Genomic heritability and genome-wide association analysis of anti-Müllerian hormone in Holstein dairy heifers

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ABSTRACT

Anti-Müllerian hormone (AMH) is an ovarian growth factor that plays an important role in regulation of ovarian follicle growth. The objectives of this study were to estimate the genomic heritability of AMH and identify genomic regions associated with AMH production in a genome-wide association (GWA) analysis. Concentrations of AMH were determined in 2,905 dairy Holstein heifers genotyped using the Zoetis medium density panel (Zoetis Inclusions, Kalamazoo, MI) with 54,519 single nucleotide polymorphism (SNP) markers remaining after standard genotype quality control edits. A linear mixed model was used to model the random effects of sampling day and genomics on the logarithm of AMH. The genomic heritability ($\pm$ standard error of the mean) of AMH was estimated to be 0.36 ± 0.03 . Our GWA analysis inferred significant associations between AMH and 11 SNP markers on chromosome 11 and 1 SNP marker on chromosome 20. Annotated genes with significant associations were identified using the Ensembl genome database (version 88) of the cow genome (version UMD 3.1; <https://www.ensembl.org/biomart>). Gene set enrichment analysis revealed that 2 gene ontology (GO) terms were significantly enriched in the list of candidate genes: G-protein coupled receptor signaling pathway (GO:0007186) and the detection of chemical stimulus involved in sensory perception (GO:0050907). The estimated high heritability and previously established associations between AMH and ovarian follicular reserve, fertility, longevity, and super-ovulatory response in cattle implies that AMH could be used as a biomarker for genetic improvement of reproductive potential.

Key words: genome-wide association, heritability, reproduction

INTRODUCTION

The rate of genetic improvement for reproductive performance in dairy cattle is relatively slow (García-Ruiz et al., 2016), partly because heritability estimates of conventional reproductive traits of dairy cattle are low, typically ranging from 0.02 to 0.17 (Berry et al., 2014). Reliable biomarkers highly correlated with reproductive performance, expressed early in life, and moderately to highly heritable have not yet been discovered. Such a discovery would be useful in identifying cattle with superior reproductive potential and designing breeding programs for faster genetic gain in reproductive efficiency to enhance profitability of the dairy industry.

Anti-Müllerian hormone (AMH) has been proposed as an important biomarker of reproductive potential of cattle. It is a growth factor produced by granulosa cells of ovarian follicles and Sertoli cells of testes, and it was first discovered to play an important role in sex differentiation during embryo development (Baarends et al., 1994; Allard et al., 2000). In adults, however, AMH is known to regulate ovarian follicle growth (Tiftik et al., 2016) as it prevents initial recruitment and the premature depletion of the follicular population in the ovary (Durlinger et al., 1999, 2002). Anti-Müllerian hormone may also inhibit FSH-induced follicular growth because it reduces the sensitivity of follicles to FSH treatments (Durlinger et al., 2001). Consequently, AMH may play a role in determining which follicles undergo selection and grow, and which follicles undergo atresia.

Our research group has shown that circulating AMH concentration is positively correlated with ovary size, number of antral follicles growing during follicular waves, and size of the ovarian reserve (total number of morphologically healthy oocytes in ovaries; Ireland et al., 2008). Anti-Müllerian hormone has been determined to predict ovarian progesterone (Ireland et al., 2009), androgen production (Mossa et al., 2010), herd longevity (Jimenez-Krassel et al., 2015), maintenance of pregnancy, and pregnancy rate in dairy cows bred on estrus (Ribeiro et al., 2014). It is also positively cor-

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related with response to superovulation (Ireland et al., 2007) and embryo production in dairy cattle (Monniaux et al., 2010; Guerreiro et al., 2014). Although highly variable among individuals, serum AMH concentration is highly repeatable within animals and not affected by the stage of estrous cycle (Ireland et al., 2008, 2011). Thus, AMH concentration is a reliable phenotypic marker, not only for size of the ovarian reserve, ovarian function, and response to superovulation, but also for fertility and herd longevity. Taken together, these findings imply that AMH may be useful for identification and subsequent genetic selection of cattle with superior reproductive potential, provided that serum AMH is at least moderately heritable.

The first objective of the present study was to estimate the genomic heritability of AMH. Our second objective was to use genome-wide association (GWA) analyses to help identify genomic regions associated with AMH using a medium-density SNP genotype panel.

MATERIALS AND METHODS

All experiments involving cattle were approved by the Institutional Animal Care and Use Committee at Michigan State University. Holstein heifers ($n = 3,252$, 11–15 mo old, located at Green Meadow Farms Inc., Elsie, MI) were each subjected to 2 intramuscular injections of PGF_{2α} spaced 11 d apart to synchronize estrous cycles. Heifers were synchronized in groups of 95 to 124 heifers once or twice a month for a total of 29 groups or sampling dates. At 96 h after the last PGF_{2α} injection, a single tail-vein blood sample was taken from each heifer to measure serum AMH concentration. Blood samples were taken beginning on April 14, 2014 (sampling date 1) and ending on December 4, 2015 (sampling date 29). Follicle hair samples were also collected at these sampling dates for genotypic analyses. Freemartins ($n = 144$) were not included in the statistical analyses.

