 ± 1.52	38.04 ± 0.25	1.59 ± 0.07	Verma *et al*. (2015)
			Spring	64.86	18.20 ± 1.51	38.19 ± 0.22	1.79 ± 0.07	
			Summer	90.96	27.89 ± 1.50	38.30 ± 0.22	2.21 ± 0.06	
	F	Adults	Winter	49.7	15.78 ± 1.14	37.49 ± 0.12	1.63 ± 0.04	Sailo *et al*. (2017)
			Spring	64.5	22.98 ± 1.14	38.39 ± 0.12	1.76 ± 0.04	
			Summer	86.44	47.30 ± 1.14	39.19 ± 0.12	2.28 ± 0.04	

Change in rectal temperature is an indicator of heat storage in an animal's body and is used to assess heat stress, which affects the growth, production, and reproduction of dairy animals (Dikmen and Hansen, 2009). Even a rise of less than 1°C in rectal temperature was enough to reduce performance in most livestock species (Liu *et al.,* 2019). Wen (2011) reported that rectal temperature was significantly higher during heat stress than during non-heat stress periods ($p<0.01$) indicating that season (THI) significantly affects the rectal temperature of dairy cows.

In cows, the rectal temperature in summer is higher than in other seasons as reported by Das, (2014), Kumar *et al*. (2015a), Sailo *et al*. (2015a) and Verma *et al*. (2015). High relative humidity reduces the efficacy of the evaporative cooling and therefore high relative humidity together with high environmental temperature lowers the capacity of the cow to maintain normal body temperature (Hill and Wall, 2014).

2.2.3 Heat Tolerance Coefficient (HTC)

Heat tolerance is the ability of the animals to withstand heat when all other factors are constant. *Bos indicus* breeds of cattle are more heat tolerant than *Bos taurus* cattle breeds (Gaugham *et al.,* 2010). To evaluate animals for their heat tolerance capacity, indices were developed by different workers some of which are given in Table 2.3.

Table 2.3. Heat tolerance indices for cattle and buffalo

Heat Tolerance Indices	Formula	Authors
Iberia Heat Tolerance Test	* HTC= 100-10 (RT-101)	Rhoad. (1944)
Gaalaa's Heat Tolerance Test	* HTC= 100-14 (RT-101)	Gaalaas. (1947)
Benezra's Coefficient of Adaptability	# BCA= RT/ 38.33 + RR/23	Benezra. (1954)
Dairy Search Index (DSI)	DSI= 0.5X1/X+ 0.3Y1/Y+ 0.2Z1/Z	Thomas *et al*. (1973)

*RT = Rectal temperature (°F); #RT = Rectal Temperature (°C); RR= Respiration rate/minute; X1, Y1 and Z1 are rectal temperature, pulse rate and respiration rate after exposure and X, Y and Z the same parameters before exposure to sun respectively.

The heat tolerance coefficient (HTC), which is a derived parameter, given by Benzera (1954) is currently used to assess the heat tolerance level in animals by taking into consideration, both the physiological indicators of heat stress i.e. respiration rate (RR) and rectal temperature (RT) together. The HTC ranged from 1.53 ± 0.06 in winter (Varma *et al.,* 2015) to 2.31 ± 0.04 in summer (Kumar *et al.,* 2015a) in Sahiwal cows, while in crossbred cows it ranged from 1.59 ± 0.07 (Varma *et al.,* 2015) in winter to 2.35 ± 0.07 (Das*,* 2014) in summer season. Lower values of HTC indicated that animal is well thermo-adaptable and higher values depicted low thermo-adaptability (Singh *et al.,* 2013).

2.3 PRODUCTION AND REPRODUCTION PERFORMANCE OF SAHIWAL AND HF CROSSBRED COWS

The means for production traits of Sahiwal and HF crossbreds, as reported in the literature by various authors are presented in Table 2.4 and the means for reproduction traits are presented in Table 2.5.

2.3.1 Performance of Sahiwal cows

The mean total lactation milk yield ranged from 1355.63 kg (Chakravarthi *et al.*,2017) to 1900 kg (Joshi *et al.,* 2001); the peak yield ranged from 6.11 kg (Chakravarthi *et al.*, 2017) to 9.20 kg (Reddy *et al.*, 2015) and the mean lactation length ranged from 235 days (Rehman and Khan, 2012) to 348.25 days (Chakravarthi *et al.*,2017) in Sahiwal cows (Table 2.4). The mean age at first service was 1053.53 days (Singh *et al.,* 2011) and the mean age at first calving ranged from 1095 days (Joshi *et al.,* 2001) to 1380 days (Dubey and Singh., 2005).Kumar and Gandhi (2011) reported significant effect of parity on all production and reproduction traits in Sahiwal cows. It was observed that first lactation reproduction traits were comparatively of longer duration than the later lactations indicating better reproductive efficiency in later lactations/age of cows.

In a study conducted by Rehman and Khan (2012) involving data on 23925 lactations of 5897 Sahiwal cows, all the productive and reproductive traits were affected ($P \leq 0.01$) by parity. The maximum milk yield was recorded for fifth parity cows.

Table 2.4. Means for production traits of Sahiwal and HF crossbreds as reported in the literature

Breed	Total lactation milk yield (kg)	Peak yield (kg)	Lactation length (days)	Author(s)
Sahiwal	1862.42 (1)	-	317.71 (1)	Javed *et al.* (2000)
Sahiwal	1900	-	315	Joshi *et al.* (2001)
Sahiwal	1823.35	-	313.78	Maurya and Saraswat (2002)
Sahiwal	-	-	280.78 (1)	Dubey and Singh (2005)
Sahiwal	-	-	240.81 (1)	Naskar *et al.* (2005)
	-	-	258.65 (2)	
	-	-	258.25 (3)	
	-	-	262.53 (4)	
	-	-	262.19 (5)	
	-	-	254.25 (P)	
Sahiwal	-	-	254	Zafar *et al.* (2008)
Sahiwal	-	6.26	-	Sharma *et al.* (2010)
Sahiwal	1793.09	-	286.32	Kumar and Gandhi (2011)
Sahiwal	1380.1 (1)	-	231 (1)	Rehman and Khan (2012)
	1520.0 (2)	-	237 (2)	
	1577.9 (3)	-	241 (3)	
	1582.7 (4)	-	239 (4)	
	1676.0 (5)	-	241 (5)	
	1590.3 (6)	-	236 (6)	
	1600.2 (7)	-	233 (7)	
	1579.6 (8)	-	231 (8)	
	1578.5 (9)	-	226 (9)	
	1503.1 (10)	-	213 (10)	
	1552.0 (P)	-	235 (P)	
Sahiwal	1780	9.20	295.54	Reddy *et al.* (2015)
	1880.39	-	-	Verma *et al.* (2016)
Sahiwal	1355.63	6.11	348.25	Chakravarthi *et al.* (2017)
HF crossbreds				
HF × Sahiwal				Bhadauria and Katpatal (2003)
3/8	1716.01 (1)	-	-	
½	2015.22 (1)	-	-	
5/8	2102.00 (1)	-	-	
¾	2101.14 (1)	-	-	
7/8	2100.53 (1)	-	-	
HF × Sahiwal	-	-	323.70 (1)	Dubey and Singh (2005)

Figures in parentheses indicate parity, P = pooled

Contd.,

Table 2.4. *Contd.,*

Breed	Total lactation milk yield (kg)	Peak yield (kg)	Lactation length (days)	Author(s)
HF × Sahiwal	2592.14 (1)	11.46 (1)	321.88 (1)	Lakshmi *et al.*(2010)
	3157.95 (2)	14.08 (2)	330.16 (2)	
	3159.42 (3)	14.75 (3)	326.09 (3)	
	3142.09 (4)	14.60 (4)	330.82 (4)	
	3268.35 (5)	14.67 (5)	327.36 (5)	
	3185.68 (6)	14.21 (6)	327.07 (6)	
	2869.89 (7)	13.48 (7)	317.63 (7)	
	2559.40 (8)	12.84 (8)	309.65 (8)	
	2615.09 (9)	12.86 (9)	323.50 (9)	
	2307.28 (10)	11.28 (10)	328.65 (10)	
	3255.63 (11)	14.99 (11)	399.38 (11)	
	2258.96 (12)	10.37 (12)	306.16 (12)	
	2864.32 (P)	13.30 (P)	329.03 (P)	
Frieswal	3165.31 (1)	-	301.74 (1)	Singh *et al.* (2014)
	3179.11 (2)	-	315.67 (2)	
	3085.24 (3)	-	313.80 (3)	
	2984.04 (4)	-	288.99 (4)	
Frieswal	4054.35 (1)	-	301.84 (1)	Singh *et al.* (2016)
Frieswal	-	-	302.64 (1)	Kakati *et al.* (2017)
	-	-	313.15 (2)	
	-	-	305.34 (3)	
	-	-	302.59 (4)	
	-	-	299.72 (5)	
	-	-	302.78 (6)	
	-	-	296.96 (7)	
	-	-	303.31 (P)	
HF × Sahiwal	3214.49	12.92	-	Kumar *et al.*(2017b)
Frieswal	2550.51 (1)	-	315.65 (1)	Kundu *et al.* (2018)
	3079.64 (2)	-	334.32 (2)	
	3240.54 (3)	-	325.53 (3)	
	3304.90 (4)	-	321.95 (4)	
	3306.64 (5)	-	319.11 (5)	
	3087.72 (6)	-	310.85 (6)	
	3190.69 (7)	-	300.52 (7)	
	3399.80 (8)	-	334.53 (8)	
	3020.04 (9)	-	326.33 (9)	
	2721.13 (10)	-	318.58 (10)	
	3090.16 (P)	-	320.74 (P)	
Frieswal	3346.17	15.21	325.90	Annual report CIRC, (2019)

Figures in parentheses indicate parity, P = pooled

Table 2.5. Means for reproduction traits of Sahiwal and HF crossbreds as reported in the literature

Breed	Age at I service (days)	Age at I calving (days)	Gestation period (days)	Service period (days)	Dry period (days)	Calving interval (days)	Author(s)
Sahiwal	-	-	-	-	198.30 (1)	-	Javed *et al*. (2000)
Sahiwal	-	1095	-	-	-	420	Joshi *et al*. (2001)
Sahiwal	-	1330.92	-	195.02 (1)	191.56 (1)	505.49 (1)	Kushwaha *et al.* (2003)
Sahiwal	-	1380.20	-	256.71 (1)	251.69 (1)	530.03 (1)	Dubey and Singh (2005)
Sahiwal	-	-	286.67 (1)	137.67 (1)	160.56 (1)	425.10 (1)	Naskar *et al.* (2005)
	-	-	286.82 (2)	122.55 (2)	127.40 (2)	406.23 (2)	
	-	-	283.69 (3)	120.45 (3)	122.61 (3)	404.31 (3)	
	-	-	284.75 (4)	116.42 (4)	131.74 (4)	398.22 (4)	
	-	-	284.97 (5)	117.06 (5)	104.39 (5)	405.24 (5)	
	-	-	285.64 (P)	123.73 (P)	133.58 (P)	408.20 (P)	
Sahiwal	-	-	-	155.33	148.61	445.92	Kumar and Gandhi (2011)
Sahiwal	1053.53	1329.25	-	-	-	-	Singh *et al*. (2011)
Sahiwal	-	-	-	178 (1)	235 1)	468 (1)	Rehman and Khan (2012)
	-	-	-	158 (2)	223 2)	445 (2)	
	-	-	-	147 (3)	215 3)	436 (3)	
	-	-	-	150 (4)	214(4)	437 (4)	
	-	-	-	146 (5)	213 5)	434 (5)	
	-	-	-	140 (6)	210 6)	428 (6)	
	-	-	-	151 (7)	224 7)	440 (7)	
	-	-	-	143 (8)	213(8)	432 (8)	
	-	-	-	143 (9)	215 9)	429 (9)	
	-	-	-	150(10)	218(10)	437 10)	
	-	-	-	151 (P)	218 (P)	438 (P)	
Sahiwal	-	1250.07	-	-	-	-	Balasubramaniam *et al*., (2013)
Sahiwal	-	-	285.12	205.0	176.79	490.58	Reddy *et al*. (2015)

Figures in parentheses indicate parity, P = pooled

Contd.,

Table 2.5. *Contd.,*

Breed	Age at I service (days)	Age at I calving (days)	Gestation period (days)	Service period (days)	Dry period (days)	Calving interval (days)	Author(s)
HF × S	-	986.00	-	-	-	-	Gaur *et al.* (2000)
HF × S			-	-	-	-	Banerjee and Banerjee (2002)
25%	-	1972.00	-	-	-	-	
50%	-	1905.00	-	-	-	-	
62.5%	-	1052.00	-	-	-	-	
75%	-	952.60	-	-	-	-	
HF × S							Banerjee and Banerjee (2003)
25%	-	-	278.00	-	-	-	
50%	-	-	281.50	-	-	-	
62.5%	-	-	280.20	-	-	-	
75%	-	-	280.60	-	-	-	
HF × S	-	1319.10	-	238.15 (1)	169.54 (1)	487.46 (1)	Dubey and Singh (2005)
HF × S	640.24	983.14	277.25 (1)	215.93 (1)	166.98 (1)	485.49 (1)	Lakshmi (2007)
	-	-	276.20 (2)	190.87 (2)	134.62 (2)	461.18 (2)	
	-	-	277.20 (3)	175.01 (3)	123.59 (3)	445.84 (3)	
	-	-	276.74 (4)	177.68 (4)	123.19 (4)	450.60 (4)	
	-	-	277.20 (5)	181.13 (5)	126.41 (5)	452.02 (5)	
	-	-	276.93 (6)	186.67 (6)	126.66 (6)	457.24 (6)	
	-	-	275.66 (7)	165.91 (7)	113.05 (7)	435.72 (7)	
	-	-	276.14 (8)	164.25 (8)	128.27 (8)	433.65 (8)	
	-	-	275.77 (9)	203.77 (9)	147.37 (9)	462.49 (9)	
	-	-	275.91 (10)	211.90 (10)	143.17 (10)	475.62 (10)	
	-	-	271.68 (11)	168.15 (11)	96.91 (12)	431.55 (11)	
	-	-	274.44 (12)	158.11 (12)	104.01 (12)	413.62 (12)	
	-	-	275.93 (P)	183.28 (P)	127.85 (P)	450.42 (P)	

Figures in parentheses indicate parity, P = pooled

Contd.,

Table 2.5. *Contd.,*

Breed	Age at I service (days)	Age at I calving (days)	Gestation period (days)	Service period (days)	Dry period (days)	Calving interval (days)	Author(s)
Frieswal	20.53 months	29.72 months	281.17 (1)	-	136.47 (1)	438.36 (1)	Singh *et al*. (2014)
	-	-	279.39 (2)	-	120.19 (2)	435.80 (2)	
	-	-	278.45 (3)	-	99.48 (3)	416.30 (3)	
	-	-	278.69 (4)	-	111.24 (4)	403.41 (4)	
	-	-	278.43 (P)	-	116.85 (P)	305.05 (P)	
Frieswal	-	-	-	169.03 (1)	139.26 (1)	439.77 (1)	Kumar *et al.* (2015b)
	-	-	-	155.06 (2)	108.72 (2)	430.76 (2)	
	-	-	-	144.68 (3)	110.47 (3)	421.61 (3)	
	-	-	-	150.45 (4)	111.87 (4)	426.99 (4)	
	-	-	-	155.89 (5)	110.71 (5)	431.55 (5)	
	-	-	-	122.00 (6)	106.51 (6)	396.37 (6)	
	-	-	-	140.55 (>7)	119.50 (>7)	414.31 (>7)	
	-	-	-	148.24 (P)	115.29 (P)	423.05 (P)	
Frieswal	-	928.07	-	-	99.40 (1)	401.33 (1)	Singh *et al.* (2016)
Frieswal	-	-	-	-	-	463.82(1)	Kakati *et al.* (2017)
	-	-	-	-	-	443.89 (2)	
	-	-	-	-	-	417.88 (3)	
	-	-	-	-	-	407.77 (4)	
	-	-	-	-	-	394.63 (5)	
	-	-	-	-	-	435.79 (6)	
	-	-	-	-	-	431.19 (P)	
HF × S	-	1092.01	-	-	-	-	Kumar *et al.* (2017b)
HF × S	-	937.28	-	-	-	-	Kundu *et al.* (2018)
Frieswal	-	970.65	-	159.95	115.36	441.96	Annual report, CIRC (2019)

Figures in parentheses indicate parity, P = pooled

Singh *et al*. (2016) evaluated the effect of genetic and non-genetic sources of variations on lactation yield in Sahiwal cows maintained at Uttar Pradesh Livestock-cum-Agriculture Farm, Lucknow, Uttar Pradesh and reported that lactation yield was significantly affected by parity ($P<0.05$).

2.3.2 Performance of HF crossbreds

The mean total lactation milk yield ranged from 2864.32 to 3346.17 kg; the peak yield from 13.30 to 15.21 kg and the mean lactation lenghth ranged from 303.21 to 325.90 days in HF crossbred cows as reported in the published literature (Table 2.4). The mean age at first calving ranged from 928.07 days (Singh *et al.,* 2016) to 1092.01 days (Kumar *et al.,* 2017b); while the gestation period, service period, dry period and calving interval ranged from 275.93 to 281.50, 148.24 to 183.28, 99.40 to 127.85 and 305.05 to 450.42 days respectively as reported by various authors (Table 2.5).

Lakshmi (2007) reported significant influence of parity on service period, dry period, and calving interval in HF crossbred cows. The cows in first parity had the longest service period (215.93 days), whereas the cows in twelfth parity had the shortest service period (158.11 days). It was found that cows in the first parity had the longest dry period (166.98 days), while those in eleventh parity had the shortest dry period (96.91 days). Similarly, the longest (485.49 days) and shortest (413.62 days) means for calving intervals were recorded for cows in parities 1 and 12, respectively.

Significant effect of parity on production traits was reported by Lakshmi *et al*. (2010) in HF Sahiwal crossbred cows of Military dairy farm, Secunderabad. The highest (3268.35 kg) and lowest (2307.28 kg) means for TLMY were obtained for the cows in parities five and ten, respectively. Similarly, cows in eleventh parity had the highest (14.99 kg) mean peak yield, while those in twelfth parity had the lowest (10.37 kg) mean. The mean lactation length was the longest in eleventh parity (399.38 days) and the shortest (306.16 days) in twelfth parity.

Singh *et al*. (2014) analyzed data of HF Sahiwal crossbred cattle maintained at Military Farm, Jammu and Kashmir, India, and reported a significant effect of parity on dry period.

Data on performance records of Frieswal cows were analyzed by Kakati *et al*. (2017) to determine the effects of genetic and non-genetic parameters on various production and reproduction traits. Parity had a significant effect on lactation milk yield and calving interval.

Kundu *et al*. (2018) analyzed lactation records of Frieswal cows from 3 Military Dairy Farms of Southern Command. Parity had a significant influence on lactation length and standard lactation milk yield.

2.4 HEAT SHOCK PROTEINS (*HSP*s)

Heat shock proteins (*HSP*s) are evolutionarily conserved family of proteins induced in a living cell in response to numerous biological stresses, including heat, high pressures, and toxic compounds. The heat shock genes are highly conserved and display low variability across species, indicating the evolutionary importance of cell protection during and after stress (Chen *et al*., 2005; Tutar *et al*., 2010). *HSP* family has a structurally common chaperone subset controlling the form of protein folding, which maintains homeostasis of proteins to stressful circumstances (Dimauro *et al*., 2016).

*HSP*s were originally identified as proteins whose expression was greatly increased by heat shock (Lindquist, 1986). As a class, *HSP*s are among the most highly expressed cellular proteins across all organisms. Heat shock proteins protect the cells when stressed by elevated temperature. Many members of these heat shock protein families are constitutively present in cells while some are expressed only after stress. The fraction of *HSP*s, however, increases to 4-6 percent of cellular proteins when cells are heated (Crevel *et al*., 2001). The thresholds for expression of *HSP*s are correlated with levels of stress that animals normally encounter (Ellis, 2006). *HSP*s are present in almost all organisms from prokaryotes to mammals (Concannon *et al*., 2003).

Mammalian *HSP*s have been classified into two groups according to their size: high molecular weight *HSP*s and small *HSP*s. The high molecular weight group includes five major families: large *HSP*s (proteins exceeding 100kDa); *HSP90* (proteins from 83-90 kDa); *HSP70* (proteins from 66-78 kDa), *HSP60* and *HSP40*.

High molecular weight *HSP*s are ATP-dependent chaperones and require co-chaperones to modulate their conformation and ATP binding. In contrast, the second group includes small *HSP*s (proteins from 15-30 kDa) which are ATP-independent chaperones (Garrido *et al*., 2001). The details of mammalian heat shock protein (*HSP*) families and their cellular location, as reported in the literature are presented in Table 2.6. Most of the *HSPs* are found in the cytosol, mitochondria, and endoplasmic reticulum of the cells.

Table 2.6. Mammalian heat shock protein (*HSP*) families and their cellular location, as reported in the literature

Heat Shock Protein	Cellular location	Reference
HSP 110	Cytosol/Nucleus	Garrido *et al*. (2001), Kregel (2002)
HSP90α	Cytosol	Whitley *et al*. (1999)
HSP90β	Cytosol	Garrido *et al*. (2001)
GRP94	Endoplasmic reticulum	Kregel, (2002)
HSP70	Cytosol/Nucleus	Whitley *et al*. (1999), Garrido *et al*. (2001)
mHSP70	Mitochondria	Kregel (2002)
GRP78	Endoplasmic reticulum	Kregel (2002)
HSP60	Mitochondria	Whitley *et al*. (1999), Garrido *et al*. (2001), Kregel (2002)
HSP47	Cytosol	Kregel (2002)
HSP40	Cytosol	Whitley *et al*. (1999), Kregel (2002)
HSP27	Cytosol	Whitley *et al*. (1999), Garrido *et al*. (2001), Kregel (2002)
HSPb12	Plasma membrane	Jee (2016)
HSP10	Mitochondria	Xu *et al*. (2014)

2.4.1 Functions of Heat Shock Proteins

*HSP*s are molecular chaperones that play a critical role in recovering cells from stress and cytoprotection, protecting the cells from subsequent insults. Through their ability to recognize nascent polypeptides, unstructured protein areas, and exposed hydrophobic stretches of amino acids, they protect stressed cells. Chaperones thus hold, translocate or refold denatured proteins and prevent their irreversible aggregation with other cell proteins (Archana *et al*., 2017).

