Association of lipid-related genes implicated in conceptus elongation with female fertility traits in dairy cattle

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ABSTRACT

Elongation of the preimplantation conceptus is a requirement for pregnancy success in ruminants, and failures in this process are highly associated with subfertility in dairy cattle. Identifying genetic markers that are related to early conceptus development and survival and utilizing these markers in selective breeding can improve the reproductive efficiency of dairy herds. Here, we evaluated the association of 1,679 SNP markers within or close to 183 candidate genes involved in lipid metabolism of the elongating conceptus with different fertility traits in US Holstein cattle. A total of 27,371 bulls with predicted transmitting ability records for daughter pregnancy rate, cow conception rate, and heifer conception rate were used as the discovery population. The associations found in the discovery population were validated using 2 female populations (1,122 heifers and 2,138 lactating cows) each with 4 fertility traits, including success to first insemination, number of services per conception, age at first conception for heifers, or days open for cows. Marker effects were estimated using a linear mixed model with SNP genotype as a linear covariate and a random polygenic effect. After multiple testing correction, 39 SNP flagging 27 candidate genes were associated with at least one fertility trait in the discovery population. Of these 39 markers, 3 SNP were validated in the heifer population and 4 SNP were validated in the cow population. The 3 SNP validated in heifers are located within or near genes *CAT*, *MYOF*, and *RBP4*, and the 4 SNP validated in lactating cows are located within or close to genes *CHKA*, *GNAI1*, and *HMOX2*. These validated genes seem to be relevant for reducing pregnancy losses, and the SNP within these genes are excellent

candidates for inclusion in genomic tests to improve reproductive performance in dairy cattle.

Key words: candidate genes, conceptus development, reproductive traits, validation study

INTRODUCTION

Elongation of the preimplantation conceptus (embryo plus extraembryonic membranes) is essential for pregnancy success in ruminants and is highly associated with pregnancy failures in dairy cattle. For instance, it has been estimated that 39% of viable blastocysts on Day 6 of development fail to elongate and maintain pregnancy by d 28 of gestation (Ribeiro, 2018). Embryonic losses during this period are substantial and cause considerable economic losses, waste of resources, and reduction in the sustainability of dairy cattle production (Ribeiro et al., 2012). Likewise, future improvements in reproductive efficiency in dairy cattle herds will require the development of strategies to minimize those embryonic losses. One option to improve reproduction efficiency is to identify genetic markers associated with early embryonic development and using them in marker-assisted selection.

To prevent embryonic losses using selection, genetic variability should exist. Estimates of heritability for superovulation records (number of structures recovered and number of good quality embryos) ranged from 0.15 to 0.27, for in vitro fertilization data (number of oocytes collected, number of cleaved embryos, number of high- and low-quality embryos, and number of transferrable embryos) varied from 0.01 to 0.21, and for pregnancy success after embryo transfer ranged from 0.02 to 0.03 (Parker Gaddis et al., 2017). According to these heritability estimates, genetic improvement for traits related to embryonic development and survival is possible. It should be noted that none of these traits are officially recorded in dairy cattle breeding programs. On the other hand, some related traits, such as daughter pregnancy rate (**DPR**), cow conception rate (**CCR**), and heifer conception rate (**HCR**), are

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routinely evaluated and included in breeding goals and selection programs. Hence, the question is whether genes relevant to conceptus development are associated with officially published fertility traits in dairy cattle.

To date, genomic regions associated with fertility traits have been found for calving to first service interval (Höglund et al., 2009; Sahana et al., 2010), interval from first service to last service (Höglund et al., 2009), days open (Schulman et al., 2008; Ortega et al., 2017), cow nonreturn rate (Höglund et al., 2009; Olsen et al., 2011), heifer nonreturn rate (Holmberg and Andersson-Eklund, 2006), daughter pregnancy rate (Cole et al., 2011; Cochran et al., 2013; Ortega et al., 2016), sire conception rate (Peñagaricano et al., 2012; Han and Peñagaricano, 2016), and HCR and CCR (Cochran et al., 2013; Ortega et al., 2016). These genomic regions generally explain only a small proportion of the genetic variance underlying reproductive performance.

Recently, Ribeiro et al. (2016b) carried out a transcriptomic analysis from conceptuses collected during the onset of elongation and found that lipid metabolism was one of the top molecular and cellular functions associated with conceptus elongation. Hence, the identification and subsequent use of SNP markers within these genes involved in lipid metabolism might improve reliability of genomic predictions for fertility traits. Therefore, the main goal of this study was to evaluate the association between markers within or around lipid-related genes implicated in conceptus elongation and reproductive performance. Three female fertility traits—DPR, HCR, and CCR—were evaluated in a large population of Holstein bulls. Two female populations, one consisting of heifers and another of lactating cows, were used to validate the associations revealed in the discovery (bull) population. Each female population (validation) had 4 fertility traits, which include success or fail at first insemination (**SF**), number of services per conception (**NSC**), fertility category (**FertC**), and age at first conception (**AFC**) for heifers or days open (**DO**) for cows.