AMH Assay

The commercially available ELISA kit for bovine AMH (MiniTube of America, Verona, WI) was used to measure serum AMH concentrations in duplicate 20-μL serum samples in cattle per kit instructions. The 2-site AMH assay has been validated (Ireland et al., 2008) for use in cattle and does not cross-react with other members of the transforming growth factor β (TGFβ) superfamily including TGFβ, bone morphogenic factor-4, inhibin, or activin (Kevenaar et al., 2006). The interassay coefficients of variation for 105 assays for low, intermediate, and high quality controls were 17,

16, and 20%, respectively. In addition, to evaluate potential AMH degradation during storage, serum AMH concentrations for 5 heifers in the study after ~1 mo storage at -80°C were determined to be 75, 55, 1,117, 436, and 208 pg/mL. When samples from these same individuals were assayed ~12 mo after storage at -80°C , AMH concentrations were 59, 48, 1,227, 435, and 191 pg/mL, respectively. These results implied that AMH is relatively stable during long-term storage.

Genotyping and Pedigree Information

A total of 2,939 Holstein heifers were genotyped for SNP markers using a Zoetis proprietary medium-density SNP panel (Zoetis Genetics, Kalamazoo, MI). Four-generation pedigree information of these animals was also retrieved to construct an additive relationship matrix. The genotypes were imputed to the standard USDA 60,671 bovine SNP set (Wiggans et al., 2016) by the USDA Animal Genomics and Improvement Laboratory (Beltsville, MD). Animal genotypes and SNP markers were retained for analysis only if they fulfilled the following criteria: missing values $<20\%$, minor allele frequency (MAF) >0.05 , and pairwise linkage disequilibrium (LD) value of $r^2 < 0.95$. The final data set used for statistical analysis after data editing included phenotypes and genotypes for $m = 54,519$ SNP markers on each of 2,905 cows and their 4-generation pedigree information.

Statistical Analysis

A linear mixed model was used to model the random effects of sampling day and genomics on the logarithm of AMH to estimate the genetic, sampling day, and residual variance components. The linear model used for analysis is provided in Equation [1]:

$$y_{ij} = \mu + d_i + a_j + e_{ij}, \quad [1]$$

where y_{ij} is the AMH phenotype taken on day i on heifer j ; μ is the overall mean; $d_i \sim NIID(\mathbf{0}, \sigma_d^2)$ is the random effect of the date of sample collection, $i = 1, 2, \dots, 29$, with $NIID$ = normally, identically, and independently distributed; and $e_{ij} \sim NIID(\mathbf{0}, \sigma_e^2)$ is the residual term for the record on animal j taken on date i . Furthermore, a_j is the genomic merit of animal j , $j = 1, 2, \dots, 2,905$, with $\mathbf{a} = \{a_j\} \sim N(\mathbf{0}, \mathbf{A}\sigma_a^2)$. Here, σ_a^2 refers to variance of the effects of date of sample collection, σ_a^2 is the additive genetic variance, and σ_e^2 is the residual variance. For pedigree-based heritability estimation, $\mathbf{A}$ was the matrix of additive genetic relation-

ships between animals. To estimate genomic heritability, $\mathbf{A}$ was replaced by $\mathbf{G} = \mathbf{Z}\mathbf{Z}'$, being the genomic relationship matrix for which elements (z_{jk}) of $\mathbf{Z}$ are characterized as in Equation [2]:

$$z_{jk} = \frac{m_{jk} - 2p_k}{\sqrt{\sum_1^m 2p_k(1 - p_k)}}. \quad [2]$$

In Equation [2], m_{jk} indicates the number of copies (0, 1, or 2) of the reference allele on SNP marker k for animal j , whereas p_k denotes the allelic frequency of the reference allele for SNP marker k . Restricted maximum likelihood was used to provide estimates of σ_a^2 , σ_d^2 , and σ_e^2 . Heritability was determined as taking the proportion of total phenotypic variation ($\sigma_a^2 + \sigma_d^2 + \sigma_e^2$) that was due to genomics (σ_a^2).