In addition to protecting cells from stress, almost all *HSP*s are constitutively expressed under normal growth conditions, where they maintain protein homeostasis by regulating protein folding quality control. The chaperone activities of heat shock proteins enable the folding of newly synthesized proteins and assist the translocation of proteins across intracellular membranes (Hartl and Hayer-Hartl, 2002).

The important functions of heat shock proteins, as reported in the literature are detailed in Table 2.7. The *HSPs* have major roles in cellular thermotolerance, apoptosis, immune-modulation, and heat stress.

2.5 HEAT SHOCK PROTEIN *(HSP)* GENES

Genes encoding the heat shock proteins are called *HSP* genes and their nomenclature is given by the HUGO Gene Nomenclature Committee. Though there are many *HSP* genes, thermotolerance is mainly correlated with *HSP70* and *HSP90* genes in Livestock species. *HSP70* is reported to be the most abundant and temperature-sensitive playing a crucial role in environmental stress and thermal adaptation (Gade *et al*., 2010). In farm animals elevated levels of expression of proteins of the *HSP70* and *HSP90* family were observed in sheep, buffalo, cattle, broilers, and goats during the summer season (Archana *et al.,* 2017). Polymorphism in the *HSP70* and *HSP90* genes have shown an association with heat tolerance, milk production, fertility, and disease susceptibility in livestock (Shergojry *et al*., 2014a; Kumar *et al*., 2015a, Bhat *et al.,* 2016). They can make ideal candidate gene markers for the selection of animals with better climate resilience, immune response, and superior performance. (Hassan *et al.,* 2019).

2.5.1 *HSP70* gene

HSP70 gene family in bovines includes *HSP70-1, HSP70-2, HSP70-3,* and *HSP70-4* gene isoforms. The bovine *HSP70-1* gene is 2369 bp long containing a 1926 bp coding sequence from base position 157 to 2082 (Gutierrez and Guerrieo, 1995; Gade *et al*., 2010). It is an intronless gene that has been mapped on to *Bos taurus* autosome 23 (BTA 23).

Table 2.7. Important functions of heat shock proteins as reported in the literature

Protein	Function	Reference
HSP27	Microfilament stabilization	Kregel (2002)
	Cellular anti-apoptotic activity	Samali and Cotter (1996) Mehlen *et al*. (1997) Arrigo (1998) Garrido *et al*. (1999) Wagstaff *et al*. (1999) Bruey *et al*. (2000) Tezel and Wax (2000)
	Increases cellular resistance against heat shock and other injuries such as those mediated by chemotherapeutic drugs and oxidative stress	Arrigo and Landry (1994) Mehlen *et al*. (1997) Arrigo (1998) Rosse *et al*. (1998)
HSP60	Refolding of proteins and prevention of aggregation of denatured proteins	Leppa and Sistonen (1997) Kregel (2002)
HSP70	Molecular chaperone, Protection of cells from heat shock-cellular anti-apoptotic activity, Thermotolerance	Samali and Cotter (1996) Leppa and Sistonen (1997) Mosser *et al*. (1997) Jaattela *et al*. (1998) Li *et al*. (2000) Kamaruddin *et al*. (2004)
HSP90	Molecular chaperone, cellular anti-apoptotic activity, regulation of steroid hormone receptors and protein translocation, Thermotolerance	Leppa and Sistonen (1997) Pandey *et al*. (2000) Kregel (2002)
HSP110	Molecular chaperone – Protein folding, Thermotolerance	Leppa and Sistonen (1997) Kregel (2002)

2.5.2 *HSP90* gene

The chaperone *HSP90* is one of the most abundant proteins in eukaryotic cells, comprising 1–2% of cellular proteins under non-stress conditions (Young *et al*., 2001). There are two major cytoplasmic *HSP90* isoforms, the inducible form (*HSP90AA1/HSP90α*) and the constitutive form (*HSP90AB1/HSP90β*), which have arisen by gene duplication (Chen *et al*., 2005).

HSP90AA1 gene is located on *Bos taurus* autosome 21 (BTA 21) and spans nearly 5368 bp comprising of 11 exons out of which the first exon does not translate. *HSP90AB1* gene has been mapped on BTA 23 and spans nearly 5883 bp comprising of 13 exons out of which the first exon does not translate. Studies have revealed that the regions from intron 7 to exon 11 of *HSP90AB1* gene are highly polymorphic.

2.6 POLYMORPHISM IN THE *HSP* GENES

2.6.1 *HSP70* gene polymorphism

Cai *et al*. (2005) identified polymorphism in selected 5' flanking region of the *HSP70* gene by using PCR-SSCP assay and determined 4 genotypes as, AA, AB, BB, and AC, whose frequencies were 58.9, 11.1, 7.78 and 22.22 respectively in dairy cows. The frequencies of alleles a, b and c were 0.739, 0.150, and 0.111 respectively.

Lamb *et al*. (2007) studied the genetic diversity by complete sequencing in a 523 bp segment of the *HSP70* gene in *Bos taurus* (Angus), *Bos indicus* (Brahman), and *Bos taurus*/*Bos indicus* crosses and found that the specific fragment of *HSP70* gene is polymorphic.

Rosenkrans *et al*. (2010) conducted an experiment to identify polymorphism in a 539 base segment of the bovine *HSP70* promoter region and reported 11 single nucleotide polymorphisms in crossbred Brahman cows.

Sodhi *et al*. (2013) characterized the *HSP70* gene in *Bos indicus*, *Bos taurus*, and buffalo breeds. Comparative sequence analysis of taurine, indicine cattle, and buffalo *HSP70* gene revealed a total of 54 gene variations among the three species in the *HSP70* gene.

Kerekoppa *et al*. (2015) investigated the polymorphism in the coding region of the *HSP70* gene in HF crossbreds and Deoni breed of cattle using PCR-SSCP technique. The analysis revealed 14 band patterns in Deoni cattle and 8 band patterns in HF crossbred cattle. Among the 14 observed band patterns, 6 bands were common to both Deoni and HF crossbred cattle, while 8 bands were unique to Deoni cattle and 2 bands were unique to HF crossbred cattle.

Bhat *et al*. (2016) subjected a 295 bp fragment of the *HSP70* gene to PCR-SSCP in Tharparkar cattle. Three SSCP patterns and consequently two alleles namely A and B were documented in the *HSP70* gene. The frequencies of the three genotypes were 0.3281 for AA, 0.3594 for AB, and 0.3125 for BB.

2.6.2 *HSP90AA1* gene polymorphism

Shergojry *et al*. (2014a) analyzed the polymorphism in the *HSP90AA1* gene using the PCR-SSCP technique in Deoni cattle. Analysis of amplicons of fragment comprising of exon 8 in the *HSP90AA1* gene revealed three unique SSCP patterns with different mobility shifts. The pattern I showed one distinct band, pattern II showed two distinct bands and pattern III showed six distinct bands, respectively. The genotype frequencies of patterns I, II, and III were 0.250, 0.639, and 0.111 respectively.

Shergojry *et al*. (2014b) studied the polymorphism in exon 9 of *HSP90AA1* gene in Deoni cows and reported two PCR-SSCP patterns with different mobility shifts, marked as Pattern I and Pattern II. The pattern I showed two distinct DNA bands, while pattern II showed three distinct DNA bands. The genotypic frequency of pattern I and pattern II was 0.153 and 0.847, respectively. The exon 10 of the *HSP90AA1* gene also revealed two PCR-SSCP patterns. The pattern I revealed two distinct DNA bands, while pattern II revealed three distinct DNA bands. The genotype frequencies of patterns I and II were 0.236 and 0.764 respectively.

Kumar *et al.* (2015a) studied the genetic polymorphism within the exon 3 of the *HSP90AA1* gene in Sahiwal cows. A 450 bp fragment covering exon 3 region of *HSP90AA1* gene was amplified and three genotypes AA, AG, and GG with respective frequencies 0.23, 0.50, and 0.27 were ascertained.

Badri *et al.* (2018) identified five single nucleotide polymorphisms in Chinese Holstein lactating cows: one in the promoter, three in the coding region, and one in the 3′-UTR region of *HSP90AA1* gene.

2.6.3 *HSP90AB1* gene polymorphism

Charoensook *et al*. (2012) conducted experiments in *Bos taurus* (crossbred Holstein Friesian) and *Bos indicus* (Thai native cattle: White Lamphun and Mountain cattle) in Thailand and found that the exons 10, 11, introns 8, 9, 10 and 11, and 3′ UTR regions of *HSP90AB1* gene were polymorphic.

Sailo *et al*. (2015a) reported polymorphism in a targeted region of 846 bp of *HSP90AB1* gene in Sahiwal cows. Further, polymorphism was also identified in the targeted regions of the *HSP90AB1* gene in Jersey crossbred cows of Assam (Sailo *et al*., 2015b). Their analysis revealed variation in exons 8 and 11 and intron 10 of the *HSPAB1* gene.

Sajjanar *et al*. (2015) used allele-specific PCR to detect nucleotide polymorphisms within the *HSP90AB1* gene in Sahiwal and Frieswal cattle. Three genotypes CC, CT, and TT were identified whose frequencies were 0.05, 0.78, and 0.17 respectively in Sahiwal and 0.20, 0.70, and 0.10 in Frieswal cattle.

2.7 ASSOCIATION STUDIES OF *HSP* GENE POLYMORPHISMS WITH PHYSIOLOGICAL, PRODUCTION AND REPRODUCTION TRAITS

2.7.1 *HSP70* gene

Cai *et al.* (2005) studied the mutations in the 5’ flanking region of the *HSP70* gene to check the association of this region with susceptibility of animals to heat stress. Four different banding patterns were observed as AA, AB, AC, and BB in heat-shocked dairy cows. The expression of *HSP70* mRNA was significantly higher in AC genotype animals as compared to the other three genotyped animals, which indicated that this polymorphic site could be related to traits of heat stress susceptibility.

Rosenkrans *et al.* (2010) amplified a 539 base segment of the bovine *HSP70* promoter region and reported single nucleotide polymorphisms some of which were associated with the milk yield and calving rates in crossbred Brahman cows. It was suggested that the promoter region of the bovine *HSP70* gene is polymorphic and may be useful in selecting cows with greater fertility.

In a study conducted by Li *et al*. (2011) on 890 Holstein Friesian cows, a G/A mutation was found in the coding region of *HSP70* gene resulting in two genotypes AA and AB, a T/C mutation was found in 3'-UTR resulting in three genotypes of CC, CD, and DD and three-point mutations were found in 3'-UTR resulting in six genotypes EE, EF, FF, EG, FG and GG. It was presumed that these mutations could be used in marker-assisted selection for anti-heat stress cows in breeding programs.

Deb *et al*. (2013) studied the promoter variants of *HSP* 70 gene by genotyping 200 Frieswal cows using the PCR-RFLP technique. It was suggested that the promoter region is polymorphic and may be useful in the selection of dairy cows for better heat tolerance. The heterozygous cytosine deletion (C-) cows had lower total milk yield, peak yield, and yield at 300 days than the wild type (CC) promoter cows. The homozygous wild types had better summer tolerance than the heterozygous deletion genotypes.

Sodhi *et al.* (2013) characterized the promoter region of the inducible *HSP70* gene in diverse breeds of Indian zebu cattle and buffaloes. A total of 11 nucleotide changes were observed in the promoter sequence across the analyzed species, 3 of these changes were located within the potential transcription factor binding domains. It was suggested that these polymorphisms could further be evaluated as molecular markers for thermotolerance in the selection of cattle.

In a study conducted by Bhat *et al*. (2016) a 295 bp fragment of the *HSP70* gene was subjected to PCR-SSCP in Tharparkar cattle. Three SSCP patterns and consequently two alleles namely A and B were documented in the *HSP70* gene. The allele A of *HSP70* was positively correlated with thermal tolerance and genotype AA was found to be superior with good heat tolerance, followed by genotype AB. They

suggested that such polymorphism could be used as an indicator for the selection of thermotolerant cattle.

2.7.2 *HSP90AA1* gene

Shergojry *et al*. (2014a) identified polymorphism in exon 8 of the *HSP90AA1* gene in Deoni cows. Three unique SSCP patterns corresponding to TT, GG, and TG genotypes were documented. Association analysis revealed that the cows having TG genotype had significantly higher Lactation milk yield (kg) when compared to cows with TT and GG genotypes. There was no difference in lactation length (days) in cows with different genotypes.

Kumar *et al.* (2015a) studied the genetic polymorphism within the exon 3 of the *HSP90AA1* gene in Sahiwal cows and explored the association with heat tolerance traits. A significant difference among the genetic variants of the *HSP90AA1* gene with heat tolerance traits was found. The AA genotype had a lower heat tolerance coefficient as compared to both AG and GG genotypes.

Kumar *et al.* (2016) identified the polymorphism in the targeted region of exon 3 of the *HSP90AA1* gene in Karan Fries cows. Association analysis indicated that the homozygous GG genotype animals had lower mean values for respiration rate, rectal temperature, and heat tolerance coefficient. It was concluded that cows with GG genotype can be favored for heat tolerance traits for better adaptation in the tropical and subtropical climate. The THI significantly influenced the physiological parameters of the cows in their study.

Badri *et al.* (2018) studied the association between the genetic variations in the promoter region of *HSP90AA1* gene and thermal resistance in Chinese Holstein cattle breeds. The genotype CC showed the highest transcription activity (expression of *HSP90AA1*) compared to the GG genotype.

2.7.3 *HSP90AB1* gene

According to a study done by Charoensook *et al.* (2012), polymorphisms in the bovine *HSP90AB1* gene were associated with heat tolerance in cattle. Mountain cattle and White Lamphun heifers recorded significantly better physiological parameters

than Holstein Friesian heifers. The association analysis revealed that the T allele within intron 3 improved heat tolerance. Allele T was exclusively found in White Lamphun animals and to 84% in Mountain cattle. Holstein Friesian heifers revealed an allele frequency of only 18%.

Sailo *et al.* (2015a) investigated the association of polymorphism of the bovine *HSP90AB1* gene with respiration rate, rectal temperature, heat tolerance coefficient, and total milk yield in Sahiwal cows. Cows with CT genotype recorded significantly lower respiration rate than CC genotype. The CT genotype also had better total milk yield. Further, Sailo *et al.* (2015b) identified polymorphism in the targeted regions of the *HSP90AB1* gene in Jersey crossbred cows of Assam and associated them with the physiological parameters. It was found that cows with CC genotype showed a significantly lower respiration rate than TT and TC genotypes.

Sajjanar *et al* (2015) studied the association of *HSP90AB1* gene polymorphism with the physiological traits like respiration rate, rectal temperature, and heat tolerance coefficient. The studies indicated that TT genotypes had a significantly higher heat tolerance coefficient and lower respiration rate values than CC and CT in both Sahiwal and Frieswal breeds. The TT genotype animals also had better production parameters in terms of total milk yield.

CHAPTER III

MATERIALS AND METHODS

Information on the livestock utilized, laboratory methods, protocols and statistical analyses followed in the present study are detailed in this chapter.

3.1 MATERIALS

3.1.1 Experimental Animals

A total of 50 Sahiwal cows (Fig 3.1) maintained at the Livestock Farm Complex, College of Veterinary Science Rajendranagar, and 50 crossbred cows (Holstein Friesian × Sahiwal crosses with 7/8 exotic inheritance, Fig 3.2) maintained at the Military Dairy Farm, Secunderabad were utilized for the present investigation.

3.1.2 Weather Conditions

Hyderabad, the capital city of Telangana State is located at 17.366°N Latitude and 78.476°E Longitude. It is situated at an elevation of 536 meters (1607 feet) above the mean sea level. The data on the weather conditions, maximum and minimum temperatures (°C), dry and wet bulb readings (°C), and relative humidity (%) during the experimental period were collected from Agriculture Climate Research Center, ARI, Hyderabad. During the present study year of 2018, the average environmental temperatures ranged from 27.92°C in December which was the coldest month to 41.24°C in May, which was the hottest month. Hyderabad experienced moderate rainfall in July, August, and September. The average annual rainfall received was 766 mm and the average relative humidity was around 65% varying from 45% during summer to 78% during the monsoon. Winters were moderately cold with temperatures ranging from 15°C to 31°C. The temperature-humidity index (THI) was calculated based on dry and wet bulb temperatures on those days, during which the physiological parameters were recorded in each season, according to the formula developed by the National Research Council (NRC), 1971.

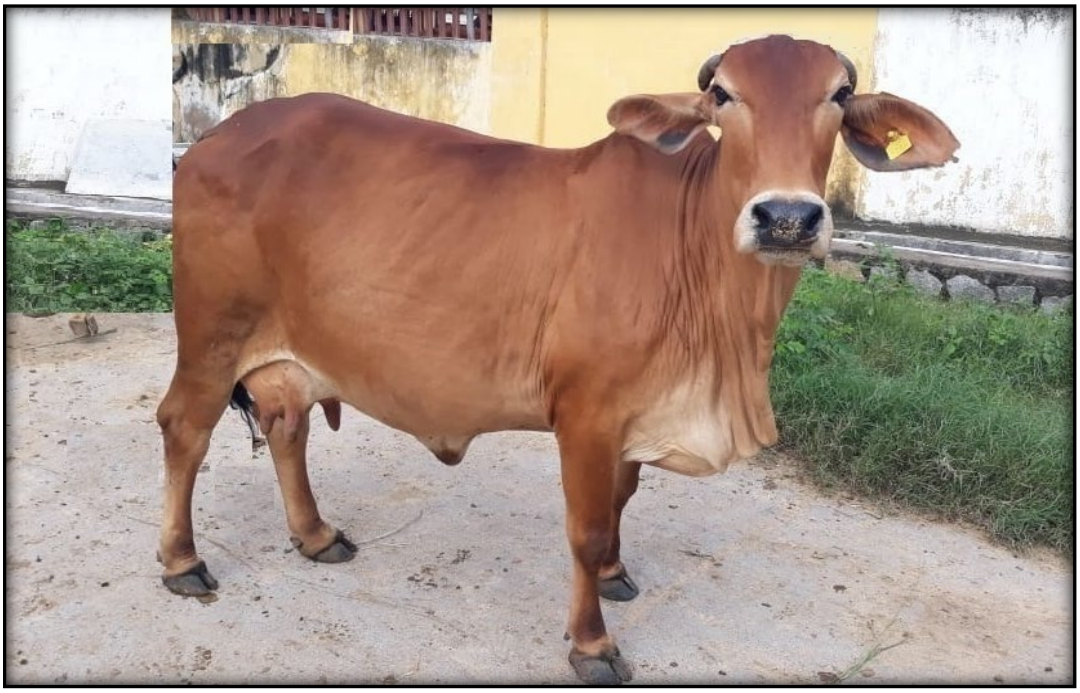

Fig 3.1. Sahiwal cow

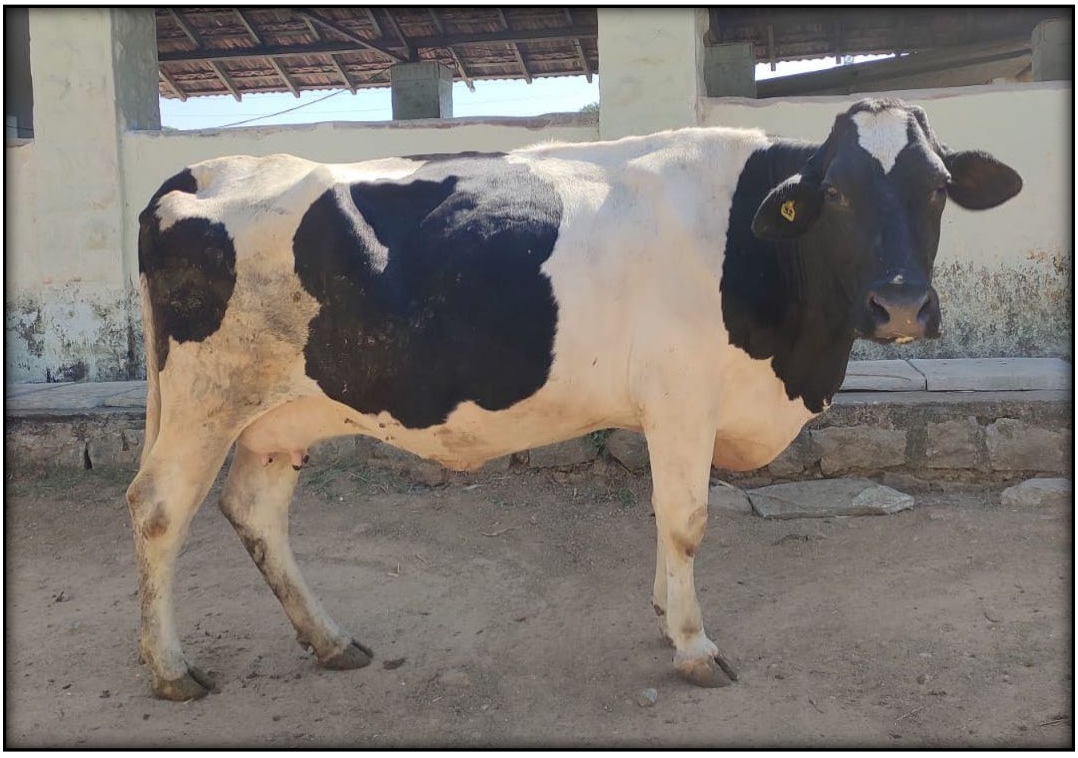

Fig 3.2. Crossbred cow

THI = 0.72 (Wb + Db) + 40.6

Where Wb is the wet-bulb and Db is the dry-bulb temperature in °C

The average THI value was calculated for each of the three seasons. The effect of season on the various physiological parameters of Sahiwal and crossbred cows was studied using univariate ANOVA taking THI as the fixed effect.

