Single-step genome-wide association for longitudinal traits of Canadian Ayrshire, Holstein, and Jersey dairy cattle

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ABSTRACT

Estimating single nucleotide polymorphism (SNP) effects over time is essential to identify and validate candidate genes (or quantitative trait loci) associated with time-dependent variation of economically important traits and to better understand the underlying mechanisms of lactation biology. Therefore, in this study, we aimed to estimate time-dependent effects of SNP and identifying candidate genes associated with milk (MY), fat (FY), and protein (PY) yields, and somatic cell score (SCS) in the first 3 lactations of Canadian Ayrshire, Holstein, and Jersey breeds, as well as suggest their potential pattern of phenotypic effect over time. Random regression coefficients for the additive direct genetic effect were estimated for each animal using single-step genomic BLUP, based on 2 random regression models: one considering MY, FY, and PY in the first 3 lactations and the other considering SCS in the first 3 lactations. Thereafter, SNP solutions were obtained for random regression coefficients, which were used to estimate the SNP effects over time (from 5 to 305 d in lactation). The top 1% of SNP that showed a high magnitude of SNP effect in at least 1 d in lactation were selected as relevant SNP for further analyses of candidate genes, and clustered according to the trajectory of their SNP effects over time. The majority of SNP selected for MY, FY, and PY increased the magnitude of their effects over time, for all breeds. In contrast, for SCS, most selected SNP decreased the magnitude of their effects over time, especially for the Holstein and Jersey breeds. In general, we identified a different set of candidate genes for each breed, and similar genes were found across different lactations

for the same trait in the same breed. For some of the candidate genes, the suggested pattern of phenotypic effect changed among lactations. Among the lactations, candidate genes (and their suggested phenotypic effect over time) identified for the second and third lactations were more similar to each other than for the first lactation. Well-known candidate genes with major effects on milk production traits presented different suggested patterns of phenotypic effect across breeds, traits, and lactations in which they were identified. The candidate genes identified in this study can be used as target genes in studies of gene expression.

Key words: milk, random regression, SNP effect, test-day

INTRODUCTION

Traits measured repeatedly during an individual's lifetime or physiological cycle are termed "longitudinal traits." Most economically important longitudinal traits in dairy cattle are lactation-related variables, such as milk, fat, and protein yields and SCS (Miglior et al., 2017; Oliveira et al., 2019c). The variability observed among repeated records for those longitudinal traits is due to changes in the physiological state of the animal over the lactation period. Such changes in the trait variation over the lactation period are likely due to differential gene expression or changes in the effects of QTL over time, as suggested by the changes in additive genetic variance and heritability over lactation (e.g., Miglior et al., 2009; Oliveira et al., 2017; Sasaki et al., 2017). In this context, a previous study showed evidence of differential sets of candidate genes underlying the phenotypic variation of longitudinal traits in different lactation stages of the Canadian Ayrshire, Holstein, and Jersey breeds (Oliveira et al., 2019b). Furthermore, gene expression studies have indicated that the amount of RNA synthesized often varies across lactations (Bionaz and Loor, 2008), and that specific genes or QTL

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can be differently expressed depending on the lactation stage (Strucken et al., 2011). Nonetheless, various studies on longitudinal traits have combined repeated records for each animal into a single estimate (e.g., average over all time points or accumulated yield), which are then used as response variables for genomic prediction of breeding values (e.g., Ding et al., 2013; Li et al., 2014; Jenko et al., 2017) and genome-wide association studies (**GWAS**; e.g., Buitenhuis et al., 2014; Raven et al., 2014; Nayeri et al., 2016).

Using a single estimate derived from repeated records when performing GWAS may compromise the understanding of the genetic architecture and underlying biological mechanisms of longitudinal traits. This approach does not enable the unraveling of genomic regions and candidate genes with differential effects along the lactation curve. For instance, a certain QTL might have a highly significant effect in a specific lactation stage (e.g., lactation peak), but when averaging its effect across the whole lactation, it might be substantially reduced, which would consequently hamper the identification of this QTL, if combining repeated records. Therefore, using statistical models that consider all the variability from repeated records can increase the statistical power to detect time-dependent QTL and, consequently, candidate genes affecting traits at specific stages of the lactation. In this context, Das et al. (2011) showed that it is possible to use random regression models (**RRM**) to exploit specific covariance functions to describe changes in SNP effects over time. By analyzing SNP associations over time, QTL with greater effects at specific times are more likely to be identified, which may help to better explain the observed phenotypic variation in longitudinal traits over time (Strucken et al., 2015). Currently, this approach is the most sophisticated and flexible tool to perform GWAS for longitudinal traits. However, despite the SNP availability and several GWAS reports for milk-related traits in dairy cattle, the use of RRM to estimate SNP effects over time remains underexplored, especially when considering different dairy cattle breeds.

Estimating SNP effects over time for various breeds is essential to identify and validate candidate genes (or QTL) associated with time-dependent effects of economically important traits across breeds, and to better understand the underlying mechanisms of lactation biology. However, this task can be difficult because the effects of QTL and the allele frequencies can differ across breeds, especially if a small number of animals is genotyped. In populations with a smaller number of genotyped animals (e.g., Canadian Ayrshire and Jersey) the use of the single-step genomic BLUP method (**ssGBLUP**; Misztal et al., 2009; Aguilar et al., 2010;

Christensen and Lund, 2010) can yield more accurate genomic EBV (**GEBV**) for estimating the SNP effects over time. Therefore, this study aimed to (1) estimate time-dependent effects of SNP associated with milk (**MY**), fat (**FY**), and protein (**PY**) yields, and SCS in the first 3 lactations of Canadian Ayrshire, Holstein, and Jersey breeds, using multiple-trait RRM and ssGBLUP; and (2) identify candidate genes associated with the analyzed traits, across lactations and breeds, and suggest their potential pattern of phenotypic effect.

MATERIALS AND METHODS

No animal care committee approval was necessary for the purposes of this study, as all information required was obtained from existing databases.