The reliability r_j^2 of $\hat{a}_j$, the genomic estimated breeding value (**GEBV**) for animal j , with GEBV being synonymous with the BLUP of a_j , was determined as in Equation [3] (Van Vleck, 1993):

$$r_j^2 = \frac{\hat{\sigma}_a^2 - c_{jj}}{\hat{\sigma}_a^2}, \quad [3]$$

where $\hat{\sigma}_a^2$ is the REML estimate of σ_a^2 under the genomic model (i.e., using $\mathbf{G}$) and $c_{jj} = \text{var}(a_j - \hat{a}_j)$ refers to the prediction error variance for $\hat{a}_j$. The GEBV for all animals, that is, $\hat{\mathbf{a}} = \{\hat{a}_j\}$, were linearly transformed to the estimated marker effects $\hat{\mathbf{g}} = \{\hat{g}_k\}$, as demonstrated by Gualdrón Duarte et al. (2014) using Equation [4]:

$$\hat{\mathbf{g}} = \mathbf{Z}'\mathbf{G}^{-1}\hat{\mathbf{a}}. \quad [4]$$

The variance of $\hat{\mathbf{g}}$ can be determined using classical mixed model theory, as also illustrated by Gualdrón Duarte et al. (2014), using Equation [5]:

$$\text{Var}(\hat{\mathbf{g}}) = \mathbf{Z}'\mathbf{G}^{-1}\mathbf{Z}\sigma_a^2 - \mathbf{Z}'\mathbf{G}^{-1}\mathbf{C}^{aa}\mathbf{G}^{-1}\mathbf{Z}, \quad [5]$$

where $\mathbf{C}^{aa} = \text{var}(\mathbf{a} - \hat{\mathbf{a}})$ is the prediction error covariance matrix of all GEBV; that is, c_{jj} is diagonal element j of $\mathbf{C}^{aa}$. Using again Gualdrón Duarte et al. (2014), the z -test statistic for testing the null hypothesis, $H_0: g_j = 0$, $j = 1, 2, \dots, m$ can be specified as in Equation [6]:

$$z_j = \frac{\hat{g}_j}{\sqrt{\text{Var}(\hat{g}_j)}}. \quad [6]$$

The corresponding P -value for the test statistic in Equation [6] is provided in Equation [7]:

$$P\text{-value}_j = 2\left[1 - \Phi(|z_j|)\right], \quad [7]$$

where Φ denotes the standard normal cumulative distribution function. P -values were converted to q -values (Storey and Tibshirani, 2003) to declare any SNP marker associations with AMH as significant based on a false discovery rate (**FDR**) of 5% (i.e., q -value < 0.05).

Number of QTL per Peak

To infer the number of QTL per region containing a peak (i.e., a q -value < 0.05), we refitted the linear mixed model in Equation [1], this time treating the most significant or peak SNP marker as fixed (Casiró et al., 2017). The model thus used was as in Equation [8]:

$$y_{ij} = \mu + \beta_{\text{peak}} g_{\text{peak},j} + d_i + a_j + e_{ij}, \quad [8]$$

where all the terms in Equation [8] are as described in Equation [1] except that β_{peak} denotes the partial regression of y_{ij} on the SNP genotype $g_{\text{peak},j}$ at the SNP marker having the smallest q -value < 0.05 on a particular chromosome. If, after fitting β_{peak} , all other SNP markers on that same chromosome do not exhibit a significant association (i.e., q -value > 0.05), this would be suggestive of no evidence of multiple QTL peaks within the corresponding genomic region (Casiró et al., 2017).

Confidence Interval of the QTL Peak

We used a cross validation strategy to estimate the 99% CI for the QTL peak position as proposed by Hayes (2013) and used by Casiró et al. (2017). In this method, we first conducted the GWA as described above to obtain the physical position ($\hat{x}$) of the most significant SNP marker with q -value < 0.05 on a particular chromosome. We then randomly divided the data set into 2 halves (1 and 2) for a particular split l , conducting an independent GWA analysis on each half to determine the physical positions ($\hat{x}_{1l}$ and $\hat{x}_{2l}$, respectively) from both analyses for the most significant SNP marker on that same chromosome. For that same split, we then computed the difference ($\hat{h}_l = \hat{x}_{1l} - \hat{x}_{2l}$) in the distance between these 2 inferred peaks. We repeated this random data split and dual GWA analysis process $n_h = 300$ times to get the standard error of half the distance [$se(\bar{h})$] between the 2 peak locations as in Equation [9]:

$$se(\bar{h}) = \sqrt{\frac{1}{4n_h} \sum_l^{n_h} \hat{h}_l^2}. \quad [9]$$

Assuming an approximate normal distribution of the peak SNP after n_h random splits of the data, we calculated the 99% CI of the width of the genomic region for the peak SNP marker as in Equation [10]:

$$99\% \text{ CI} = \hat{x} \pm 2.58se(\bar{h}); \quad [10]$$

note that in Equation [10], 2.58 represents the 99.5th percentile of a standard normal distribution.