MATERIALS AND METHODS

Candidate Genes

In a previous gene expression study (Ribeiro et al., 2016b), transcervical uterine flushings of single-ovulating lactating dairy cows ($n = 160$) on d 15 after AI resulted in the recovery of preimplantation conceptuses ($n = 65$) in different stages of elongation, ranging from 1 to 60 mm in length. Recovered conceptuses were categorized based on length as ovoid (1–4 mm), tubular (5–19 mm), or filamentous (20–60 mm), and total RNA was extracted from individual conceptuses. Good qual-

ity RNA (integrity number >7.5) from a subsample of conceptuses from each group (ovoid = 8; tubular = 17; and filamentous = 17) was subjected to transcriptomic analysis using the GeneChip Bovine Genome Array (Affymetrix, Santa Clara, CA). In total, 1,611 transcripts were differentially expressed in at least 1 of the 3 pairwise comparisons among experimental groups, which revealed important biological events associated with the onset of elongation of the preimplantation conceptus.

Pathway and functional analyses of the differentially expressed transcripts revealed that lipid metabolism was one of the top molecular and cellular functions associated with conceptus elongation (Ribeiro et al., 2016b). Specifically, 194 genes found to be related directly or indirectly to lipid metabolism and associated with elongation of bovine conceptus were used as candidate genes in this study (Supplementary File S1; <https://doi.org/10.3168/jds.2019-17068>). The details of sampling, nucleic acid isolation, and analysis of transcriptome from conceptuses cells is described in Ribeiro et al. (2016b). The annotation information of candidate genes was obtained from the Ensembl database using the R package “biomaRt” (Durinck et al., 2009).

Discovery Population

To further investigate the results of the microarray study using a genome-wide approach, a total of 27,371 Holstein bulls with PTA records for HCR, CCR, and DPR were used as the discovery population. The trait HCR is defined as the percentage of inseminated heifers that become pregnant at each service (heifer's ability to conceive); CCR is defined as the percentage of inseminated cows that become pregnant at each service (lactating cow's ability to conceive); and DPR is defined as the percentage of nonpregnant cows that become pregnant during each 21-d period (a cow's overall ability to start cycling again, show heat, conceive, and maintain a pregnancy). The Holstein bulls' PTA records and corresponding reliabilities were retrieved from July 2018 US national genetic evaluation from the Council on Dairy Cattle Breeding website (<https://www.uscdcb.com>). The mean reliabilities for DPR, CCR, and HCR were 0.83, 0.82, and 0.74, respectively; all bulls had reliability values >0.70 for the 3 traits. The descriptive statistics of these traits are shown in Table 1.

Validation Populations

Two Holstein female populations consisted of 1,122 heifers and 2,138 lactating cows from a large commercial farm in Florida were used as validation populations

Table 1. Descriptive statistics of fertility traits in the discovery and validation Holstein populations

Population and trait ¹	N	Mean	SD	Minimum	Maximum
Discovery: Bulls					
DPR (DEBV)	27,371	−3.09	20.403	−81.9	80.91
CCR (DEBV)	27,371	−0.31	25.25	−101.5	100.3
HCR (DEBV)	27,371	4.61	19.17	−76.65	82.27
Validation: Heifers					
SF (%)	1,126	55	49	0	1
NSC (no.)	1,122	2.18	1.92	1	12
AFC (d)	1,122	428.38	58.22	358	786
FertC	1,126	1.62	0.76	1	4
Validation: Cows					
SF (%)	2,346	39	49	0	1
NSC (no.)	2,138	2.32	1.69	1	9
DO (d)	2,138	122.49	55.58	47	303
FertC	2,346	1.93	0.92	1	4

¹DPR, daughter pregnancy rate; DEBV, deregressed breeding values; CCR, cow conception rate; HCR, heifer conception rate; SF, success at first insemination; NSC, number of services per conception; AFC, age at first conception; FertC, fertility category; DO, days open.

