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Simple Summary: Fertility in Holstein dairy cows is a critical factor influencing herd profitability. In the past, genetic selection focused primarily on improving traits related to milk production. Today, selection indexes in dairy cattle also include fertility-related traits. A problem for fertility improvement is the identification of traits that are easily measured as indicators of reproductive performance. Measurement of serum Anti-Müllerian hormone (AMH) has been proposed as a marker for fertility in Holstein cows, as well as single nucleotide polymorphisms (SNPs) associated with AMH. However, the heat stress (HS) that occurs in the summer of semi-arid regions appears to affect the predictability of the AMH marker(s). In the current study, serum AMH was proved as a marker for fertility in cows subjected to a reproductive management program during summer but only if the heat stress did not exceed a moderate threshold. Interestingly, four SNPs were confirmed as genetic markers associated with reproductive performance in Holstein cows exposed to moderate or severe HS. These SNPs were in the genes *AMH, IGFBP1, LGR5* and *TLR4*, which are involved in physiological processes that regulate ovarian activity and thermotolerance.

Abstract: Anti-Müllerian hormone (AMH) has been proposed as biomarker for fertility in cattle, yet this associative relationship appears to be influenced by heat stress. Objective was to test serum AMH and AMH-related single nucleotide polymorphisms (SNPs) as markers potentially predictive of reproductive traits in heat-stressed dairy cows. The study included 300 Holstein cows that were genotyped using BovineSNP50 (54,000 SNP) after blood samples were collected and DNA extracted. A genome wide association study was then executed. Nine intragenic SNPs within the pathways that influence the *AMH* gene were found important with multiple comparisons adjustment test ($p < 1.09 \times 10^{-6}$). A further validation study was performed in an independent Holstein cattle population, which was divided into moderate (MH; n=152) and severe heat-stressed (SH; n=128) groups, and subjected to a summer reproductive management program. Serum AMH was confirmed as a predictor of fertility measures ($p < 0.05$) in MH but not in SH group. Cows were genotyped using the TaqMan allelic discrimination method and qPCR, which revealed four SNPs as predictive markers for serum AMH ($p < 0.01$), reproductive traits ($p < 0.01$) and physiological variables ($p < 0.05$). These SNPs were in the genes *AMH, IGFBP1, LGR5* and *TLR4*. In conclusion, both serum AMH concentrations and AMH-related SNPs appear to be associated with fertility traits in dairy cows managed during summer.

Keywords: AMH; fertility; Holstein cows; markers; SNPs

1. Introduction

Fertility is important to the efficiency of dairy herds, as it accounts for one of the major costs of production. In the last century, selection programs in dairy cattle systems were focused primarily on improving milk yield traits [1], which caused a reduction in the genetic merit for fertility [2]. The high-production dairy cows that comprise the current population were produced with great success using this selection strategy [3]. However, the progressive decline in dairy cow fertility stimulated remarkable change in dairy cow selection indices in the past 25 years. Therefore, modern indices include functional traits such as those that are specifically designated to enhance reproduction and health, in addition to traits related to milk production [1,3,4].

Currently, the combination of reproductive management techniques and breeding selection programs have improved on-farm reproduction and accelerated the rate of fertility genetic progress in dairy cows [5–7]. To incorporate reproductive features into breeding programs, one of the biggest challenges has been measuring phenotypes with a reasonable heritability. For instance, heritability estimates for mostly fertility traits are usually low. Another important issue for many fertility traits is to identify easy-to-measure phenotypic variables or genetic markers that correlate to relevant fertility variables [8].

Evaluations of new measurable traits, such as endocrine or molecular markers for reproductive phenotypes, offer potential new tools to help genetic improvement of fertility in cattle [9]. Then, a phenotypic trait with high variability, heritability, and repeatability that is also associated with fertility could be a candidate to include in selection strategies [10]. The Anti-Müllerian hormone (AMH) has emerged as potential biomarker for fertility in dairy cows [11]. Concentrations of AMH in cattle have been reported to have high variability among animals and high repeatability within individual animals [12], and it is considered as a moderately heritable trait (0.36 to 0.46) [13]. Therefore, circulating AMH is proposed as reproductive management tool. The success of this tool has been reported in breeding and superovulation protocols in dairy heifers and lactating cows [14].

Novel elements that should be investigated to improve fertility in dairy cows include the range of circulating AMH and its threshold values, as well as genome-wide association studies (GWAS) that identify single nucleotide polymorphisms (SNPs) linked to phenotypic variations in AMH concentrations. The establishment of an AMH threshold, and an test executable on a dairy farm, would enable farmers to selectively breed cows with elevated AMH levels, in order to increase the reproductive efficiency in their herds [15]. Similarly, GWAS technologies make possible to identify functional genes associated with serum AMH that are directly or indirectly involved in reproductive processes, as well as to recognize specific molecular markers that could play a role enhancing fertility-related physiological events [16].

Efficacy of endocrine and molecular markers as predictors appear to be highly influenced by climate, especially in semi-desert regions that can induce heat-stressed climatic conditions [17,18]. Heat stress (HS) during summer is a major factor responsible to disrupt several reproductive events. High environmental temperatures affect heat dissipation in lactating dairy cows leading to an increase in body temperature, which is strongly associated with impaired reproduction [19]. HS affects a number of reproductive processes, including the formation of the corpus luteum, ovarian follicular growth, gonadotropin production, oocyte competence, embryonic growth, and uterine endometrial responses [20,21].

Cellular and molecular impairments appear to be part of the response of lactating diary cows to heat stress. When oocytes are exposed to heat shock during maturation, it hinders their ability to reorganize their microtubules and microfilaments and damaged spindle apparatus [22]. Oocytes that were exposed to HS showed an increase in both apoptotic gene expression and production of reactive oxygen species (ROS), which decreased the oocyte's capacity to split and develop into a blastocyst [23,24]. Taken together, heat stress-induced alterations in oocytes might be associated with fertilization failure, embryo development defects, and consequently low conception rates and (or) high embryo mortality rates [19].

Therefore, our objective was to evaluate associations of serum AMH concentrations and AMH-related single nucleotide polymorphisms (SNPs) with reproductive performance in Holstein dairy cows exposed to heat-stressed environmental conditions.

2. Materials and Methods

The Institutional Animal Care and Use Committee of the Insituto Tecnologico de Sonora approved all procedures performed in animals (Approval code 2017-0079).

2.1. Location and experimental population

Three neighboring dairy farms in the Yaqui Valley Sonora, Mexico collaborated in the current study, and they were managed as intensive milk production systems. The geographic coordinates are 27°21' North Latitude and 109°54' West Longitude. The site is located 46 meters above sea level. Local climate ranges from dry to semi-humid, with summer rains, an average of 371.6 mm of precipitation annually, and an a yearly mean temperature of 23 °C. During the summer, this zone can record ambient temperatures above 48 °C, and a temperature-humidity index (THI) that often exceeds 85 units.