Proportion of Genetic Variance Explained by the Significant Region

The proportion of genetic variance explained by each of the 2 regions associated with AMH was determined by refitting the model in Equation [1], but this time partitioning $\mathbf{a}$ into 2 sets of genetic effects for each animal: (1) the contribution of the cumulative effects of the SNP markers falling within the 99% CI of the peak SNP into $\mathbf{a}_{peak} = \mathbf{Z}_{peak}\mathbf{g}_{peak}$ and that of the remaining markers in $\mathbf{a}_{-peak} = \mathbf{Z}_{-peak}\mathbf{g}_{-peak}$. In other words, we partitioned $\mathbf{a} = [\mathbf{a}_{peak} \quad \mathbf{a}_{-peak}]'$ and $\mathbf{Z} = [\mathbf{Z}'_{peak} \quad \mathbf{Z}'_{-peak}]'$. Hence, we defined $var(\mathbf{a}_{peak}) = \mathbf{G}_{peak}\sigma_{a(peak)}^2$ and $var(\mathbf{a}_{-peak}) = \mathbf{G}_{-peak}\sigma_{a(-peak)}^2$ with $\mathbf{G}_{peak} = \mathbf{Z}_{peak}\mathbf{Z}'_{peak}$ and $\mathbf{G}_{-peak} = \mathbf{Z}_{-peak}\mathbf{Z}'_{-peak}$, such that the proportion of genetic variance accounted for by the 99% CI peak region is estimated as in Equation [11]:

$$\frac{\hat{\sigma}_{a(peak)}^2}{\hat{\sigma}_{a(peak)}^2 + \hat{\sigma}_{a(-peak)}^2}, \quad [11]$$

where $\hat{\sigma}_{a(peak)}^2$ is the REML estimate of $\sigma_{a(peak)}^2$, and $\hat{\sigma}_{a(-peak)}^2$ is the REML estimate of $\sigma_{a(-peak)}^2$. A likelihood ratio test was used to test the importance of the peak region by comparing -2 times the log restricted log-likelihood for the model with 2 genetic variance components $[\sigma_{a(peak)}^2 \text{ and } \sigma_{a(-peak)}^2]$ to that from a null model that only modeled genetic variability outside of the peak 99% CI region $[\sigma_{a(-peak)}^2]$ to a 50:50 mixture of a chi-squared distribution with 0 and 1 df, respectively (Self and Liang, 1987).

Estimation of variance components and GWA analysis was done using the gwaR (<https://github.com/steibelj/gwaR>) package in R. All analyses were conducted using R version 3.2.0 (<http://www.R-project.org/>).

Identification of Candidate Genes and Enrichment Analysis

Annotated genes falling within the corresponding 99% CI for peak SNP associations were retrieved from the Ensembl genome database version 88 of the cow genome (version UMD 3.1; <https://www.ensembl.org/biomart>) using the “GenomicRanges” package in R (Lawrence et al., 2013). This list of candidate genes was submitted to The Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8; Huang et al., 2009) to retrieve the corresponding gene ontology (GO) terms related to biological pathways of all the genes, and to perform gene set enrichment analysis to discover biological pathways that were significantly enriched in the list of candidate genes.

RESULTS AND DISCUSSION

Descriptive Statistics

Serum concentrations of AMH in the dairy heifers had a mean of 438.50 pg/mL and a standard deviation of 604.33 pg/mL. The median AMH concentration was 333.3 pg/mL, with individual AMH values ranging from 2 to 14,350 pg/mL. These concentrations were comparable to those reported in other studies in Holsteins (Guerreiro et al., 2014; Ribeiro et al., 2014), although significant variation exists between breeds of cattle (Batista et al., 2014; Ribeiro et al., 2014) because of differences in reproductive physiology and genetic potential between breeds. The distribution of AMH concentration and logarithmically transformed AMH concentration (the latter demonstrating a far better approximation to normality) are depicted in Figure 1a and b. Hence, all subsequent analyses are based on the logarithm of AMH.

Heritability Inference and Prediction Accuracies

The heritability estimate ($\pm$ SE) for AMH based on pedigree information was 0.43 ± 0.07 , whereas the estimate of genomic heritability of AMH was 0.36 ± 0.03 . The higher pedigree-based heritability may be due to overestimation of additive relationships by the $\mathbf{A}$ matrix compared with $\mathbf{G}$ matrix or due to imperfect LD between SNP markers and the QTL controlling the trait. This difference is consistent with those observed in other dairy genetic studies (Veerkamp et al., 2011; Haile-Mariam et al., 2013). Nevertheless, these heritability estimates for AMH are the highest reported for any trait related to reproduction in female cattle (Berry et al., 2014). Gobikrushanth et al. (2018) estimated a similar genomic heritability for AMH (0.46 ± 0.31) in

Canadian Holstein cows, albeit with a substantially larger standard error, likely due to their smaller sample size ($n = 198$). The estimated variance components associated with the day of blood sample collection, genetic and residual variance components, and heritability estimates for both models are provided in Table 1.

The average reliability of genomic prediction of GEBV for the 2,905 animals was 55.7% and ranged from

46.5 to 67.2%. Moderate to high reliabilities of GEBV were expected considering the reasonably large genomic heritability estimate for AMH. Reliabilities of genomic prediction are known to increase with the number of animals genotyped, marker density, and the heritability of the trait under selection. The mean reliability for genomic predictions in our study exceeded the expected mean reliability of 36% for genomic predictions based

Figure 1. Distribution of (a) serum concentrations and (b) log-transformed serum concentrations of anti-Müllerian hormone (AMH) in Holstein heifers. Color version available online.