(calving date: from 2012 to 2017). Information related to reproductive performance (date and success of breedings) was retrieved from the herd management software (PCDART, Dairy Records Management Systems, Raleigh, NC). Pregnancy diagnosis of all cows and heifers was performed by rectal palpation and ultrasonography examination of the uterus 45 ± 3 d after breeding. Four reproductive traits, including SF, NSC, FertC, and AFC (heifers) or DO (cows), were used to validate the findings obtained in the discovery population. The trait SF was coded as SF = 1 if the heifer/cow became pregnant at the first insemination, otherwise SF = 0. The trait DO was computed as the interval between the previous calving date and the date of the breeding that resulted in a positive pregnancy diagnosis. The minimum and maximum of DO after data editing was 47 and 303, respectively. Fertility categorization was based on pregnancy success after breeding as follow: 1 = pregnant at the first breeding; 2 = pregnant at the second or third breeding; 3 = pregnant at fourth or more breeding; and 4 = fail to become pregnant. Pregnancy status for FertC was based on diagnosis at 45 d after breeding.

Genotype Data

Genome-wide data for 312,614 SNP markers were available for the 27,371 Holstein bulls. The SNP data were kindly provided by the Cooperative Dairy DNA Repository (CDDR; Columbia, MO). This set of SNP markers is available in the BovineHD Genotyping BeadChip (Illumina Inc., San Diego, CA; Wiggans et al., 2016). Of the 194 differentially expressed genes involved in lipid metabolism, 183 were marked by 1,708 SNP in the 312k SNP panel. These SNP were

located either within the candidate genes (i.e., genomic sequence between the start of the first exon and the end of the last exon) or at most 10 kb upstream or downstream of the candidate genes. The distance of 10 kb was used to capture proximal regulatory and other functional regions that may lie outside, but close to, the candidate genes. The validation populations were genotyped with 60,671 SNP markers. Of 1,708 candidate SNP, 208 SNP were found in the 60k panel and the remaining 1,500 SNP were imputed using the 312k panel with the software “FImpute” (Sargolzaei et al., 2014). After quality control of candidate SNP for minor allele frequency (exclusion of SNP with minor allele frequency <0.01) in both male and female populations, 1,679 SNP remained for the association study.

Statistical Analysis

Discovery Phase. The association analyses between the 1,679 SNP and the fertility traits were conducted using a mixed linear model. Because a bull's PTA includes both pedigree information and daughter phenotypic performance, there is a risk that SNP would be associated on the basis of parent average rather than own performance. Therefore, deregressed Holstein bull proofs (DEBV) were computed as described in Nayeri et al. (2017). To assess the significance of each SNP, the following linear mixed model was used:

$$\mathbf{y} = 1\mu + \mathbf{Xb} + \mathbf{Zu} + \mathbf{e},$$

where $\mathbf{y}$ is the vector of DEBV, μ is the intercept, $\mathbf{b}$ is the vector of SNP substitution effects, $\mathbf{u}$ is the vector of random polygenic effects, $\mathbf{e}$ is the vector of random residual effects, $\mathbf{X}$ is the genotype matrix relating DEBV

and SNP genotypes coded as 0, 1, and 2 for *BB*, *AB*, and *AA*, respectively, and **Z** is a design matrix relating bulls to observations. The 2 random effects **u** and **e** were distributed as $\mathbf{u} \sim N(0, \mathbf{G}\sigma_a^2)$ and $\mathbf{e} \sim N(0, \mathbf{R}^{-1}\sigma_e^2)$, where σ_a^2 and σ_e^2 are the genomic and residual variances, respectively, **G** is the genomic relationship matrix constructed using roughly 300k SNP markers across the genome, and **R** is a diagonal matrix with its elements representing reliabilities of PTA records. Given that SNP within genes are not fully independent because of linkage disequilibrium, Bonferroni correction was performed based on the number of candidate genes (183) instead of total number of SNP. A *P*-value ≤ 0.0003 ($0.05/183$) was used as threshold for denoting significant associations in the discovery population. These analyses were carried out using the software SNP1101 tool (Sargolzaei, 2014).

Validation Phase. For SF, NSC, AFC, and FertC in heifers, the statistical model included year-season of first insemination and age at first service as fixed effects. Similarly, for SF, NSC, DO, and FertC in cows, the year-season of calving, parity, and clinical disease status in the first 21 d postpartum were considered fixed effects. To control for population stratification, a genomic relationship matrix was constructed using roughly 60k SNP markers across the genome and fed to the model to fit the polygenic effect. All of these association analyses were also performed using the SNP1101 tool. Given the stringent criterion used in the discovery population, a threshold of *P*-value ≤ 0.05 was used in the validation study. Therefore, if a SNP showed a significant association in the discovery population (*P*-value ≤ 0.0003) and showed a significant association (*P*-value ≤ 0.05) with the same effect direction in any of the validation populations, then the candidate gene marked by the SNP was considered to be associated with reproductive performance.