Three hundred lactating and spring-calved Holstein cows with average body weight of 635.1 ± 32.5 kg, body condition score from 2.5 to 3.5, and 4 to 6 yrs-old were used in this study. Cows received a similar management and were kept in shaded stables with free access to a water source and a commercial trace mineral supplement. Floor and shade areas were provided according to the Holstein cows requirements. A mixed diet was supplied twice daily, which was designed to meet requirements for dairy cows with average body weight of 650 kg and daily milk production of 30 kg. This ration's average composition was 3.5% fat and 3.2% crude protein.

2.2. Blood sampling for AMH

Blood samples were collected early in the summer by puncturing the coccygeal vein using serum tubes, which were promptly placed on ice. After clotting, samples were centrifuged for 30 min at room temperature (18 to 22 °C). Subsequently, serum samples were stored at -20 °C until analysis. Serum AMH concentrations were measured using the Minitube of America AMH-bovine specific immunoassay (AMH Fertility AssayTM), which has a sensitivity of 0.04 pg/mL and an intra-assay coefficient of variation of 2.2%.

2.3. SNP data and quality control

An additional blood sample of 3 ml was drawn from each cow through venipuncture of the coccygeal vein using disposable sterile syringes. Five drops of the collected whole blood were spotted on Fast Technology for Analysis of Nucleic Acids cards (FTA®), which were sent to Neogen AgriGenomics (Lincoln, NE) for DNA extraction and genotyping. The SNP panel BovineSNP50, which contains 53,218 highly informative SNPs evenly dispersed throughout the whole bovine genome, was used to acquire genotypes of each cow.

PLINK v1.07 software was used to implement quality control for the SNPs that were used in genotype to phenotype association analyses. Only SNPs that met the following criteria were included in analyses: A call rate of greater than 95% or a false discovery rate of less than 5%, a missing genotype frequency of less than 5%, a minor allele frequency (MAF) of more than 5%, and no-deviation from Hardy-Weinberg equilibrium (p-value of Chi-square goodness-of-fit test greater than 0.05, $X^2 > 0.05$). The analyses also excluded SNPs with uncertain loci or those on the sex chromosomes. After quality control, a total of 45,832 SNPs were retained for further analyses.

2.4. Genome-wide association study (GWAS)

Batch effects/stratification of the test input data were corrected by using principal component analysis (PCA) option. The genomic analysis was then performed using a single-locus mixed model

to study associations between genotypes of each SNP marker with serum AMH level as a phenotypic observation (i.e., single-marker SNP GWAS). The software SNP & Variation Suite v8 (SVSv8; Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com) was used for this purpose by fitting all SNP simultaneously.

The additive mixed model used was: $\mathbf{y = X\beta + Za + e}$, where $\mathbf{y}$ was the vector of phenotypic observations (AMH), $\mathbf{X}$ was the incidence matrix of fixed effects, $\mathbf{\beta}$ was the vector of fixed effects, $\mathbf{Z}$ was the design matrix of random additive genetic effects, $\mathbf{a}$ was the vector of random additive genetic effects, and $\mathbf{e}$ was the vector of residual effects. This model assumed that $a \sim N(0, G\sigma^2_a)$ and $e \sim N(0, I\sigma^2_e)$, where σ^2_a was the additive genetic variance, σ^2_e was the residual variance component, G was the genomic relationship matrix and I was the identity matrix.

2.5. Multiple testing adjustment

The *p*-values obtained from the genomic analyses were subjected to the Bonferroni correction test ($b=\alpha/n$), which assumed independence between SNPs to adjust for multiple comparisons. The experiment-wise error was $\alpha=0.05$ and the number of tests (n) was taken to be the number of the useful SNPs (n=45,832). Therefore, the 5% genome-wide threshold was equivalent to a nominal *p*-value of 1.09×10^{-6}, corresponding to 5.96 on a $-\log10(P\text{-value})$ scale.

2.6. Validation population

An independent Holstein cattle population (n=280) was selected to perform the validation study for the candidate SNP markers previously identified as associated with serum AMH. Cows were selected from two neighboring dairy herds in the Yaqui Valley; they were spring-calved Holstein, with average body weight of 651.3 ± 34.7 kg, mean body condition score of 3.0 ± 0.5, and aged 4 to 7. Cows were randomly assigned to one of two experimental periods. The moderate heat-stressed (MH; n=152) group included cows managed during late spring and early summer (AT= 28.1 ℃, RH= 55.8% and THI= 75.7 units), whereas the severe heat-stressed (SH; n=128) group included cows managed during middle and late summer (AT= 30.6 ℃, RH= 71.4% and THI= 82.2 units). Level of heat stress was established according to the classification described by Collier et al. [25]: (1) absence of heat stress (< 68 units), (2) heat tress threshold (68–72 units), (3) moderate heat stress (73–79 units), and (4) severe heat stress (> 79 units).

2.7. Reproductive management

Cows were subjected to an ovulation synchronization protocol that included the insertion of an intravaginal device for delayed progesterone release (CIDR®, Pfizer, Mexico) at day 0 plus an intramuscular (im) administration of 0.01 mg of GnRH (Fertagyl®, Intervet, Mexico). The CIDR was removed 7 days later and then 25 mg im of prostaglandin F2a (PGF2a, Lutalyse®, Pfizer, Mexico) was applied. The cows were given a second dose of 0.01 mg of GnRH at day 9, and 24 hours later they were artificially inseminated (FTAI; day 10). At 30 days following FTAI, ultrasound scanning was used to diagnose pregnancy. The number of pregnant cows divided by the total number of FTAI services at day 30 was used to compute the conception rate (CR).

A trans-rectal ultrasonography inspection was performed in cows utilizing a 7.5 MHz transducer (Sonosite MicroMaxx TM®, Bothell, WA, USA) to assess the health of their reproductive system, mainly uterine body and horns. Ovarian activity was also evaluated through measuring dominant follicle diameter (FOL) on day 7 of the synchronization protocol, as well as Corpus Luteum diameter (CL) on day 14 after AI.

2.8. Physiological traits and climatic data

During the course of the study, both rectal temperature (RT; °C) and respiratory rate (RR; breaths/min) were measured at 1200 h two times per week. The RT was obtained using a digital thermometer (TES- 1310®) with a contact sounding line (type K; 9-cm large), which was inserted into the animal to touch the rectal mucosa, while the RR was determined by visually counting the

intercostal movements. A blood sample was collected at the beginning of the study to measure serum AMH as described above.

Data on the ambient temperature (AT; °C) and relative humidity (RH; %) were obtained from the REMAS website (http://www.siafeson.com/remas) by a local meteorological station (Network of Automatic Meteorological Stations of Sonora). Daily records from both AT and RH were used to calculate the temperature-humidity index (THI), by processing the following equation: THI = (0.8 AT) + [(RH/100) (AT 14.4)] + 46.4 [26].