Phenotypic, Genotypic, and Pedigree Data

The Canadian Dairy Network (**CDN**; Guelph, ON, Canada) provided the December 2012 pedigree, genotypes, and the MY, FY, PY, and SCS test-day records (phenotypes) from the first 3 lactations of Ayrshire, Holstein, and Jersey breeds. Phenotypic quality control was performed to remove outlier test-day records for each trait (mean $\pm$ 3 SD, within contemporary group, which were defined by the combination of herd and test-day), and contemporary groups that were formed from fewer than 3 animals. Details on the criteria and number of test-day records removed in each step can be found in Oliveira et al. (2019a). The average MY, FY, PY, and SCS over DIM estimated for the Ayrshire, Holstein, and Jersey breeds are shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-16821>).

Initially, the number of animals genotyped with low-density (<20K) SNP panels was 300, 2,270, and 202; with medium-density (>20K and <60K) SNP panels was 944, 17,105, and 1,157; and with high-density (>60K) SNP panels was 749, 2,459, and 158 for Ayrshire, Holstein, and Jersey, respectively. Analyses in this study were performed using the 50K SNP panel. Therefore, only SNP present on the 50K panel were kept for the animals genotyped using high-density panels. In addition, animals genotyped with low- and medium-density panels were imputed by CDN to 50K using the FImpute software (Sargolzaei et al., 2011) for each breed separately. Details about the genotype imputation process performed by CDN, as well as the average and minimum accuracy of imputation for the Canadian breeds, are presented in Larmer et al. (2014). After the imputation process, only genotyped animals born before December 2012 were kept in the analyses and used to perform genotypic quality control

Table 1. Number of animals included in the pedigree file, herd-test-day (HTD), number of animals with phenotypes in each lactation, genotyped animals, and SNP used in the analyses for Ayrshire, Holstein, and Jersey breeds

Trait ¹	Ayrshire	Holstein	Jersey
MY, FY, and PY			
Pedigree	204,429	5,085,542	157,718
HTD	104,985	2,258,332	70,629
Phenotypes ²			
Lactation 1	129,912 (7.57)	3,791,813 (7.32)	89,693 (6.77)
Lactation 2	130,094 (5.13)	3,789,780 (5.03)	89,685 (4.84)
Lactation 3	130,112 (3.79)	3,795,917 (3.40)	89,623 (3.48)
Genotypes ³	1,827 (634)	20,663 (8,517)	1,427 (849)
SNP ⁴	38,096	40,658	34,500
SCS			
Pedigree	195,099	4,983,668	154,123
HTD	91,101	2,091,168	64,199
Phenotypes			
Lactation 1	121,974 (6.88)	3,682,503 (6.84)	86,102 (6.29)
Lactation 2	122,091 (4.76)	3,679,049 (4.74)	86,187 (4.50)
Lactation 3	122,214 (3.57)	3,686,040 (3.23)	86,257 (3.27)
Genotypes	1,827 (634)	20,663 (8,517)	1,427 (849)
SNP	38,096	40,658	34,500

¹Milk (MY), fat (FY), and protein (PY) yields in the first, second, and third lactations were analyzed separately from SCS in the first, second, and third lactations.

²Number of animals with phenotypes (average number of phenotypes per animal within parentheses).

³Total number of genotyped animals (bulls within parentheses).

⁴SNP were spread along the 29 bovine autosomes.

(within breed). Then, SNP with Mendelian conflicts, call rate <0.95, minor allele frequency (**MAF**) <0.01, and with a difference between observed and expected heterozygosity frequency >0.15 (Wiggans et al., 2009) were removed using the preGSf90 software (Aguilar et al., 2014). The total number of animals included in the pedigree file, herd-test-days, phenotyped animals per lactation, genotyped animals, and SNP that remained in the analyses for each breed after quality control are shown in Table 1.

Statistical Analyses

Prediction of Breeding Values. Breeding values for random regression coefficients of each animal were estimated using ssGBLUP and RRM for each breed separately. The same RRM currently used in the official genetic evaluations performed by CDN were used in this study: one considering MY, FY, and PY in the first 3 lactations (i.e., 9-trait model); and another considering SCS in the first 3 lactations (i.e., 3-trait model). Since 2016, SCS has usually been analyzed separately from the production traits to improve the stability of SCS proofs published by CDN (CDN, 2016). Both RRM included herd-test-day as fixed effect, a fixed regression for age-parity-season of calving, and random regressions for herd-year of calving, and animal additive genetic and permanent environmental effects, as

routinely implemented by CDN in Canada (Interbull, 2018). All regressions were modeled using the fourth order Legendre orthogonal polynomials (Kirkpatrick et al., 1990) on DIM (which ranged from 5 to 305 d).

In ssGBLUP analyses, the pedigree-based relationship matrix (**A**) traditionally used in the mixed model equations is replaced by the **H** matrix, which combines the pedigree and genomic information. In summary, the **H** matrix can be described as (Aguilar et al., 2010; Christensen and Lund, 2010):

$$\mathbf{H}^{-1} = \mathbf{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & \tau(0.95\mathbf{G} + 0.05\mathbf{A}_{22})^{-1} - \omega\mathbf{A}_{22}^{-1} \end{bmatrix},$$

where **G** is the genomic relationship matrix [calculated using the first method presented in VanRaden (2008) and the observed allele frequencies], **A** is the pedigree-based relationship matrix (considering animals up to 10 generations back and accounting for inbreeding coefficients), and $\mathbf{A}_{22}^{-1}$ is the inverse of the section of **A** related to the genotyped animals. To make **A** and **G** more compatible, the optimal values for the scaling factors (τ and ω) reported by Oliveira et al. (2019a), using the same populations, were used in this study. The values used for τ and ω are summarized in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-16821>). Solutions of the mixed model equations were

obtained using the BLUP90IOD software (Miszta et al., 2002, 2014), with a modified preconditioner for high-order multiple-trait RRM (Ignacio Aguilar, Instituto Nacional de Investigacion Agropecuaria, Canelones, Uruguay, personal communication). Prediction reliability and bias of breeding values for each trait, lactation, and breed, as well as complete description of the statistical models used in this study are detailed in Oliveira et al. (2019a).