3.1.3 Management Practices

Both Sahiwal and Crossbred herds were provided with standard housing. The animals were given balanced ration comprising of both green and dry fodder. Adequate quantities of concentrates, based on their maintenance and production requirements were given to the milch animals. Regular deworming and vaccination schedules were followed. The Sahiwal farm was established in the year 2015 with the main objective of conservation and propagation, while the military dairy farm, Secunderabad was one of several such breeding farms established by the Government of India in different agro-climatic regions with a primary objective to supply milk to the defense forces. In both the farms, selective breeding for higher lactation milk yield was employed.

3.1.4 Physiological parameters

Data about the physiological parameters, respiration rate (RR) and rectal temperature (RT) of each animal under the present study were recorded twice daily for 30 days in each of the three seasons *i.e* during May for summer, August for rainy and from mid-December to mid-January for winter season respectively and the average was taken as final reading for each cow in association analysis. The timings of recording the physiological parameters were 8 AM and 2 PM.

3.1.4.1 Respiration rate

Respiration rate of each animal was recorded by visual observation of the inward and outward movement of flank for one minute without disturbing the animal. One inward and outward movement was counted as one respiration and respiration

rate was expressed in number per minute. Care was taken during the measurement of respiration that the breathing sound which should be clear and not raspy or obstructed.

3.1.4.2 Rectal temperature

Rectal temperature was recorded by a clean, lubricated digital thermometer by gently inserting into the rectum using a twisting motion and left in contact with the rectal mucosa for about two minutes. Rectal temperature was recorded in °C.

3.1.4.3 Heat Tolerance Coefficient

Heat Tolerance Coefficient (HTC) based on respiration rate and rectal temperature was calculated for each animal using the formula given by Benezra (1954), as detailed below.

$$HTC = \left[\frac{BT}{38.33}\right] + \left[\frac{RR}{23}\right]$$

In the equation, denominator 38.33 (BT/38.33) is defined as the normal body temperature (°C), and the denominator 23 (RR/23) is considered to be the normal respiration rate (breaths/minute) in cattle under ideal conditions. The rectal temperature is taken as representative of the body temperature in the present study. The lower the value determined by the equation, the higher the degree of adaptability.

Two way analysis of variance was employed to study the effect of genetic group and season on the physiological parameters. The mathematical model is as follows.

$Y_{ijk} = \mu + G_i + S_j + e_{ijk}$

Where, Y_{ijk} = record on n^{th} cow during the j^{th} season under i^{th} genetic group

μ = overall mean,

G_i = effect of the i^{th} genetic group (i = 1 and 2 for Sahiwal and crossbreds, respectively)

Sj = Effect of j^{th} season (j = 1 for summer, 2 for rainy and 3 for winter season)

e_{ijk} = random error assumed to be distributed normally and independently with mean zero and variance σ^2_e.

3.1.5 Production and Reproduction traits

Data on each animal about different aspects like Animal number, Sire number, Dam number, Date of birth, Date of calving, Lactation length and Lactation milk yield, *etc*., were collected from the history sheet/daily farm registers maintained at the concerned farms. The various production and reproduction traits like Total Lactation Milk Yield (TLMY), Peak Yield (PY), Lactation length (LL), Age at first service (AFS), Age at first calving (AFC), Gestation Period (GP), Service Period (SP), Dry Period (DP) and Calving Interval (CI) were calculated from the available data in both Sahiwal and crossbred cows.

Two way analysis of variance was employed to study the effect of genetic group and parity on various production and reproduction traits using the following mathematical model.

$Y_{ijk} = \mu + G_i + P_j + e_{ijk}$

Where, Y_{ijk} = record on n^{th} cow belonging to j^{th} parity under i^{th} genetic group

μ = overall mean,

G_i = effect of the i^{th} genetic group (i = 1 and 2 for Sahiwal and crossbreds, respectively)

Pj = Effect of j^{th} parity (j = 1 to 6)

e_{ijk} = random error assumed to be distributed normally and independently with mean zero and variance σ^2_e.

Significant differences between the means of different breeds and parities were tested by Duncan's Multiple Range Test (DMRT). Values were considered significant at $P \leq 0.05$ and presented as means ± standard errors.

3.2 METHODS

3.2.1 General Laboratory Preparation

For undertaking the molecular study, the laboratory facilities available at the Department of Animal Genetics and Breeding, College of Veterinary Science, Rajendranagar, Hyderabad were utilized.

Molecular grade reagents were used for the preparation of all solutions and buffers. The reagents and labware were procured from GeNei (Bangalore), Himedia (Mumbai), Merck (Mumbai), and Sigma (USA). All the aqueous solutions were prepared using double distilled water and wherever necessary, solutions were autoclaved at 121°C and 15 lbs pressure for 15 minutes.

Glassware was soaked in neutral detergent (Labolene) overnight, scrubbed, and washed thoroughly under running tap water. Then they were washed with de-ionized water, single distilled water, and finally rinsed with double distilled water, air-dried, packed and sterilized in a hot air oven at 160°C for 2 hours. Filter assemblies, caps wrapped in double paper, micropipette tips, deep well plates, troughs, microfuge tubes, *etc.* were sterilized by autoclaving at 121°C and 15 lbs pressure for 15 minutes.

3.2.2 Collection of Blood Samples

About 10 ml of peripheral blood was collected aseptically from each of the representative cows from the external jugular vein into a sterile vacutainer tube containing 0.5 M EDTA. The tube was shaken gently to facilitate thorough mixing of blood with the anticoagulant. After collection, the samples were labeled, kept instantly in an icebox, brought to the laboratory, and stored at 4 °C until further processing.

3.2.3 Isolation of Genomic DNA

Genomic DNA of each of the animals was isolated from the blood samples using the standard phenol-chloroform extraction method as described by Green and Sambrook (2012), with minor modifications. All the chemicals and reagents used for DNA isolation are enlisted in Appendix A and the protocols followed are detailed in the following paragraphs.

A) Isolation of WBCs

- 10 ml of whole blood was transferred into a centrifuge tube. To this, double the quantity of chilled RBC lysis buffer was added. The contents were mixed thoroughly and incubated on in ice for 10 minutes with intermittent shaking to allow complete lysis of RBCs.
- The tubes were then centrifuged at 4000 rpm for 10 minutes at room temperature. The blackish supernatant containing the lysed RBCs was discarded carefully, while the WBC pellet was retained.
- To remove the unlysed RBC, the white cell pellet was resuspended in the RBC lysis buffer and centrifuged further at 4000 rpm for 10 minutes. The above two steps were repeated 2-3 times till the WBC pellet became nearly clear white without any reddish tinge.

B) Digestion of proteins

- After the RBCs were lysed satisfactorily, DNA extraction buffer was added at 3 ml per 10 ml of blood and gentle vortexing was done to dissolve the WBC pellets in the buffer properly. They were incubated at 37°C in a water bath for 30 minutes.
- A 10% solution of sodium dodecyl sulfate (SDS) was added at 200 µl per 10 ml blood, mixed carefully by shaking/inverting the tubes several times. Vigorous tilting was avoided to prevent damage to exposed DNA and bubble formation.
- About 40µl proteinase-K (25µg/µl) per 10 ml of blood was added and mixed properly. The tubes were then incubated at 50°C in a water bath overnight.

C) Removal of proteins and precipitation of genomic DNA

- After the overnight proteinase-K digestion, the liquid became clear without any clump of WBCs. Then, an equal volume (approx. 3 ml) of Tris-saturated phenol (pH >8) was added in the tubes. It was mixed thoroughly by repeated gentle inversion of the tubes for 10 minutes.
- The tubes were centrifuged at 4000 rpm for 20 minutes at room temperature. After centrifugation, the upper aqueous phase containing DNA was transferred

very carefully to another batch of 15 ml centrifuge tubes using 1000 µl wide-bore pipette tips without disturbing the protein layers. The lower organic phase contained phenol, cell lysate, protein, etc. and the white, thin interphase layer contained proteins.

- An equal volume (approx. 3 ml) of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the separated aqueous phase and mixed properly in the same way followed by centrifugation at 4000 rpm for 10 minutes.
- Similarly, the upper aqueous phase was again transferred to another batch of 15 ml tubes; an equal volume of (approx. 3 ml) of chloroform: isoamyl alcohol (24:1) was added, mixed in the same manner, followed by centrifugation at 4000 rpm for 10 minutes.
- The upper aqueous phase was transferred into a fresh tube and 3M sodium acetate ($1/10^{th}$ volume) was added and mixed gently. Two volumes of chilled ethanol or isopropanol at room temperature was added; After this step, the DNA started to precipitate. The tubes were left at room temperature for 5 minutes to allow complete precipitation of DNA.
- The tubes containing DNA were shaken very carefully to transform DNA threads into white pelleted form. These DNA pellets were spooled out with the help of 1000 µl wide-mouth pipette tips and transferred into sterile 1.5 ml Eppendorf tubes.
- Then, 500 µl of 70% ethanol was added in each Eppendorf tube; DNA pellets were washed properly by gentle shaking of the tubes and centrifuged at 10000 rpm for 10 minutes at 4°C in a microcentrifuge.
- The ethanol was discarded by inversion and the above step was repeated twice to remove any salt from the DNA pellets.
- The DNA pellets were air-dried by placing the Eppendorf tubes on a blotting paper in an inverted manner for 1 hour. Finally, when the alcohol was fully evaporated and DNA pellets dried, they were dissolved in 100 µl nuclease-free water (NFW) and stored in the refrigerator at 4°C until further use.

3.2.4 Evaluation of Purity, Quality, and Concentration of DNA

3.2.4.1 Purity of DNA

The purity of genomic DNA samples was assessed by measuring the optical densities (OD) at 260 nm and 280 nm against blank using Nanodrop (Thermo Fisher Scientific). The ratio of OD260 to OD280 was taken as an indicator of the purity of the isolated DNA, as per Green and Sambrook (2012). The samples which gave the OD ratio between 1.7 and 1.9 were assessed as good and used for PCR amplification and further work.

3.2.4.2 Quality of DNA

The quality of genomic DNA was checked by horizontal submarine agarose gel electrophoresis. Agarose of 0.8% (w/v) was dissolved in 1X TBE buffer by heating. The agarose solution was cooled to 45℃ and poured into the casting gel tray after adding ethidium bromide (10 mg/ml). The gel was allowed to solidify and the comb was removed. The gel casting tray was submerged in a gel tank having 1X TBE buffer. The isolated DNA samples were mixed with $1/6^{th}$ volume of 6X gel loading dye and loaded gradually into each well. Electrophoresis was carried out at 70V for 45 minutes at room temperature and the gel was carefully removed from the chamber, visualized under a UV transilluminator and photographed in a gel documentation system (Syngene, UK). The DNA samples showing an intact thick band without any smearing were used for further study. After checking the quality, the isolated DNA was diluted to 50-100 ng/µl with NFW and stored at 4°C for further analysis. Agarose gel electrophoresis reagents used in the present study are detailed in Appendix B.

3.2.4.3 Concentration of DNA

The concentration of genomic DNA was obtained using Nanodrop (Thermo Fisher Scientific) and expressed in µg/µl for all the cows under study.

3.2.5 Amplification of the *HSP* gene fragments

Six fragments, two from each of *HSP70*, *HSPAA1,* and *HSPAB1* genes were amplified using polymerase chain reaction (PCR) technique under appropriate conditions.

3.2.5.1 PCR Primers

Six pairs of primers (procured from BioServe Biotechnologies Pvt Ltd, Hyderabad) specific for the desired regions of *HSP70*, *HSPAA1,* and *HSPAB1* genes as available in the literature were used to amplify the targeted regions. The details of primer sequences, length of the primer (bp), melting temperature (Tm), the region of the gene covered by the individual primer, and expected amplicon size (bp) are presented in Table 3.1.

3.2.5.2 Preparation of PCR reaction mixture

The PCR reaction mixture was prepared using the following components for a 12.5 µl reaction.

Components	Volume (µl)
Taq Polymerase (5 units/µl)	0.125
dNTPs (10 mM)	0.8
Primer-Forward (10 pM)	1.0
Primer-Reverse (10 pM)	1.0
10X Taq buffer	2.5
Nuclease-free water (NFW)	6.075
Template DNA	1.0
Total	12.5

The master mix of all the above components except genomic DNA was prepared in a 1.5 ml sterile Eppendorf tube for the required number of reactions. After the addition of each of the components, they were mixed properly by gentle pipetting. Taq DNA polymerase enzyme was added at last to prevent loss of enzyme activity and just before that 10X PCR buffer was added to avoid the formation of bubbles in the

Table 3.1. Sequence of primers, their respective number of bases, target regions and amplicon sizes of bovine *HSP* gene fragments

Gene	Fragment		Sequence (5'-3')	Length (bp)	Melting temperature (Tm, °C)	Region of the genome covering	Amplicon Size (bp) expected	Reference(s)
HSP70	I	F	AAACATGGCTATCGGCATCGACCT	24	57	Initial coding region	295	Bhat *et al.* (2016)
		R	AGGCTTGTCTCCGTCGTTGATGA	23	57			
	II	F	CTAAGGTGCAGGTGAGCTACAAAG	24	57	Initial coding region	220	
		R	TTGATGATCCTCAGCACGTTCAGC	24	57			
HSP90AA1	I	F	GCGTCATCACGTGTCATCTT	20	52	Exon 3	450	Kumar *et al.* (2015a)
		R	CCT CCTTTGGGGTTCCAGT	19	53			
	II	F	CCCATGGGAACAGTTGAGTG	20	54	Exon 8	539	Shergojry *et al.* (2014a)
		R	GCTTTAAGCTCCTTTTAAGTTCG	23	52			
HSP90AB1	I	F	AGTGAGTATCTTTTGCCCTAATG	23	52	Part of Intron 7, exon 8 and part of intron 9	459	Sailo *et al.* (2015a)
		R	TCTCCTCTAACCGAATGAAAAA	22	51			
	II	F	GCTGCTGCGCTATCACACG	19	52	Part of exon 10, intron 10 and exon 11	387	
		R	GCCCTCCTTGGTCACAGA	18	51			

F = Forward; R = Reverse

mixture while mixing the other components. The master mix was then mixed properly by tapping, followed by a brief spin. An aliquot of 11.5 µl of master mix per sample was drawn into thin-walled PCR tubes and 1 µl (50-100ng) of template DNA was added for making 12.5 µl.

All these steps were carried out on ice under a sterile environment. Utmost care was taken to eliminate any chance of contamination in PCR. A negative control containing all the components except template DNA was also used. The PCR tubes were then put into a pre-programmed thermo cycler (Prima-Duo, Himedia labs) for amplification. After completion of the PCR program, the products were stored at 4°C until further use.

3.2.5.3 PCR program conditions

Customized program conditions specific for each of the fragments were stored in the thermo cycler. The best possible program condition giving the finest amplification of a particular fragment was decided by trial and error of several combinations of temperature and time. The PCR cycling conditions for different fragments of *HSP* genes used are given below.

Steps	Temperature (°C)	Time
Initial denaturation	95	5 min
Cyclic Denaturation	95	30 sec
Primer Annealing*	50-51	35-45 sec
Cyclic Extension	72	30 sec
Steps 2 to 4 were repeated for 35 cycles		
Final extension	72	10 min
Hold	4	Forever

*Annealing temperature and time for each primer were different as detailed below.

Primer set for gene	Annealing temperature (°C)	Annealing time (seconds)
HSP70 Fragment I and II	50	35
HSP90AA1 Fragment I and II	51	45
HSP90AB1 Fragment I and II	50.5	40

The annealing temperatures ranged from 50 to 51°C and the annealing time ranged from 35-45 seconds depending on the primers used. The PCR tubes were kept in a thermal cycler and the program was executed. Each PCR amplification program took about 2 hours. At the end of the PCR, the tubes were taken out and stored at -20 °C until further use.

3.2.5.4 Confirmation of amplicons

The amplified products were checked by horizontal submarine agarose gel electrophoresis. A 2% (w/v) agarose gel was prepared in 1X TBE and 5 µl of the amplicons mixed with 1 µl of 6X gel loading dye, was loaded into a well. A 100 bp DNA ladder marker was also loaded alongside in a separate well to determine the exact size of the amplicons. The electrophoresis was performed at 80V for 1 hour and the gel was visualized under a UV transilluminator to detect the desired fragments and photographed in a gel documentation system (Syngene, UK).

3.2.6 SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP)

The presence of variation in the six different fragments of *HSP* genes was screened using the single-strand conformation polymorphism (SSCP) technique. Formamide denaturing dye mix and polyacrylamide gel (12% concentration) were made for SSCP study.

3.2.6.1 Preparation and running of PAGE

Different ratios of acrylamide and bisacrylamide were tried in different concentrations to prepare the polyacrylamide gel as per Green and Sambrook (2012). Finally, it was found that 12% polyacrylamide gel electrophoresis was giving the best resolution of SSCP bands for different fragments and so, the same concentration was followed for all the gels. The composition of the formamide denaturing dye mix and polyacrylamide gel is given in Appendix –C.

3.2.6.2 Procedure for SSCP

The following steps were used to prepare and run the gel:

- The gel plates, spacers, and comb were soaked in detergent or soap water rubbed carefully and washed properly first in running tap water, then in distilled water to remove any grease and dirt greasiness present on them. They were air-dried, rinsed with methanol, and dried finally.
- Two 1 mm spacers were placed at both left and right margin of the plate and the notched plate was placed over them aligning its four margins with those of the rectangular plate. Clamps were attached to the left and right margin of the plates above the position of the spacers. The flat end of the glass plates was sealed by a rubber gasket. The gel plate assembly was then kept standing at an angle of 45° by some backside support in such a way that the notched plate faced the user.
- In the meantime, the gel mix was prepared by adding the required components (acrylamide: bisacrylamide (49:1) solution, ammonium persulfate (APS) 10%, Glycerol, 1X TBE, and TEMED) in a beaker. TEMED was added at the last moment as it accelerates the polymerization of the gel at a very fast rate. The freshly prepared gel mix was poured carefully into space in-between the two plates avoiding trapping of air bubbles inside. The comb was inserted immediately and the gel was allowed to polymerize at room temperature for about 1 hour.
- After polymerization, the comb and the clamps were removed with care. The gel plates were assembled in the apparatus with the notched plate facing the buffer reservoir. Both the upper and lower buffer tanks were filled with 1X TBE. The wells were washed properly using a gel loading tip to remove the unpolymerised gel mix, if any, from the wells. A pre-run was made at a constant 15-20 mA current for 30 minutes to bring the gel up to the operating temperature.
- About 5 µl of PCR products were taken in sterile PCR tubes and 15 µl of formamide denaturing dye was added and mixed properly to each sample. The tubes were sealed by parafilm and placed in a water bath heated to 95ºC for 5 minutes. Immediately they were snap cooled on ice for 15 -20 minutes.
- The denatured products were then loaded into the gel and electrophoresis was carried out at 4°C at a constant 15-20 mA current and 110V for 10-12 hrs.

3.2.6.3 Silver staining of SSCP gel

Silver staining of SSCP gel was undertaken by following the protocol of Bassam *et al*. (2007) with minor modifications. The steps followed in silver staining were as follows:

- The pair of gel plates sandwiching the gel was taken out from the assembly and the upper plate was separated carefully avoiding breakage of gel and plates.
- The first lane of the gel was marked by notching slightly at the upper left corner of the gel. The gel along with the lower plate was placed on a plastic staining tray of suitable size and the lower plate was removed by flooding with distilled water with utmost care to prevent breakage of gel.
- Then distilled water was removed and the gel was fixed by pouring 300 ml of freshly prepared 10% ethanol in the tray. The gel was agitated slowly for 5 minutes until the tracking dye was no longer visible.
- Ethanol was discarded and 300 ml of 1% nitric acid was added to the tray and was placed on the gel rocker for 30 minutes.
- The gel was then washed carefully with double distilled water three times for 3 minutes each.
- 300 ml 0.1% (w/v) silver nitrate was added to the tray and similarly placed on a rocker for 30 minutes, after which, the silver nitrate was poured out of the tray.
- The gel was rinsed with double distilled water twice for 30 seconds each to remove all the traces of silver nitrate and other chemicals.
- The gel was then developed by pouring 300 ml freshly prepared 3% (w/v) sodium carbonate solution containing 450 µl 37% formaldehyde (added at the last minute). It was shaken gently until satisfactory visualization of brownish bands. Immediately, the development was stopped by pouring 200 µl 10% glacial acetic acid.

3.2.6.4 Documentation of SSCP patterns

SSCP patterns could be visualized with the naked eye, but for better documentation, it was seen under white light in trans illuminator and photographed. The different patterns observed were recorded for further analysis. The most common

band pattern identified was named as A. If there are more bands, in addition to the common bands, they were marked as B, C, etc., depending on the band pattern.