Figure 2. Distribution of reliability of genomic EBV of anti-Müllerian hormone (AMH) using 54,519 SNP markers on 2,905 Holstein heifers. Color version available online.

on a reference population of 3,000 animals and a trait heritability of 0.35, as expected by Hayes et al. (2009). The distribution of prediction accuracies for the 2,905 animals is provided in Figure 2. Hence, genetic selection on AMH could improve reproductive potential in cattle, provided that a positive genetic correlation exists between AMH and economically relevant reproduction traits.

It has previously been established that AMH has a moderate phenotypic correlation with the average and maximal number of embryos collected per cow ($r = 0.49$, $P < 0.001$; and $r = 0.58$, $P < 0.0001$, respectively) (Monniaux et al., 2010). Jatón et al. (2016) demonstrated that genetic correlations between the number of collectible and viable embryos and milk production traits were low, ranging between -0.12 and -0.29 , and concluded that the genetic selection of cattle that produce a large number of embryos without compromising milk production is plausible. Given that our estimated heritability of AMH is more than twice that (0.14) estimated for the number of embryos by Jatón et al. (2016), indirect selection on serum AMH in young animals could allow earlier and more accurate selection for

the number of embryos in both cows and bulls, provided that the genetic correlation between the 2 traits is large (Woolliams and Smith, 1988). Although superovulatory traits are not yet included in the dairy breeding goals, estimating breeding values for AMH would be useful for breeders who are interested in maximizing their efficiency of embryo production by selecting animals as donors based on their genetic potential for superovulation.

GWA Study

Significant associations between AMH and SNP markers were found in 2 distinct regions of the genome, one within each of chromosomes 11 and 20. The most significant association was with SNP marker Hapmap41435-BTA-115556 on BTA11 at position 95,026,013 bp, whereas the sole significant association on BTA20 was with SNP marker ARS-BFGL-NGS-110286 at position 25,689,084 bp. Genome-wide association inferences are best illustrated by a Manhattan plot [plot of $-\log_{10}(P\text{-values})$ versus genomic location] overlain with a statistical significance threshold reference line,

Table 1. Estimates ($\pm$ SE) of variance components and heritability of anti-Müllerian hormone (AMH) from models based on pedigree information and genomic information

Model	Genetic variance	Sampling day variance	Residual variance	Heritability
Pedigree-based	0.34 ± 0.05	0.02 ± 0.01	0.42 ± 0.04	0.43 ± 0.07
SNP marker-based	0.28 ± 0.01	0.02 ± 0.01	0.48 ± 0.02	0.36 ± 0.03

Figure 3. Manhattan plot of $-\log_{10} P$ -values versus genomic location for logarithm of serum concentrations of anti-Müllerian hormone (AMH) in dairy Holstein heifers. Horizontal reference line corresponds to false discovery rate of 5% superimposed. Color version available online.

as provided in Figure 3. This plot demonstrates significant associations between AMH and 11 SNP markers within 1 genomic region on BTA11 and 1 SNP marker on BTA20 based on an FDR of 5%. The names of all significant SNP markers and their corresponding P -values are provided in Table 2.

Inferring Number of QTL

To assess whether evidence of more than one QTL might exist within the particular genomic region of 11 significant SNP markers on BTA11, the most sig-

nificant SNP marker, Hapmap41435-BTA-115556, was included as a fixed effect in the model. Upon doing so, none of the remaining SNP markers on BTA11 reached the level of statistical significance (5% FDR). This suggests that all significant SNP associations on BTA11 were not due to the presence of multiple QTL; however, we also recognize that this procedure may not be very sensitive to detecting multiple QTL within the same region for markers in high LD with each other. Because there was only one significant marker association on BTA20, this process was not considered within that region.

Table 2. Significant marker associations from genome-wide association analysis of logarithm of anti-Müllerian hormone (AMH) in dairy Holstein heifers (SNP identifiers can be found at <http://bioinformatics.tecnoparco.org/SNPchimp/>)

SNP ID	BTA	Position (bp)	P -value	q -value	Minor allele frequency
BTA-17666-no-rs	11	92,059,274	1.59×10^{-6}	0.007	0.18
ARS-BFGL-NGS-118517	11	93,546,324	1.29×10^{-6}	0.007	0.47
Hapmap38572-BTA-115523	11	94,071,468	5.10×10^{-7}	0.003	0.22
BTA-115525-no-rs	11	94,101,466	7.77×10^{-9}	0.0001	0.20
BovineHD1100027436	11	94,202,912	2.00×10^{-6}	0.008	0.22
Hapmap46766-BTA-115526	11	94,446,742	7.92×10^{-7}	0.005	0.22
Hapmap41435-BTA-115556	11	95,026,013	5.94×10^{-9}	0.0001	0.18
ARS-BFGL-NGS-12334	11	95,516,698	4.65×10^{-7}	0.00386	0.37
Hapmap53866-rs29019867	11	95,568,110	1.40×10^{-8}	0.00018	0.14
Hapmap47514-BTA-115564	11	96,074,475	7.87×10^{-9}	0.00013	0.16
ARS-BFGL-NGS-114094	11	99,413,363	2.63×10^{-7}	0.00279	0.10
ARS-BFGL-NGS-110286	20	25,689,084	1.64×10^{-6}	0.00794	0.46

