Genotypes Within the Prolactin and Growth Hormone Insulin-Like Growth Factor-I Pathways Associated with Milk Production in Heat Stressed Holstein Cattle

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ABSTRACT. The association of single nucleotide polymorphisms (SNPs) within genes of the prolactin (PRL), growth hormone (GH) and insulin-like growth factor-I (IGF1) pathways with 305-day milk yield (MY305) were evaluated in heat-stressed Holstein cows. Ambient temperature and relative humidity were used to calculate a temperature-humidity index (THI), which revealed that heat stress conditions existed in the Yaqui Valley, Sonora, México from May through October of 2011 and 2012. Cows (n = 573) were genotyped for 179 tag SNPs within 43 candidate genes in the PRL and GH/IGF1 pathways. Seven SNPs within *7 genes (AVPR1A, Furin, IGFBP5, IGFBP6, PMCH, PRLR and STAT5B)* were found to be associated with MY305 ($P < 0.05$); therefore, their effects were used to estimate a molecular breeding value (MBV). The correlation between the

MBV and MY305 was 0.21 ($P < 0.001$) and the adjusted coefficient of determination (R^2) was 4%, whereas correlation between MBV and the estimated breeding value for MY305 was 0.25 ($P < 0.001$) with a R^2 of 6%. Heritability estimate for MY305 was 0.41 ± 0.12. The MBV estimated was positive, but weakly associated with MY305. The small amount of additive and phenotypic variation explained by the MBV was most likely due to the few number of SNPs and the complexity of the trait, particularly under extreme weather conditions. In conclusion, SNPs within the PRL and GH/IGF1 pathways were associated with MY305; however, the MBV estimated with these SNPs was not yet suitable to use in genetic selection procedures due to the small amount of variation explained for MY305.

KEY WORDS: Prolactin; Heat Stress; Milk yield; SNPs; MBV.

INTRODUCTION

One of the major challenges for dairy cattle in tropical, subtropical and semi-arid regions is heat stress. Environmental conditions such as high ambient temperatures, high relative humidity and prolonged periods of solar radiation, compromises the ability of dairy cows to dissipate heat. This condition causes behavioral changes such as increased water consumption and decreased feed intake, which in turn, results in losses in reproductive efficiency and milk production (Accorsi et al., 2002; West, 2003; Bernabucci et al., 2010). Persistent exposure to heat stress leads cows to an altered physiological state known as acclimation, which is a process largely controlled by the endocrine system that involves changes in plasma concentration of several hormones and somatomedins like prolactin (PRL), growth hormone (GH), insulin-like growth factor-I (IGF1), thyroid hormones, glucocorticoids, and mineral corticoids (Collier et al., 2008; Bernabucci et al., 2010). The PRL hormone plays a key role in lactogenesis in ruminants and some reports indicate that the synthesis and secretion of PRL by the anterior pituitary gland is sensitive to changes in ambient temperature, specifically higher levels of this hormone are observed during summer due to its influence in regulation of body fluids and its relationship with seasonal hair growth (Alamer, 2011). In addition to PRL, other important biological molecules altered during heat stress are GH and IGF1. These hormones are members of the somatotropic axis which regulate metabolism and physiology of mammalian growth, however, they are affected by the negative energy balance derived from the reduction of feed intake in fresh cows that is exacerbated by heat stress (De Rensis and Scaramuzzi, 2003; Bonakdar et al., 2010).

The genetic development of heat tolerant dairy cattle is complex; however, with advancements of molecular and genomic technologies it is posible to identify DNA markers (SNPs) within candidate genes that underlie quantitative trait loci (QTL) that contribute to the favorable phenotypic variations of animals that have fitness in heat stress conditions (Bonakdar et al., 2010; Shaji et al., 2015). The hypothesis of this study, was that a molecular breeding value (MBV) constructed with DNA markers within genes of the PRL and GH/IGF1 pathways may have the potential to predict 305-day milk yield in heat stressed Holstein dairy cows. To test this hypothesis, 179 tag SNP were identified in 43 genes within these pathways. The objectives of the study were to estimate an MBV using these SNPs and evaluate their association with 305-day milk yield in heat-stressed Holstein cows. Another objective was to estimate the amount of additive and phenotypic variability explained by the MBV for 305-day milk yield in heat-stressed lactating Holstein cows on 3 dairy farms in Sonora, México.