RESULTS

Discovery Population

Thirty-nine SNP located within or close to 27 different genes (*ACSS2*, *APC*, *APP*, *ATP9A*, *B4GALT1*, *BAX*, *CAT*, *CERS4*, *CHKA*, *CUX1*, *EPHX2*, *FNBP1*, *GNAI1*, *GPAM*, *GPAT3*, *HLF*, *HMOX2*, *LPCAT2*, *LPCAT4*, *MYOF*, *NCOR1*, *PEMT*, *PLA2G7*, *RBP4*, *SLC27A6*, *SSFA2*, and *TGM2*) were associated (*P*-value ≤ 0.0003) with at least one fertility trait in the discovery population after multiple testing correction (Figure 1; Supplemental File S1; <https://doi.org/10.3168/jds.2019-17068>). A total of 11 SNPs were associated with DPR, 20 were associated with CCR, and 21 were associated with HCR.

The 11 SNP associated with DPR are within or close to 8 genes: *APP*, *CERS4* (2 SNP), *B4GALT1*, *FNBP1*, *TGM2*, *BAX*, *HMOX2* (2 SNP), and *CHKA* (2 SNP). The 20 SNP significantly associated with CCR are located inside or at most 10 kb apart from 15 genes: *APP*, *GNAI1*, *SLC27A6*, *B4GALT1*, *APC*, *TGM2*, *ATP9A*, *LPCAT2*, *BAX*, *PLA2G7*, *HMOX2* (4 SNP), *CUX1*, *RBP4*, *GPAM*, and *CHKA* (3 SNP). The 21 SNP related to HCR are within or near 16 genes: *SSFA2*, *GPAT3*, *SLC27A6*, *EPHX2*, *LPCAT4*, *ACSS2* (2 SNP), *TGM2*, *CAT*, *LPCAT2*, *HLF* (2 SNP), *NCOR1* (2 SNP), *PEMT*, *HMOX2*, *CUX1*, *MYOF*, and *RBP4* (3 SNP).

Two SNP, rs137538530 and rs137538530, located on BTA13 and BTA25, were associated with DPR, CCR, and HCR. These SNP are located within the *TGM2* and *HMOX2* genes, respectively. Because DPR and CCR are highly correlated traits, of the 11 SNP associated with DPR, 8 were also associated with CCR with the same SNP effect direction as for DPR. Two SNP were associated with both DPR and HCR (rs137538530 and rs137538530), and 5 SNP (rs137538530, rs109674646, rs109164874, rs110852551, rs42672508) were associated with CCR and HCR. In all cases, the allele substitution effects were in the same direction for DPR and either HCR or CCR (Supplemental File S1; <https://doi.org/10.3168/jds.2019-17068>).

Heifer Validation Population

The estimates of genomic heritability in the heifer population for SF, NSC, AFC, and FertC were 0.010 ± 0.007 , 0.030 ± 0.010 , 0.040 ± 0.011 , and 0.020 ± 0.005 , respectively ($\pm$ SEM). The correlations between SNP effects and the number of common SNP among fertility traits between discovery and validation data sets are given in Table 2. The correlation of SNP solutions between fertility traits ranged from -0.05 (between FertC and HCR) to 0.11 (between NSC and DPR). Of the 21 SNP significantly associated with HCR in the discovery population, 3 SNP within or near the *CAT*, *MYOF*, and *RBP4* genes were significantly ($P \leq 0.05$) related to either NSC, SF, or FertC in the heifer population (Table 3). The direction of the SNP substitution effects in the heifer population supported the findings obtained in the bull population; hence, these 3 SNP were considered to be validated (Table 3; Supplemental File S1; <https://doi.org/10.3168/jds.2019-17068>).

Interestingly, the SNP on BTA15 at position 65.8 Mb in *CAT* was significantly associated with all 3 fertility traits in the discovery population and was also related to NSC ($P = 0.04$) and tended to be associated ($P \leq 0.10$) with AFC and FertC (Table 3). The SNP on BTA26 at position 14.8 Mb in *MYOF* was associated

Figure 1. Circular Manhattan plot of the SNP investigated. The 3 circles, from inside to outside, represent daughter pregnancy rate, cow conception rate, and heifer conception rate, respectively. Labels in the outer circle correspond to gene names; x-axis = genomic position of SNP in the genome; y-axis = statistical significance. The red dashed lines indicate the threshold for statistical significance.