Confidence Interval of the Peak SNP Association on Chromosomes 11 and 20

Based on the procedures adapted from Casiró et al. (2017), we determined that the 99% CI of the peak SNP on BTA11 ranged from 92,879,023 to 97,160,473 bp, a span of roughly 4.28 Mbp. Our SNP panel contained 68 SNP markers from that region which accounted for 7.9% of the total genetic variance. The genetic variance for this region was determined to be significant ($P = 1.7 \times 10^{-7}$) based on a likelihood ratio test. Similarly, the 99% CI of the peak significant marker on BTA20 (ARS-BFGL-NGS-110286) ranged from 25,002,762 to 26,362,752 bp, a span of roughly 1.35 Mbp. This chromosomal region contained 25 SNP markers and accounted for 5.25% of the genetic variance, which was also deemed to be significant ($P = 0.007$) based on a likelihood ratio test. Therefore, the proportion of total genetic variance jointly explained by both regions in BTA11 and BTA20 was 13.25%.

Since the original submission of this paper, a concurrent GWA study (Gobikrushanth et al., 2018) has identified 640 significant SNP marker associations spread over 12 chromosomes with serum AMH drawn from 589 mature Canadian Holstein cows. In that study, 513 of the 640 associations substantially overlapped with our own region of inferred associations on BTA11, although we did infer a narrower region using a cross validation approach, as explained above, in part due to our substantially larger sample sizes. Their greater number of declared associations could be partly attributed to primarily imputed associations as the cows in the Gobikrushanth et al. (2018) study were genotyped with a lower density panel (~26K) imputed in 2 steps to the Illumina Bovine 50K panel (Illumina Inc., San Diego, CA) and then to the Bovine HD 777K panel (Illumina Inc.) using an Irish Holstein-Friesian reference population. Their GWA analysis was conducted such that for the SNP marker association being tested, none of the effects of the other markers on the same chromosome were simultaneously fitted; conversely, our SNP marker association tests were adjusted for all other SNP markers, including those on the same chromosome as the marker of interest. Furthermore, their P -value threshold for declaring significant associations was less stringent (about one order of magnitude) than our corresponding threshold even though their number of marker tests was far greater.

In humans, Perry et al. (2016) determined that 3 SNP variants close to the AMH gene locus on chromosome 19 were associated with log serum AMH. Their GWA analyses also inferred a sex interaction effect because no SNP markers were deemed associated with AMH in females. As our study consisted of heifers only,

we could not assess whether such an interaction existed for serum AMH levels in cattle. Considering AMH as an indicator of reproduction traits, we also reviewed GWA studies for reproduction traits in Holstein to find any evidence of overlap of associations with our results. A GWA study on superovulatory traits in dairy cattle (Jaton et al., 2018) concluded that 26 of 36 significant marker associations with total number of embryos and all 14 significant markers associated with the number of viable embryos were located between 92,918,581 and 97,926,706 bp on BTA11. That study, in conjunction with ours, further strengthens the hypothesis that AMH has a common QTL with superovulatory response traits in cattle, with both traits being regulated by common genes, given that strong phenotypic associations between the 2 traits have already been well established.

Candidate Genes and Functional Enrichment

The 99% CI of the most significant association on BTA11 (92,879,023 to 97,160,473 bp) contained 81 potential candidate genes, whereas the corresponding region on BTA20 (25,002,762 to 26,362,752 bp) contained 9 potential candidate genes based on the Ensembl genome database v. 88 of the cow genome. Therefore, 90 genes were submitted to DAVID to retrieve functional GO information; 69 genes were successfully mapped to the DAVID *Bos taurus* GO database (Supplemental Tables S1 and S2; <https://doi.org/10.3168/jds.2018-14798>). Gene set enrichment analysis using DAVID resulted in identification of 2 GO terms (biological pathways) significantly enriched (P -values $< 10^{-13}$) in the list of candidate genes compared with the background genes of *Bos taurus* (Table 3). These terms were the “G-protein coupled receptor signaling” (GO:0007186) and “detection of chemical stimulus involved in sensory perception” (GO:0050907) pathways. The former pathway begins with receptor–ligand interaction and ends with the regulation of a downstream cellular process (e.g., transcription). The latter is involved in a series of events in which a chemical stimulus is received and converted into a molecular signal as part of sensory perception. Although it is unclear at this point how these pathways specifically affect serum AMH concentration, it is apparent that the genes involved in cell-to-cell signaling mechanisms are key regulators of AMH. Some of the other interesting GO terms associated with candidate genes include “male gonad development” (GO:0008584), “multicellular organism aging” (GO:0010259), “steroid hormone mediated signaling pathway” (GO:0043401), “positive regulation of male gonad development” (GO:2000020), “negative regulation of female gonad development” (GO:2000195), and