MATERIALS AND METHODS

Data

Data were collected from 573 Holstein dairy cows. Observations were collected during 2011 and 2012 from three dairy farms located in Blocks 910 and 1114 of the Yaqui Valley, Sonora, México (27°21'N 109°54W). Each farm contributed the following number of animals for the study: farm 1 (n = 252), farm 2 (n = 89), and farm 3 (n = 232). Cows had body condition scores ranging from 2.5 to 3.5 (1 = very thin, 5 = very fat) (Kadarmideen and Wegmann, 2003). Test-day milk yield records were used to calculate an adjusted 305-day milk yield (MY305) per cow in kg. The MY305 was calculated by multiplying milk production levels by an adjustment factor that depended on days in milk and the age of the cow. These factors were obtained from the Dairy Herd Improvement Association (McDaniel et al., 1965; Norman et al., 1973).

Management and health status

As part of a routine management of all farms during summer, cows were subjected to showers with cooling systems as was described for the control group in the study of Leyva-Corona et al. (2016) to minimize the detrimental effects of heat stress. Cooling systems had 16 sprinklers (with an approximate water distribution of 15L per cow/cycle) and three ~ 367 watts electrical fans located 2.73 meters above the floor. Cows were maintained in shaded holding pens with free access to water and an average of 8.5 m^2 of shade per cow. In farms 1 and 3, each holding pen contained approximately 50 cows, meanwhile in farm 2, the holding pens contained 25 to 30 animals. Cows were fed twice a day with a ration consisting of 75% alfalfa hay and 25% corn silage that supplied their nutritional requirements according to guidelines established by the NRC (2001), for lactating Holstein cows with an average weight of 650 kg and producing approximately 30 kg/d of milk with average composition of 3.5% fat and 3.2% protein.

Health records were obtained from the cows of the three dairies and were compiled into one variable known as health status that was recorded categorically and coded as 0 for no disease diagnosis and 1 for any disease diagnosis. Metritis diagnosis was performed with a portable ultrasound system (SonoSite, Inc., Bothell, WA) at day 40 postpartum, while for the diagnosis of subclinical mastitis, the qualitative California mastitis test (Laboratorios Sanfer, S.A. de C.V., Obregón, México) was used. Summary statistics (Table 1) were calculated for each health status group using PROC MEANS of SAS (version 9.4; SAS Institute Inc., USA).

Table 1. Summary statistics per health status group (0 is for no disease diagnosis, 1 for any disease diagnosis) in lactating Holstein cows in Sonora, MX.

Health	Variable	N	Mean	SD	Minimum	Maximum
0	MY305[1], kg	545	6412.22	1392.10	2863.8	10787
	Years of age	544	5.34	1.94	2	13
	Number of lactation	544	3.14	1.85	1	11
	DIM[2]	545	307	47	116	471
1	MY305[1], kg	28	5514.45	1580.72	2314.72	8029.77
	Years of age	28	4.99	1.75	2	10
	Number of lactation	28	2.82	1.59	1	8
	DIM[2]	28	308	75	122	441

[1]MY305 = 305-day milk yield. [2]DIM = Days in milk

Temperature and humidity index (THI)

The THI was calculated and provided by the Instituto Tecnológico de Sonora for the years 2011 and 2012. The climatic records were obtained thru Sonora, México Agro-climatic Station Network, available through www.agroson.org México. This calculation was based on the equation:

$$THI = 0.81(T°C) + RH/100(T°C - 14.4) + 46.4$$

where THI represented the temperature and humidity index, $T°C$ was the temperature in degrees Celsius and RH was the percentage of relative humidity (Hahn, 1999). Index values were calculated for each hour of each day (24 times), and then, were used to calculate monthly averages to plot the trajectory of the THI thru the years of study.