Derivation of SNP Effects from Additive Random Regression Coefficients. The SNP solutions for the random regression coefficients were calculated using postGSf90 software (Aguilar et al., 2014). In summary, the postGSf90 software back-solves the additive genomic random regression coefficients (i.e., the GEBV for the additive random regression coefficients) to SNP effects. In a simple way, the back-solving process can be briefly described, for each trait, lactation, and breed, as follows (Wang et al., 2014):

$$\hat{\mathbf{u}}_c = \mathbf{M}'[\mathbf{M}\mathbf{M}']^{-1}\widehat{\mathbf{GEBV}}_c,$$

where $\hat{\mathbf{u}}_c$ is the vector of estimated SNP solutions for the c th random regression coefficient; $\mathbf{M}$ is the matrix that contains the centered genotypes (i.e., 1, 0, and -1 representing AA, Aa, and aa, respectively), and $\widehat{\mathbf{GEBV}}_c$ is the vector of GEBV for the c th random regression coefficient estimated by the ssGBLUP, which contains the c th random regression coefficient from all genotyped animals.

Thereafter, the SNP solutions for all random regression coefficients ($c = 1, 2, \dots, 5$) of the same SNP k were combined into a vector $\left[\hat{\mathbf{u}}_k = [\hat{u}_{k1} \hat{u}_{k2} \hat{u}_{k3} \hat{u}_{k4} \hat{u}_{k5}]'\right]$,

and used to estimate the SNP effects for all DIM (from 5 to 305 d) as $\mathbf{SNP}_k = \mathbf{T}\hat{\mathbf{u}}_k$, where $\mathbf{SNP}_k$ is the vector that contains the SNP effects estimated for every DIM of the k th SNP, $\mathbf{T}$ is a matrix of covariates for each DIM, associated with the Legendre orthogonal polynomials, and $\hat{\mathbf{u}}_k$ is the vector of SNP solutions for all random regression coefficients related to the k th SNP.

Selection of Relevant SNP and Cluster Analyses. The SNP were selected for further investigation based on the magnitude of their effects. In this context, the SNP were ranked according to the magnitude of their daily effects (considering all DIM in the same rank), for each trait, lactation, and breed. The top 1% of SNP that showed a high magnitude of SNP effect in at least one DIM were selected as relevant SNP to be further investigated. The strategy for selecting the top 1% SNP has been implemented in several GWAS (Silva et al., 2014; Abdalla et al., 2016; Mastrangelo et al.,

2018), and seems to be sufficient to show changes in SNP effects over time between breeds.

To facilitate the visualization of results and posterior discussion, the SNP selected as relevant were clustered according to the trajectory of their SNP effects over time. Therefore, an algorithm was created in R (R Development Core Team, 2016) to assign each SNP to 1 of 5 pre-established clusters ($\mathbf{C}$; defined in preliminary analysis based on graphical inspection and biological knowledge of the traits; results not shown): C1 = constant SNP effect over time; C2 = SNP effect increases over time; C3 = SNP effect decreases over time; C4 = SNP effect increases in the middle of lactation; and C5 = SNP effect decreases in the middle of lactation. In brief, the created algorithm estimates the average of the daily SNP effect for 3 different lactation stages: (1) from 5 to 95 DIM; (2) from 96 to 215 DIM; and (3) from 216 to 305 DIM, as previously defined in Oliveira et al. (2019b), and then assigns the SNP to their most likely cluster based on the pattern of those averages. Averages that did not differ by more than 10% from one to another lactation stage were considered as constant over time. No significance tests were performed on these differences, because the estimated pattern of the lactation curve depends on the combination of all Legendre coefficients, making the test of individual coefficients not very useful.

To describe the differences among clusters, the average of the coefficient of variation ($\mathbf{CV}$) was estimated for each cluster. The CV of the k th SNP was calculated as follows:

$$CV_k (\%) = \frac{s_k}{\bar{X}_k} \times 100,$$

where s_k is the standard deviation for the k th SNP over time, and $\bar{X}_k$ is the k th SNP mean.

Gene Identification: Gene Mapping, Overrepresentation Analysis, and Candidate Genes

Positional candidate genes were mapped using the Biomart tool (Kinsella et al., 2011) embedded in the Ensembl Genes database version 94 (<http://useast.ensembl.org/index.html>), using the bovine genome UMD 3.1 as the reference genome. Genes located within ± 250 kb from the location of the selected SNP were used in the overrepresentation analysis (**ORA**).

The ORA was performed using the Medical Subject Headings (**MeSH**) vocabulary to retrieve annotations that appear more frequently in a selected gene group relative to their occurrence by chance in a set of refer-

ence genes (Nelson et al., 2004). The statistical significance (P -value) of this overrepresentation analysis was assessed by a hypergeometric test (Adams and Skopek, 1987):

$$P = \sum_{i=k}^{\min(M,n)} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}},$$

where N is the total number of genes that were analyzed for each trait (reference genes), M is the total number of selected genes, n is the total number of genes in the MeSH term under study, k is the number of selected genes that belong to the MeSH term under study, and $\binom{y}{x} = \frac{y!}{x!(y-x)!}$ is the binomial coefficient. To increase the power of test, all lactations from the same trait and breed were analyzed together in the ORA. The ORA was performed using the MeSH ORA tool (Tsuyuzaki et al., 2015), available in the Bioconductor package of the R software (R Development Core Team, 2016).

Genes retrieved from significant ($P < 0.01$) MeSH terms related to milk production traits were considered candidate genes for the traits, lactations, and breeds that they were identified. To understand the biological processes related to the studied traits and to define the most likely functional candidate genes, complete gene functions were obtained from the National Center for Biotechnology Information database (NCBI; www.ncbi.nlm.nih.gov/gene/) and Animal QTL Database (QTLdb; www.animalgenome.org/cgi-bin/QTLdb/BT/index).