2.9. SNP marker genotyping

An additional blood sample was collected using Vacutainer tubes with EDTA (EDTA-Na2; Venoject®, Terumo, Lakewood, CA, USA), by puncturing the jugular vein from each cow at the beginning of the study. The samples were centrifuged at 3500 RMP for 15 min. Then, 200 μl of leukoplatelet layer was removed by pipet and refrigerated at −20 °C. Next, the DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kits; QIAGEN, Hilden, Germany) and following the manufacturer's instructions. The concentration and purity of the DNA were determined using a NanoDrop automated spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The nine significant SNPs reported in this study were genotyped using the TaqMan method for allelic discrimination and RT-qPCR (StepOneTM, Applied Biosystems, Foster City CA, USA), in accordance with the methods described by Castillo-Salas et al. [27]. The PCR was carried out using the StepOne Real-Time PCR System from Thermo Fisher Scientific (Massachusetts, USA). Finally, genotyping and analysis of PCR data were performed using StepOne software (Life Technologies Corporation, version 2.3).

2.10. Statistics for the validation analysis

Mean values of the analyzed traits were calculated using the MEANS and FREQ procedures for continuous and categorical variables, respectively. The assumptions of normality and homogeneity of variances were tested through the UNIVARIATE procedure. Allele and genotype frequencies for each polymorphism and deviations from Hardy-Weinberg equilibrium were obtained using the ALLELE program.

A mixed effects statistical model was used to perform an associative study using the MIXED and GLIMMIX procedures for continuous (AMH, FOL, CL, RT and RR) and categorical variables (CR), respectively. For the statistical processing of the information, the SAS (Statistical Analysis System) program version 9.4 was used, which included the procedures for genetic studies [28]. The statistical model included the fixed effects of the SNP genotype, herd and lactation, as well as the covariant of days in milk, and the random effect of sire.

If genotype term was confirmed as significant source of variation ($p < 0.05$) in the associative statistical analysis, then LSMEANS procedure was performed to make comparisons between average genotypic values, considering the Bonferroni adjustment [29]. The allele substitution effects (i.e., effect of substituting one allele for another within the population) was determined using a regression model that included the allelic effect as a covariate [29]. Additive genetic effects were obtained using the procedures described by Luna-Nevárez et al. [30].

3. Results

3.1. Climate and AMH sampling

During the validation study, moderate HS characterized the climatic conditions in late spring and early summer (i.e., THI ranged from 72 to 79 units), whereas severe heat stressed conditions prevailed in mid- and late summer (i.e., THI ranged from 79 to 84), as observed in **Figure 1**.

Average values for serum AMH, reproductive traits and physiological variables differed (p < 0.05) in cows managed during moderate heat stress compared with those cows exposed to severe heat stress (**Table 1**). A decrease in serum AMH, FOL, CL and CR was observed when the THI

surpassed the threshold of 79 units, whereas an slight increase was detected in the physiological traits RT and RR.
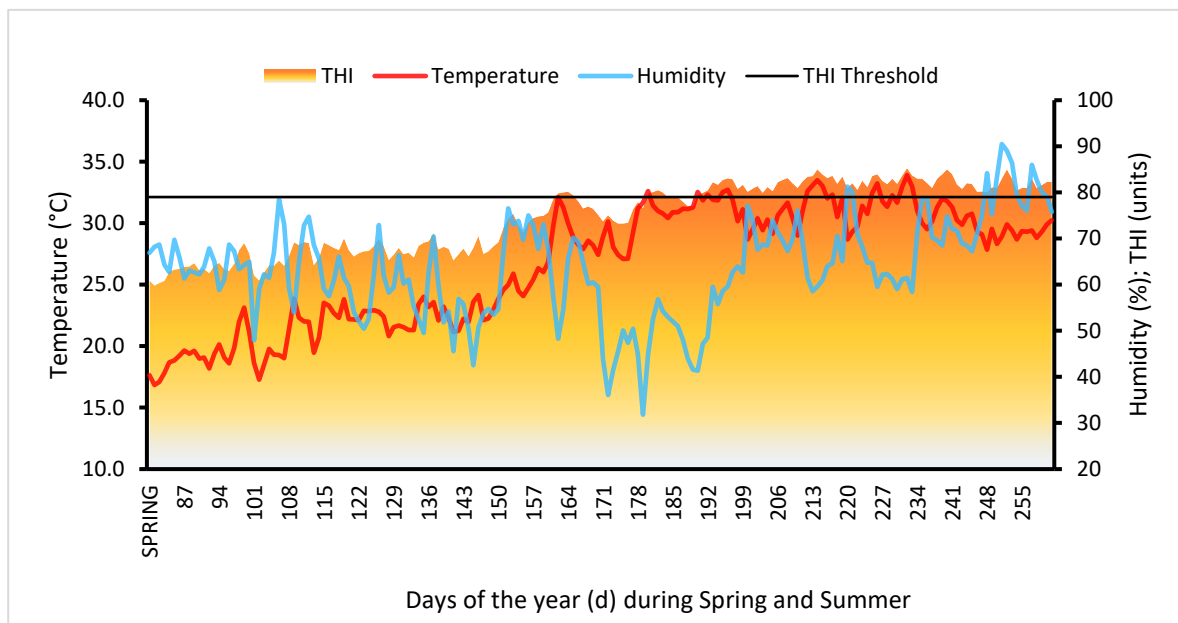
Figure 1. Ambient temperature (AT, °C), relative humidity (RH, %), and temperature-humidity index (THI, units) during the study. The straight line indicates the threshol between moderate and severe heat stress.

Table 1. Serum AMH, reproductive traits and physiological variable in Holstein cows according to heat-stressed environmental conditions.

Variable	Moderate heat stress (n=152; THI = 72-79)	Severe heat stress (n=128; THI > 79)
AMH (ng/ml)	417.26 ± 4.51 [a]	136.94 ± 4.03 [b]
FOL (mm)	12.95 ± 0.93 [a]	10.15 ± 1.02 [b]
CL (cm)	2.68 ± 0.02 [a]	1.26 ± 0.04 [b]
CR (%)	52.63 [a]	37.50 [b]
RT (°C)	38.21 ± 1.25 [a]	38.73 ± 3.05 [b]
RR (breaths/min)	63.35 ± 3.14 [a]	67.14 ± 4.96 [b]

AMH = Serum Anti-Müllerian hormone; FOL = Follicle diameter; CL = Corpus Luteum diameter; CR = Conception rate; RT = Rectal temperature; RR = Respiratory rate. [a, b] Different literals between columns indicate a significant statistical difference (p < 0.05).