3.2.6.5 Genotype and allele frequencies

Genotype frequencies for variant genotypes were calculated using the formula:

$$Genotype\ frequency = \frac{No.of\ animals\ with\ specific\ genotype\ (AA, AB\ or\ BB)}{Total\ No.of\ animals}$$

Allele frequencies were calculated as follows:

$$Allele\ frequency\ of\ A = AA + \frac{1}{2}\ AB$$

$$Allele\ frequency\ of\ B = BB + \frac{1}{2}\ AB$$

Where,

AA and *BB* = Genotype frequencies of homozygotes

AB = Genotype frequency of heterozygote

A and *B* = Allele frequencies

3.2.7 Association analysis of SSCP polymorphism with physiological, production and reproduction traits

Statistical analysis was performed to study the association of each SSCP genotype on physiological, production, and reproduction traits in Sahiwal and crossbred cows. Data on physiological traits was corrected for season effect and used for association analysis. The univariate GLM model of SPSS 25 was used to perform the analysis according to the following statistical model:

$Y_{ijk} = \mu + G_i + P_j + e_{ijk}$

Where, Y_{ijk} = dependent variable (respiration rate, rectal temperature, heat tolerance coefficient, total lactation milk yield, peak yield, lactation length, gestation period, service period, dry period and calving interval)

μ = overall mean,

G_i = effect of i^{th} SSCP genotype (i= 1…n)

Pj= Effect of j^{th} parity of the animal at the time of blood collection (j= 1…n)

e_{ijk}= random error assumed to be distributed normally and independently with mean zero and variance σ^2_e.

For Age at first service (AFS) and Age at first calving (AFC), the following statistical model was used.

$Y_{ij} = \mu + G_i + e_{ij}$

Where, Y_{ij} = dependent variable (AFS/AFC)

μ = overall mean

G_i = effect of i^{th} SSCP genotype

e_{ij} = random error, assumed to be distributed normally and independently with mean zero and variance σ^2_e.

Significant differences between the means of different genotypes and parities were tested by Duncan's Multiple Range Test (DMRT). Values were considered significant at $P \leq 0.05$ and presented as means ± standard errors.

CHAPTER IV
RESULTS

In the present study, a total of 50 purebred Sahiwal cows from the Livestock Farm Complex, College of Veterinary Science, Rajendranagar, Hyderabad, and 50 crossbred cows maintained at the Military Dairy Farm, Secunderabad were utilized to identify polymorphisms in specific fragments of bovine heat shock protein genes - *HSP70*, *HSP90AA1,* and *HSP90AB1* by SSCP technique. An attempt was also made to study the association between these heat shock protein gene polymorphisms and physiological, production, and reproduction traits. The results obtained are presented below.

4.1 WEATHER CONDITIONS

Means of weather parameters such as maximum and minimum temperatures, dry and wet bulb readings, relative humidity and temperature-humidity index (THI) values in each season during which physiological parameters were recorded are shown in Table 4.1.

Table 4.1. Mean weather parameters and THI recorded during the study period

Parameter	Season					
	Summer		Rainy		Winter	
	Mean	S.E	Mean	S.E	Mean	S.E
Maximum temperature (°C)	41.24	0.16	29.87	0.36	27.92	0.16
Minimum temperature (°C)	21.34	0.35	17.42	0.16	16.02	0.47
Dry bulb reading (°C)	35.03	0.71	26.61	0.37	23.02	0.60
Wet bulb reading (°C)	24.85	0.29	23.58	0.14	19.19	0.30
THI	83.71	0.01	71.37	0.00	66.69	0.01
Relative humidity (%)	44.26	1.65	78.10	2.24	72.26	0.90

The mean maximum temperatures (°C) were 41.24 ± 0.16, 29.87 ± 0.36 and 27.92 ± 0.16; mean minimum temperatures were 21.34 ± 0.35, 17.42 ± 0.16 and 16.02 ± 0.47 during summer, rainy and winter seasons respectively, while the respective relative humidity (%) was 44.26 ± 1.65, 78.10 ± 2.24 and 72.26 ± 0.90. Temperature-

humidity index was found to be 83.71, 71.37, and 66.69 during summer, rainy, and winter seasons respectively.

4.2 PHYSIOLOGICAL PARAMETERS

The mean physiological parameters *viz.* respiration rate (RR), rectal temperature (RT), and heat tolerance coefficient (HTC) recorded in the morning and afternoon and percentage increase between both periods, during various seasons in Sahiwal and crossbred cows are presented in Table 4.2.

Table 4.2: Mean physiological parameters recorded during various seasons in Sahiwal and crossbred cows

Season	Sahiwal					Crossbreds				
	8 AM		2 PM		% change	8 AM		2 PM		% change
	Mean	SE	Mean	SE		Mean	SE	Mean	SE	
Respiration Rate (RR)										
Summer	23.16	0.15	32.88	0.24	41.97	29.94	0.36	59.78	0.33	99.67
Rainy	20.88	0.19	24.79	0.32	18.73	23.68	0.29	28.76	0.44	21.45
Winter	18.23	0.31	21.76	0.25	19.36	19.58	0.36	24.78	0.44	26.56
Rectal Temperature (RT °C)										
Summer	38.19	0.02	38.79	0.02	1.54	38.61	0.03	39.71	0.03	2.77
Rainy	38.01	0.02	38.39	0.02	0.98	38.34	0.03	38.98	0.03	1.64
Winter	37.94	0.02	38.26	0.02	0.83	37.58	0.03	37.92	0.03	0.89
Heat Tolearnce Coefficient (HTC)										
Summer	2.00	0.01	2.44	0.01	22.0	2.30	0.01	3.64	0.02	58.26
Rainy	1.90	0.01	2.08	0.02	9.47	2.03	0.01	2.27	0.02	11.82
Winter	1.74	0.01	1.99	0.02	14.36	1.83	0.01	2.07	0.02	13.11

The mean RR in Sahiwal cows at 8 AM was 23.16 ± 0.15, 20.88 ± 0.19 and 18.23 ± 0.31 in summer, rainy and winter seasons and the same was increased to 32.88 ± 0.24, 24.79 ± 0.32 and 21.76 ± 0.25 by 2 PM. The percentage change was 41.97, 18.73 and 19.36 in summer, rainy and winter seasons, respectively.

The mean RR in crossbred cows at 8 AM was 29.94 ± 0.36, 23.68 ± 0.29 and 19.58 ± 0.36 in summer, rainy and winter seasons and the same was increased to 59.78 ± 0.33, 28.76 ± 0.44 and 24.78 ± 0.44 by 2 PM. The percentage change was 99.67, 21.45 and 26.56 in summer, rainy and winter seasons, respectively.

The mean RT in Sahiwal cows at 8 AM was 38.19 ± 0.02, 38.01 ± 0.02 and 37.94 ± 0.02 in summer, rainy and winter seasons and the same was increased to 38.79 ± 0.02, 38.39 ± 0.02 and 38.26 ± 0.02 by 2 PM. Similarly in crossbred cows, the mean RT at 8 AM was 38.61 ± 0.03, 38.34 ± 0.03 and 37.58 ± 0.03 in summer, rainy and winter seasons which increased to 39.71 ± 0.03, 38.98 ± 0.03 and 37.92 ± 0.03, respectively by afternoon.

The mean HTC in Sahiwal cows at 8 AM in summer, winter and rainy seasons was 2.00 ± 0.01, 1.90 ± 0.01 and 1.74 ± 0.01 and the same was increased to 2.44 ± 0.01, 2.08 ± 0.02 and 1.99 ± 0.02 by 2 PM. The percentage change was 22.0, 9.47 and 14.36 in summer, rainy and winter seasons ,respectively. In crossbred cows , the mean HTC at 8 AM in summer, winter and rainy seasons was 2.30 ± 0.01, 2.03 ± 0.01 and 1.83 ± 0.01 and the same was increased to 3.64± 0.01, 2.27 ± 0.02 and 2.07 ± 0.02 by 2 PM. The percentage increase was 58.26, 11.82 and 13.11 in summer, rainy and winter seasons, respectively. The percentage change in RR, RT and HTC was higher in crossbreds as compared to Sahiwal cows in all the seasons, more so in summer season in the present study.

The analysis of variance and means for physiological parameters (average of readings recorded at 8 AM and 2 PM) in Sahiwal and crossbred cows are presented in Tables 4.3 and 4.4, respectively. The effect of genetic group and season (THI) was found to be highly significant ($P \leq 0.01$) on all the physiological parameters studied with the crossbreds recording significantly higher values when compared to Sahiwal cows. The effect of interaction between genetic group and season was also found to be significant.

The study also revealed a significant influence of season on RR, where highest RR was recorded in summer (44.58 ± 0.38), followed by rainy (25.94 ± 0.34) and winter (21.90 ± 0.37) seasons in crossbred cows while lower RR was recorded in

Table 4.3. Analysis of variance of physiological parameters in Sahiwal and crossbred cows

Source of variation	d.f	MSS		
		RR	RT	HTC
Genetic group	1	3313.36**	6.29**	6.61**
Season (THI)	2	6488.40**	20.66**	13.04**
Genetic group*Season	2	1656.36**	7.32**	3.32**
Error	294	7.15	0.06	0.01

**Significant (P≤0.01)

Table 4.4. Means for physiological parameters in Sahiwal and crossbred cows

Effect	n	RR		RT (°C)		HTC	
		Mean	SE	Mean	SE	Mean	SE
Overall	300	27.48	0.15	38.44	0.02	2.20	0.01
Genetic group							
Sahiwal	150	24.16[b]	0.21	38.29[b]	0.03	2.05[b]	0.02
Crossbreds	150	30.81[a]	0.22	38.58[a]	0.03	2.35[a]	0.02
Season							
Summer (THI=83.71)	100	36.57[a]	0.27	38.87[a]	0.03	2.60[a]	0.02
Rainy (THI=71.37)	100	24.66[b]	0.26	38.47[b]	0.02	2.08[b]	0.02
Winter (THI=66.69)	100	21.22[c]	0.27	37.96[c]	0.03	1.91[c]	0.02
Genetic group*Season							
Sahiwal-Summer	50	28.56	0.36	38.52	0.03	2.25	0.02
Sahiwal-Rainy	50	23.38	0.33	38.23	0.03	2.01	0.02
Sahiwal-Winter	50	20.54	0.38	38.13	0.02	1.89	0.02
Crossbreds-Summer	50	44.58	0.38	39.22	0.03	2.96	0.02
Crossbreds-Rainy	50	25.94	0.34	38.72	0.02	2.14	0.02
Crossbreds-Winter	50	21.90	0.37	37.80	0.03	1.94	0.02

Means with dissimilar superscripts in a column differ significantly (P>0.95)

winter (20.54 ± 0.38), followed by rainy (23.38 ± 0.33) and summer (28.56 ± 0.36) seasons in Sahiwal cows. Overall means for rectal temperature (ºC) were 38.29 ± 0.03 in Sahiwal and 38.58 ± 0.03 in crossbreds. The mean RT recorded in Sahiwal was 38.52 ± 0.03, 38.23 ± 0.03 and 38.13 ± 0.02; while the same in crossbred cows was 39.22 ± 0.03, 38.72 ± 0.02 and 37.80 ± 0.03 during summer, rainy and winter seasons respectively. Overall means for HTC were 2.05 ± 0.02 and 2.35 ± 0.02 in Sahiwal and crossbred cows, respectively. Higher values of heat tolerance coefficients (2.60 ± 0.02) were observed in summer when THI was above 83, as compared to other seasons in the present study.

4.3 PRODUCTION TRAITS

Results of the analysis of variance of production traits are presented in Table 4.5 while corresponding means are given in Table 4.6.

The mean TLMY was 1768.32 ± 109.67 and 2983.45 ± 78.32 kg in Sahiwal and crossbred cows, respectively (Table 4.6). Total lactation milk yield was significantly ($P \leq 0.01$) affected by genetic group and parity with crossbreds recording higher TLMY (2983.45 ± 78.32 kg). The highest and lowest TLMY was recorded for cows in parities three (2681.42 kg) and one (2215.50 kg), respectively.

The PY was also significantly ($P \leq 0.01$) affected by genetic group and parity with crossbreds recording higher PY (14.92 ± 0.36 kg) than Sahiwal cows (10.17 ± 0.50 kg). The highest and lowest PY was recorded for cows in parities one (11.45 kg) and five (13.66 kg), respectively.

However, genetic group and parity had a non significant effect on lactation length. The mean lactation length in Sahiwal and crossbred cows in the present study was 304.41 ± 13.00 and 324.71 ± 9.29 days respectively.

Table 4.5. Analysis of variance of various production traits in Sahiwal and crossbred cows

Source of Variation	d.f	MSS		
		TLMY	PY	LL
Genetic group	1	100642631.96**	1539.523**	28079.446
Parity	5	1322433.40**	31.793**	5163.306
Error	357	555510.72	11.634	7809.075

**Significant (P≤0.01)

Table 4.6. Means for various production traits in Sahiwal and crossbred cows

Effect	n	TLMY (kg)		PY (kg)		LL (days)	
		Mean	SE	Mean	SE	Mean	SE
Overall	364	2375.88	83.92	12.54	0.38	314.56	9.95
Genetic group							
Sahiwal	121	1768.32[b]	109.67	10.17[b]	0.50	304.41	13.00
Crossbreds	243	2983.45[a]	78.32	14.92[a]	0.36	324.71	9.29
Parity							
1	110	2215.50[c]	74.53	11.45[c]	0.34	322.66	8.84
2	92	2365.50[b]	81.49	12.75[b]	0.37	294.67	9.66
3	74	2681.42[a]	91.01	13.64[a]	0.42	304.57	10.79
4	44	2644.33[a]	120.92	13.49[a]	0.55	302.91	14.34
5	26	2623.23[a]	155.75	13.66[a]	0.71	320.25	18.47
6	18	2407.78[b]	186.32	13.62[a]	0.85	315.15	22.09

Means with similar superscripts in a column do not differ significantly (P≤0.05)

4.4 REPRODUCTION TRAITS

The results of the analysis of variance for the effects of genetic group and parity on various reproduction traits in the present study are given in Table 4.7 and the means are presented in Table 4.8.

Table 4.7. Analysis of variance of various reproduction traits in Sahiwal and crossbred cows

Source of Variation	d.f	MSS	d.f	MSS		
		GP		SP	DP	CI
Genetic group	1	382.36	1	148.00	106492.77**	17967.18
Parity	5	398.82	5	8501.37	3331.16	2986.70
Error	357	232.62	347	10727.77	5087.55	8080.43

**Significant (P≤0.01)

Table 4.8. Means (days) for various reproduction traits in Sahiwal and crossbred cows

	n	GP		n	SP		DP		CI	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE
Overall	364	276.52	1.72	354	181.84	11.77	147.24	8.11	421.92	10.22
Genetic group										
Sahiwal	121	277.71	2.24	115	182.60	15.51	167.47[a]	10.68	430.23	13.46
Crossbreds	243	275.34	1.60	239	181.09	10.91	127.01[b]	7.51	413.61	9.47
Parity										
1	110	276.81	1.53	100	202.61	10.36	156.40	7.13	434.62	8.99
2	92	273.69	1.67	84	176.84	11.33	138.96	7.80	428.72	9.83
3	74	279.28	1.86	63	167.15	13.19	134.67	9.08	429.44	11.45
4	44	278.19	2.47	37	163.24	17.57	133.21	12.10	418.86	15.25
5	26	272.86	3.19	24	180.50	22.10	152.27	15.22	432.02	19.18
6	18	280.54	3.81	16	183.32	26.68	148.86	18.37	419.50	23.15

Means with dissimilar superscripts in a column differ significantly (P>0.95)

The means for the gestation period, service period, dry period and calving interval in Sahiwal cows were 277.71 ± 2.24, 182.60 ± 15.51, 167.47 ± 10.68, and 430.23 ± 13.46 days while the same in crossbred cows were 275.34 ± 1.60, 181.09 ± 10.91, 127.01 ± 7.51 and 413.61 ± 9.47 days respectively (Table 4.8). The dry period was significantly (P≤0.01) affected by genetic group, while parity had a non-significant effect on the gestation period, service period, dry period, and calving interval. The dry period was significantly longer in Sahiwal cows (167.47 days) than the crossbreds.

The mean age at first service and age at first calving were 641.34 ± 17.24 and 947.26 ± 19.67 days, respectively in crossbred cows, while these means could not be estimated in Sahiwal cows due to paucity of records.

4.5 MOLECULAR GENETIC STUDIES

4.5.1 Genomic DNA Extraction and Evaluation

DNA was extracted from blood samples of the experimental cows by the Phenol chloroform method reported by Green and Sambrook (2012) with minor modifications. The quality of isolated genomic DNA was checked on 0.8% agarose gel by electrophoresis. DNA was visualized by the gel documentation system which revealed all screened samples having a single intact band without smearing near the wells. The cow wise concentration of DNA and yield of genomic DNA, obtained in the present study are detailed in Table 4.9. The overall means for the purity and concentration of genomic DNA were 1.76 ± 0.01 and 1.774 ± 0.052 µg/µl in Sahiwal and 1.75 ± 0.01 and 1.813 ± 0.046 µg/µl in crossbred cows respectively. The OD values of DNA varied from 1.60 to 1.89 among the Sahiwal cows, and from 1.54 to 1.92 among the crossbred cows. Similarly, the yield of the DNA in Sahiwal cows ranged from 0.884 to 2.455 µg/µl and from 0.896 to 2.651µg/µl in crossbred cows.

Table 4.9: Genomic DNA quantification in Sahiwal and crossbreds using NanoDrop.

Sample No.	Sahiwal		Crossbreds	
	OD260/OD280 ratio	Yield of DNA (µg/µl)	OD260/OD280 ratio	Yield of DNA (µg/µl)
1	1.82	0.884	1.75	2.145
2	1.76	0.995	1.72	2.238
3	1.82	1.565	1.77	1.968
4	1.81	1.655	1.74	1.946
5	1.79	1.889	1.58	1.254
6	1.83	1.965	1.84	1.864
7	1.81	2.411	1.86	1.954
8	1.80	2.121	1.64	1.564
9	1.84	2.125	1.84	1.998
10	1.76	1.966	1.56	0.960
11	1.64	2.455	1.64	1.145
12	1.66	2.147	1.66	1.256
13	1.70	1.456	1.70	1.963

Table 4.9. *Contd.,*

Sample No.	Sahiwal		Crossbreds	
	OD260/OD280 ratio	Yield of DNA (µg/µl)	OD260/OD280 ratio	Yield of DNA (µg/µl)
14	1.60	2.189	1.54	0.896
15	1.77	1.826	1.77	1.856
16	1.68	1.115	1.88	2.120
17	1.76	1.561	1.76	1.928
18	1.71	1.456	1.82	1.968
19	1.82	1.541	1.76	1.568
20	1.85	1.841	1.82	1.741
21	1.86	1.458	1.81	1.758
22	1.65	2.104	1.79	1.852
23	1.74	2.156	1.83	1.887
24	1.78	2.189	1.81	1.632
25	1.76	2.102	1.80	1.695
26	1.75	1.967	1.84	1.689
27	1.69	2.112	1.72	1.625
28	1.85	1.988	1.64	1.568
29	1.89	2.330	1.72	1.658
30	1.85	1.963	1.74	1.565
31	1.74	1.317	1.78	1.965
32	1.68	1.251	1.64	1.854
33	1.75	1.666	1.92	1.960
34	1.72	1.654	1.66	2.140
35	1.77	1.865	1.74	2.132
36	1.74	1.958	1.74	1.985
37	1.88	1.698	1.79	2.136
38	1.85	1.256	1.78	1.824
39	1.65	1.364	1.74	1.456
40	1.68	2.145	1.75	1.887
41	1.85	1.884	1.88	1.958
42	1.87	1.966	1.77	2.256
43	1.78	1.986	1.74	2.112
44	1.75	1.758	1.58	1.999
45	1.72	1.856	1.85	1.869
46	1.75	1.584	1.82	1.958
47	1.72	1.465	1.76	1.565
48	1.77	1.884	1.82	2.651
49	1.74	1.365	1.81	1.989
50	1.71	1.256	1.79	1.695
Overall mean	1.76	1.774	1.75	1.813
SE	0.01	0.052	0.01	0.046

4.5.2 PCR-Amplification of different fragments of *HSP* genes

The PCR reactions were set for all the animals (50 each of Sahiwal and crossbreds) with six sets of species-specific primers available in the literature for the amplification of different segments of *HSP70, HSP90AA1,* and *HSP90AB1* genes. Amplification of desired size was noticed in all the tested samples. The representative figures showing the PCR amplified products of *HSP70* gene fragments I and II, showing the sizes of 295 bp and 220 bp are presented in figures 4.1 and 4.2, respectively.

The representative figures showing the PCR amplified products of *HSP90AA1* gene fragments I and II, confirming the sizes of 450 bp and 539 bp are presented in figures 4.3 and 4.4, respectively while those of *HSP90AB1* gene, fragments I and II, depicting confirmation of the literature reported sizes of 459 bp and 387 bp are shown in figures 4.5 and 4.6, respectively.

4.5.3 PCR-SSCP of different regions of *HSP* Genes

The PCR products of different fragments of *HSP* genes for all the samples were screened using the SSCP technique to detect polymorphism and the results obtained are presented in the following paragraphs.

The PCR-SSCP polyacrylamide gel images of fragments I and II of *HSP70, HSP90AA1, and HSP90AB1* genes for Sahiwal and crossbred cows are presented in figures 4.7 to 4.12. Polymorphism was identified basing on the band pattern observed in the SSCP gels after silver staining. The most common band pattern identified was named as A. If there are more bands, in addition to the common bands, they were marked as B, C, etc., depending on the band pattern. The allelic frequencies and genotypic frequencies obtained at each of the loci in both the genetic groups are detailed in Tables 4.10 and 4.11, respectively.