RESULTS AND DISCUSSION

Selection of the Most Relevant SNP and Cluster Analysis

The number of selected SNP, their average SNP effects, and average of CV estimated for each cluster are shown in Figure 1 for all traits, lactations, and breeds. A total of 381, 407, and 345 SNP were selected as the most relevant (top 1%) for each trait/lactation of the Ayrshire, Holstein, and Jersey breeds, respectively. In general, most SNP selected for MY, FY, and PY increased the magnitude of their effects over time (i.e., most SNP were clustered in C2) for all breeds. In contrast, for SCS, most selected SNP decreased the magnitude of their effects over time (i.e., most SNP were clustered in C3), especially for the Holstein and Jersey breeds, which might coincide with a higher prevalence

of mastitis in early lactation for those breeds (Dego and Tareke, 2003). No SNP whose magnitude of effect decreased in the middle of lactation (C5) were found, for any of the traits, lactations, or breeds. In addition, no SNPs whose magnitude of effect increased in the middle of lactation (C4) were found for FY in the Ayrshire and Jersey breeds, or for SCS in the first lactation of all breeds.

Except for SCS in the first lactation of all breeds and FY in the first lactation of the Holstein breed, only a few SNP were found to have approximately constant effects over DIM (Figure 1). These results, combined with the different trajectory of SNP effects over time shown by the majority of SNP, suggest a differential phenotypic effect over time, which is in agreement with Strucken et al. (2011). For all traits, the average SNP effect was higher for Holstein than for the Ayrshire and Jersey breeds, which is likely due to the higher average MY in Holstein (i.e., scale effect). These findings are in agreement with Oliveira et al. (2019b), who reported genomic windows with a higher proportion of the explained genetic variance in the Holstein breed compared with the Ayrshire and Jersey breeds, and with Pryce et al. (2010), who found that the average variation explained by the significant SNP was higher in Holstein than in Jersey. Considering the average effects across selected SNP for all lactations, a single SNP changed the daily MY, FY, PY, and SCS by approximately 12.4 g, 0.4 g, 0.4 g, and 1.4, respectively, in the Ayrshire breed; by 39.4 g, 1.7 g, 1.0 g, and 7.8, respectively, in the Holstein breed; and by 7.4 g, 0.4 g, 0.2 g, and 0.9, respectively, in the Jersey breed. For instance, this means that the QTL associated with an average SNP would be responsible for about 0.50 kg (i.e., 1.7 g $\times$ 301) of FY in one lactation of the Holstein breed, for example. This is in agreement with Strucken et al. (2012), when studying German Holstein Friesian cows, who concluded that one marker could change overall FY by approximately 0.46 kg.

Especially for MY in the Holstein breed, the average SNP effect was higher in C2 for the first lactation, and in C4 for the second and third lactations, compared with the other clusters (Figure 1). This finding suggests that some genomic regions with a large effect in MY have an increasing effect over DIM in the first lactation, and higher effects in the middle of lactation in the second and third lactations. Thus, genomic regions with large effects in MY seem to have higher persistency in their SNP effect in the first lactation than in the second and third lactations, which could be related to the lower lactation peak and higher lactation persistency found in the first lactation of the Holstein breed compared with later lactations (Stanton et al., 1992; Márquez et al., 2016). Similar results were found for

Figure 1. Number of selected SNP, average (μ) of their effect, and coefficient of variation (CV) in percentage, estimated in each cluster for all traits and lactations of Ayrshire, Holstein, and Jersey breeds. Cluster 1 = constant SNP effect over time; cluster 2 = SNP effect increases over time; cluster 3 = SNP effect decreases over time; and cluster 4 = SNP effect increases in the middle of lactation. Traits: milk (MY1, MY2, MY3), fat (FY1, FY2, FY3), and protein (PY1, PY2, PY3) yields, and SCS (SCS1, SCS2, SCS3) in the first, second, and third lactations, respectively.

FY in the Holstein breed, whose higher average SNP effect was observed in C1 compared with the other SNP clusters, especially for the second and third lactations (Figure 1). Similar average SNP effects were estimated among different clusters for all other traits and breeds. As expected, higher CV were estimated in C2 and C3 than in the other clusters (Figure 1). The trajectory of SNP effects over DIM estimated for the top 10 SNP of each cluster for each trait and lactation are shown in Supplemental Figures S2, S3, and S4 (<https://doi.org/10.3168/jds.2019-16821>), for the Ayrshire, Holstein, and Jersey breeds, respectively.

ORA and Candidate Genes

The ORA is used to retrieve genes that appear more frequently in a selected group relative to their occurrence by chance (Nelson et al., 2004). Currently, several GWAS have used ORA to reduce the number of candidate genes and, consequently, to find the most likely candidate genes related to traits of interest in various species, including cattle, swine, horses, and chicken (Morota et al., 2015, 2016; Oliveira et al., 2019b). In this study, the ORA reduced the number of candidate genes, which consequently facilitated the interpretation

Figure 2. Number of common candidate genes for milk yield in the different lactations of Ayrshire, Holstein, and Jersey breeds. Cluster 1 = constant SNP effect over time; cluster 2 = SNP effect increases over time; cluster 3 = SNP effect decreases over time; and cluster 4 = SNP effect increases in the middle of lactation.

of the results. The number of candidate genes found in common after the ORA for MY, FY, PY, and SCS in different lactations is shown in Figures 2, 3, 4, and 5, respectively. A summary of the total number of candidate genes found for each cluster is given in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-16821>).