Table 2. Number of significant SNP (diagonal; bold), correlations between SNP solutions (above the diagonal), and number of common significant SNP (below diagonal) for reproduction traits in the discovery and validation populations [heifers (H) or cows (C)]

Trait ¹	DPR	CCR	HCR	SF_H	NSC_H	AFC_H	FertC_H	SF_C	NSC_C	DO_C	FertC_C
DPR	11	0.88	0.45	−0.01	0.11	0.07	0.05	−0.06	0.08	0.11	0.08
CCR	8	20	0.57	−0.02	0.09	0.06	0.04	−0.07	0.09	0.12	0.09
HCR	2	5	21	0.04	0.01	−0.02	−0.05	−0.06	0.06	0.08	0.07
SF_H	0	0	2	87	−0.75	−0.82	−0.94	0.36	−0.32	−0.41	−0.40
NSC_H	0	0	1	38	89	0.85	0.84	−0.39	0.29	0.38	0.41
AFC_H	0	0	0	24	50	101	0.92	−0.43	0.43	0.55	0.51
FertC_H	0	1	2	56	45	30	79	−0.36	0.33	0.42	0.41
SF_C	1	3	3	3	5	15	3	113	−0.68	−0.76	−0.91
NSC_C	0	1	2	2	2	17	2	47	88	0.87	0.83
DO_C	0	0	2	3	5	1	2	19	24	57	0.94
FertC_C	0	1	2	3	5	17	2	74	67	22	106

DPR, daughter pregnancy rate; CCR, cow conception rate; HCR, heifer conception rate; SF, success at first insemination; NSC, number of services per conception; AFC, age at first conception; FertC, fertility category; DO, days open.

Table 3. The SNP solutions and *P*-values of SNP significantly associated with fertility traits in the discovery population and then validated in the validation populations¹

Group and gene	Chromosome	Position	SNP	Criteria	Discovery population				Validation population				
					Freq	DPR	CCR	HCR	Freq	SF	NSC	AFC/DO	FertC
Heifers <i>CAT</i>	15	65813540	rs41603739	Effect	0.57	0.86	1.32	1.62	0.47	0.05	-0.35	-9.42	-0.11
				<i>P</i> -value		0.024	0.002	4.1E-07		0.281	0.041	0.075	0.096
	26	14814944	rs41659834	Effect	0.25	1.12	1.40	1.50	0.18	0.11	-0.28	-5.51	-0.16
<i>MYOF</i>				<i>P</i> -value		0.003	0.001	2.7E-06		0.050	0.200	0.409	0.058
	26	14945816	rs42672480	Effect	0.66	-0.85	-1.25	-1.25	0.79	-0.10	0.36	9.60	0.19
				<i>P</i> -value		0.017	0.002	3.2E-05		0.054	0.085	0.129	0.018
Cows <i>GNAI1</i>	4	41244123	rs42275710	Effect	0.59	1.38	1.95	1.35	0.60	0.04	-0.33	-6.59	-0.16
				<i>P</i> -value		0.003	0.0002	0.0004		0.116	0.005	0.078	0.004
	25	3647714	rs41255150	Effect	0.33	1.30	1.76	1.02	0.35	0.06	-0.08	-0.24	-0.07
				<i>P</i> -value		0.002	0.0002	0.003		0.055	0.503	0.949	0.243
	29	46255705	rs136330584	Effect	0.71	1.21	1.78	0.46	0.64	0.08	-0.12	-4.64	-0.15
				<i>P</i> -value		0.002	0.0001	0.169		0.005	0.298	0.213	0.008
<i>CHKA</i>	29	46260264	rs134425982	Effect	0.29	-1.58	-1.86	-0.50	0.36	-0.08	0.13	4.78	0.15
				<i>P</i> -value		8.5E-05	6.23E-05	0.139		0.005	0.277	0.200	0.008

¹Freq, frequency of the reference allele; DPR, daughter pregnancy rate; CCR, cow conception rate; HCR, heifer conception rate; SF, success at first insemination; NSC, number of services per conception; AFC, age at first conception; DO, days open; FertC, fertility category.

($P = 0.05$) with SF and tended to be associated ($P = 0.06$) with FertC (Table 3). The SNP on BTA26 at position 14.9 Mb in *RBP4* was associated ($P \leq 0.05$) with SF and FertC (Table 3).

Cow Validation Population

The estimates of genomic heritability in the cow population for SF, NSC, DO, and FertC were 0.010 ± 0.005 , 0.040 ± 0.016 , 0.030 ± 0.011 , and 0.030 ± 0.011 , respectively. The correlation of SNP solutions between fertility traits in the bull population and fertility traits in the cow population ranged from -0.07 (between CCR and SF) to 0.12 (between CCR and DO). Out of 39 significant SNP in the discovery population, 4 SNP within the *GNAI1*, *HMOX2*, and *CHKA* genes were significantly associated with at least one of the fertility traits in the cow population (Table 3). These SNP are located on chromosomes 4, 25, and 29. The substitution effects of these SNP were in the expected direction based on the SNP effects observed in the discovery population.