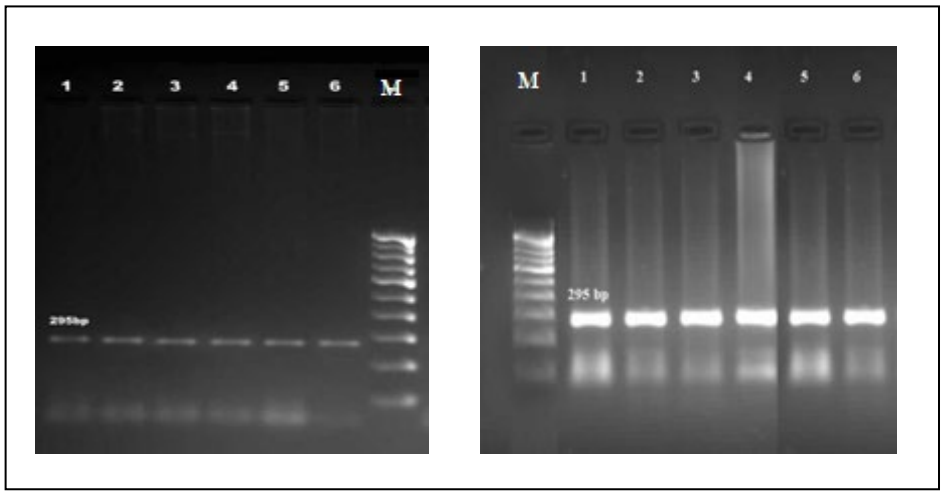

Fig 4.1. Agarose gel electrophoresis image showing PCR amplified product of Fragment I (295 bp) of *HSP70* gene
Lane 1-6 (L): Sahiwal, Lane 1-6 (R): Crossbred cows
Lane M: 100 bp ladder

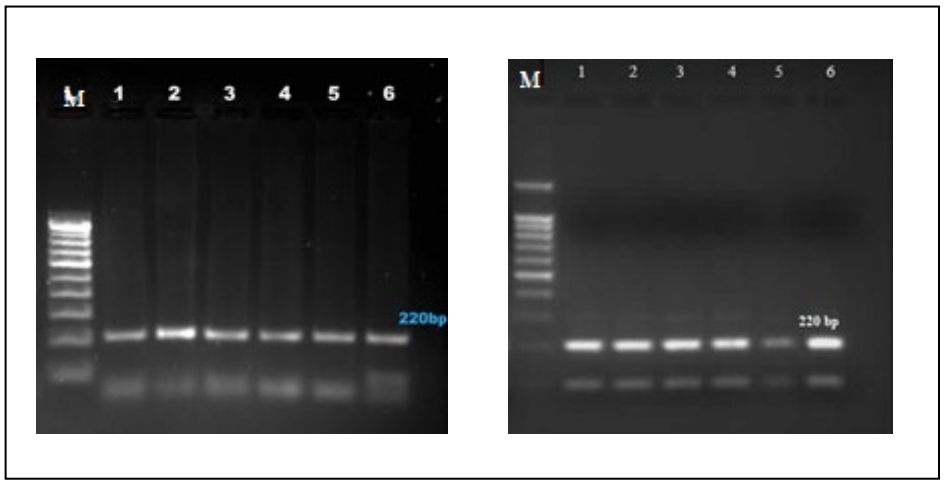

Fig 4.2. Agarose gel electrophoresis image showing PCR amplified product of Fragment II (220 bp) of *HSP70* gene
Lane 1-6 (L): Sahiwal, Lane 1-6 (R): Crossbred cows
Lane M: 100 bp ladder

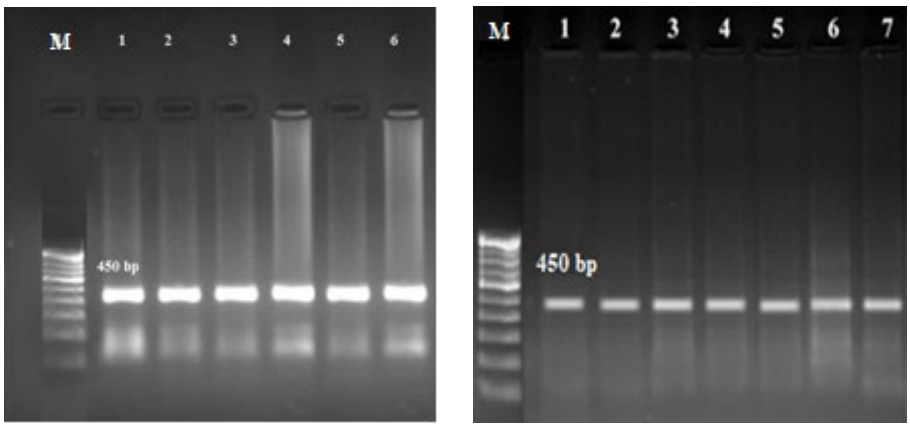

Fig 4.3. Agarose gel electrophoresis image showing PCR amplified product of Fragment I (450 bp) of *HSP90AA1* gene
Lane 1-6 (L): Sahiwal, Lane 1-7 (R): Crossbred cows
Lane M: 100 bp ladder

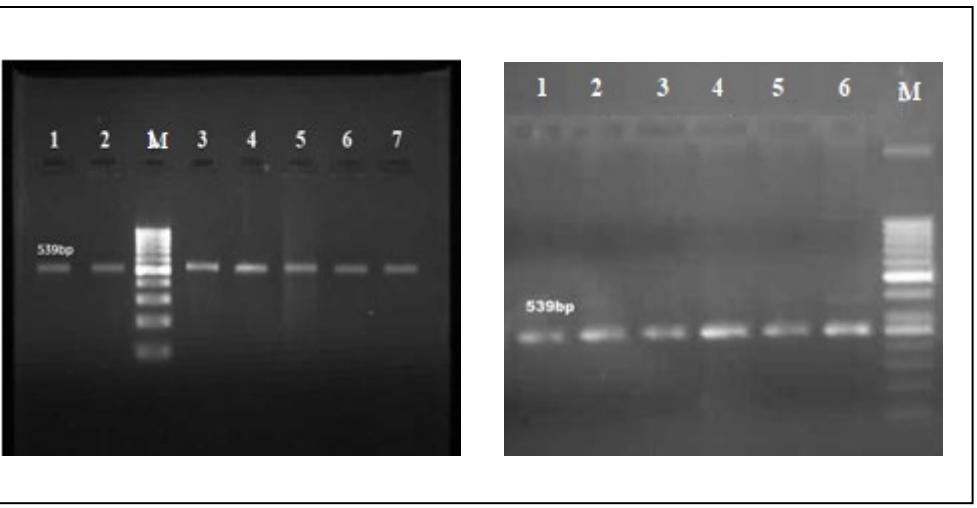

Fig 4.4. Agarose gel electrophoresis image showing PCR amplified product of Fragment II (539 bp) of *HSP90AA1* gene
Lane 1-7 (L): Sahiwal, Lane 1-6 (R): Crossbred cows
Lane M: 100 bp ladder

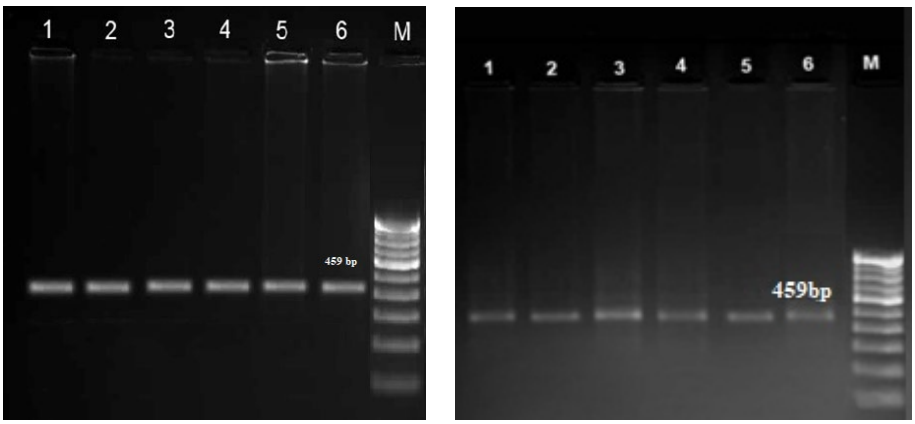

Fig 4.5. Agarose gel electrophoresis image showing PCR amplified product of Fragment I (459 bp) of *HSP90AB1*gene
Lane 1-6 (L): Sahiwal, Lane 1-6 (R): Crossbred cows
Lane M: 100 bp ladder

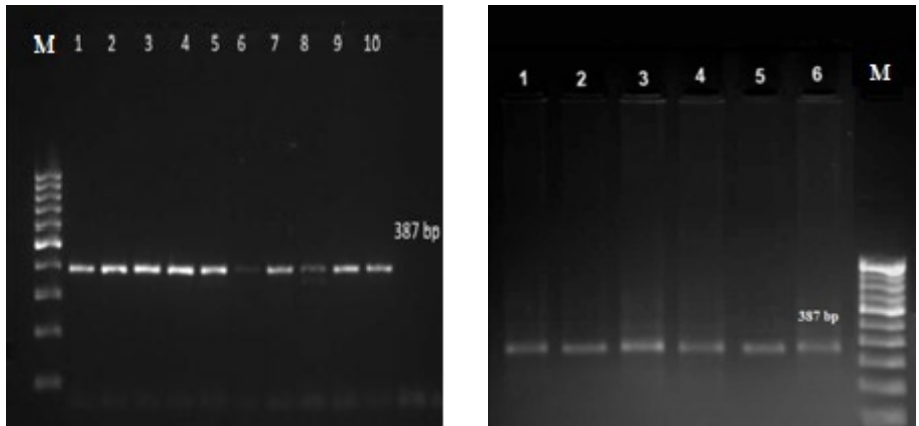

Fig 4.6. Agarose gel electrophoresis image showing PCR amplified product of Fragment II (387 bp) of *HSP90AB1*gene
Lane 1-10 (L): Sahiwal, Lane 1-6 (R): Crossbred cows
Lane M: 100 bp ladder

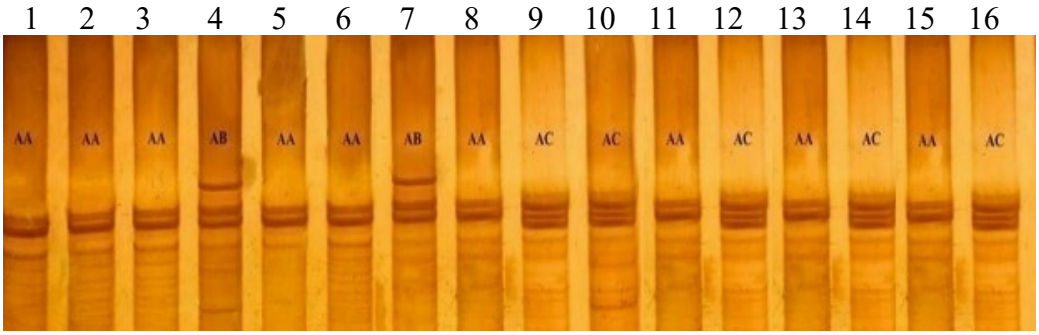

Fig 4.7. Polyacrylamide gel electrophoresis image showing PCR-SSCP patterns for Fragment I of *HSP70* gene
Lane 1-8 Sahiwal; Lane 9-16 Crossbred cows

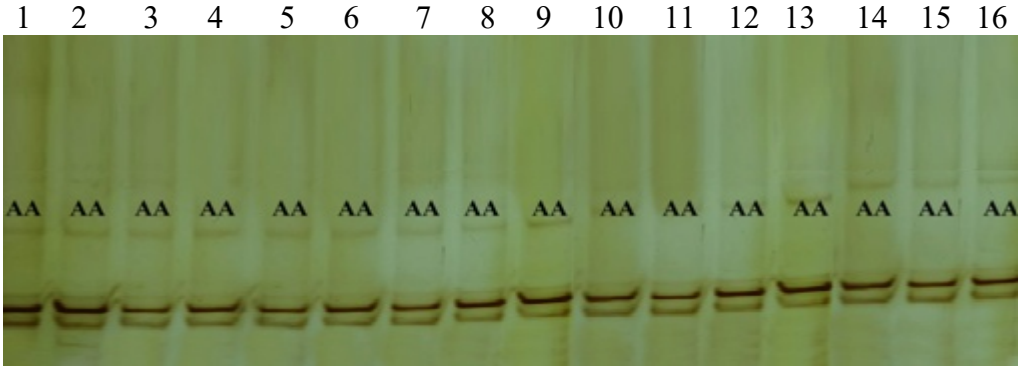

Fig 4.8. Polyacrylamide gel electrophoresis image showing PCR-SSCP patterns for Fragment II of *HSP70* gene
Lane 1-8 Sahiwal; Lane 9-16 Crossbred cows

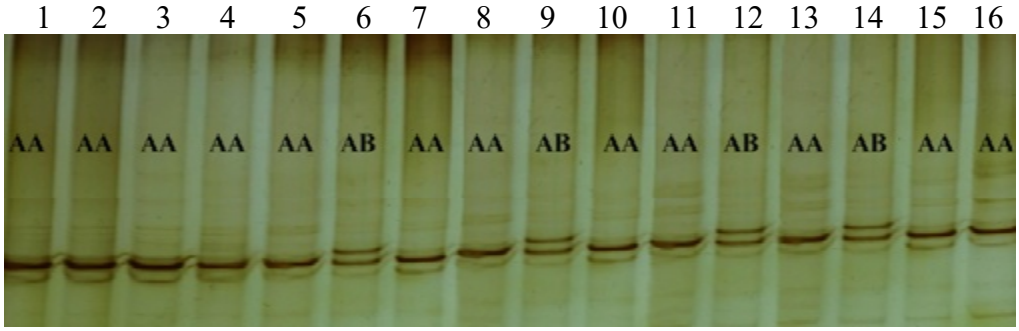

Fig 4.9. Polyacrylamide gel electrophoresis image showing PCR-SSCP patterns for Fragment I of *HSP90AA1* gene.
Lane: 1-8 Sahiwal; Lane: 9-16 Crossbred cows

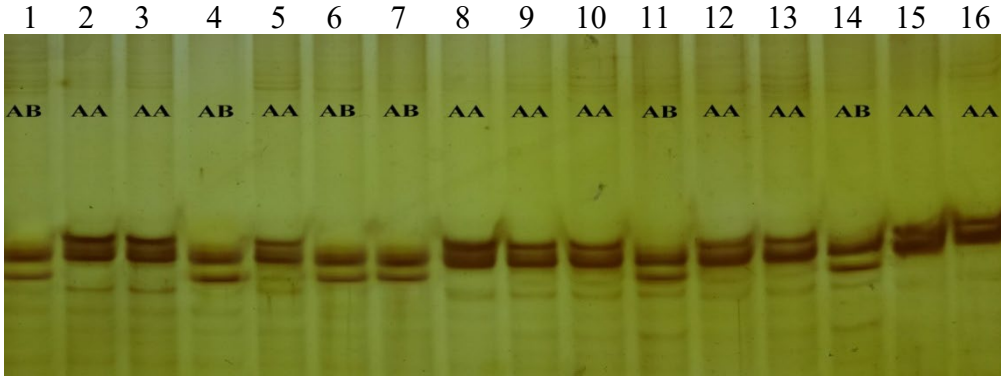

Fig 4.10. Polyacrylamide gel electrophoresis image showing PCR-SSCP patterns for Fragment II of *HSP90AA1* gene.
Lane 1-8 Sahiwal; Lane 9-16 Crossbred cows

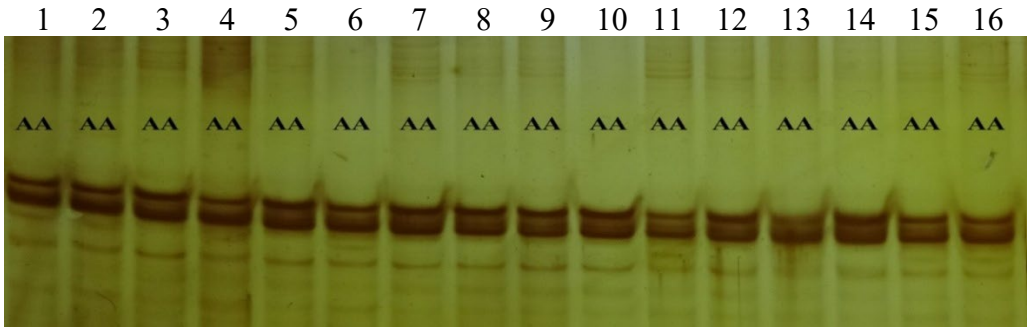

Fig 4.11. Polyacrylamide gel electrophoresis image showing PCR-SSCP patterns for Fragment I of *HSP90AB1* gene.
Lane 1-8 Sahiwal; Lane 9-16 Crossbred cows

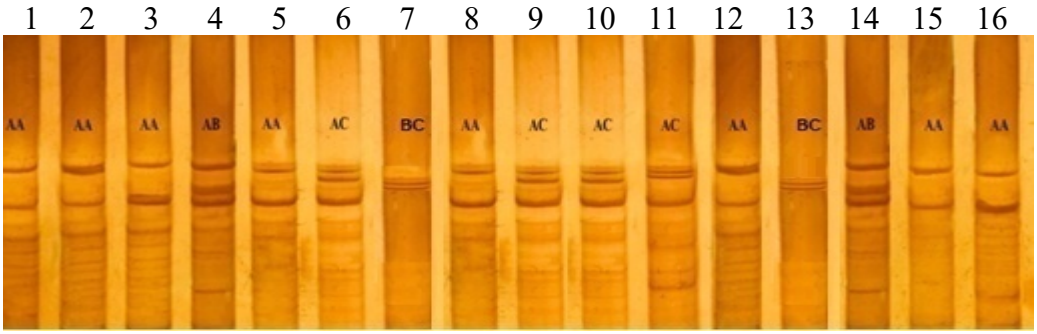

Fig 4.12. Polyacrylamide gel electrophoresis image showing PCR-SSCP patterns for Fragment II of *HSP90AB1* gene.
Lane: 1-8 Sahiwal; Lane: 9-16 Crossbred cows

Table 4.10. Allele frequencies of *HSP* genes in Sahiwal and crossbred cows

Gene Fragment	Sahiwal			Crossbreds		
	A	B	C	A	B	C
HSP70 Fragment I	0.79	0.21	-	0.81	-	0.19
HSP70 Fragment II	1.00	-	-	1.00	-	-
HSP90AA1 Fragment I	0.78	0.22	-	0.84	0.16	-
HSP90AA1 Fragment II	0.85	0.15	-	0.81	0.19	-
HSP90AB1 Fragment I	1.00	-	-	1.00	-	-
HSP90AB1 Fragment II	0.78	0.14	0.08	0.59	0.23	0.18

Table 4.11. Frequencies of SSCP genotypes in *HSP* genes in Sahiwal and crossbred cows

Gene Fragment	Sahiwal				Crossbreds			
	AA	AB	AC	BC	AA	AB	AC	BC
HSP70 Fragment I	0.58	0.42	-	-	0.62	-	0.38	-
HSP70 Fragment II	1.00	-	-	-	1.00	-	-	-
HSP90AA1 Fragment I	0.56	0.44	-	-	0.68	0.32	-	-
HSP90AA1 Fragment II	0.70	0.30	-	-	0.62	0.38	-	-
HSP90AB1 Fragment I	1.00	-	-	-	1.00	-	-	-
HSP90AB1 Fragment II	0.64	0.20	0.08	0.08	0.32	0.28	0.26	0.14

4.5.3.1 PCR-SSCP of *HSP70* gene fragments

Fragment I (295 bp) of the *HSP70* gene was found to be polymorphic in both Sahiwal and crossbred cows. In Sahiwal, two SSCP genotypes namely AA and AB were documented. Consequently, at this locus two alleles namely A and B with allelic frequency of 0.79 and 0.21 were present. Among 50 samples, 29 showed pattern AA and 21 showed pattern AB. The frequencies of AA and AB genotypes were estimated to be 0.58 and 0.42, respectively (Fig 4.7).

In crossbred cows, two SSCP patterns AA and AC were documented. The frequencies of AA and AC genotypes were found to be 0.62 and 0.38, while the corresponding allelic frequencies of A and C alleles were 0.81 and 0.19 respectively (Fig 4.7).

HSP70 gene fragment II (220 bp) was found to be monomorphic in both Sahiwal and crossbred cows (Fig 4.8).

4.5.3.2 PCR-SSCP of *HSP90AA1* gene fragments

Fragment I (450 bp) of *the HSP90AA1* gene was found to be polymorphic in both Sahiwal and crossbred cows. Two SSCP genotypes namely AA and AB were documented and consequently at this locus two alleles A and B were present in both genetic groups with allelic frequency of 0.78 and 0.22 in Sahiwal and 0.84 and 0.16 in crossbred cows. The frequencies of AA and BB genotypes were estimated to be 0.56 and 0.44 in Sahiwal and 0.68 and 0.32 in crossbred cows, respectively (Fig 4.9).

PCR-SSCP of Fragment II (539 bp) of the *HSP90AA1* gene revealed two different SSCP patterns AA and AB in both Sahiwal and crossbred cows. Consequently, at this locus two alleles namely A and B with allelic frequency of 0.85 and 0.15, respectively in Sahiwal and 0.81 and 0.19, respectively in crossbred cows were present. The frequencies of AA and BB genotypes were estimated to be 0.70 and 0.30 in Sahiwal and 0.62 and 0.38 in crossbred cows, respectively (Fig 4.10).

4.5.3.3 PCR-SSCP of *HSP90AB1* gene fragments

PCR-SSCP of Fragment I (459 bp) of the *HSP90AB1* gene revealed a single SSCP pattern AA in both Sahiwal and crossbred cows and was found to be monomorphic (Fig 4.11).

Fragment II (387 bp) of the *HSP90AB1* gene was found to be polymorphic in both Sahiwal and crossbred cows. The results showed four different SSCP patterns (AA, AB, AC, and BC) corresponding to three allelic variants (A, B, and C) in both genetic groups studied (Fig 4.12). The frequencies of the alleles A, B and C were 0.78, 0.14 and 0.08 in Sahiwal and 0.59, 0.23 and 0.18 in crossbred cows, respectively.