Table 3. Biological pathways significantly associated with log serum anti-Müllerian hormone (AMH) concentrations in heifers

Gene ontology terms	No. of genes matched/total	Fold enrichment	P-value	False discovery rate
GO:0007186 G-protein coupled receptor signaling pathway	24/69	7.78	3.0×10^{-15}	3.5×10^{-12}
GO:0050907 Detection of chemical stimulus involved in sensory perception	13/69	21.02	6.3×10^{-13}	7.5×10^{-10}

“gamete generation” (GO:0007276), among many others. One potential limitation of this GO analysis is that all of these candidate genes derive from only 2 narrowly defined regions of the genome (i.e., a 4.28-Mbp region on BTA11 and a 1.35-Mbp region on BTA20). Hence, any such inferred associations may be biased if nonrandom associations exist between genomic locations and functional processes of the resident genes. All candidate genes and their corresponding GO terms retrieved from DAVID are listed in Supplemental Tables S1 (BTA11) and S2 (BTA20; <https://doi.org/10.3168/jds.2018-14798>).

We propose that all the genes lying within the 99% CI for the peak SNP are potential candidates for understanding molecular mechanisms for the regulation of AMH. A literature search of candidate genes revealed that several of these candidates (*DENND1A*, *NR5A1*, *NR6A1*, *PTGS1*, and *NDUFA8* on BTA11; and *FST* on BTA20) have a plausibly important role in AMH regulation in cattle based on previous studies as subsequently outlined. The most significant association (Hapmap41435-BTA-115556) with AMH in our study was located within the intronic region of the *DENND1A* gene. This gene has a role in membrane trafficking and receptor turnover. Welt et al. (2012) determined that the marker rs10986105, also located within *DENND1A*, had the most significant association with polycystic ovarian syndrome (PCOS) for European and Chinese human females. It is well known that women suffering from PCOS are subfertile (Franks, 1995), have a large number of ovarian follicles (Hughesdon, 1982) and abnormally high serum AMH concentrations (Pigny et al., 2003), and exhibit high RNA expression of the *DENND1A* gene in thecal cells and enhanced circulating concentrations of androgens and progesterone (McAllister et al., 2015). These phenotypic characteristics in women are similar to those in cattle having a high antral follicle count, which is also associated with reduced reproductive performance and shortened herd longevity (Jimenez-Krassel et al., 2017) as well as high circulating concentrations of testosterone (Mossa et al., 2010) and progesterone (Ireland et al., 2009), and high circulating AMH concentrations (Ireland et al., 2008). In addition, *DENND1A* was identified as a candidate gene on BTA11 in a recent GWA study involving superovulatory responses (total number of embryos and the

number of viable embryos) in Canadian Holstein dairy cows (Jaton et al., 2018). This linkage with our study is particularly significant because, again, AMH concentration is known to be positively correlated with the response to superovulation in dairy cows (Monniaux et al., 2010).

The prostaglandin-endoperoxide synthase 1 (*PTGS1*) gene (commonly known as COX-1) is responsible for the conversion of arachidonic acid into PGH₂, a precursor of all forms of prostaglandins such as PGE₂ and PGF_{2α} (Arosh et al., 2002). Prostaglandins are well known to play an important role in ovulation and fertilization (Armstrong, 1981) by either contracting or relaxing the muscles of the oviduct, leading to rapid transport of gametes and zygote (Wijayagunawardane et al., 2001). Although *PTGS1* is constantly expressed in many tissues and cell types (Asselin et al., 1997), the concentration of *PTGS1* mRNA consistently increases in the oviduct from uterine pole toward the ovarian pole, independent of the stage of estrous cycle (Gauvreau et al., 2010), suggesting its significance in the oviduct, which serves as the site of fertilization and transports the gametes and zygote to the uterus. Moreover, *PTGS1* has been associated with the number of collectible embryos and number of viable embryos in Canadian Holsteins (Jaton et al., 2018). As AMH has been identified as an important marker of superovulatory response (Monniaux et al., 2010; Souza et al., 2015), these findings suggest an important role for *PTGS1* in the regulation of AMH and superovulation.

Our study also identified 2 microRNAs, miR-181a and miR181b, that have been shown to be induced by AMH in a mouse model (Hayes et al., 2016). In vivo treatment of AMH induces expression of miR-181a in mouse ovary that targets activin receptor (ACVR2A) leading to disruption of activin effects on follicular growth. Furthermore, AMH also induces miR-181b expression that targets adenylate cyclase 9 (ADCY9), decreasing cyclic AMP levels and resulting in suppression of FSH signaling. Our results, coupled with the fact that similar results were also found in human granulosa-like tumor cell line (KGN cells; Hayes et al., 2016), suggest that molecular mechanisms of AMH are conserved across species.