The SNP on BTA4 at position 41.2 Mb within *GNAI1* was significantly associated with CCR and HCR in the bull population and was also associated with NSC and FertC ($P \leq 0.02$) and tended to be associated ($P = 0.08$) with DO in the cow population (Table 3). The SNP on BTA25 at 36.5 Mb within *HMOX2* was associated with SF ($P = 0.05$; Table 3). Moreover, 2 SNP within *CHKA*, located on BTA29 at 46.2 Mb, were significantly associated ($P = 0.02$) with SF and showed marginal associations ($P = 0.07$) with FertC (Table 3).

DISCUSSION

Conception rate in dairy cows, measured as the proportion of cows diagnosed pregnant after a breeding, has remained low and stagnant for many years in North American herds, averaging between 30 and 35% (Ribeiro, 2018). The reduced efficiency in pregnancy establishment after breeding is mainly explained by a high incidence of early pregnancy losses, as the rate of synchronous ovulation and fertilization of oocytes after breeding is relatively high, above 70% (Ribeiro, 2018). Thus, development of strategies to minimize the occurrence of early pregnancy losses has great potential to improve reproductive efficiency of lactating cows, which is critical for profitability and sustainability of dairy production worldwide (Ribeiro et al., 2012). A conceivably small but relevant portion of the variation in pregnancy success is explained by the genetics of the cow, the genetics of the breeding sire, and the resulting genetics of the embryo (Butler, 2013; Cole et al.,

2016; Han and Peñagaricano, 2016; Ortega et al., 2016; Ribeiro, 2018).

Using a large population of Holstein bulls as our discovery population, we were able to identify 1,679 SNP located within or close to 183 of 194 candidate genes. Our candidate genes were all associated with lipid metabolism of conceptus cells, in which transcript expression increased ($n = 118$ genes; range: 50 to 351% increase in expression) or decreased ($n = 76$ genes; range: 50 to 143% decrease in expression) during the onset of elongation of the bovine conceptus; that is, in the transition from ovoid to tubular and from tubular to filamentous (Ribeiro et al., 2016b). All sires in our discovery population had reliabilities greater than 70% for PTA of HCR, CCR, and DPR. These 3 traits are routinely evaluated in US dairy cattle and included in all merit indices (VanRaden et al., 2018). Combining the information of candidate SNP and PTA records in the bull (discovery) population, we were able to identify 39 SNP significantly associated with at least 1 of the 3 fertility traits. These SNP were within or around 27 genes likely relevant for pregnancy establishment and maintenance, and they represent polymorphisms likely important for fertility in Holsteins.

To validate the findings obtained in the discovery population, we evaluated the 39 SNP in a population of Holstein lactating cows and in a population of Holstein heifers. We collected phenotypic information on 4 fertility traits in the lactating cow population (SF, DO, NSC, FertC) and 4 fertility traits in the heifer population (SF, AFC, NSC, and FertC). All of these fertility traits are economically relevant and commonly measured in commercial herds, and they are all associated with pregnancy success. Moreover, for lactating cows, we collected detailed information related to incidence of clinical diseases postpartum, which have a strong influence on subsequent reproductive performance (Ribeiro and Carvalho, 2017). Inclusion of health postpartum in our models helped to explain the variability in fertility phenotypes and likely enhanced our ability to identify genetic markers truly associated with inherited fertility in lactating cows.

Out of the 39 SNP found to be associated with fertility traits in the sire population, 7 SNP located within or close to 6 genes were validated in lactating cows (*CHKA*, *GNAI1*, *HMOX2*) or heifers (*CAT*, *MYOF*, *RBP4*). Interestingly, all validated genes had intermediate to high transcript expression in preimplantation conceptus cells, and their expression increased 150 to 321% during the onset of elongation, in the transition from ovoid to tubular and from tubular to filamentous (Ribeiro et al., 2016b). Two of the validated SNP were located close to each other and within the *CHKA* gene

located on BTA29. The remaining genes had one validated SNP each. The SNP rs41255150 in *HMOX2* is a synonymous variant, whereas the other 6 SNP are intronic variants.