As expected, due to the number of phenotypic records available and SNP selected for each breed (Table 1), the number of candidate genes identified was, in general, higher for the Holstein breed, followed by the Ayrshire and Jersey breeds, respectively. With the exception of SCS for the Holstein and Jersey breeds, the highest number of candidate genes was found in C2 for all traits and breeds, which suggests that the majority of genes related to milk-related traits have the phenotypic effect increased over DIM. In general, the same candidate gene was not found in more than one cluster, which suggests that SNP associated with the same candidate gene have similar SNP effect patterns over time, and that the method used to cluster the SNPs was efficient. In accordance with Oliveira et al. (2019b), similar genes were found across different lactations for the same trait of a same breed (Figures 2 to 5). For some of the candidate genes, the suggested pattern of phenotypic effect changed among lactations (i.e., some

candidate genes were found in different clusters in different lactations; Supplemental Tables S3 to S6; <https://doi.org/10.3168/jds.2019-16821>). Among lactations, candidate genes (and the suggested phenotypic effect over time) identified for the second and third lactations were more similar to each other than to those identified for the first lactation. This may be related to the fact that the activity level of different genes that contribute to milk-related traits in different lactations depends on the metabolic changes happening in the individual (Strucken et al., 2011), and in the first lactation, cows are still growing; that is, the mammary gland and digestive tract are under development (Taylor et al., 2003).

The number of candidate genes found in common for each breed is shown in Figure 6. Similar to the findings reported by Oliveira et al. (2019b), a different set of candidate genes was identified for each breed. From the different sets of candidate genes found, only 2 were found across all 3 breeds. Considering only the candidate genes identified in more than one breed, they were more similar between Ayrshire and Holstein breeds. This reinforces the hypothesis that differences exist in the underlying lactation biology and genes associated with milk-related traits across breeds or populations. Detailed information regarding the candidate genes

Figure 3. Number of common candidate genes for fat yield in the different lactations of Ayrshire, Holstein, and Jersey breeds. Cluster 1 = constant SNP effect over time; cluster 2 = SNP effect increases over time; cluster 3 = SNP effect decreases over time; and cluster 4 = SNP effect increases in the middle of lactation.

mapped through Ensembl for the SNP selected in each cluster after the ORA is given in Supplemental Tables S3, S4, S5, and S6 (<https://doi.org/10.3168/jds.2019-16821>), for MY, FY, PY, and SCS, respectively. Only selected candidate genes will be discussed in detail in this paper.

Candidate Genes for MY

The number of candidate genes in common was higher between Ayrshire and Holstein breeds (Figure 6). Among the candidate genes in common for MY were *ACO2*, *BOP1*, *DGAT1*, *FBXL6*, *IGLL1*, and *SLC5A1* (for Ayrshire and Holstein), *KPNA7* (for Ayrshire and Jersey), *GNG12* and *SLC25A4* (for Holstein and Jersey), and *COL4A3* (for all breeds). However, only *ACO2*, *KPNA7*, and *SLC25A4* were found in the same clusters for the mentioned breeds. The *BOP1*, *DGAT1*, *FBXL6*, *IGLL1*, and *SLC5A1* genes were grouped in C2 for Ayrshire (all lactations) and Holstein (only in the first lactation). For the second and third lactations of the Holstein breed, these candidate genes were grouped in either C1 or C4. The *GNG12* gene was found in C3 for the Holstein breed and in C2 for the Jersey breed. In addition, *COL4A3* was found in C2 for the Ayrshire

and C1 for the Holstein and Jersey breeds. Details regarding the candidate genes found in each cluster for MY are shown in Supplemental Table S3 (<https://doi.org/10.3168/jds.2019-16821>).

No candidate genes with constant suggested effect over DIM (i.e., candidate genes associated with SNP clustered in C1) were found for MY in the Ayrshire breed. Genes such as *IGFBP3*, *ACO2*, *ANGPTL4*, *FASN*, *CCL28*, *PRLR*, and *BTN1A1* showed an increasing effect over time for all 3 lactations. For instance, *BTN1A1* has been linked to the secretion of milk lipid droplets because it is highly expressed in the mammary epithelium during lactation (Jeong et al., 2009). The *CCL28* gene has been reported to play a role in lactation persistence in Canadian Holstein cattle (Do et al., 2017), which may explain its increased effect over time here for the Ayrshire breed. As discussed by Tong et al. (2018), milking frequency affects lactation persistency, expression of the PRL receptor (*PRLR*), and the milk response to prolactin inhibition (Bernier-Dodier et al., 2011; Lacasse et al., 2011). Therefore, it is possible that some factors modulating milk production are affecting prolactin secretion or the responsiveness of the mammary gland to this hormone (Tong et al., 2018).

Figure 4. Number of common candidate genes for protein yield in the different lactations of Ayrshire, Holstein, and Jersey breeds. Cluster 1 = constant SNP effect over time; cluster 2 = SNP effect increases over time; cluster 3 = SNP effect decreases over time; and cluster 4 = SNP effect increases in the middle of lactation.

The genes *MAP2*, *DGAT1*, *IGLL1*, and *SLC5A1* showed an increasing effect over DIM (C2) and were suggested as candidate genes only for the second and third lactations of the Ayrshire breed. These genes are well known to affect MY and other milk-related traits (Nilsen et al., 2009; Huang et al., 2012). Cerri et al. (2012), investigating the effects of lactation and pregnancy on endometrial gene expression on day 17 of the estrous cycle and pregnancy, reported that *IGLL1* was upregulated by lactation. The casein group (i.e., *CSN1S1*, *CSN1S2*, and *CSN3* genes) was suggested as a candidate with increased phenotypic effect in the middle of lactation, only in the first lactation. Interestingly, *CSN1S1* was suggested to have a decreasing effect over DIM in the second lactation. Strucken et al. (2012) reported that the largest effects for the casein genes occurred at the beginning of the lactation, but they did not find statistical significance for MY. In addition, Strucken et al. (2012), studying how the influence of genomic regions changes during the first 60 DIM in German Holstein Friesian cattle, showed that *DGAT1* had increasing effects for MY.

Some genes were identified to be affecting all production traits (i.e., MY, FY, and PY) in all lactations of Ayrshire, with differential phenotypic effect patterns,

such as *ANGPTL4*, which is known to play an important role in lipid metabolism. Wang et al. (2018) investigated variations of serum *ANGPTL4* in periparturient and early lactation dairy cows and reported that serum *ANGPTL4* concentrations peaked at parturition, and declined rapidly over the following 2 wk, indicating that energy balance changes associated with parturition contributed significantly to this effect. Koltes and Spurlock (2012) reported that the abundance of *ANGPTL4* mRNA expression increased 2- to 6-fold with declining energy balance. According to those authors, the consistency and magnitude of the increase in *ANGPTL4* mRNA across multiple models of altered energy balance identifies it as an adipokine that is uniquely responsive to changes in energy balance in the lactating dairy cow. In our study, depending on the trait, this gene was clustered in different groups (e.g., C1 or C2).