3.2. AMH as endocrine marker during summer

In late spring and early summer, high correlations (r = 0.71 to 0.75, $p < 0.05$) were observed between serum AMH and reproductive traits (i.e., FOL and CL; **Table 2**). However, this relationship diminished during summer when the THI surpassed 79 units, as correlations dropped (r < 0.30) due to the severe heat stress. A similar associative relationship trend was observed between serum AMH and physiological traits (i.e., RT and RR) through the study, although correlations were mostly not significant.

The simple regression analysis detected serum AMH as predictor for the reproductive traits FOL ($R^2 = 0.56$; $p < 0.01$) and CL ($R^2 = 0.52$; $p < 0.01$) but only for cows managed under moderate heat stress (THI = 72 to 79 units; **Figures 2a, 2b**). When the THI increased above 79 units, the severe heat stress appeared to affect the ability of serum AMH to predict the ovarian traits FOL and CL (**Figures 2c, 2d**).

Table 2. Pearson correlations between serum Anti-Müllerian hormone (AMH) with ovarian and physiological traits in Holstein cows according to climatic experimental groups (HS = heat stress).

Variable	Moderate HS (*p*-value)	Severe HS (*p*-value)
FOL (mm)	0.7508 (<0.0001)	0.2567 (0.0837)
CL (cm)	0.7188 (<0.0001)	0.1160 (0.2155)
RT (°C)	0.3427 (0.0142)	0.0958 (0.2671)
RR (breaths/min)	0.2135 (0.1938)	0.0634 (0.5728)

FOL = Follicle diameter; CL = Corpus Luteum diameter; RT = Rectal temperature; RR = Respiratory rate.
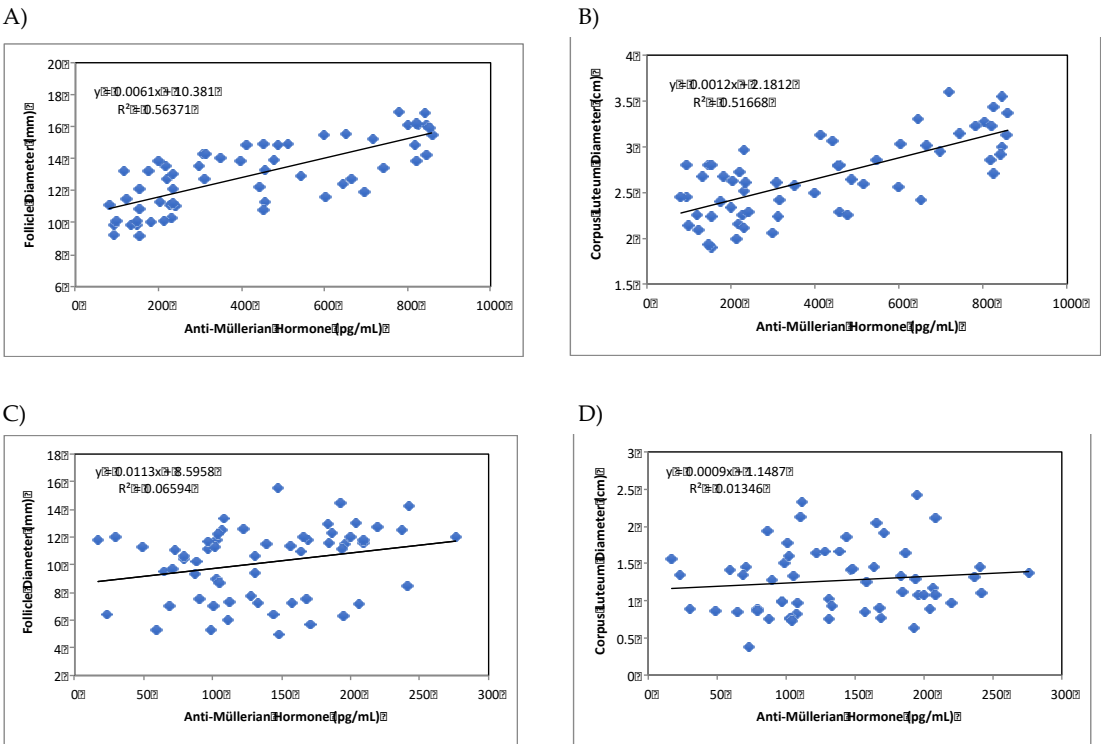

Figure 2. Regression relationships between Anti-Müllerian hormone (AMH) and ovarian variables according to heat-stressed group. A) Follicle diameter (moderate HS), B) Corpus Luteum diameter (moderate HS), C) Follicle diameter (severe HS), D) Corpus Luteum diameter (severe HS).

3.1. Genome-wide association study

After applying quality control criteria, 45,832 SNPs were interpreted useful and were used in GWAS analysis. Then, the single-locus mixed model identified 81 genome- wide SNPs associated with serum concentrations of AMH (p < 0.00001). However, only 16 of these SNPs surpassed multiple testing correction because they were significant at lower p-value than the Bonferroni adjusted threshold (i.e., 1.09×10^{-6}; **Figure 3**). From these 16 SNPs, nine were located within genes (i.e., intronic), whereas the remaining seven SNPs showed an intergenic position as observed in **Table 3**.
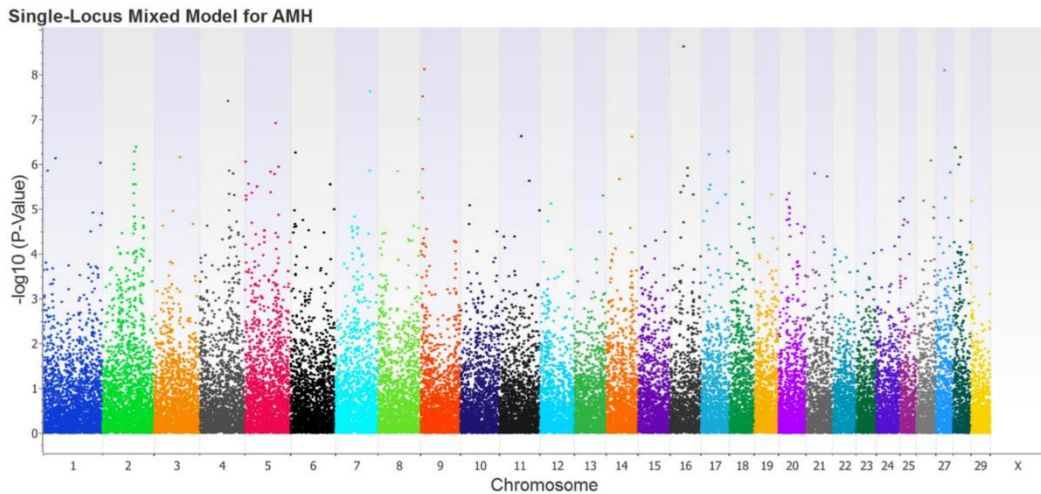
Figure 3. Manhattan plot from single-marker GWAS for Anti-Mülleriam hormone (AMH) serum concentrations in Holstein cows managed during summer in a semi-desert region.