4.5 ASSOCIATION ANALYSIS OF *HSP* GENE POLYMORPHISM

Results of association analysis of different SSCP genotypes identified in the present study with physiological, production and reproduction traits in both genetic groups are presented here under. The two fragments which showed monomorphic band pattern in PCR-SSCP were not considered for association studies.

4.5.1 Physiological traits

The analysis of variance to determine the effect of *HSP70 Fragment I* genotypes and parity on the physiological traits in Sahiwal and crossbred cows is presented in Table 4.12, while the means are presented in Table 4.13. Genotype had no significant effect on all the physiological parameters studied in both Sahiwal and crossbred cows.

Table 4.12. Analysis of variance of *HSP70 Fragment I* in Sahiwal and crossbred cows for physiological traits

Source of variation	Sahiwal				Crossbreds			
	d.f	MSS			d.f	MSS		
		RR	RT	HTC		RR	RT	HTC
HSP70 Fragment I	1	0.79	0.01	0.01	1	4.21	0.01	0.01
Parity	3	2.05	0.03	0.01	5	3.39	0.03	0.01
Error	45	2.02	0.02	0.01	43	4.67	0.04	0.01

Table 4.13. Means of *HSP70 Fragment I* genotypes and parity effects for physiological traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	RR		RT (°C)		HTC		n	RR		RT (°C)		HTC	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	24.14	0.21	38.29	0.02	2.05	0.01	50	30.96	0.38	38.57	0.04	2.35	0.02
Genotype														
AA	29	24.27	0.27	38.30	0.03	2.05	0.01	31	31.30	0.46	38.57	0.04	2.37	0.02
AB	21	24.01	0.32	38.28	0.03	2.04	0.01	-	-	-	-	-	-	-
AC	-	-	-	-	-	-	-	19	30.62	0.57	38.58	0.05	2.34	0.03
Parity														
1	13	24.65	0.39	38.35	0.04	2.07	0.02	5	31.55	1.25	38.66	0.12	2.38	0.06
2	11	24.29	0.43	38.30	0.05	2.05	0.02	7	30.86	0.97	38.50	0.09	2.35	0.04
3	18	23.82	0.34	38.25	0.04	2.03	0.01	12	30.82	0.72	38.53	0.07	2.35	0.03
4	8	23.81	0.51	38.26	0.05	2.03	0.02	9	31.33	0.82	38.65	0.08	2.37	0.04
5	-	-	-	-	-	-	-	9	30.32	0.81	38.65	0.08	2.33	0.04
6	-	-	-	-	-	-	-	8	29.37	0.82	38.60	0.08	2.29	0.04

The results obtained from the analysis of variance for the effect of *HSP90AA1 Fragment I* genotypes on the physiological traits in Sahiwal and crossbred cows are presented in Table 4.14 and the means are presented in Table 4.15.

Table 4.14. Analysis of variance of *HSP90AA1 Fragment I* in Sahiwal and crossbred cows for physiological traits

Source of variation	Sahiwal				Crossbreds			
	d.f	MSS			d.f	MSS		
		RR	RT	HTC		RR	RT	HTC
HSP90AA1 Fragment I	1	0.70	0.02	0.001	1	5.47	0.01	0.01
Parity	3	1.97	0.03	0.004	5	3.47	0.03	0.01
Error	45	2.02	0.02	0.004	43	4.64	0.04	0.01

Table 4.15. Means of *HSP90AA1 Fragment I* genotypes and parity effects for physiological traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	RR		RT (°C)		HTC		n	RR		RT (°C)		HTC	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	24.15	0.21	38.29	0.02	2.05	0.01	50	30.92	0.38	38.57	0.04	2.35	0.02
Genotype														
AA	28	24.27	0.28	38.27	0.03	2.05	0.01	34	31.31	0.44	38.58	0.04	2.37	0.02
AB	22	24.03	0.31	38.31	0.03	2.04	0.01	16	30.53	0.60	38.57	0.06	2.33	0.03
Parity														
1	13	24.63	0.39	38.35	0.04	2.07	0.02	5	31.57	1.25	38.67	0.12	2.38	0.05
2	11	24.31	0.43	38.30	0.04	2.05	0.02	7	30.87	0.97	38.50	0.09	2.35	0.04
3	18	23.83	0.34	38.25	0.04	2.03	0.01	12	30.79	0.71	38.53	0.07	2.35	0.03
4	8	23.84	0.51	38.27	0.05	2.03	0.02	9	31.11	0.83	38.65	0.08	2.36	0.04
5	-	-	-	-	-	-	-	9	30.28	0.81	38.64	0.08	2.33	0.04
6	-	-	-	-	-	-	-	8	29.27	0.82	38.60	0.08	2.28	0.04

Similarly the results of the analysis of variance conducted to study the influence of *HSP90AA1 Fragment II* genotypes and parity on the physiological traits in Sahiwal and crossbred cows are given in Table 4.16 while the means for the corresponding traits are presented in Table 4.17.

Table 4.16. Analysis of variance of *HSP90AA1 Fragment II* in Sahiwal and crossbred cows for physiological traits

Source of variation	Sahiwal				Crossbreds			
	d.f	MSS			d.f	MSS		
		RR	RT	HTC		RR	RT	HTC
HSP90AA1 Fragment II	1	4.55	0.02	0.01	1	3.79	0.01	0.01
Parity	3	3.08	0.03	0.01	5	2.91	0.03	0.01
Error	45	1.94	0.02	0.01	43	4.68	0.04	0.01

Table 4.17. Means of *HSP90AA1 Fragment II* genotypes and parity effects for physiological traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	RR		RT (°C)		HTC		n	RR		RT (°C)		HTC	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	23.97	0.24	38.28	0.03	2.04	0.01	50	30.95	0.38	38.57	0.04	2.35	0.02
Genotype														
AA	35	24.38	0.25	38.30	0.03	2.06	0.01	31	31.27	0.45	38.57	0.04	2.37	0.02
AB	15	23.56	0.45	38.25	0.05	2.02	0.02	19	30.63	0.58	38.57	0.06	2.34	0.03
Parity														
1	13	24.80	0.40	38.36	0.04	2.08	0.02	5	31.33	1.26	38.66	0.12	2.37	0.06
2	11	23.89	0.50	38.27	0.05	2.03	0.02	7	30.86	0.97	38.50	0.09	2.35	0.04
3	18	23.72	0.34	38.24	0.04	2.03	0.01	12	30.90	0.70	38.53	0.07	2.35	0.03
4	8	23.47	0.56	38.24	0.06	2.01	0.02	9	31.23	0.82	38.65	0.08	2.37	0.04
5	-	-	-	-	-	-	-	9	30.42	0.79	38.64	0.07	2.33	0.03
6	-	-	-	-	-	-	-	8	29.37	0.82	38.60	0.08	2.29	0.04

The analysis of variance on the physiological traits in Sahiwal and crossbred cows for the influence of *HSP90AB1 Fragment II* genotypes is presented in Table 4.18. The corresponding means are presented in Table 4.19. The results of ANOVA revealed that genotypes of these gene fragments have not significantly influenced the physiological traits in both Sahiwal and crossbred cows.

Table 4.18. Analysis of variance of *HSP90AB1 Fragment II* in Sahiwal and crossbred cows for physiological traits

Source of variation	Sahiwal				Crossbreds			
	d.f	MSS			d.f	MSS		
		RR	RT	HTC		RR	RT	HTC
HSP90AB1 Fragment II	1	1.44	0.02	0.003	3	2.25	0.02	0.01
Parity	3	1.71	0.03	0.004	5	3.09	0.03	0.01
Error	45	2.03	0.02	0.004	41	4.86	0.04	0.01

Table 4.19. Means of *HSP90AB1 Fragment II* genotypes and parity effects for physiological traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	RR		RT (°C)		HTC		n	RR		RT (°C)		HTC	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	24.02	0.37	38.27	0.04	2.04	0.02	50	30.85	0.42	38.57	0.04	2.35	0.02
Genotype														
AA	32	24.34	0.30	38.32	0.03	2.05	0.01	16	30.98	0.64	38.62	0.06	2.36	0.03
AB	10	23.70	0.49	38.22	0.05	2.02	0.02	14	30.78	0.69	38.55	0.06	2.35	0.03
AC	4	23.55	0.80	38.31	0.08	2.02	0.03	13	31.56	0.68	38.53	0.06	2.38	0.03
BC	4	24.48	0.89	38.23	0.09	2.06	0.04	7	30.09	1.09	38.58	0.10	2.32	0.05
Parity														
1	13	24.70	0.41	38.37	0.04	2.07	0.02	5	31.12	1.35	38.65	0.13	2.36	0.06
2	11	24.11	0.59	38.26	0.06	2.04	0.03	7	30.51	1.03	38.51	0.10	2.34	0.05
3	18	23.63	0.55	38.22	0.06	2.02	0.02	12	30.77	0.77	38.52	0.07	2.35	0.03
4	8	23.63	0.68	38.23	0.07	2.02	0.03	9	31.63	0.92	38.66	0.09	2.39	0.04
5	-	-	-	-	-	-	-	9	30.75	0.81	38.63	0.08	2.35	0.04
6	-	-	-	-	-	-	-	8	29.25	0.85	38.61	0.08	2.28	0.04

4.5.2 Production traits

The results of the analysis of variance carried out to study the influence of *HSP70 Fragment I* genotypes on the production traits in Sahiwal and crossbred cows are presented in Table 4.20 while the means for the corresponding traits are presented in Table 4.21. Genotype had a significant effect on total lactation milk yield and peak yield, with AA genotype having higher TLMY (3005.01 kg) in crossbred cows and with AA genotype recording the highest peak milk yield of 10.77 kg in Sahiwal cows. Parity also significantly affected total lactation milk yield and lactation length in crossbred cows with the highest milk yield recorded in the fourth parity.

The results of the analysis of variance to study the effect of *HSP90AA1 Fragment I* genotypes on the production traits in Sahiwal and crossbred cows are presented in Table 4.22 and the means are presented in Table 4.23. The effect of genotype was significant on lactation length in Sahiwal cows with AA genotype having longer LL (295.62 days). In crossbred cows, genotype had significant effect on total lactation milk yield and peak yield with AA genotype recording higher values for both traits in crossbred cows. Parity also had a highly significant effect on total lactation milk yield and lactation length in crossbred cows.

The analysis of variance on the production traits in Sahiwal and crossbred cows for the influence of *HSP90AA1 Fragment II* genotypes is presented in Fig 4.24. The corresponding means are presented in Table 4.25. The effect of genotype was found to be non significant in both genetic groups, while parity significantly influenced the total lactation milk yield and lactation length in crossbred cows.

Table 4.20. Analysis of variance of *HSP70 Fragment I* in Sahiwal and crossbred cows for production traits

Source of variation	Sahiwal				Crossbreds			
	d.f	MSS			d.f	MSS		
		TLMY	PY	LL		TLMY	PY	LL
HSP70 Fragment I	1	10303.09	30.92*	27626.56	1	1057929.04*	24.03	816.44
Parity	3	572351.84	15.62	17701.15	5	975489.94**	14.74	589.76*
Error	45	512529.37	6.44	7333.80	43	294660.70	7.61	226.04

*Significant (P≤0.05);** Significant (P≤0.01)

Table 4.21. Means of *HSP70 Fragment I* genotypes and parity effects for production traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	N	TLMY		PY		LL		n	TLMY		PY		LL	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	1775.70	107.38	9.96	0.39	266.91	12.84	50	2836.10	95.14	13.58	0.48	328.28	2.64
Genotype														
AA	29	1760.88	135.75	10.77[a]	0.49	291.17	291.17	31	3005.01[a]	115.50	14.39	0.59	332.97	3.20
AB	21	1790.51	162.69	9.15[b]	0.58	242.65	242.65		-	-	-	-	-	-
AC	-	-	-	-	-	-	-	19	2667.19[b]	143.72	12.78	0.73	323.59	3.98
Parity														
1	13	1964.88	198.72	10.62	0.71	317.79	23.77	5	2581.30[c]	314.81	13.27	1.60	336.23[ab]	8.72
2	11	1606.12	216.06	9.47	0.77	240.52	25.85	7	3399.18[a]	243.41	16.36	1.24	337.94[a]	6.74
3	18	1971.79	170.33	11.19	0.60	280.33	20.37	12	3239.35[ab]	179.80	14.12	0.91	321.28[c]	4.98
4	8	1560.01	258.45	8.56	0.92	228.99	30.92	9	3499.99[a]	205.56	16.97	1.04	322.81[c]	5.69
5	-	-	-	-	-	-	-	9	3306.44[a]	203.23	14.52	1.03	312.73[d]	5.63
6	-	-	-	-	-	-	-	8	3050.13[b]	205.56	14.26	1.04	330.96[b]	5.69

Means with similar superscripts in a column do not differ significantly (P≤0.05)

Table 4.22. Analysis of variance of *HSP90AA1 Fragment I* in Sahiwal and crossbred cows for production traits

	Sahiwal				Crossbreds			
Source of variation	d.f	MSS			d.f	MSS		
		TLMY	PY	LL		TLMY	PY	LL
HSP90AA1 Fragment I	1	704913.70	15.04	36331.99*	1	1691990.35*	36.33*	812.24
Parity	3	555410.54	13.15	14302.84	5	945402.40**	14.30	592.92**
Error	45	497093.58	6.80	7140.35	43	277974.87	7.29	226.15

*Significant (P≤0.05);** Significant (P≤0.01)

Table 4.23. Means of *HSP90AA1 Fragment I* genotypes and parity effects for production traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	TLMY		PY		LL		n	TLMY		PY		LL	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	1759.15	104.68	10.05	0.39	268.24	12.55	50	2808.13	94.21	13.46	0.48	327.89	2.69
Genotype														
AA	28	1879.78	137.36	10.60	0.51	295.62[a]	16.46	34	3025.11[a]	108.84	14.46[a]	0.56	332.65	3.10
AB	22	1638.52	153.53	9.49	0.57	240.85[b]	18.40	16	2591.15[b]	146.20	12.45[b]	0.75	323.14	4.17
Parity														
1	13	1956.74	195.70	10.52	0.72	313.82	23.45	5	2597.33[c]	305.81	13.34	1.57	336.25[a]	8.72
2	11	1615.74	212.78	9.60	0.79	245.22	25.50	7	3408.80[a]	236.44	16.40	1.21	337.95[a]	6.74
3	18	1941.69	167.70	11.24	0.62	279.64	20.10	12	3210.51[a]	174.88	14.00	0.90	321.25[c]	4.99
4	8	1522.44	250.56	8.82	0.93	234.28	30.03	9	3382.87[a]	202.81	16.43	1.04	320.11[c]	5.78
5	-	-	-	-	-	-	-	9	3270.39[a]	197.73	14.37	1.01	312.68[d]	5.64
6	-	-	-	-	-	-	-	8	2995.00[b]	199.67	14.00	1.02	329.61[b]	5.70

Means with similar superscripts in a column do not differ significantly (P≤0.05)

Table 4.24. Analysis of variance of *HSP90AA1 Fragment II* in Sahiwal and crossbred cows for production traits

	Sahiwal				Crossbreds			
Source of variation	d.f	MSS			d.f	MSS		
		TLMY	PY	LL		TLMY	PY	LL
HSP90AA1 Fragment II	1	489298.73	0.75	675.90	1	708004.13	7.32	383.66
Parity	3	744049.27	13.21	11416.68	5	999205.14**	13.63	555.06*
Error	45	501885.02	7.11	7932.70	43	303869.25	8.05	237.43

*Significant (P≤0.05);** Significant (P≤0.01)

Table 4.25. Means of *HSP90AA1 Fragment II* genotypes and parity effects for production traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	TLMY		PY		LL		n	TLMY		PY		LL	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	1707.48	123.80	10.03	0.47	268.94	15.56	50	2837.11	97.55	13.64	0.50	328.48	2.73
Genotype														
AA	35	1841.91	125.71	10.19	0.47	273.94	15.80	31	2974.37	114.64	14.09	0.59	331.67	3.20
AB	15	1573.05	227.87	9.86	0.86	263.94	28.65	19	2699.86	148.53	13.20	0.76	325.28	4.15
Parity														
1	13	2017.72	203.34	10.63	0.77	317.84	25.56	5	2479.25^{c}	319.67	12.85	1.65	333.60^{ab}	8.94
2	11	1470.35	253.30	9.38	0.95	237.73	31.85	7	3392.85^{a}	247.18	16.29	1.27	337.64^{a}	6.91
3	18	1923.69	173.04	11.31	0.65	284.06	21.75	12	3285.80^{a}	177.99	14.42	0.92	322.82^{c}	4.98
4	8	1418.17	285.08	8.80	1.07	236.13	35.84	9	3456.25^{a}	208.75	16.79	1.07	321.69^{c}	5.83
5	-	-	-	-	-	-	-	9	3364.50^{a}	200.01	14.90	1.03	314.65^{d}	5.59
6	-	-	-	-	-	-	-	8	3045.61^{b}	208.75	14.21	1.07	330.74^{b}	5.83

Means with similar superscripts in a column do not differ significantly (P≤0.05)

The analysis of variance for the effect of *HSP90AB1 Fragment II* genotypes on the production traits in both genetic groups is presented in Fig 4.26 while the means for the same are presented in Fig 4.27. Genotype had a significant effect on total lactation milk yield with BC genotype having higher TLMY in crossbred cows. Parity also significantly affected the total lactation milk yield and lactation length in crossbred cows.

Table 4.26. Analysis of variance of *HSP90AB1 Fragment II* in Sahiwal and crossbred cows for production traits

Source of variation	Sahiwal				Crossbreds			
	d.f	MSS			d.f	MSS		
		TLMY	PY	LL		TLMY	PY	LL
HSP90AB1 Fragment II	3	21838.06	4.748	6181.58	3	167359.29*	14.00	669.67
Parity	3	571244.16	14.215	10923.50	5	770163.52*	9.96	579.89*
Error	43	535083.96	7.131	7886.11	41	326471.05	7.54	205.47

*Significant (P≤0.05);** Significant (P≤0.01)

Table 4.27. Means of *HSP90AB1 Fragment II* genotypes and parity effects for production traits in Sahiwal and crossbred cows

Effect	Sahiwal							Crossbreds						
	n	TLMY		PY		LL		n	TLMY		PY		LL	
		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	1720.05	187.88	10.71	0.69	269.83	22.81	50	2917.24	110.04	14.13	0.53	328.21	2.76
Genotype														
AA	32	1809.03	153.71	9.58	0.56	269.62	18.66	16	2841.54^{b}	165.54	12.99	0.80	337.83	4.15
AB	10	1737.93	251.42	11.13	0.92	283.11	30.52	14	2777.69^{b}	178.50	13.09	0.86	319.22	4.48
AC	4	1663.05	411.04	10.67	1.50	309.15	49.90	13	2915.93^{ab}	175.47	14.57	0.84	328.23	4.40
BC	4	1670.18	454.36	11.44	1.66	217.46	55.16	7	3133.79^{a}	282.17	15.88	1.36	327.56	7.08
Parity														
1	13	1980.79	211.03	10.27	0.77	317.88	25.62	5	2575.90^{c}	349.26	13.62	1.68	328.25^{bc}	8.76
2	11	1535.53	301.57	10.43	1.10	238.12	36.61	7	3436.88^{a}	266.31	16.67	1.28	338.67^{a}	6.68
3	18	1891.37	282.62	12.23	1.03	283.69	34.31	12	3406.85^{a}	199.50	15.25	0.96	321.08^{de}	5.00
4	8	1472.51	347.38	9.89	1.27	239.65	42.17	9	3392.17^{a}	239.29	16.09	1.15	325.08^{cd}	6.00
5	-	-	-	-	-	-	-	9	3459.70^{a}	209.03	15.51	1.00	316.18^{e}	5.24
6	-	-	-	-	-	-	-	8	3066.06^{b}	220.10	14.38	1.06	332.85^{b}	5.52

Means with similar superscripts in a column do not differ significantly (P≤0.05)

4.5.3 Reproduction traits

The analysis of variance for the effect of *HSP70 Fragment I* genotypes on the reproduction traits in Sahiwal and crossbred cows is presented in table 4.28, while the means for the traits studied are presented in Table 4.29. Genotype had a significant effect on service period in crossbred cows with AC genotype recording the highest SP. Parity had a significant effect on the service period in Sahiwal cows.

The results obtained from the analysis of variance for the effect of *HSP90AA1 Fragment I* genotypes on the reproduction traits in Sahiwal and crossbred cows are presented in Table 4.30 and the means are presented in Table 4.31. The effect of genotype was non significant on the reproductive traits studied while parity had a significant effect on the service period in Sahiwal cows.

The results of the analysis of variance conducted to study the influence of *HSP90AA1 Fragment II* genotypes on the reproduction traits in Sahiwal and crossbred cows are given in Table 4.32 while the means for the corresponding traits are presented in Table 4.33. The effect of genotype was significant on the service period with AB genotype having a longer service period in crossbred cows.

The analysis of variance on the reproduction traits in Sahiwal and crossbred cows for the influence of *HSP90AB1 Fragment II* genotypes is presented in Table 4.34. The corresponding means are presented in Table 4.35. The effect of the genotype was highly significant ($P \leq 0.01$) on calving interval and significant ($P \leq 0.05$) on the service period in Sahiwal cows. Cows with genotype BC had a longer calving interval. Parity also significantly influenced service period, dry period, and calving interval. In crossbred cows, genotype had a significant effect on age at first service. The AA genotype had a lower age at first service.