SNP discovery and genotypes

Forty-three candidate genes (see **Table S1**) within the PRL and GH/IGF1 pathways were studied. These genes were selected based on their physiological function, involvement in milk production and documented heat stress responsiveness (Accorsi et al., 2002; Etherton, 2003). For DNA extraction, blood samples (3 ml) were obtained by caudal venipuncture of each cow using sterile syringes. This sample was spotted on nucleic acid cards (GeneSeek, Inc., Lincon, NE). Genotyping was then completed using several multiplex SNP assays and the Sequenom Mass Array platform (GeneSeek, Inc., Lincon, NE). Linkage disequilibrium regions between the genotyped polymorphisms were analyzed using HaploView software (Barrett et al., 2005). A range from 2 to 50 SNPs were found within each gene and yielded a panel of 179 tag SNPs (see **Table S2**).

SNP effects

The associative analysis between genotypes and phenotypes for MY305 were performed using the mixed procedure of SAS. The statistical model was:

$$y = Xb + Za + e$$

where y corresponded to the 305-day milk yield observation of each cow, b was the vector of unknown fixed effects, and a was the vector of unknown random sire effects. Fixed effects included SNP genotype (0, 1 or 2) modelled as a categorical variable, farm, lactation number, health status and calving month; days in milk were included as a linear covariate. X and Z were known incidence matrices relating observations in y to both fixed and random effects, and e was the vector of unknown residual errors. Associations between each SNP and 305-day milk yield were reported based on their significance ($P < 0.05$). Each single SNP effect was estimated using two distinct approaches. In the first approach, the SNP genotype was included as a covariate to determine the allele substitution effect. In the second approach, the SNP genotype was included in the model as a categorical variable and orthogonal contrasts were used to estimate additive effects (Cochran et al., 2013). The false discovery rate (FDR) or Q-value was calculated to control for false positives using PROC MULTTEST (Benjamini and Hochberg, 1995).

Molecular breeding value (MBV)

The MBV was calculated only for the cows that had a genotyping rate of 100% for all the SNPs that showed a significant independent association ($P < 0.05$) with MY305 (n = 538 cows). The MBV estimation involved the summation of the additive genotype effect of each locus and was performed using the Animal Breeder Tool Kit (Golden et al., 1992). Pearson's correlation between 305-day milk yield and the MBV, as well as the EBV for MY305 and the MBV were calculated using PROC CORR in SAS (version 9.4; SAS Institute Inc., USA). A regression-prediction analysis using PROC MIXED was used to estimate a full and a reduced model in order to calculate the amount of phenotypic variation explained by the MBV. The full model was:

$$y = \mu + X_{DIM}\beta_{DIM} + X_{MBV}\beta_{MBV} + X_{Lac.n}\beta_{Lac.n} + X_{H.Stat}\beta_{H.Stat} + X_{Farm}\beta_{Farm} + e$$

where y was the dependent variable of 305-day milk yield, μ was the population mean, X_{DIM} was the linear covariate for days in milk, β_{DIM} was the slope for the variable days in milk, X_{MBV} was the linear covariate for MBV, β_{MBV} was the slope for the variable MBV, $X_{Lac.n}\beta_{Lac.n}, X_{H.Stat}\beta_{H.Stat}, X_{Farm}\beta_{Farm}$ were the incidence matrixes for the categorical variables number of lactations, health status and farm with vectors for fixed effects respectively and e was the vector of residual effect or error term.

The reduced model constructed only with the dependent variable and the MBV was:

$$y = \mu + X_{MBV}\beta_{MBV} + e$$

where y was defined as the dependent variable of 305-day milk yield, μ was the population mean, X_{MBV} was the linear covariate for MBV and β_{MBV} was the slope for the variable MBV, and e was the vector of residual effect or error term.