Table 3. Significant SNPs ($p < 1.09 \times 10^{-6}$) from a single-marker genome-wide association study (GWAS) with Anti-Müllerian hormone (AMH) in Holstein cows managed in a warm environment.

SNP ID [1]	Variant [2]	BTA [3]	Position [4]	Gene [5]	Alleles [6]	Var [7]	*p*-Value [8]
rs41807005	Intergenic	16	34′845050	--------	T/C	0.162	2.35×10^{-9}
rs109740021	Intergenic	9	9′329501	--------	A/G	0.157	7.53×10^{-9}
rs42416336	Intronic	27	23′354986	*LONRF1*	A/C	0.152	8.16×10^{-9}
rs876084180	Intronic	7	21′401999	*AMH*	A/C	0.143	2.38×10^{-8}
rs136263395	Intergenic	9	4′423733	--------	T/C	0.141	3.11×10^{-8}
rs445674221	Intronic	4	76′133069	*IGFBP1*	A/G	0.139	3.85×10^{-8}
rs8193046	Intronic	8	108′833985	*TLR4*	T/C	0.131	9.86×10^{-8}
rs42849475	Intronic	5	1′087211	*LGR5*	A/G	0.129	1.20×10^{-7}
rs42338999	Intergenic	11	58′684365	--------	T/C	0.124	2.38×10^{-7}
rs135450328	Intergenic	14	68′794329	--------	A/C	0.123	2.46×10^{-7}
rs478504266	Intronic	2	89′091847	*SGO2*	G/A	0.119	4.15×10^{-7}
rs137194049	Intronic	28	4′632166	*DISC1*	T/C	0.118	4.24×10^{-7}
rs135441773	Intronic	2	84′744596	*SLC39A10*	T/G	0.117	5.22×10^{-7}
rs110893810	Intronic	17	73′804897	*RSPH14*	T/C	0.116	5.24×10^{-7}
rs136745124	Intergenic	6	12′650752	--------	A/G	0.115	5.49×10^{-7}
rs43475092	Intergenic	3	68′078177	--------	T/C	0.114	7.04×10^{-7}

[1] SNP reference of the NCBI; [2] SNP chromosome variant; [3] *Bos taurus* autosomal chromosome number; [4] SNP position within the chromosome; [5] candidate gene symbol (*LONRF1* = LON peptidase N-terminal domain and ring finger 1; *AMH* = Anti-mullerian hormone; *IGFBP1* = Insulin-like growth factor binding protein 1; *TLR4* = toll-like receptor 4; LGR5 = Leucine rich repeat containing G protein-coupled receptor 5; *SGO2* = Shugoshin 2; *DISC1* = DISC1 scaffold protein; *SLC39A10* = Solute carrier family 39 member 10; *RSPH14* = Radial spoke head 14 homolog). [6] alleles from the SNP; [7] percentage of trait variance explained by the SNP; [8] SNP statistical significance.

3.2. SNPs selection

From the 16 genome-wide SNPs significantly associated with AMH serum levels, the 9 intragenic SNPs were selected for use in a validation study because they are located within genes. However, only 4 intragenic SNPs did not deviate from the Hardy–Weinberg equilibrium (HWE, $X^2 > 0.05$) and met the criteria for minor allele frequency higher than 10% (MAF > 0.10). These 4 SNPs were rs876084180, rs445674221, rs42849475 and rs8193046, which were in the genes *AMH, IGFBP1, LGR5* and *TLR4* (**Table 4**). Therefore, only these 4 SNPs were further tested for validation as molecular markers through a genotype to phenotype association study.

Table 4. Identification, gene name, favorable SNP allele, allele frequencies, and Hardy–Weinberg equilibrium analysis for genomic SNPs associated with serum AMH concentrations.

SNP ID [1]	Gene [2]	F. Allele [3]	Allele Frequency [4]		HWE Test [5]	HWE p-Value [6]
			A	C		
rs876084180	*AMH*	T	0.35	0.65	0.32	0.46
			A	G		
rs445674221	*IGFBP1*	T	0.53	0.47	2.63	0.18
rs42849475	*LGR5*	T	0.24	0.76	1.25	0.31
rs8193046	*TLR4*	T	0.39	0.61	0.86	0.39

[1] SNP reference of the NCBI; [2] Gene symbol name; [3] F. Allele = allele with the favorable effect on phenotype; [4] Frequency of both alleles within cow population; [5] Hardy–Weinberg equilibrium "χ^2" test value; [6] "χ^2" test p-value with 1 degree of freedom and $\alpha = 0.05$.

3.3. SNP markers validation

The least square means for AMH and reproductive traits according to SNP genotypes are reported in **Table 5**. The SNP rs876084180 was associated with serum AMH, FOL and CL ($p < 0.01$), whereas the SNP rs42849475 was associated with ovarian traits and CR ($p < 0.05$). Interestingly, the SNPs rs445674221 and rs8193046 were predictors of serum AMH ($p < 0.0001$) and all reproductive traits ($p < 0.01$). The SNPs rs445674221 and rs8193046 were also associated with the physiological traits RT and RR ($p < 0.01$), whereas the SNPs rs876084180 and rs42849475 were associated only with RT ($p < 0.05$) as observed in **Table 6**.

From the SNPs rs876084180, rs445674221, rs42849475 and rs8193046, the most favorable genotypes were AA, GG, GG and AA, respectively, because they were associated with a better reproductive and physiological response. According to the allele substitution effects, the SNP rs876084180 had the highest allele contribution ($p < 0.0001$) on serum AMH levels. However, the SNPs rs445674221 and rs8193046 were the highest contributors for the reproductive traits FOL, CL and CR ($p < 0.1$). Interestingly, these two SNPs were also the most beneficial contributors for the physiological traits RT and RR ($p < 0.01$), as they were associated with a slight reduction in both variables.

A significant additive fixed effect was confirmed for the SNPs rs876084180, rs445674221, rs42849475 and rs8193046, suggesting that the sum of their individual effects was equal to their combined allele effects.

Table 5. Least-square means ± SE according to SNP's marker genotypes for serum AMH and reproductive traits in Holstein cattle from a validation population.