The *NR5A1* gene encodes steroidogenic factor 1 (SF-1), which is known to bind to 2 binding sites within

the promoter region of AMH gene (Giuli et al., 1997; Watanabe et al., 2000) and regulates AMH expression (Jin et al., 2016). In addition, the SF-1 binding site to the AMH promoter is known to be essential for sex- and cell-specific AMH promoter activity (Giuli et al., 1997) and transcription of key genes involved in sexual development and reproduction. Similarly, *NR6A1* (also known as germ cell nuclear factor, *GCNF*), which encodes an oocyte-specific transcription repressor (Cooney et al., 1998), belongs to the same family as *NR5A1* and is known to play an important role in embryo development and folliculogenesis (Pangas and Rajkovic, 2006).

The *NDUFA8* gene encodes a subunit of enzyme NADH dehydrogenase (ubiquinone), which is located in the mitochondrial inner membrane and transfers electrons with high redox potential from NADH to ubiquinone, thus playing an important role in energy metabolism (Held et al., 2012). Gene expression of *NDUFA8* is downregulated in competent bovine embryos (O'Shea et al., 2012). However, this gene is highly expressed in human heart, skeletal muscle, and fetal heart (Triepels et al., 1998). Moreover, a GWA study in cattle with brisket disease revealed NADH dehydrogenase as a candidate gene involved in hypertension (Newman et al., 2011). Recently, AMH concentration has been found to be negatively correlated with ultrasonographic diameter of the aorta, whereas high AMH levels have been linked to the absence of cardiovascular disease in men (Dennis et al., 2013). In addition, female offspring of beef cows fed a mild nutrient-restricted diet during the first trimester of pregnancy not only had significantly larger aortic diameters and higher blood pressure but also lower circulating AMH concentrations compared with controls (Mossa et al., 2013). Taken together, these studies in humans and cattle potentially demonstrate a previously unstudied link between AMH concentrations and cardiovascular health.

The *FST* gene encodes the protein follistatin, which binds to members of the TGF- β superfamily, particularly activin. Follistatin was first discovered in follicular fluid and known to bind to activins and inhibit activin-induced secretion of FSH from pituitary gland (Ying, 1988). Follistatin and activin interaction is widely known as the autocrine/paracrine regulator of various physiological processes in reproduction, including folliculogenesis and maturation of oocytes (Muttukrishna et al., 2004). Furthermore, increased abundance of *FST* mRNA transcript is positively associated with competence of fertilized bovine oocytes to develop into embryos (Patel et al., 2007; Lee et al., 2009). Follistatin enhances AMH signaling in luciferase reporter assays (Kawagishi et al., 2017), implying that it may have a role in regulation of AMH bioactivity.

Future Research Questions

A limitation of our study is that all the data were collected from a single farm and consisted of females only. It would be informative to conduct a GWA with a larger data set consisting of both sexes to determine whether there is a sex interaction effect, as found in humans, or whether the genetic correlation in AMH between bulls and cows is different from unity. The existence of such an interaction could affect genomic selection programs for AMH. Second, widespread application of AMH as a diagnostic tool to select superior dairy animals for reproductive traits requires further research involving animals in different stages of productive life and under different management conditions. Future studies should be considered to infer the phenotypic and genetic correlations of AMH with economically relevant traits in the dairy industry, especially superovulatory traits, as anticipated by our results in conjunction with those of Jatón et al. (2018). A multiple trait analysis could assess whether AMH could be used for indirect genetic improvement of reproduction in cattle without any deleterious effects on production traits, for example.

Candidate genes identified in this study also require further study. Some of these genes have already been studied extensively in the past. For example, *NR5A1* knockout mice showed complete adrenal and gonadal agenesis (Luo et al., 1994), and mutations in this gene caused ovarian insufficiency (Lourenço et al., 2009). However, validation of other genes should be tested by a series of experiments. First, fine sequencing should be done in the proposed genomic regions to detect the causative QTL. These studies could be followed by differential gene expression studies and knockout experiments to confirm the role of candidate genes in the regulation of AMH. Moreover, a gene network analysis using publicly available scientific data sets on genes and pathways can reveal important protein-protein and gene-gene interactions among the candidate genes of AMH. These studies can provide important information to design gene knockout experiments to explore drug targets for treatment of subjects with unusually high or low AMH concentration.

CONCLUSIONS

Serum concentration of AMH is a complex trait (controlled by several QTL) like most other quantitative traits. We estimated a heritability (0.36 ± 0.03) for AMH that was large relative to economically important reproductive traits. The moderate to high heritability of AMH along with the known positive association of AMH concentration with numerous indirect and direct

measures of fertility in cattle indicate that AMH might be useful to indirectly improve predictions of genetic merit of some reproductive traits. Our GWA study, in conjunction with another recent GWA study on superovulatory responses in dairy cattle, further indicates considerable overlap between the genes that influence serum AMH and those that affect superovulatory traits in cattle. Future studies are needed to further establish the genetic correlation of AMH with superovulation and other economically important traits in dairy cattle.

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