Estimated Breeding Value and Heritability for 305-day milk yield

A mixed model was implemented using ASREML 3.0 (Gilmour et al., 2009) in order to estimate breeding values (EBV) and the heritability of 305-day milk yield. The general model equation was:

$$y = Xb + Za + e$$

where y was the vector of 305-day milk yield, b was the vector of fixed effects, and a was the vector of random effects which included the individual cow. Fixed effects included farm, lactation number, health status and calving month. The variable days in milk were included as linear covariate. The X and Z were known incidence matrices relating records in y to both fixed and animal random effects in b and a, and e was the vector of random residual effects. The pedigree file included a total of 971 individuals. In order to estimate the amount of additive variance explained by the MBV, a final regression-prediction analysis using PROC MIXED was used. The model was:

$$y = \mu + X_{MBV}\beta_{MBV} + e$$

where y was the EBV for 305-day milk yield obtained from the previous model, μ was the population mean, X_{MBV} was the covariate for **MBV** and β_{MBV} was the slope for the variable MBV, and e was the vector of residual effect or error term. It is important to mention that this model was implemented using only cows that had a valid observation for the MBV.

RESULTS

Temperature humidity index (THI)

Estimated THI values revealed that cows in this study were exposed to heat stress conditions from approximately May until October during both years of the study (Figure 1), varying from light (72-79 units) to moderate stress (80-89 units) according to the classification described by Armstrong (1994).

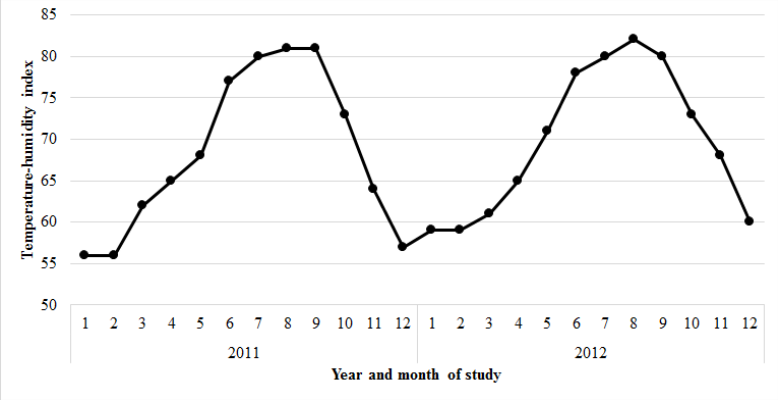

Figure 1: Mean monthly temperature-humidity index from January of 2011 to December of 2012 in the Yaqui Valley, Sonora, MX.

SNP effects

Seven SNPs within 7 genes were associated (P < 0.05) with 305-day milk yield. These genes were the Arginine vasopressin receptor 1A *(AVPR1A)*, *Furin*, Insulin-like growth factor binding proteins 5 and 6 *(IGFBP5, IGFBP6)*, Pro-melanin concentrating hormone *(PMCH)*, Prolactin receptor *(PRLR)* and the Signal Transducer and Activator of Transcription 5B (*STAT5B)*. Table 2 shows the significance level (P-value), chromosomal location and additive effect for each SNP.

Table 2. SNPs within the PRL and GH-IGF1 pathways associated with 305-day milk yield in heat stressed lactating Holstein cows in Sonora, MX.

Gene	Chr[1]	Location (Mb)	SNP	*P*	FDR[2]	Alleles	Additive effect (Kg)
IGFBP5	2	10.5	rs208989155	0.02	0.03	**A** / G	494.97
AVPR1A	5	50.5	rs210011420	0.02	0.03	**T** / C	286.75
IGFBP6	5	27.0	rs211039223	0.02	0.03	**C** / T	332.42
PMCH	5	66.3	rs135033882	0.01	0.03	**A** / T	37.92
STAT5B	19	42.9	rs384930401	0.01	0.03	**G** / A	373.34
PRLR	20	39.1	rs135164815	<0.01	0.03	**A** / G	222.93
FURIN	21	22.2	rs381099643	0.03	0.03	**G** / A	253.11

Favorable alleles are bolded.[1]Chr = chromosome. [2]FDR = false discovery rate.

MBV

The molecular breeding value was calculated for 538 cows that were genotyped for all the SNPs that were found to have a significant association with MY305 (Figure 2). The resulting MBV had an average value of 2575.92 and ranged from 623.45 to 4008.88. Within the full model, all variables included were statistically significant for predicting MY305, this model had an adjusted coefficient of determination (R^2) of 47.2%. The reduced model constructed with our variable of interest (MBV) as an independent variable had an adjusted R^2 of 4%, this value represents the percentage of the phenotypic variance explained by the MBV.