SNP ID [1]	Trait [2]	Least-Square Means by Genotype ± SE [3]			p-Value [4]	*AlleleSE*	*AdditiveFE*
		AA	AC	CC			
rs876084180	AMH	501.26 ± 45.3 [a]	382.49 ± 37.9 [b]	152.94 ± 19.2 [c]	<0.0001	165.12	174.16*
	FOL	13.01 ± 1.06 [a]	11.95 ± 1.04 [b]	10.17 ± 0.98 [c]	0.0010	1.37	1.42*
	CL	2.53 ± 0.17 [a]	1.92 ± 0.16 [ab]	1.47 ± 0.19 [b]	0.0086	0.50	0.53*
	CR	45.20 ± 3.67 [a]	36.75 ± 3.32 [a]	40.65 ± 4.01 [a]	0.6519	2.21	2.27
		AA	AG	GG			
rs445674221	AMH	141.80 ± 19.6 [a]	267.39 ± 23.2 [a]	442.65 ± 37.8 [b]	0.0009	146.01	150.42*
	FOL	10.63 ± 0.96 [a]	11.04 ± 1.05 [a]	13.91 ± 1.12 [b]	0.0068	1.60	1.64*
	CL	1.24 ± 0.09 [a]	1.82 ± 0.07 [b]	2.89 ± 1.18 [c]	0.0087	0.72	0.77*
	CR	34.50 ± 0.47 [a]	45.15 ± 0.46 [b]	56.25 ± 0.42 [c]	<0.0001	10.55	10.87*
rs42849475	AMH	291.55 ± 26.7 [a]	342.16 ± 37.5 [a]	401.29 ± 39.2 [a]	0.1506	52.76	54.87*
	FOL	9.86 ± 0.87 [a]	11.16 ± 1.09 [b]	12.64 ± 1.26 [b]	0.0079	1.34	1.39*
	CL	1.38 ± 0.07 [a]	1.92 ± 1.05 [b]	2.24 ± 1.90 [b]	0.0096	0.38	0.43*
	CR	39.70 ± 3.07 [a]	46.20 ± 3.59 [b]	50.25 ± 4.36 [b]	0.0188	4.91	5.27*
rs8193046	AMH	474.67 ± 38.7 [a]	379.23 ± 37.3 [b]	158.34 ± 14.2 [c]	<0.0001	151.78	158.16*
	FOL	13.28 ± 1.29 [a]	11.37 ± 0.94 [b]	10.14 ± 0.94 [c]	0.0065	1.52	1.57*
	CL	2.71 ± 2.02 [a]	1.86 ± 1.45 [b]	1.20 ± 1.08 [c]	0.0012	0.67	0.75*
	CR	54.15 ± 3.67 [a]	45.70 ± 3.29 [a]	36.75 ± 3.18 [b]	0.0034	8.45	8.70*

[1] SNP reference of the NCBI; [2] phenotypic traits (AMH = serum Anti-Mullerian hormone, ng/ml; FOL = Follicular diameter, mm; CL = Corpus Luteum diameter, cm; CR = Conception rate, %); [3] least-square means according to SNP genotype ± SE ([a,b,c] indicate statistical difference among genotypes at); [4] *p*-value = statistical significance; [5] Allele substitution effect; [4] Additive fixed estimated effect.

Table 6. Least-square means ± SE according to SNP markers genotypes for physiological traits in Holstein cattle from a validation population.

SNP ID [1]	Trait [2]	Least-Square Means by Genotype ± SE [3]			*p*-Value [4]	*Allele SE*	*Additive FE*
		AA	AC	CC			
rs876084180	RT	38.14 ± 2.98 [a]	38.25 ± 2.67 [a]	38.79 ± 3.39 [b]	0.0316	-0.28	0.32*
	RR	63.67 ± 5.22 [a]	65.44 ± 5.19 [a]	66.35 ± 5.19 [a]	0.0928	-1.63	1.69*
		AA	AG	GG			
rs445674221	RT	38.89 ± 3.02 [a]	38.46 ± 2.96 [b]	38.15 ± 2.77 [c]	0.0094	-0.34	0.37*
	RR	68.32 ± 4.98 [a]	65.01 ± 4.67 [b]	61.96 ± 4.12 [c]	0.0067	-3.06	3.18*
rs42849475	RT	38.81 ± 3.54 [a]	38.39 ± 2.69 [b]	38.26 ± 3.82 [b]	0.0235	-0.24	0.27*
	RR	65.79 ± 4.28 [a]	64.03 ± 5.02 [a]	62.99 ± 4.67 [a]	0.2138	-1.35	1.40*
rs8193046	RT	38.16 ± 2.65 [a]	38.49 ± 3.08 [b]	38.93 ± 3.19 [c]	0.0076	-0.35	0.38*
	RR	61.24 ± 5.09 [a]	64.96 ± 5.16 [b]	68.95 ± 4.87 [c]	0.0052	-3.69	3.85*

[1] SNP reference of the NCBI; [2] phenotypic traits (RT = rectal temperature, °C; RR = respiration rate, breaths/min); [3] least-square means according to SNP genotype ± SE ([a,b,c] indicate statistical difference among genotypes); [4] *p*-value = statistical significance; [5] Allele substitution effect; [4] Additive fixed estimated effect.

3.4. SNP effects on reproductive phenotypes

A significant improvement in serum AMH and reproductive traits ($p < 0.05$) was observed as the number of favorable genotypes of the four genome-wide significant SNP markers increased from 0 to 4. Serum AMH differed in cows having 0 to 1, 2 to 3 and 4 favorable genotypes; FOL differed among all favorable genotype categories; CL differed between 0, 1, 2 to 3 and 4 favorable genotypes; and CR differed in cows having 0, 1 to 3 and 4 favorable genotypes (**Figures 4a, 4b, 4c, 4d**).
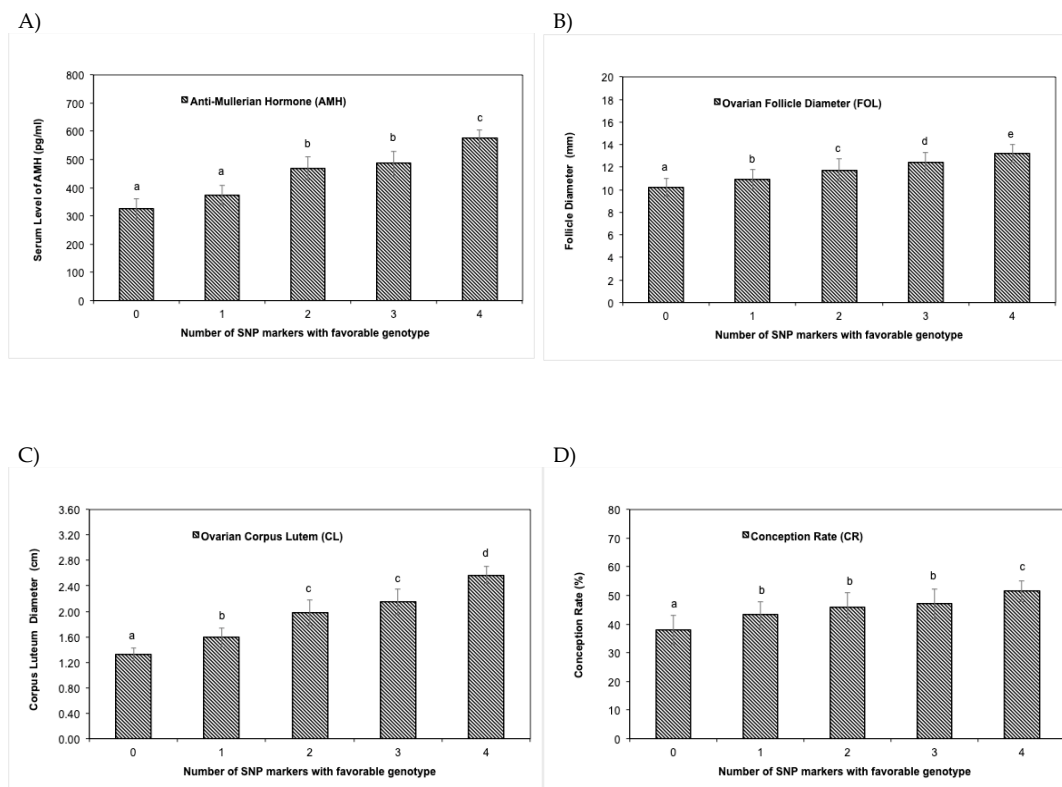
Figure 4. Average values for serum AMH concentrations and reproductive traits (± SE) during the expeimental period in Holstein cows according to the number of favorable genotype SNP markers (i.e., 0, 1, 2, 3 or 4). A) Serum AMH concentrations (pg/ml); B) Follicle diameter (mm); C) Corpus Luteum diameter (cm); D) Conception rate (%).