Table 4.28 . Analysis of variance of *HSP70 Fragment I* in Sahiwal and crossbred cows for reproduction traits

Source of variation	Sahiwal						Crossbreds								
	d.f	MSS	d.f	MSS			d.f	MSS		d.f	MSS	d.f	MSS		
		GP		SP	DP	CI		AFS	AFC		GP		SP	DP	CI
HSP70 Fragment I	1	198.03	1	5938.75	49.17	6479.94	1	15735.54	66.84	1	50.29	1	11706.66*	1000.06	6742.10*
Parity	3	65.84	3	9770.98*	4389.00	4579.50	-	-	-	5	212.97	5	2380.81	1052.90	2263.27
Error	45	172.52	39	2562.80	1955.24	1776.09	48	14843.33	19754.89	43	308.94	39	2630.71	1700.65	1856.07

*Significant (P≤0.05)

Table 4.29. Means of *HSP70 Fragment I* genotypes and parity effects for reproduction traits in Sahiwal and crossbred cows

	Sahiwal										Crossbreds				
	n	GP (days)		n	SP (days)		DP (days)		CI (days)		n	AFS (days)		AFC (days)	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE
Overall	50	279.25	0.97	44	149.85	8.16	140.56	7.13	438.31	6.79	50	645.73	17.75	947.55	20.48
Genotype															
AA	29	281.30	2.49	25	161.74	10.40	139.48	9.09	425.89	8.66	31	627.45	21.88	946.35	25.24
AB	21	277.19	2.98	19	137.96	12.12	141.64	10.58	450.73	10.09	-	-	-	-	-
AC	-	-	-	-	-	-	-	-	-	-	19	664.00	27.95	948.74	32.24
Parity															
1	13	281.39	3.65	13	192.99[a]	14.05	144.15	12.28	463.12	11.70	-	-	-	-	-
2	11	275.90	3.96	11	145.10[b]	15.28	168.64	13.35	421.13	12.72	-	-	-	-	-
3	18	279.10	3.13	14	136.60[b]	13.71	127.09	11.98	448.19	11.42	-	-	-	-	-
4	8	280.60	4.74	6	124.70[b]	20.83	122.36	18.19	420.81	17.34	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Means with similar superscripts in a column do not differ significantly (P≤0.05)

Table 4.29 *Contd.,*

Crossbreds										
Overall	n	GP (days)		n	SP (days)		DP (days)		CI (days)	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	274.83	3.08	46	202.29	9.20	157.03	7.39	418.56	7.73
Genotype										
AA	31	275.99	3.74	30	183.29[b]	11.05	151.48	8.88	404.14[b]	9.28
AC	19	273.66	4.65	16	221.28[a]	14.47	162.58	11.63	432.97[a]	12.15
Parity										
1	5	269.72	10.19	5	185.00	29.76	158.15	23.93	442.53	25.00
2	7	262.83	7.88	7	247.80	23.01	136.49	18.50	428.12	19.33
3	12	277.80	5.82	11	212.77	18.48	174.87	14.85	418.10	15.52
4	9	275.59	6.66	7	185.33	20.94	162.83	16.84	421.17	17.59
5	9	265.25	6.58	8	192.42	20.42	170.54	16.42	421.87	17.16
6	8	277.74	6.66	8	186.83	20.94	150.83	16.84	416.50	17.59

Means with dissimilar superscripts in a column differ significantly (P>0.95)

Table 4.30. Analysis of variance of *HSP90AA1 Fragment I* in Sahiwal and crossbred cows for reproduction traits

Source of variation	Sahiwal						Crossbreds								
	d.f	MSS	d.f	MSS			d.f	MSS		d.f	MSS	d.f	MSS		
		GP		SP	DP	CI		AFS	AFC		GP		SP	DP	CI
HSP90AA1 Fragment I	1	9.44	1	4388.03	229.76	36.03	1	8413.84	1629.74	1	517.13	1	10320.02	2097.53	4006.27
Parity	3	64.39	3	8944.92*	4276.76	5162.70	-	-	-	5	211.35	5	2142.72	1149.05	2333.11
Error	45	176.71	39	2602.56	1950.61	1941.32	48	14995.86	19722.33	43	296.66	39	2671.50	1668.38	1936.54

*Significant(P≤0.05)

Table 4.31. Means of *HSP90AA1 Fragment I* genotypes and parity effects for reproduction traits in Sahiwal and crossbred cows

Effect	Sahiwal										Crossbreds				
	n	GP (days)		n	SP (days)		DP (days)		CI (days)		n	AFS		AFC	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE
Overall	50	279.57	1.97	44	44	150.56	8.18	140.66	7.08	436.24	50	646.35	18.56	945.06	21.29
Genotype															
AA	28	280.02	2.59	25	24	160.68	10.65	138.34	9.22	437.16	34	632.44	21.00	951.18	24.08
AB	22	279.13	2.89	19	20	140.45	11.90	142.97	10.30	435.32	16	660.25	30.61	938.94	35.11
Parity															
1	13	281.20	3.69	13	13	191.30^{a}	14.16	144.41	12.26	464.01	-	-	-	-	-
2	11	276.13	4.01	11	11	147.10^{b}	15.40	168.34	13.33	420.08	-	-	-	-	-
3	18	279.46	3.16	14	14	138.55^{bc}	13.68	127.12	11.84	444.51	-	-	-	-	-
4	8	281.51	4.72	6	6	125.29^{c}	20.99	122.77	18.17	416.36	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Means with similar superscripts in a column do not differ significantly (P≤0.05)

Table 4.31 *Contd.,*

Crossbred cows										
Overall	n	GP (days)		N	SP (days)		DP (days)		CI (days)	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE
	50	273.93	3.08	46	203.18	9.45	158.24	7.47	418.42	8.04
Genotype										
AA	31	277.72	3.56	32	184.97	10.89	150.03	8.60	407.07	9.27
AB	19	270.13	4.78	14	221.39	15.22	166.45	12.03	429.76	12.96
Parity										
1	5	270.60	9.99	5	185.26	30.00	157.26	23.71	443.55	25.54
2	7	263.36	7.72	7	247.96	23.19	135.96	18.33	428.73	19.74
3	12	276.22	5.71	11	212.16	18.68	176.94	14.76	415.71	15.90
4	9	273.80	6.63	7	191.40	21.33	165.57	16.85	424.95	18.16
5	9	263.28	6.46	8	191.87	20.63	172.44	16.30	419.68	17.56
6	8	277.03	6.52	8	192.90	21.33	153.57	16.85	420.28	18.16

Table 4.32. Analysis of variance of *HSP90AA1 Fragment II* in Sahiwal and crossbred cows for reproduction traits

Sahiwal							Crossbreds								
Source of variation	d.f	MSS	d.f	MSS			d.f	MSS		d.f	MSS	d.f	MSS		
		GP		SP	DP	CI		AFS	AFC		GP		SP	DP	CI
HSP90AA1 Fragment II	1	31.45	1	68.16	1666.69	814.58	1	856.72	2337.53	1	162.27	1	13585.09*	1917.47	2116.77
Parity	3	50.26	3	6009.99	4537.28	4446.09	-	-	-	5	203.40	5	1607.96	1132.89	2178.51
Error	45	176.22	39	2713.33	1913.76	1921.36	48	15153.30	19707.59	43	306.00	39	2575.46	1673.67	1992.11

*Significant($P \leq 0.05$)

Table 4.33. Means of *HSP90AA1 Fragment II* genotypes and parity effects for reproduction traits in Sahiwal and crossbred cows

Overall	Sahiwal										Crossbreds				
	n	GP (days)		n	SP (days)		DP (days)		CI (days)		n	AFS		AFC	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE
	50	280.15	2.32	44	150.73	10.41	135.46	8.74	432.90	8.76	50	640.32	17.93	945.57	20.45
Genotype															
AA	35	279.07	2.36	32	152.56	9.80	144.48	8.23	439.20	8.25	31	644.58	22.11	952.61	25.21
AB	15	281.23	4.27	12	148.91	19.63	126.45	16.49	426.60	16.52	19	636.05	28.24	938.53	32.21
Parity															
1	13	280.82	3.81	13	192.78	15.11	147.70	12.69	466.50	12.71	-	-	-	-	-
2	11	277.17	4.75	11	144.36	19.47	159.53	16.35	413.70	16.38	-	-	-	-	-
3	18	279.91	3.24	14	138.96	15.40	121.63	12.93	441.04	12.96	-	-	-	-	-
4	8	282.70	5.34	6	126.84	24.18	112.98	20.30	410.36	20.35	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.33 *Contd.,*

Crossbred cows										
	n	GP (days)		n	SP (days)		DP (days)		CI (days)	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE
Overall	50	275.67	3.10	46	203.74	9.23	158.00	7.44	417.267	8.119
Genotype										
AA	31	273.59	3.64	30	183.21[b]	10.68	150.29	8.61	409.163	9.393
AB	19	277.75	4.71	16	224.27[a]	14.70	165.71	11.85	425.372	12.932
Parity										
1	5	270.03	10.14	5	198.18	29.45	162.57	23.74	450.04	25.90
2	7	262.18	7.84	7	247.49	22.77	136.06	18.35	429.38	20.02
3	12	279.33	5.65	11	204.84	17.18	173.13	13.85	409.59	15.18
4	9	275.73	6.62	7	192.18	20.93	165.40	16.87	423.87	18.41
5	9	267.16	6.35	8	193.52	20.22	172.08	16.30	417.36	17.78
6	8	277.27	6.62	8	186.83	20.72	150.83	16.70	416.50	18.22

Means with dissimilar superscripts in a column differ significantly (P>0.95)

Table 4.34. Analysis of variance of *HSP90AB1 Fragment II* in Sahiwal and crossbred cows for reproduction traits

Source of variation	Sahiwal						Crossbreds							
	d.f	MSS	d.f	MSS			d.f	MSS		d.f	MSS	d.f	MSS	
		GP		SP	DP	CI		AFS	AFC		GP		SP	DP	CI
HSP90AB1 Fragment II	3	112.20	3	6883.97*	2182.26	11599.43**	3	46971.74*	21600.94	3	215.99	3	3155.22	1973.40	2554.64
Parity	3	94.85	3	13680.25**	5180.53*	2256.39*	-	-	-	5	209.46	5	3441.89	900.44	2880.56
Error	43	177.32	37	2303.68	1885.31	1106.73	46	12767.39	19206.50	41	309.51	37	2865.16	1653.19	1943.27

*Significant(P≤0.05)

Table 4.35. Means of *HSP90AB1 Fragment II* genotypes and parity effects for reproduction traits in Sahiwal and crossbred cows

Effect	Sahiwal										Crossbreds				
Overall	n	GP (days)		n	SP (days)		DP (days)		CI (days)		n	AFS		AFC	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE
	50	278.50	3.42	44	119.67	13.79	131.32	12.47	450.75	9.56	50	654.12	16.80	956.85	20.61
Genotype															
AA	32	280.29	2.80	28	170.34^{a}	10.67	148.38	9.65	433.08^{b}	7.40	16	569.63^{c}	28.25	900.81	34.65
AB	10	279.21	4.58	8	134.32^{b}	20.11	114.52	18.19	404.72^{c}	13.94	14	652.86^{b}	30.20	955.57	37.04
AC	4	283.73	7.48	4	107.03^{c}	27.71	117.19	25.07	441.27^{b}	19.20	13	675.85^{ab}	31.34	962.46	38.44
BC	4	270.76	8.27	4	67.00^{d}	30.95	145.20	27.99	523.94^{a}	21.45	7	718.14^{a}	42.71	1008.57	52.38
Parity															
1	13	281.99	3.84	13	201.67^{a}	14.03	148.37^{a}	12.69	462.81^{a}	9.73	-	-	-	-	-
2	11	274.08	5.49	11	104.54^{b}	20.69	157.41^{a}	18.72	439.50^{b}	14.34	-	-	-	-	-
3	18	277.94	5.14	14	94.48^{bc}	20.65	114.57^{b}	18.68	466.36^{a}	14.31	-	-	-	-	-
4	8	279.97	6.32	6	78.00^{c}	27.13	104.95^{b}	24.54	434.34^{b}	18.80	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Means with similar superscripts in a column do not differ significantly (P≤0.05)

Table 4.35 *Contd.,*

Crossbred cows									
	n	GP (days)		n	SP (days)		DP (days)		CI (days)	
		Mean	SE		Mean	SE	Mean	SE	Mean	SE
Overall	50	274.31	3.39	46	199.21	11.01	156.11	8.36	420.92	9.07
Genotype										
AA	16	278.53	5.10	15	192.85	16.12	149.56	12.24	413.04	13.28
AB	14	268.56	5.50	14	176.92	16.84	141.11	12.79	400.42	13.87
AC	13	276.82	5.40	12	215.72	17.08	173.12	12.97	420.44	14.07
BC	7	273.33	8.69	5	211.34	31.18	160.64	23.68	449.79	25.68
Parity										
1	5	265.69[b]	10.75	5	190.07	33.12	158.70	25.16	452.75	27.28
2	7	263.05[b]	8.20	7	255.18	25.31	138.10	19.22	440.97	20.84
3	12	276.79[a]	6.14	11	202.82	20.21	173.75	15.35	415.93	16.64
4	9	277.27[a]	7.37	7	183.94	23.78	162.73	18.06	413.65	19.58
5	9	266.94[b]	6.44	8	186.76	20.97	173.57	15.93	414.36	17.27
6	8	278.86[a]	6.78	8	193.53	23.37	153.75	17.75	428.23	19.25

Means with similar superscripts in a column do not differ significantly (P≤0.05)

CHAPTER V
DISCUSSION

The present investigation on Sahiwal and crossbred cows was undertaken to catalogue the genetic polymorphism in specific fragments of heat shock protein (*HSP*) genes using polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP) technique and to study the association of those polymorphisms with physiological, milk production and reproduction traits.

5.1 WEATHER CONDITIONS

Data collected from Agriculture Climate Research Center, ARI, Hyderabad during the experimental period revealed that the maximum temperatures recorded in the summer season go well beyond 40 °C for most days during the month of May. The temperatures were moderate in rainy and winter seasons. The temperature-humidity index (THI) calculated was 83.71 for summer, which indicated a moderate level of thermal stress on the animals as classified by Habeeb *et al.* (2018). Upadhyay *et al*. (2008) also found that THI > 78 requires greater efforts to dissipate heat resulting in reduced production and reproduction performance in dairy cattle. The THI was 71.37 and 66.69 in rainy and winter seasons respectively which was within the comfort range of the animals.

5.2 PHYSIOLOGICAL PARAMETERS

The means for all the physiological parameters *viz.* respiration rate (RR), rectal temperature (RT), and heat tolerance coefficient (HTC) recorded in the morning (8 AM) and afternoon (2 PM) and percentage increase between both periods, during various seasons in both the genetic groups indicate that, the means for RR, RT and HTC increased during the afternoon in all the seasons, and the percentage change was higher in crossbreds during summer.

The analysis of variance and means for physiological parameters (average of readings recorded at 8 AM and 2 PM) indicated significant effect of genetic group and season on the respiration rate, rectal temperature, and heat tolerance coefficient. Sahiwal cows had a lower overall mean respiration rate (24.16 ± 0.21, number/minute) than

crossbred cows (30.81 ± 0.22). *Bos indicus* cattle maintained lower respiration rates than *Bos taurus* cattle, at all temperatures. A lower respiration rate under hot weather identifies animals with lesser discomfort. Respiration rate generally increases with THI and is a reliable physiological parameter for assessing heat stress in dairy cattle (Dalcin *et al.,* 2016). Published literature also revealed higher respiration rates in cattle during summer, when THI values increase (Kumar *et al*., 2015a; Verma *et al*., 2015, Das, 2014 and Sailo *et al*., 2017). The increase in respiration rate in summer as compared to other seasons could be due to increased demand for oxygen by tissues under stressful conditions (Hall and Guyton, 2011).

Mean respiration rates were 28.56 ± 0.36, 23.38 ± 0.33 and 20.54 ± 0.38 in Sahiwal cows and 44.58 ± 0.38, 25.94 ± 0.34 and 21.90 ± 0.37 in crossbred cows during summer, rainy and winter seasons, respectively. Several authors also reported higher respiration rates in Sahiwal (Kumar *et al*., 2015a; Sailo *et al*., 2015b; Verma *et al*., 2015 and Das, (2014) and HF crossbred cattle (Deb *et al.,* 2013; Verma *et al*., 2015; Das, 2014 and Sailo *et al*., 2017) during summer as compared to winter season. Increased respiration is an important physiological response, which aids in dissipation of excess body heat by vaporizing more moisture in the expired air (Das, 2014 and Atkins *et al*., 2018). Therefore, lower RR indicates an improved thermotolerance. The change in the RR during summer season was observed to be higher in crossbreds than in indigenous cows indicating lower thermotolerance in crossbreds and is in confirmation with the findings of Sailo *et al*. (2017).

In the present investigation, the overall means for rectal temperature (ºC) were 38.29 ± 0.03 and 38.58 ± 0.03 in Sahiwal and crossbred cows respectively, which were well within the normal physiological range for cattle. Higher rectal temperatures (38.96 ± 0.03) were recorded in summer season (THI=83.71), while the lowest rectal temperatures (37.96 ± 0.03) were recorded in winter season (THI=66.69). Kumar *et al*. (2015), Sailo *et al*. (2015b), Verma *et al*. (2015) and Das, (2014) also reported higher mean rectal temperatures during summer in purebred Sahiwal and HF crossbred cows. The increase in rectal temperature was observed to be more in crossbreds during summer as compared to Sahiwal cows. Higher rectal temperatures during summer season may be due to excessive

heat production due to increased metabolic rate, especially in lactating cows. The basal metabolic rate of zebu cattle is generally lower compared to *Bos taurus* which may be one reason for maintaining a stable rectal temperature during different seasons and exhibiting increased heat tolerance when exposed to higher ambient temperature during summer season.

Similarly higher values of heat tolerance coefficients were observed in summer when THI was above 83, as compared to the other two seasons in the present study. The HTC was found to be 2.25 ± 0.02 and 2.96 ± 0.02 during summer, in Sahiwal and crossbred cows, respectively. Kumar *et al*. (2015), Sailo *et al*. (2015a), Verma *et al*. (2015) and Das, (2014) also reported higher HTC values for crossbreds in north India during summer, indicating the lower adaptability of crossbred cows over the indigenous cows which are more thermotolerant. The indigenous cows thus possess climate-resilient traits and can withstand the high temperatures in the face of climate change.

5.3 MEAN PERFORMANCE OF PRODUCTION TRAITS

The present investigation revealed that the total lactation milk yield and peak yield were significantly ($P \leq 0.01$) affected by genetic group with the crossbred cows recording higher total lactation milk yield (2983.45 ± 78.32 kg) and peak yield (14.92 ± 0.36 kg) as compared to Sahiwal cows. Many authors have reported that crossbred cows have higher total lactation milk yield than indigenous cows (Dubey and Singh, 2005; Reddy *et al*., 2015 and Chakravarthi *et al.*, 2017).

The mean total lactation milk yield in Sahiwal (1768.32 kg) obtained in the present study is in agreement with the means reported by Kumar and Gandhi (2011) for the animals maintained at three different farms in northern India and Reddy *et al.* (2015) for animals maintained in Tirupati, located in Andhra Pradesh state of Southern India. However, Javed *et al.* (2000), Joshi *et al.* (2001), Maurya and Saraswat (2002), and Verma *et al.* (2016), reported higher means, while Rehman and Khan (2012) and Chakravarthi *et al.* (2017) observed means lower than those obtained in the present investigation. The mean total lactation milk yield in crossbred cows (2983.45 kg) obtained in the present study was almost similar to the means reported by Lakshmi *et al.*

(2010). However, Kumar *et al.* (2017b), Kundu *et al.* (2018), and Annual report CIRC, (2019) observed means higher than those obtained in the present investigation. Varying lactation lengths, in addition to the geographical location and the different managemental practices, followed in the respective farms might be the reasons for the differences in the total lactation milk yields.

The mean peak yield in Sahiwal cows in the present investigation was 10.17 kg which was higher than the mean peak yield reported by Sharma *et al.* (2010), Reddy *et al.* (2015), and Chakravarthi *et al.* (2017). The mean (14.92 kg) peak yield in crossbred cows obtained in the present study was in accordance with that reported by Lakshmi *et al.* (2010), Kumar *et al.* (2017b) and Annual report CIRC, (2019) in Holstein Friesian × Sahiwal cows.

Though the effect of the genetic group was found to be non-significant on the lactation length in the present study, the mean lactation length in crossbred cows was found to be higher (324.71 days) than in Sahiwal cows (304.41 days). The results obtained were within the wide range (235 to 348.25 days) as observed from published literature on Sahiwal and HF crossbred cows (Rehman and Khan, 2012 and Reddy *et al.*, 2015). The crossbreds, in the present investigation, recorded higher peak yield (4.75 kg more than Sahiwal), lactation length (20.3 days longer than Sahiwal), and consequently, the elevated TLMY (1215.13 kg higher than Sahiwal).

The analysis revealed a significant ($P \leq 0.01$) influence of parity on total lactation milk yield in the present study with the mean increasing from first to third parity, followed by a decreasing trend in the later parities. The highest and lowest total lactation milk yields were recorded for the cows in parities three (2681.42 kg) and one (2215.50 kg), respectively. A perusal of the published literature also revealed a significant effect of parity on total lactation milk yield in Sahiwal cows (Kumar and Gandhi, 2011, Rehman and Khan, 2012 and Singh *et al*., 2016) and in HF crosses (Lakshmi *et al*., 2010, Kakati *et al*., 2017 and Kundu *et al*., 2018). Consistent increase in total lactation milk yield up to the third lactation might be due to the development occurring in the body, particularly in the mammary glands with advancement of age.

5.4 MEAN PERFORMANCE OF REPRODUCTION TRAITS

The overall mean gestation period, service period, dry period and calving interval obtained in the present study were 276.52 ± 1.72, 181.84 ± 11.77, 147.24 ± 8.11 and 421.92 ± 10.22 days respectively. The genetic group had non significant effect on above reproduction traits except dry period, while parity had a non-significant effect on the gestation period, service period, dry period, and calving interval.