Choline kinase α (*CHKA*) is a mammalian enzyme that catalyzes the phosphorylation of choline to phosphocholine to produce the major membrane phospholipid phosphatidylcholine. Wu et al. (2008) generated a mouse lacking *CHKA* and concluded that *CHKA* is an essential gene for early embryonic growth. Transcript expression of *CHKA* in bovine conceptus cells increased 2.8-fold with the onset of elongation (Ribeiro et al., 2016b) and is likely critical for the rapid synthesis of phosphatidylcholine and cell biomass required by the elongating conceptus. Sahana et al. (2010) identified a genomic region on BTA29 at position 46.9–51.9 Mb, close to *CHKA*, that had significant associations with number of inseminations, interval from first to last insemination, and fertility index in Holstein dairy cattle. Moreover, Frischknecht et al. (2017) detected a significant SNP on BTA29, approximately 1 Mb downstream of *CHKA*, that was associated with days to first service in Brown Swiss cattle. These studies corroborate our results, in which the 2 validated SNP in *CHKA* were highly associated with DPR and CCR in the bull population and with SF and FertC in the lactating cow population.

Heterotrimeric G proteins are composed of subunits α , β , and γ , and are responsible for transducing signals from G protein-coupled receptors in the plasma membranes to second messengers in the cytoplasm, thus having a wide variety of functions in multiple tissues (Álvarez et al., 2015). The *GNAI1* gene (G-protein subunit alpha I1) encodes one of the α subunits from the G α i family, which is known for its inhibitory effect on adenylyl cyclase activity. Multiple reproductive hormones, including neuropeptides, gonadotropins, and prostaglandins, bind to G protein-coupled receptors in target tissues and, therefore, use G proteins for downstream signaling. Recently, *GNAI1* was also associated with progesterone-associated networks involved in ovulation (Yang et al., 2018). Moreover, de novo mutations in *GNAI1* have been associated with developmental disorders in humans (Deciphering Developmental Disorders Study, 2017). In our study, the validated SNP located in *GNAI1* was highly associated with all 3 fertility traits in the sire population (DPR, CCR, and HCR), and was associated with NSC, FertC, and DO in the lactating cow population. Interestingly, the peak on BTA4 at 41.2 Mb within the *GNAI1* gene identified in our study overlaps with significant regions reported for number of inseminations per conception in Nordic Holstein, Danish Jersey, and Nordic Red cattle

(Höglund et al., 2014), conception rate in Chinese and Nordic Holsteins (Liu et al., 2017), and pregnancy rate in Brahman heifers (Porto-Neto et al., 2015).

On BTA25, our study detected a synonymous SNP at 3.65 Mb near *HMOX2*; this marker was associated with all 3 fertility traits evaluated in Holstein bulls (DPR, CCR, and HCR) and with SF in lactating cows. Frischknecht et al. (2017) also reported a SNP on BTA25 at 1.5 Mb, associated with days to first service and interval between first and last insemination in Brown Swiss cattle. Heme oxygenase 2 is 1 of 2 heme oxygenases (*HMOX1* and *HMOX2*) responsible for the degradation of heme into biliverdin, iron, and carbon monoxide, and it is involved in sensing of oxygen levels and regulation of oxidative stress in multiple tissues (Muñoz-Sánchez and Cháñez-Cárdenas, 2014). Whereas *HMOX2* is considered to be constitutively expressed in cells, *HMOX1* is considered to be an inducible protein. In preimplantation bovine conceptuses, expression of *HMOX2* was 3.3-fold greater than expression of *HMOX1* (Ribeiro et al., 2016b). Heme is a prosthetic group in multiple hemoproteins such as cyclooxygenases, peroxidases, and catalases, which are critical for conceptus development and lipid metabolism (Ribeiro et al., 2016a). Nonetheless, excessive levels of free heme in the tissue environment can cause cellular damage (Kumar and Bandyopadhyay, 2005). Therefore, expression and function of heme oxygenases are critical to maintaining adequate levels of heme in the tissues and hemoprotein function.

A SNP located at 65.8 Mb on BTA15 was associated with HCR and CCR in the sire population and with NSC, AFC, and FertC in the heifer population. This SNP is within the *CAT* gene. Normally, the body is protected by a wide range of antioxidant systems acting in concert with intracellular enzymes such as catalase (*CAT*), which remove superoxides and peroxides before they react with metal catalysts to form more reactive compounds (Miller et al., 1993). During elongation of the preimplantation conceptus, the rapid proliferation and intense metabolism of conceptus cells might generate a high level of reactive oxygen species in the uterine lumen, which need to be controlled by enzymes such as catalase. A SNP close to this region was previously identified on BTA15 at 64.1 Mb for days to first service in Brown Swiss cattle (Frischknecht et al., 2017). In addition, Peñagaricano et al. (2012) reported the presence of a SNP 251 kb from the 65.8 Mb region on BTA15 that was significantly associated with sire conception rate in Holstein dairy cattle.