For the Holstein breed, our results suggested that *COL4A3* has a constant effect over DIM for all 3 lactations. In addition, *IGLL1* and *SLC5A1* showed a constant effect over DIM but only for the second and third lactations. The candidate genes *GNG12* and *TNFRSF1A* were suggested to have a decreasing effect over DIM for all 3 lactations. Interestingly, genes on BTA14 are well known for their effects in MY; for

Figure 5. Number of common candidate genes for SCS in the different lactations of Ayrshire, Holstein, and Jersey breeds. Cluster 1 = constant SNP effect over time; cluster 2 = SNP effect increases over time; cluster 3 = SNP effect decreases over time; and cluster 4 = SNP effect increases in the middle of lactation.

example, *BOP1*, *CPSF1*, *CYHR1*, *DGAT1*, *EEF1D*, *FBXL6*, *FOXH1*, *LRRC24*, *PTK2*, *RECQL4*, *TONSL*, *TRAPPC9*, and *VPS28* had increasing effects over DIM in the first lactation and a higher effect in the middle of lactation in the second and third lactations. This suggests that the pattern of phenotypic effect for these genes also changes across lactations, even though they remain important in all lactations. The *EEF1D* gene has previously been associated with fat percentage (Jiang et al., 2010), which is a trait dependent on the level of MY and FY across lactation. The importance of this gene for various milk traits and breeds highlights its importance in lactation biology. In addition, the different pattern of phenotypic effect suggested for genes on BTA14 may explain the higher average SNP effect in C2 for the first lactation and in C4 for the second and third lactations. Differences compared with the conclusions presented by Strucken et al. (2012) for the *DGAT1* gene are probably because of the short period studied by those authors and the use of MY averaged over the first 3 lactations. Thus, Strucken et al. (2012) could not observe suggested decrease in phenotypic expression after the middle of lactation for the second and third lactations.

Wang et al. (2015a) investigated the expression pattern of the clock circadian regulator network and selected metabolic genes simultaneously in mammary gland, liver, and subcutaneous adipose tissue to better understand the transition from late gestation to early lactation. The authors reported that the expression of *PPARA* was lower at -10 d but increased after parturition. However, Schäff et al. (2013) reported that *PPARA* expression decreased continuously after parturition, whereas we observed an effect increase in the middle of lactation (C4) for Holstein.

For the Jersey breed, the *SLC34A2*, *STAT5A*, *IGF1R*, *KPNA7*, and *SLC5A2* genes showed increasing effects over DIM in all 3 lactations (C2). The *STAT5A* gene, a member of the JAK-STAT signaling pathway, has been associated with proliferation, secretory differentiation, and survival of mammary epithelial cells and milk synthesis (Furth et al., 2011; Liu et al., 2012; Ao et al., 2015). Additionally, *STAT5A* mediates the pathway to regulate milk protein synthesis, mammary gland epithelial cell differentiation (Faraci-Orf et al., 2006; Buser et al., 2007), and mastitis susceptibility (Usman et al., 2014). Interestingly, the pattern of phenotypic effect suggested for *ACTB* changed completely

Figure 6. Number of common candidate genes in the different breeds for milk (a), fat (b), and protein (c) yields, and SCS (d).

among lactations, as it had a suggested constant effect over time in the first lactation (C1), a decreasing effect in the second lactation (C3), and a higher effect in the middle of lactation, in the third lactation (C4). The *IKBKB*, *NRG1*, and *ANXA11* genes were suggested as candidate genes with increasing effect over time for the second and third lactations. In agreement with Oliveira et al. (2019b), no potential effect of *DGAT1* gene was found for MY in the Jersey breed.

Candidate Genes for FY

No common candidate genes were identified among all 3 breeds (Figure 6). Among the candidate genes in common for FY between 2 breeds were *ACO2*, *PTK2*, and *TRAPPC9* (for Ayrshire and Holstein); *KPNA7* (for Ayrshire and Jersey); and *BOP1* and *DGAT1* (for Holstein and Jersey). In this context, *ACO2*, *KPNA7*, *BOP1*, and *DGAT1* were found in the same clusters for the mentioned breeds. However, *PTK2* and *TRAPPC9* were grouped in C2 for the Ayrshire (found only in the first lactation) and C1 for the Holstein breed (all lactations). Details regarding the candidate genes found in each cluster for FY are shown in Supplemental Table S4 (<https://doi.org/10.3168/jds.2019-16821>).

The *CSN1S1* and *PTK2* genes were suggested as candidate genes only for the first lactation of the Ayr-

shire breed, and they had suggested constant (C1) and increasing (C2) effects over DIM, respectively. The *ACP6*, *IGFBP3*, *ANGPTL4*, *SLC5A1*, *DCXR*, *FASN*, and *PYCR1* genes had increasing effects over DIM (C2), for all 3 lactations. Bionaz and Looor (2008) studied mammary tissue mRNA expression across changes in physiological state, including different lactation stages, and they reported that *FASN* is associated with fatty acid synthesis and desaturation. Moreover, these authors reported that the peak expression of *FASN* was around 60 DIM.