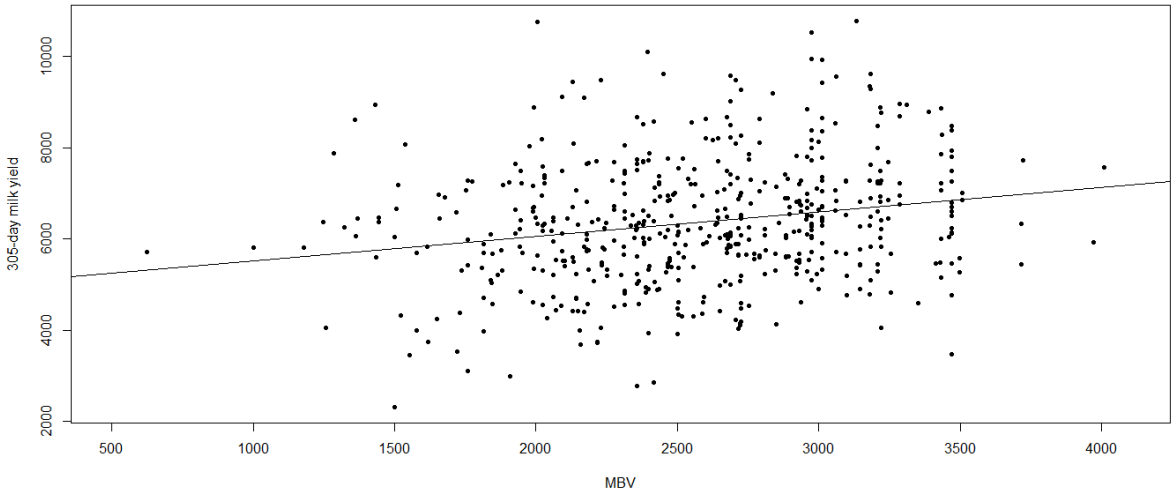

Figure 2. Scatter plot and regression of MY305 versus MBV in lactating Holstein cows in Sonora, MX. Slope = 0.54 kg, Pearson correlation = 0.21.

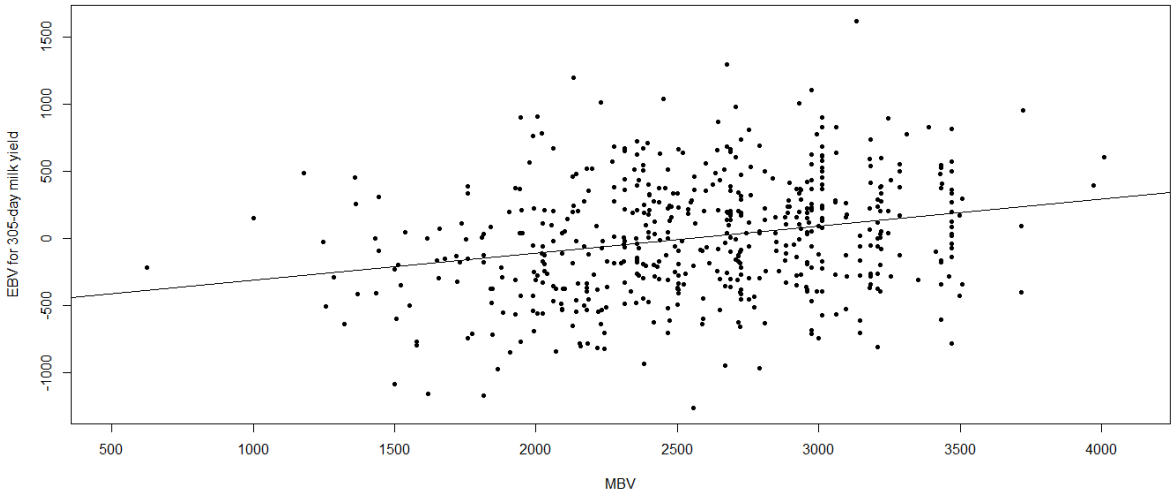

Figure 3. Scatter plot and regression of the EBV for MY305 versus MBV in lactating Holstein cows in Sonora, MX. Slope = 0.20 kg, Pearson correlation = 0.25.