Two regions on BTA26 at approximately 15 Mb associated with HCR in Holstein bulls were then validated in the analysis of SF and FertC using the heifer popula-

tion. These 2 regions harbor the retinol binding protein 4 (*RBP4*) and myoferlin (*MYOF*) genes. Notably, Cole et al. (2011) identified a region close to (within 199 kb) the *MYOF* and *RBP4* genes that was associated with DPR, sire calving ease, and daughter calving ease. The nuclear receptor peroxisome proliferator-activated receptor gamma (*PPARG*) was thought to be central for lipid metabolism and elongation of the preimplantation conceptus in dairy cows (Ribeiro et al., 2016a,b). Although no SNP within or close to *PPARG* was associated with fertility traits in the discovery population, SNP in *PPARG* were statistically associated with fertility traits of heifers (9 SNP) and cows (3 SNP). Moreover, to act as a nuclear receptor, *PPARG* needs to form a dimer with retinoid X receptor (*RXR*) after binding of their ligands, fatty acids, and retinol, respectively. Thus, metabolism of retinol in utero is also important for conceptus development, and *RBP4* could play an essential role in bioavailability of retinol in the uterine lumen (Ribeiro et al., 2018). In fact, expression of *RBP4* in the endometrium of dairy heifers increased in the period preceding conceptus elongation (Mullen et al., 2012) and, in conceptus cells, expression of *RBP4* increased 94% with the onset of elongation (Ribeiro et al., 2016b).

In addition to the 7 validated SNP, the remaining 32 SNP identified in the discovery population should not be ignored. They could be validated in other populations, and this deserves further research. In fact, several genes in this list seem to be relevant for fertility. For instance, the 2 genes *CUX1* (cut-like homeobox) and *BAX* (*BCL2*-associated X protein) have strong evidence in the literature supporting their function in reproduction. In females, *BAX* has been identified as a pro-apoptotic gene (Lazzari et al., 2011) that, when deleted, results in increased oocyte and follicle numbers in mice (Greenfeld et al., 2007). In males, however, the absence of *BAX* results in infertility. It has been also shown that a homozygous mutant mouse for *CUX1* had severely reduced male fertility (Luong et al., 2002).

Fertility traits are among the most complex, lowly heritable, and difficult to adequately measure traits. Therefore, the power of any association study to detect genetic variants related to fertility traits is probably not high. Additionally, the probability of detecting the same region in different studies becomes smaller. Also, trait definitions and strategies for editing phenotypic data, especially with respect to the handling of outlier records, differ among studies. Pryce et al. (2010) conducted a whole-genome association mapping for pregnancy rate using data from Holstein cattle and validated the results in both Holstein and Jersey populations. Notably, they could not confirm any SNP related to

pregnancy rate in the validation populations. Visscher et al. (2012) argued that low power probably explains why these effects have not been robustly detected. Nonetheless, this indicates the challenges related to mapping and validating associations for reproduction traits and emphasizes the importance of our successful validation analyses.

In this study, different genes were validated separately for traits measured in cows and heifers. This is consistent with other genome-wide association studies in which phenotypes are separated into cow and heifer traits (Höglund et al., 2009). The correlations between SNP solutions for heifer and cow fertility traits varied from 0.29 to 0.41. These findings agree with previous studies that investigated correlations between fertility traits measured in heifers and cows (Oltenacu et al., 1991; Jamrozik et al., 2005). Jamrozik et al. (2005) found a genetic correlation between cows and heifers of 0.60 for nonreturn rate and 0.76 for NSC in Canadian Holstein cattle. Oltenacu et al. (1991) reported a genetic correlation between heifer and cow first-service conception rate of 0.59 for Swedish Red and White. Therefore, we believe that the genetic and physiological mechanisms regulating fertility traits in heifers and cows are not essentially the same and should be studied separately.

CONCLUSIONS

The association of 1,679 SNP located within or close to 183 genes involved in lipid metabolism of conceptus cells with fertility traits in dairy cattle was investigated. A total of 39 SNP within or close to 27 genes were significantly associated with at least one fertility trait (DPR, CCR, and HCR) in the discovery population. Seven SNP located within 6 genes (*CAT*, *MYOF*, *RBP4*, *GNAI1*, *HMOX2*, and *CHKA*) were confirmed in the validation populations. The validated genes for fertility traits are likely important for reducing pregnancy losses. The SNP within genes confirmed in the present study are good candidates for inclusion in genomic tests of fertility traits in dairy cattle.

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