For the Holstein breed, the *BOP1*, *DGAT1*, *FBXL6*, *OPLAH*, *PTK2*, and *TRAPPC9* genes had constant effect over DIM (C1) for all 3 lactations. However, as suggested by the average SNP effect estimated in the clusters, some important genes on BTA14 had different phenotypic effect patterns in the first lactation compared with second and third lactations. Those genes were *CPSF1*, *CYHR1*, *FOXH1*, *LRRC24*, *RECQL4*, *SLC39A4*, *TONSL*, and *VPS28*, and they had an increasing effect over DIM in the first lactation and a constant effect in the second and third lactations. For instance, *CPSF1* and *CYHR1* have been reported to be associated with milk fat components (Buitenhuis et al., 2014; Jiang et al., 2014; Nayeri et al., 2016); *FOXH1* has also been linked to milk-related traits (Nayeri et al., 2016). The *ADIPOR2* gene showed an

increasing effect in the first and second lactations and a decreasing effect over DIM in the third lactation. Weber et al. (2016) reported that *ADIPOR2* expression changed from late gestation to early lactation. Ohtani et al. (2011) investigated the expression of *ADIPOR2* in various bovine tissues and mammary glands among different lactation stages, and the effects of lactogenic hormones (insulin, dexamethasone, and prolactin) and growth hormone in cultured bovine mammary epithelial cells (BMEC). They reported that in BMEC, lactogenic hormones and growth hormone upregulated *ADIPOR2* mRNA expression. Loor et al. (2006) studied temporal gene expression profiles during the dry period and early lactation in liver of Holstein cows. They identified 85 genes with expression patterns that were affected by level of energy intake prepartum over time. Restricted energy intake prepartum resulted in more pronounced upregulation of genes with key functions in hepatic fatty acid oxidation that were also identified in our study (*ADIPOR2*), and ad libitum feeding upregulated several genes associated with liver triacylglycerol synthesis (*DGAT1*) and proinflammatory cytokines (*TNFAIP3*). However, in our study, *TNFAIP3* was associated with SCS. Other well-known candidate genes were suggested to have the same phenotypic effect pattern over DIM in all 3 lactations, such as *AGPAT3*, *APP*, *ACO2*, *DNTT*, *FAS*, *IFIT3*, and *IFIT5*, which had an increasing effect over DIM, and *EEF1D*, which had a suggested decreasing effect over DIM for all 3 lactations.

Similar to results for Holstein, the *BOP1* and *DGAT1* genes were suggested to have a constant effect over DIM for all 3 lactations in the Jersey breed. Those results corroborate with those of Oliveira et al. (2019b), which also reported the *BOP1* and *DGAT1* genes as being important along lactation for FY in Jersey. The *SLC34A2*, *SLC25A11*, and *IKBKB* genes were found to have increasing effects over DIM in all 3 lactations. In contrast, the *IGFBP2* and *FTO* genes were suggested to have increasing effects over DIM only for the second and third lactations. Zielke et al. (2013) investigated the biological role of *FTO* (fat mass and obesity associated gene) on milk composition in German Holstein cattle. They reported that higher-frequency SNP alleles and haplotypes within the *FTO* gene increased FY and PY by up to 2.8 and 2.2 kg per lactation, respectively. According to those authors, the pleiotropic effects observed suggest a biological function on the usage of energy resources and the control of energy balance rather than directly affecting fat and protein synthesis. Clempson et al. (2012) reported that polymorphisms in *IGFBP2* were associated with the circulating patterns of glucose, insulin, and BHB, particularly around second calving. Interestingly, for the Jersey breed, we observed that this gene was identified in second and

third parities for FY. Clempson et al. (2012) also reported associations between the *IGFBP2* SNP and 305-d yield in the first lactation, as well as trends of association with DIM in first and second lactations in UK dairy cows.

Candidate Genes for PY

Differently from the other traits, more candidate genes were in common for PY between the Ayrshire and Jersey than for the Ayrshire and Holstein breeds (Figure 6). Among the candidate genes found were *ACO2* (for Ayrshire and Holstein), and *COL4A3*, *CSN1S1*, *NPC1*, *PDIA3*, *RPL37* (for Ayrshire and Jersey). In summary, the *ACO2*, *COL4A3*, *NPC1*, and *PDIA3* candidate genes were found in the same clusters for the mentioned breeds. In contrast, *CSN1S1* was grouped in C2 and C3 for the Ayrshire breed (in the first and second lactations, respectively), and C1 for the Jersey breed (all lactations). The *RPL37* gene was grouped in C2 and C4, for the Ayrshire and Jersey breeds, respectively. Details regarding the candidate genes found in each cluster for PY are shown in Supplemental Table S5 (<https://doi.org/10.3168/jds.2019-16821>).

The majority of candidate genes found for PY were suggested to have an increasing effect over DIM. Specifically for the Ayrshire breed, *IGFBP3*, *ACO2*, *ANGPTL4*, *IGLL1*, *SLC5A1*, *EIF1*, *FASN*, and *PDIA3* genes were suggested as candidate genes for all 3 lactations. Interestingly, *CSN1S1* was suggested as a candidate gene with a decreasing effect over DIM for the second lactation. Strucken et al. (2012) found statistical significance in the decreasing gene expression of *CSN1S1* over time for PY, when studying the first 60 DIM of German Holstein Friesian cattle.

For the Holstein breed, *GNG12* was suggested as a candidate gene with constant effect over DIM, for the first lactation. The *APP*, *ACO2*, *PLCB4*, and *BoLA* genes were suggested to have an increasing effect over DIM for all 3 lactations. The bovine major histocompatibility complex (*BoLA*) genes encode proteins involved in the presentation of extracellular antigens to CD4⁺ T cells (Ellis et al., 1999), and are known to have a significant role in the development of immunological response and disease resistance (Stear et al., 2001; Ellis and Codner, 2012). Various studies have associated the *BoLA* genes with clinical mastitis, SCS, and antibody-mediated and cell-mediated immune responses in Holstein cattle (Wu et al., 2010; Yoshida et al., 2012; Thompson-Crispi et al., 2014). Interestingly, we identified an association of *BoLA* with MY, PY and FY, but not with SCS. Pokorska et al. (2018) also identified a higher MY for a specific allele in Holstein cattle. The *BOP1*, *DGAT1*, *EEF1D*, *FBXL6*, and *PTK2* genes were suggested as

candidate genes only for the first lactation. In addition, it is important to notice that the *AGPAT1*, *BMP5*, and *CYP21* genes were suggested as candidate genes for the first and second lactations, and that *FAS*, *IFIT3*, and *IFIT5* were suggested as candidate genes for the second and third lactations; all had an increasing effect over DIM. Our findings regarding the potential pattern of phenotypic effect of the candidate genes identified for PY disagree with those reported by Lu and Bovenhuis (2019), in a recent study analyzing 1,829 Dutch Holstein first-parity cows. In that study, the authors used 4 different GWAS approaches to analyze PY, which included separate GWAS for specific lactation stages, and GWAS using repeatability models (assuming both SNP effects constant during lactation or genotype by lactation stage interaction); they concluded that there was no evidence that their effects changed during lactation.