4. Discussion

Anti-Müllerian hormone (AMH) has been suggested as potential biomarker associated with ovarian function and fertility traits in dairy cows [13,31]. AMH has been positively associated with the total number of healthy oocytes and follicles in the cow, and this relationship is influenced by climate [17]. An increase in temperature and humidity lead to heat stress (HS) in lactating dairy cows, which affect oocyte quality and development, follicular growth and embryo viability [32,33]. Severe heat-stressed conditions appear to compromise the predictive ability of AMH as physiological marker. However, the reliability of AMH as molecular marker appears to be unaffected by adverse environmental conditions. In the current study, we detected that serum AMH appeared to be an endocrine marker for summer reproductive response in Holstein cows, but only when HS did not exceed a moderate threshold. Furthermore, we identified four SNPs associated with serum AMH as molecular markers for reproductive traits, which also appeared to be predictors of physiological traits indicative of HS tolerance.

In northwestern Mexico, the Yaqui Valley experiences extremely hot weather conditions characterized by an average THI that varies from 75.7 units in late spring to 82.2 units at the end of summer. These THI values indicate heat-stressed climatic conditions progressing from moderate to severe during summer, and creating a unique hot weather favorable to study heat stress influence on physiological and molecular markers in dairy cattle [18,34]. Therefore, further studies are proposed to elucidate the ability of circulating AMH concentrations to predict fertility in Holstein dairy cows exposed to HS. In addition, the study of AMH polymorphisms as predictors for reproductive performance and thermotolerance, appears to be a viable strategy for genetic improvement of Holstein cows managed under warm environments.

Endocrine and molecular technologies have been proposed to identify practical and reliable markers for fertility in cattle. The circulating AMH concentration is proposed as potential candidate marker due to this trait having high variability, repeatability and heritability [35–37]. In the current study, we first tested serum AMH as endocrine marker in Holstein cows managed from late spring until the end of the summer. Serum AMH collected at the beginning of a reproductive management program was associated with higher FOL, CL and CR, and resulted as predictor for ovarian response ($p < 0.05$) under moderate HS. However, this predictive ability was not detected in Holstein cows when HS progressed from moderate to severe during summer.

We observed a significant reduction in average serum AMH in Holstein cows after HS increased from moderate to severe (417.26 ± 4.51 to 136.94 ± 4.03 pg/mL). According to Gobikrushanth et al. [15] AMH concentrations in dairy cows are classified as low (≤140 pg/mL), intermediate (>140 to ≤450 pg/mL) or high (>450 pg/mL). In our study, serum AMH dropped from intermediate to low levels as the summer season progressed. A significant decrease in follicular growth, ovulation rate and embryo quality was observed in cows averaging serum AMH below 400 pg/ml [17]. In another study, cows with high and intermediate plasma AMH concentrations were 1.42 and 1.51 times more likely to be associated with pregnant than cows with low plasma AMH concentrations within 84 days following the start of the mating season [38].

High temperatures and humidity in cattle affect ovarian hormone concentrations (i.e., AMH, FSH, LH, etc.), uterine environment, and follicular development, which compromises oocyte competence and early embryonic development [39–41]. Fertilization process and embryonic development were disrupted by heat stress leading to a 30-35% decrease in conception rates in dairy cows [6]. Reductions in hematocrit and modifications in water balance during heat stress may attribute to the decline in hormone concentrations within peripheral blood [42].

After executing GWAS, we detected 16 SNPs associated with serum AMH that were significant at the Bonferroni adjusted *p*-value. Only 9 of these SNPs were selected as candidate marker because

they were located within a gene. From these SNPs, 4 of them surpassed the quality control criteria and were tested through a genotype to phenotype association study. Such SNPs were rs876084180, rs445674221, rs42849475 and rs8193046, which were within the genes *AMH, IGFBP1, LRG5* and *TLR4*. These SNP were then validated as molecular markers for fertility traits and thermotolerance in Holstein cows subjected to a reproductive management program during the summer.

Nawaz et al. [13] reported candidate genome-wide SNPs on BTA11 and BTA20 associated with AMH concentrations and located in the genes *DENND1A, NR5A1, NR6A1, PTGS1, NDUFA8* and *FST*, which have plausible roles on fertility and AMH regulation. The *DENND1A* gene was also described as a candidate gene associated with total number of collected and viable embryos in superovulated Canadian Holstein cows [43]. Gobikrushanth et al. [15] reported genome-wide SNPs associated with variation in serum AMH in Holstein cows, one within the *AMH* gene (BTA7) and four in the genes *SCAI* and *PPP6C* (BTA11), and *FGF18* and *EEF2K* (BTA20). A similar GWAS study for AMH concentration in cows from several dairy breeds detected a single quantitative traits locus (QTL) on BTA11. This QTL included candidate genes linked to fertility-related phenotypes, some of them previously reported (*NR5A1, HSPA5, CRB2, DENND1A* and *NDUFA8*) [38].

The four genome-wide SNPs validated in the current study as markers associated with fertility traits and thermotolerance in Holstein cows are located in the genes *AMH, IGFBP1, LRG5* and *TLR4*. The SNPs rs876084180 is located within the *AMH* (Anti-Müllerian hormone) gene. AMH is a dimeric glycoprotein synthesized by granulosa cells within growing preantral and antral follicles [44]. This protein is considered a potential marker of the ovarian follicular reserve and healthy follicular population in cows [31,35], because it is highly variable, repeatable and heritable [35–37].