Genetic parameters

Additive genetic and phenotypic variances as well as heritability were estimated for 305-day milk yield. Our study revealed a heritability estimate of 0.41 with a standard error of 0.12 for 305-day milk yield (Table 3). The correlation between the MBV and the MY305 EBV was 0.25 ($P < 0.0001$) while regression of the MY305 EBV on the MBV is shown in Figure 3. The percentage of the additive variance explained by the MBV (represented by the adjusted R^2) was 6%.

Table 3. Genetic parameters for 305-day milk yield in lactating Holstein cows in Sonora, MX. without using the MBV as a fixed effect.

	MY305[1] (kg^2)	SE (kg)
σ^2_a	439980	140380
σ^2_e	621100	124430
σ^2_p	1061080	66803
h^2	0.41	0.12

[1] MY305 = 305-day milk yield

DISCUSSION

Based on the trajectory of the THI thru the years of study, it should be noted that the environmental conditions during the summer in the Yaqui Valley exceeded 72 THI units, which is considered the threshold to initiate heat stress in dairy cattle (Armstrong, 1994). These results agree with other studies conducted in this geographical region of México that described that the THI can be moderate and reach around 85 units during summer (Rivera-Acuña et al., 2015). To help mitigate the effect indicated by the THI on dairy cattle, cooling systems are used in northwest México (Leyva-Corona et al., 2016); however, it has also been reported that even with the use of cooling systems, milk yield decreases by 10 to 15% when ambient temperature causes heat stress (Dunshea et al., 2013). These previous findings have shown the increased need to find new strategies that could allow the improvement of milk production in these climate zones; concomitantly, Collier et al. (2008) described the important role of genetics in the bovine heat stress response. In parallel with this review of heat-stress responsive endocrine pathways, we previously reported suppressed serum concentrations of PRL in Holstein cows being milked in the summer in the Yaqui Valley (Leyva-Corona et al., 2016). Serum levels of IGF1 were also influenced by cooling strategies on these dairy farms. These results were from an intensive endocrine study of heat-stressed Holstein cows; however, for the much larger scale genotype to phenotype association study of the current effort, it was unfeasible to obtain intensive endocrine data for 573 lactating cows.

Our findings revealed a SNP (rs210011420) located at the *AVPR1A* gene associated with 305-day milk yield in heat stressed Holstein cows. To our knowledge, no previous research regarding this genotype to phenotype association exists. The AVPR1A gene is located on bovine chromosome 5 (BTA5) and is involved in regulation of systemic arterial pressure. The previous could be the reason of its involvement in the physiological response to heat stress in cattle, because when cows are subjected to heat stress, the blood flow is shifted to peripheral tissues for cooling (West, 2003). Moreover, the protein AVPR1A is a receptor for the arginine vasopressin hormone (*AVP)*, which is a diuretic protein involved in the secretion and ejection of milk during lactation (Nussey et al., 1987), which could explain its association with 305-day milk yield.

Other genetic variants that were found to be associated with 305-day milk yield were rs208989155 and rs211039223, located within the genes IGFBP5 and IGFBP6, respectively. Their association may be explained by the role of these binding proteins in the somatotropic axis, since the activity of IGF1 (i.e., free vs bound form in circulation) in milk production and cell proliferation during lactation is regulated by the IGFBP protein family (Accorsi et al., 2002). Specifically, it has been reported that IGFBP5 is an important regulator of apoptosis in the mammary gland and that IGFBP6 shows a decrease in its expression during lactation (Accorsi et al., 2002; Fenwick et al., 2008).

This research reported evidence of the association between the SNP (rs381099643) in the *Furin* gene and 305-day milk yield. The association may be explained by *Furin* involvement in the post-translational processing of growth hormone release hormone (*GHRH)* and, indirectly, in the synthesis and secretion of GH (Posner et al., 2004), which could affect nutrient mobilization and cell proliferation during lactation. *Furin* is involved in the activation of precursor proteins through the cleavage of a single or paired basic amino acid residue (Khatib and Sfaxi, 2012; Maruotti et al., 2012). Previous research investigated *Furin* in lactating cows (Cánovas et al., 2010), but an association with milk production traits was not reported.