The mean gestation period in Sahiwal was 277.71 days which was lower than the mean obtained by Reddy *et al*. (2015), while the mean gestation period in crossbred cows was 275.34 days which was similar to the mean reported by Lakshmi (2007) in HF × Sahiwal cows of the military dairy farm, Secunderabad. Effect of parity was found to be non-significant on the gestation period. Lakshmi (2007) also found a non-significant effect of parity on the gestation period.

The mean service period was 182.60 and 181.09 days in Sahiwal and crossbred cows respectively. Published literature revealed that the service period ranged from 123.73 to 205 days in Sahiwal cows (Naskar *et al.*, 2005 and Reddy *et al*., 2015) and from 148.24 to 183.28 days in HF × Sahiwal cows (Lakshmi, 2007 and Kumar *et al.*, 2015b). Effect of parity was found to be non-significant on service period in the present study. However, Lakshmi, (2007) reported a significant effect of parity on the service period with the lowest and highest means obtained for cows in parities 12 and 1, respectively.

The mean dry period in Sahiwal cows was 167.47 days which was within the wide range (133.58 to 176.79 days) as observed from the published literature on Sahiwal cows (Naskar *et al.*, 2005 and Reddy *et al*., 2015). The mean dry period of 127.01 days in HF crosses obtained in the present study corroborated with the findings of Lakshmi (2007) in HF × Sahiwal cows. However, lower means were reported by Singh *et al*. (2014) and Kumar *et al.* (2015b) in crossbred cows. In the present investigation, influence of parity was found to be non-significant on dry period, however significant effect of parity was reported by Lakshmi (2007) and Singh *et al*. (2014) in HF × Sahiwal cows.

The mean calving interval in Sahiwal was 430.23 days which was more than the mean obtained by Naskar *et al.* (2005), while the mean calving interval in crossbred cows was 413.61 days which was less than the mean reported by Lakshmi (2007), Singh *et al*. (2014), Kumar *et al.* (2015b), Kakati *et al.* (2017) and Annual report, CIRC, (2019) in HF × Sahiwal cows. Parity was found to have non-significant effect on the calving interval. However Lakshmi (2007) and Kakati *et al.* (2017) reported a significant effect of parity on calving interval, which was attributed to the variations in the age and management conditions provided at different times.

The mean age at first service and age at first calving was 641.34 ± 17.24 and 947.26 ± 19.67 respectively in crossbred cows. Lakshmi (2007) also reported similar means for AFS and Kundu *et al.* (2018) for AFC in HF × Sahiwal cows.

In the present investigation, the effect of genetic group was found to be significant on total lactation milk yield and peak yield, which are traits of economic importance in dairy cows, with the crossbreds having higher milk yield. Crossbreds also recorded lower service period, dry period, and calving interval. Overall performance of crossbreds was found to be better than the Sahiwal cows. However, the crossbreds are more prone to heat stress as evidenced by their higher values for physiological parameters and higher percentage increase in the summer season.

5.5 MOLECULAR GENETIC STUDIES

5.5.1 Genomic DNA

The overall mean absorbance ratio of genomic DNA was 1.76 ± 0.01 in Sahiwal cows while it was 1.75±0.01 in crossbred cows, which is similar to the ratios recommended for pure DNA preparations free from protein. DNA with an optical absorbance ratio of 1.80 to 2.00 is considered to be of good quality without any protein contamination (Green and Sambrook, 2012). The yield of DNA in Sahiwal cows ranged from 0.884 to 2.455 µg/µl and from 0.896 to 2.651µg/µl in crossbred cows respectively. Agarose gel electrophoresis of DNA samples isolated revealed an intact single band for each of the DNA samples, which indicated that there was no shearing of genomic DNA.

5.5.2 PCR-Amplification of different fragments of *HSP* genes

Though there are many *HSP* genes, thermotolerance is mainly correlated with *HSP70* and *HSP90* genes in Livestock species (Archana *et al.,* 2017). Polymorphism in the *HSP70* and *HSP90* genes have shown an association with heat tolerance, milk production, fertility, and disease susceptibility in livestock (Shergojry *et al*., 2014a; Kumar *et al*., 2015a and Bhat *et al.,* 2016).

In the present study, the DNA template of each sample was used to amplify the two specific fragments each of *HSP70, HSP90AA1,* and *HSP90AB1* genes. One of the most critical factors is the annealing temperature which affects both specificity and yield of the PCR product. The primer sets for *HSP70, HSP90AA1,* and *HSP90AB1* genes were optimized at 50°C, 51°C, and 50.5°C respectively. At lower annealing temperatures, non-specific products were formed while at higher than optimal temperature yield of the product was reduced. So the temperatures and time for annealing were optimized for PCR amplification of the different fragments of *HSP* genes.

5.5.3 PCR-SSCP of different regions of *HSP* genes

The PCR-SSCP technique was used to detect the polymorphism in different targeted regions of *HSP70, HSP90AA1,* and *HSP90AB1* genes.

5.5.3.1 PCR-SSCP of *HSP70* gene fragments

In the present investigation, PCR-SSCP of *HSP70* Fragment I (295 bp) was found to be polymorphic and revealed two SSCP patterns namely AA and AB in Sahiwal. The frequencies of AA and AB genotypes were 0.58 and 0.42, respectively. In crossbred cows, two SSCP patterns AA and AC were documented, the frequencies of which were found to be 0.62 and 0.38 respectively. Polymorphism was observed in different regions of *HSP70* gene by many authors in various genetic groups (Cai *et al*., 2005; Lamb *et al*., 2007; Rosenkrans *et al.,* 2010 and Sodhi *et al*., 2013).

These findings are in agreement with previous studies of Bhat *et al.* (2016) as they detected three SSCP patterns and two allelic variants with a frequency of 0.59 and 0.41, respectively for the same 295 bp fragment of *HSP70* gene in Tharparkar cattle.

Kerekoppa *et al.*(2015) reported a still high polymorphism in the coding region of *HSP70* gene with 8 and 14 band patterns in HF crossbreds and Deoni cattle.

HSP70 gene fragment II (220 bp) was found to be monomorphic in both Sahiwal and crossbred cows in the present study. Similar findings were reported by Bhat *et al.* (2016) in Tharparkar cattle.

5.5.3.2 PCR-SSCP of *HSP90AA1* gene fragments

Fragment I (450 bp) of *HSP90AA1* gene studied comprised the exon 3, which was found to be polymorphic in both Sahiwal and crossbred cows. Two SSCP genotypes namely AA and AB were documented in the present study whose frequencies were estimated to be 0.56 and 0.44 in Sahiwal and 0.68 and 0.32 in crossbred cows respectively.

Kumar *et al.* (2015a) also observed a higher amount of polymorphism in the same 450 bp fragment and detected three genotypes AA, AG, and GG with respective frequencies of 0.23, 0.50, and 0.27 within the exon 3 of *HSP90AA1* gene in Sahiwal cows.

In the present study, PCR-SSCP of Fragment II (539 bp) of *HSP90AA1* gene revealed two different SSCP patterns AA and AB in both Sahiwal and crossbred cows. However, Shergojry *et al*. (2014a) analyzed the polymorphism in the same region (exon 8) of *HSP90AA1* gene using the PCR-SSCP technique in Deoni cattle and revealed three unique SSCP genotypes with frequencies of 0.250, 0.639 and 0.111 respectively.

Shergojry *et al*. (2014b) also found two SSCP patterns in exon 9 region of *HSPAA1* gene with frequencies of 0.153 and 0.847 and two PCR-SSCP patterns in exon 10 with frequencies 0.236 and 0.764 respectively in Deoni cows. Also Badri *et al.* (2018) identified five single nucleotide polymorphisms in Chinese Holstein lactating cows: one in the promoter, three in the coding region, and one in 3′-UTR region of *HSP90AA1* gene.

5.5.3.3 PCR-SSCP of *HSP*90*AB1* gene fragments

PCR-SSCP of Fragment I (459 bp) of *HSP90AB1* gene was monomorphic while Fragment II (387 bp) was polymorphic in both Sahiwal and crossbred cows in the present study. Four different SSCP patterns corresponding to three allelic variants (A, B amd C) with frequencies of 0.78, 0.14 and 0.08 in Sahiwal, and 0.59, 0.23 and 0.18 in crossbred cows respectively were found. Current results are in agreement with previous studies reported by Charoensook *et al*. (2012), Sailo *et al*. (2015a), Sailo *et al*. (2015b) and Sajjanar *et al*. (2015) who also found polymorphism in *HSPAB1* gene. Sajjanar *et al.* (2015) also reported three different genotypes CC, CT and TT with frequencies of 0.05, 0.78 and 0.17 respectively in Sahiwal and 0.20, 0.70 and 0.10 in Frieswal cattle. The monomorphism observed in Fragment I of *HSP90AB1* could be due to the small sample size of 50 animals in the present study which may not elucidate polymorphism reliably.

5.6 ASSOCIATION ANALYSIS OF *HSP* GENE POLYMORPHISM

The current work was carried out to study the association of SSCP genotypes of different *HSP* genes with physiological, production, and reproduction traits in Sahiwal and crossbred cows.

5.6.1 Association with physiological traits

The SSCP genotypes of Fragment I of *HSP70* gene had no significant effect on physiological parameters studied in both Sahiwal and crossbred cows. However, Bhat *et al*. (2016) found allele A of *HSP70* to have positive correlation with thermal tolerance and genotype AA demonstrated superior heat tolerance. Various other authors also suggested that polymorphism in the *HSP70* gene could be used in genetic improvement programmes of cattle for heat tolerance (Cai *et al.*, 2005; Li *et al*., 2011; Deb *et al*., 2013 and Sodhi *et al.,* 2013).

The SSCP genotypes of Fragment I and II of the *HSP90AA1* gene had no significant effect on the physiological parameters studied in both Sahiwal and crossbred cows. However, Kumar *et al.* (2015a) found that AA genotype of Fragment I of *HSPAA1* gene had lower heat tolerance coefficient as compared to AG and GG genotypes in Sahiwal cows and GG genotype had lower mean respiration rate, rectal temperature and HTC in Karan Fries cows (Kumar *et al.*, 2016)

The SSCP genotypes of Fragment II of the *HSP90AB1* gene had no significant effect on the physiological parameters in both Sahiwal and crossbred cows. However, previous authors reported that cows with CT genotype had significantly lower respiration rate than CC genotype in Sahiwal (Sailo *et al.,* 2015a); cows with CC genotype had significantly lower respiration rate than TT and TC genotypes in Jersey crossbred cows of Assam (Sailo *et al.,* 2015b). Sajjanar *et al.* (2015) also found that TT genotypes had significantly lower respiration rate values than CC and CT in both Sahiwal and Frieswal breeds.

In the present investigation, the genotypes of different *HSP* gene fragments have not significantly influenced the physiological traits in both Sahiwal and crossbred cows which need to be validated with larger sample size.

5.6.2 Association with production traits

The SSCP genotypes AA of Fragment I of *HSP70* gene had significantly higher peak yield of 10.77 kg in Sahiwal cows and higher total lactation milk yield in crossbred cows. The present investigation established a relationship between the *HSP70* Fragment I genotypes and production traits, with allele A having a positive effect on milk yield. It may be suggested that AA genotype may be incorporated in marker-assisted selection to increase milk yield in both Sahiwal and crossbred cows. Rosenkrans *et al.* (2010) also reported polymorphisms in the promoter region of the *HSP70* gene, some of which were associated with the milk yield in crossbred Brahman cows which may be useful in selecting cows.

The SSCP genotypes of Fragment I of *HSP90AA1* gene obtained in the present study had a significant effect on lactation length with AA genotype having higher lactation length in Sahiwal cows and higher total lactation milk yield and peak yield in crossbred cows. The genetic polymorphisms in Fragment I of *HSP90AA1* gene and its association with milk production traits reveal their importance as a potential genetic marker for milk production traits in Sahiwal and crossbred cows.

The SSCP genotypes of Fragment II of *HSP90AB1* gene obtained in the present study had a significant effect on the TLMY, with the BC genotype having higher total

lactation milk yield. Sajjanar *et al.* (2015) also reported that TT genotype animals had better production parameters in terms of total milk yield in Sahiwal and Frieswal breeds.

5.6.3 Association analysis with reproduction traits

The SSCP genotypes of Fragment I of *HSP70* gene had a non-significant effect on the reproductive traits studied in both Sahiwal and crossbred cows except on service period in crossbreds. Crossbred cows with AA genotype had a longer service period. Rosenkrans *et al.* (2010) also reported polymorphisms in the promoter region of the *HSP70* gene, some of which were associated with calving rates in crossbred Brahman cows which may be useful in selecting cows with greater fertility.

The differences obtained in various traits due to different genotypes of fragment II of the *HSP90AA1* gene were not statistically significant in Sahiwal cows, whereas in crossbred cows the effect of genotype was significant only on service period with AB genotype having longer service period.

The SSCP genotypes of Fragment II of *HSP90AB1* gene obtained in the present study had a significant effect on calving interval and service period in Sahiwal cows and on the AFS in crossbred cows. Sahiwal cows with genotype BC had a shorter service period and longer calving interval while crossbred cows with AA genotype had lower AFS.

Though these genetic polymorphisms reveal their importance as genetic markers in selecting cows for better production and reproductive performance, further research is needed to establish the physiological mechanisms by which these polymorphisms determine the association between genotypes and performance of cows. The SSCP patterns obtained in the present study for various fragments of *HSP* genes and their association analysis with physiological parameters revealed no significant differences between the genotypes suggesting that a larger sample size with a wide genetic base may be needed to elucidate the association of polymorphism. Also, more number of markers within the *HSP* genes need to be studied to establish the associations.

CHAPTER VI
SUMMARY

The present investigation was carried out on 50 purebred Sahiwal cows of Livestock farm complex, Rajendranagar and 50 crossbred cows of Military Dairy Farm, Secunderabad to study genetic polymorphism in different fragments of heat shock protein (*HSP*) genes through Polymerase Chain Reaction and Single-stranded conformation polymorphism (PCR-SSCP) technique and to correlate the polymorphism with the physiological, production and reproduction traits.

Genetic group and season significantly affected the physiological parameters (average of readings recorded at 8 AM and 2 PM) studied. Crossbreds recorded higher mean respiration rate, rectal temperature and heat tolerance coefficient. The mean RR, RT and HTC were 24.16 ± 0.21 (number/minute), 38.29 ± 0.03 °C and 2.05 ± 0.02 in Sahiwal and 30.81 ± 0.22, 38.58 ± 0.03 and 2.35 ± 0.02 in crossbred cows, respectively. The mean respiration rates were 36.57 ± 0.27, 24.66 ± 0.26 and 21.22 ± 0.27 (number/minute) and mean rectal temperatures were 38.87 ± 0.03, 38.47 ± 0.02, and 37.96 ± 0.03 °C, during summer, rainy and winter seasons, respectively. Similarly, higher values of heat tolerance coefficients were observed in summer when THI was above 83, as compared to other seasons.

The effect of genetic group was found to be significant on total lactation milk yield and peak yield, with the crossbreds having higher production performance than Sahiwal cows. The mean total lactation milk yield, peak yield and lactation length were 1768.32 ± 109.67 kg, 10.17 ± 0.50 kg, and 304.41 ± 13.00 days in Sahiwal cows and 2983.45 ± 78.32 kg, 14.92 ± 0.36 kg, 324.71 ± 9.29 days in crossbred cows, respectively. The means for the reproduction traits, *viz*, gestation period, service period, dry period and calving interval were 277.71 ± 2.24, 182.60 ± 15.51, 167.47 ± 10.68 and 430.23 ± 13.46 days in Sahiwal and 275.34 ± 1.60, 181.09 ± 10.91, 127.01 ± 7.51 and 413.61 ± 9.47 days in crossbred cows, respectively.

Genomic DNA of the experimental animals was isolated by phenol-chloroform extraction method and used for PCR after evaluating the quality and quantity. Six fragments, two each of *HSP70, HSP90AA1*, and *HSP90AB1* genes were amplified and the amplicons were confirmed on 1.5% agarose gel with standard DNA ladder.

The amplified segments of the *HSP* genes were subjected to Single-Stranded Conformation Polymorphism (PCR-SSCP) technique to detect polymorphism. Out of the six fragments studied, two (Fragment II of *HSP70* and Fragment I of *HSP90AB1* gene) were monomorphic while four fragments revealed polymorphism in both Sahiwal and crossbred cows. The PCR-SSCP of Fragment I of *HSP70* gene revealed two genotypes AA and AB in Sahiwal cows and two genotypes AA and AC in crossbred cows. The corresponding allele frequencies of A and B in Sahiwal cows were 0.79 and 0.21, and the allele frequencies of A and C in crossbred cows, were 0.81 and 0.19, respectively.

The PCR-SSCP of Fragment I of *HSP90AA1* gene yielded two conformational patterns AA and AB corresponding to two allelic variants A and B in both Sahiwal and crossbred cows. The allele frequencies of A and B were 0.78 and 0.22, and 0.84 and 0.16 in Sahiwal and crossbred cows, respectively. The *HSP90AA1* fragment II yielded two genotypic patterns AA and AB corresponding to two allelic variants with frequencies of 0.85 and 0.15, and 0.81 and 0.19 in Sahiwal and crossbred cows, respectively. Fragment II of *HSP90AB1* gene yielded four SSCP patterns AA, AB, AC, and BC corresponding to three allelic variants A, B, and C whose allelic frequencies were 0.78, 0.14, 0.08, and 0.59, 0.23, 0.18 in Sahiwal, and crossbred cows, respectively. The present investigation indicated that the SSCP technique is a valuable tool for the identification of genetic polymorphism.

PCR-SSCP patterns of different fragments of HSP genes were correlated with the physiological, productive, and reproductive traits in both Sahiwal and crossbred cows. It was observed that *HSP70* Fragment I genotype AA had higher peak milk yield in Sahiwal cows while the same genotype had higher total lactation milk yield, lower service period,

and calving interval in crossbred cows. The association analysis of SSCP patterns of the fragment I of *HSP90AA1* gene revealed that genotype AA had higher lactation length in Sahiwal cows, and higher total lactation milk yield and peak yield in crossbred cows.

The effect of *HSP90AA1 Fragment II* genotypes was non-significant in Sahiwal cows, while in crossbred cows, AB genotype had a longer service period. The association analysis of SSCP patterns of the fragment II of the *HSP90AB1* gene revealed that cows with genotype BC had a longer calving interval in Sahiwal while BC genotype had higher total lactation milk yield and AA genotype had a lower age at first service in crossbred cows.

In conclusion, the differences found between the different SSCP genotypes of *HSP* genes in production and reproduction traits indicated their possible role in marker-assisted selection (MAS). However, the SSCP patterns obtained in the present study for various fragments of *HSP* genes and their association analysis with physiological parameters did not reveal significant differences among the genotypes indicating that a larger population of cows with a wide genetic base may be needed to elucidate the association of polymorphism.

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APPENDIX - A

COMPOSITION OF THE REAGENTS FOR DNA ISOLATION

RBC lysis buffer:

1.6M Sucrose	20 ml
0.1M Tris (pH 7.5)	1 ml
0.5M MgCl2	1 ml
Triton X- 100	1 ml
Sterile double distilled Water	up to 100 ml

1.6M Sucrose:

Sucrose	87.54 g
Distilled water	100 ml

0.1M Tris (pH 7.5):

Tris base	1.21 g
Distilled water	100 ml

0.5M MgCl2:

MgCl2	2.38 g
Distilled water	50 ml

DNA Extraction buffer:

1M Tris (pH 8.0)	1 ml
5M NaCl	8 ml
0.5M EDTA (pH 8.0)	0.4 ml
Distilled water	up to 500 ml

1M Tris (pH 8.0):

Tris base	12.11 g
Distilled water	100 ml

5M NaCl:

NaCl	14.6 g
Distilled water	50 ml

0.5M EDTA:

EDTA	18.61 g
Distilled water	100 ml

10% SDS:

SDS	10 g
Distilled water	100 ml

3M Sodium acetate (pH 5.2):

Sodium acetate	20.4 g
Distilled water	50 ml

APPENDIX – B

COMPOSITION OF THE REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

5X TBE Electrophoresis buffer:

Tris base	54 g
Boric acid	27.5 g
EDTA	4.15 g
Distilled water	up to 1000 ml

Agarose (2%):

Agarose	2 g
TBE (1×)	100 ml

Ethidium Bromide:

Ethidium Bromide	100 mg (2.5 mg/ml)
Distilled water	1 ml

6X Gel loading buffer:

Bromophenol blue	0.0025 g
Sucrose	4 g
Distilled water	10 ml

APPENDIX- C

COMPOSITION OF THE REAGENTS FOR SINGLE STRAND CONFORMATION POLYMORPHISM

Formamide denaturing dye mix:

Formamide (95%)	9.5 ml
Bromophenol blue (0.025%)	25 mg
Xylene cyanol (0.025%)	25 mg
0.5M EDTA	400 µl
Distilled water	100 µl

Solutions/reagents for PAGE (12%):

Acrylamide: Bisacrylamide (49:1) solution:

Acrylamide	49 g
Bisacrylamide	1 g
Distilled water	100 ml

Ammonium persulfate (APS) 10%:

Ammonium persulfate	0.1 g
Distilled water	1 ml

Polyacrylamide gel (12%):

Acrylamide: Bisacrylamide (49:1)	9.6 ml
Glycerol	1.5 ml
APS (10%)	100 µl
TEMED	75 µl
1x TBE	28.3 ml

Solutions/reagents for silver-staining:

Fixative / stop solution (10%):

Ethanol	50 ml
Distilled water	up to 500 ml

Oxidative solution (1%):

Nitric acid	5 ml
Distilled water	up to 500 ml

Silver-staining solution (0.1%):

Silver nitrate	0.5 g
Distilled water	up to 500 ml

Developer solution:

Sodium carbonate	15 g
Formaldehyde	750 µl
Distilled water	500 ml