In our study, results suggested that the SNPs rs876084180 in the gene *AMH* was a predictor for reproductive traits (i.e., FOL, CL and CR), because AMH regulates follicular growth and oocyte competence, as well as fertilization and embryo development. Such reproductive functions of AMH appear to be due to its close relationship with the FSH signaling pathway, which regulate and promote ovarian follicle development [45]. In addition, the ability of AMH to predict RT is because the direct participation of this protein within TGFβ pathway, which is involved in architecture remodeling of cells exposed to heat shock [46].

The SNP rs445674221 is within the gene *IGFBP1*. This gene encodes a protein primarily produced in hepatocytes and renal cells, which is a key modulator of the IGF1. The IGFBP1 is released into the bloodstream by the liver and binds to IGF1 more strongly than its receptor IGFR1 to control the bioavailability and activity of IGF1 [29]. The IGF1 peptide is essential in the regulation of reproductive functions by acting synergistically with gonadotropins [47]. This peptide plays an important role in oocyte development, follicular growth, oocyte maturation, ovulation, luteal function, and also prevent cellular apoptosis and enhance proliferation in the ovary [48–50].

Under heat stress, IGFBP1 transports IGF1 to the membrane of the oocyte allowing the interaction with its receptor (IGF1R), which induces activation of PI3K/AKT signal pathway [51]. Then, AKT inactivates caspases (CASP-3 and CASP-9) [52], and regulates the levels of the BCL-2 family proteins, which reduce cellular alterations caused by heat stress (i.e., increase in ROS and H_2O_2) [53]. As per activation of the receptor by the IGF1 ligand, the functionality of the mitochondrial membrane is stabilized, the continuation of apoptosis in the oocyte is deferred, and the cell survival pathways are activated [54,55]. Addition of IGF1 (12.5 to 25 ng/ml) to oocytes cultured at 41 °C tended to minimize the reduction in both mitochondrial activity and developmental competence, two processes affected by heat stress [56,57].

The SNP rs42849475 is within the gen *LGR5*. This gene is expressed in the epithelial surface of the ovarian stem cells [58], as well as in the surface of the Corpus Luteum [59]. It could explain the association of *LGR5* gene with the diameter of FOL and CL in the current study. Similarly, this gene is also expressed in the uterine epithelia, where appears to collaborate in uterine functions needed to support implantation and pregnancy establishment [60]. It seems to explain the relationship between *LGR5* gene and CR.

LGR5 is a potential target of the Wnt signaling pathway [61], which promotes cell proliferation and cell survival during heat stress. The proliferative mechanism has been involved in the growth of

the mammary gland in dairy cows [62]. This pathway is also associated with energy metabolism, a critical function required to maintain a thermal balance within the body once the animal is exposed to heat stress [63]. It appeared to support effects of *LGR5* gene on thermotolerance-related traits.

The SNP rs8193046 is in the gene *TLR4*. This gene is a crucial mediator of the inflammation-like response in the uterus triggering the production of regulatory T (Treg) cells, which are required to facilitate and promote embryo implantation [64]. Treg cells suppress anti-fetal effector responses and support the process of uterine vascular remodeling, facilitating strong placental development that supports growth and survival of the fetus [65,66]. It provides evidence of the mechanisms through that *TLR4* is helping to improve conception rate as observed in the current study.

As part of the immune response against pathogens and external stressors, *TLR4* induces the expression of pro-inflammatory cytokines [67], which are regulated by nuclear factor kappa-B (NF-κB) expression [68,69]. NF-κB is an important intracellular signaling protein that has a critical role in preventing heat stress-induced early apoptosis, and instead promotes growth and survival in cells exposed to heat-stressed conditions [70]. *TLR4* has been associated with a reduction in both lypolitic action and adypocite mobilization in Holstein cows exposed to heat stress as a body response to minimize heat load [71,72]. It appeared to explain the associative relationship between *TLR4* gene and thermotolerance traits observed in the current study.

The allele substitution effects detected in the current study supported the concept of the favorable contribution of the genes *AMH, IGFBP1, LGR5* and *TLR4* to reproductive and physiological traits evaluated in the current study. This positive effect of the four validated genes was confirmed by the improvement in serum AMH, FOL diameter, CL diameter and conception rate as the number of SNPs with favorable genotypes increased from 0 to 4. A previous report also involving GWAS and a validation study identified 3 genes (i.e., *TLR4, GRM8* and SMAD3) as favorable markers for milk yield and thermotolerance in Holstein cows managed in a heat stressed environment [18].

The identification of genetic makers associated with variations in circulating AMH concentrations in Holstein cows exposed to HS would be potentially helpful to identify and select future elite genetic merit cows with greater potential to respond successfully in summer reproductive programs [15]. The discovery and validation of novel molecular markers associated with serum AMH appeared to be a promissory strategy to identify dairy cows with superior fertility during summer. Selection of these cows within a genetic improvement program could lead to faster genetic gain in reproductive efficiency in dairy production systems within in semiarid regions [13,31]. As stated by Hansen et al. [73], selecting for genetic thermotolerance makes possible to reduce the impact of HS on reproduction. Genomic studies in Australian Holstein cows reported higher breeding values for fertility in cows that were genetically more thermotolerant [74].

A limitation of the current study is the low number of cows included in the GWAS analysis, which combined with the multiple-testing statistical adjustment may reduce the number of significant SNPs detected [75]. In addition, due to the polygenic effect of AMH a reduced variation explained by the markers is expected. Fertility is a highly polygenic trait that is influenced by thousands of SNP effects [15,76], and the candidate genes we detected appeared to be a small number of the many that slightly contribute to phenotypic variance of serum AMH concentrations. Interestingly, our validation study in an independent Holstein population confirmed the ability of these genes as predictors for fertility and thermotolerance traits. Because the likelihood of an SNP being significant in two different populations is low, validating the effects of SNPs in independent animal populations seems to be the most reliable method for determining the significance of candidate SNPs [77,78].

5. Conclusions

Identification of physiological and genetic markers for fertility in Holstein cows exposed to warm climate conditions during summer could be a favorable strategy for dairy producers. Anti-Müllerian hormone (AMH) has been recognized as potential blood marker associated with reproductive performance in Holstein cows; however, there is limited information on its effectiveness as a marker under extreme climatic conditions of heat. In the current study, we provided evidence

that serum AMH concentration was an endocrine biomarker for summer reproductive response but only when heat stress did not exceed a moderate threshold. Interestingly, molecular analysis revealed four SNPs associated with serum AMH concentrations as genetic markers to improve fertility traits and thermotolerance in dairy cows managed under severe summer weather conditions. However, further GWAS analyses including extensive dairy populations are recommended in order to identify and validate more candidates SNPs associated with serum AMH. Also, additional studies using these markers in the processes to estimate breeding values for fertility in Holstein cows exposed to different heat stress intensities could be helpful in dairy cattle genetic improvement programs.

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