Previous research has not identified SNP within the *PMCH* gene to be associated with milk production traits. In our study, one SNP (rs135033882) was found to be associated with 305-day milk yield. This association may be explained by the effect of this gene on energy status, which could lead to changes in energy balance in heat-stressed cows. The *PMCH* gene is involved in regulation of energy homeostasis and could be a defense mechanism against energy deficiency (Beerda et al., 2008). It should be noted that energy balance decreases rapidly within the first 100 days in milk in high-yield lactating Holstein cows (Hüttmann et al., 2009).

Another polymorphism associated with 305-day milk yield was rs384930401, which is located in the STAT5B gene. Similar results were provided by He et al. (2011), who indicated that a SNP within the STAT5B gene was significantly associated with the EBV for milk production in Chinese Holstein cattle.

An important candidate gene identified as being associated with 305-day milk yield was *PRLR*. The relevance of *PRLR* is due to its role in milk production and heat stress response (Collier et al., 2008, Lü et al., 2010). The SNP rs135164815 within the *PRLR* gene that was associated with milk yield in our research was located in exon 2, position 39.1 Mb on BTA20. This result is similar to the reported by Zhang et al. (2008), who reported that polymorphisms in exons 3 and 7 of the *PRLR* gene were associated with milk yield in Holstein cattle. In contrast,

other studies found a *PRLR* mutation in exon 10 that introduces a premature stop codon and is considered a candidate for slick hair coat genotype and heat tolerance in Senepol cattle. Specifically, this SNP (39.1 Mb) in the *PRLR* gene was a single-base deletion in exon 10 that introduced a premature stop codon at the protein position 462 (Leu) yielding a loss of 120 C-terminal amino acids from the long isoform of the receptor (Littlejohn et al., 2014).

Previous research identified a "phenotype characterized by development of a very short, sleek hair coat that is inherited as if controlled by a single dominant gene", the slick gene (Olson et al., 2003). In that study, it was reported that Holstein dairy cattle with the slick haplotype exhibit higher milk yields than non-slick contemporaries. This is of particular interest for dairy farming, where most genetic selection has occurred in heat-intolerant *Bos taurus* breeds (Littlejohn et al., 2014). Moreover, *PRLR* is found at the same locus, BTA20, as other DNA markers used to map the slick gene in *Bos taurus* cattle. Within this region, two genes, *PRLR* (38.0 Mb) and sperm flagellar 2 (SPEF2) (38.4 Mb) were found. The *SPEF2* and *PRLR* genes are also involved in reproduction and milk production. Additionally, values of integrated haplotype scores indicated that the region between *PRLR* and *SPEF2* is a target of recent selection (Huson et al., 2014).

Our study revealed a heritability estimate of 0.41 ± 0.12 for 305-day milk yield (Table 3). Other studies reported heritabilities ranging from 0.29 to 0.37 for milk yield (Cohen-Zinder et al., 2005, Raven et al., 2013). Calculation of a moderate heritability confirms the important contribution of genetics to milk yield in Holstein cows. The MBV estimated from SNPs within the PRL and GH/IGF1 pathways genes was weakly associated with 305-day milk yield and with the EBV for 305-day milk yield in Holstein cows from Sonora, México. Although an alternative MBV was also calculated using all available SNPs, this estimate was not adequate, perhaps due to the lack of relationship between most of the variants with MY305. The predictive ability of the MBV for the phenotypic and the additive variances was extremely low, probably due to the few number of SNPs included in its estimation and the complexity of the trait, specially under heat stress conditions.

CONCLUSION

In conclusion, we found association between seven SNPs within the PRL and GH/IGF1 pathways and 305-day milk yield, therefore, the result of this study support our hypothesis that a molecular breeding value (MBV) constructed with DNA markers within the PRL and GH/IGF1 pathways may have the potential to predict 305-day milk yield in heat stressed Holstein dairy cows; however, due to the small amount of additive and phenotypic variability explained by the MBV, its implementation is not yet feasible in genetic selection procedures.

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