For the Jersey breed, the casein group (i.e., *CSN1S1*, *CSN1S2*, and *CSN3* genes), *STATH*, and *PRLR* genes were identified as having a constant effect over DIM for the first lactation. The *SLC34A2*, *TLR4*, *PDIA3*, and *NPC1* genes were suggested as candidate genes with increasing effects over DIM, for all 3 lactations. The *TLR4* gene, which was identified to be important for MY and PY, had been previously associated with lactation persistency in Canadian Holstein bulls (Sharma et al., 2006), as well as MY, FY, and fat and protein percentage and other milk composition traits (Viale et al., 2017; Zhou et al., 2017). The *IKBKB* and *NRG1* genes were found to have an increasing effect over DIM only for the second and third lactations. Interestingly, *RPL37* was suggested to have a higher effect in the middle of lactation in the first lactation. The gene *MAPK1*, which was identified for all 4 traits in this study, regulates milk protein synthesis (Lu et al., 2012).

Candidate Genes for SCS

Only a few candidate genes were identified in common for SCS between the breeds (Supplemental Table S6; <https://doi.org/10.3168/jds.2019-16821>). Among the important candidate genes identified for SCS in more than one breed were *EEF1D*, *SNCA*, *TNFRSF1A* (for Ayrshire and Holstein); *ABCG2* (for Holstein and Jersey); and *TRAPPC9* (for all 3 breeds). Interestingly, all candidate genes identified for SCS were found in different clusters among the mentioned breeds. In this context, the *EEF1D* gene was found either in C1 or C2 for the Ayrshire, and in C3 for the Holstein breed. The *SNCA* and *TNFRSF1A* genes were found in C2 for the Ayrshire, and in either C1 or C3 for the Holstein breed. The *ABCG2* gene was found in C1 or C3 for the Holstein and in C4 for the Jersey breed. The *TRAPPC9*

gene was found in C1 or C3 for the Ayrshire, C1 or C2 for the Holstein, and C2 or C3 for the Jersey breed. Details regarding the candidate genes found in each cluster for SCS are shown in Supplemental Table S6 (<https://doi.org/10.3168/jds.2019-16821>).

For Ayrshire, *TUBB2B* and *SLC5A2* were suggested as candidate genes with increasing effect over DIM, for all lactations, whereas *ACTB* gene was suggested as a candidate gene with decreasing effect over DIM, for all lactations. Interestingly, the *ACP6*, *TNFAIP3*, *CAPN3*, and *TRAPPC9* genes were suggested as candidate genes with constant effect in the first lactation and with decreasing effect in the second and third lactations. In the same way, the *EEF1D* gene was suggested as a candidate gene with constant effect in the first lactation, whereas it seemed to increase its effect in the second and third lactations.

For the Holstein breed, genes such as *BOP1*, *DGAT1*, *EEF1D*, *FBXL6*, *C4BPA*, and *C4BPB* were suggested as candidate genes with higher effects in the middle of lactation, for all 3 lactations. However, although the *TNFRSF1A* and *SNCA* genes also seemed to have a higher effect in the middle of lactation for the second and third lactations, they had constant effects in the first lactation. The *ABCG2*, *FAM13A*, and *HTRA4* genes were suggested as candidate genes with constant effect in the first and second lactations and with higher effects in the middle of lactation for the third lactation. The ATP-binding cassette sub-family G member 2 (*ABCG2*) gene has been associated with various milk-related traits in Holstein and other dairy breeds, including milk production (Pausch et al., 2017), coagulation properties (Cecchinato et al., 2015), and fatty acid composition (Li et al., 2016). In addition, various signature of selection studies have identified this gene as playing an important role in milk production traits (e.g., Rothammer et al., 2013; Zhao et al., 2015). Li et al. (2016) reported an association between *FASN*, *ABCG2*, and *IGF1* on milk fatty acid composition. Wei et al. (2012) concluded that *ABCG2* plays an important role in mammary epithelial cell proliferation and that functional polymorphisms in this gene may influence the cellular compartment of the mammary gland and potentially milk production. As there is an inferred positive correlation between mammary cell numbers and milk production, mammary progenitor cells may be important in renewing damaged mammary cells during lactation (Capuco and Ellis, 2005), which could also explain its differential association with SCS during the lactation. Mani et al. (2009) investigated the differential expression and localization of lipid transporters in the bovine mammary gland during the pregnancy-lactation cycle and reported that *ABCG2* mRNA abundance was significantly increased after parturition and declined

from the lactating to the nonlactating state in the bovine mammary gland. In accordance with this hypothesis, for the Holstein breed, *ABCG2* was identified in C1, whereas, for Jersey, it was clustered in C4 (higher effect in the middle of lactation). Do et al. (2017) suggested the association of this gene with lactation persistence. The *NRG1* gene has been strongly correlated with the progression of *Staphylococcus aureus* subclinical mastitis (Song et al., 2016). The *SOCS3* gene, identified for SCS in our study, was previously thought to have a primary role in apoptosis and tissue remodeling during involution through programmed cell death and tissue remodeling, initiated after the termination of lactation (Sutherland et al., 2007). Arun et al. (2015) reported that the levels of *SOCS3* expression increased from pregnancy to involution.

For the Jersey breed, the *SLC25A4* gene was suggested to have a constant effect and the *SLC34A2* and *PRLR* genes were suggested to have increasing effects over DIM, for all 3 lactations. Prolactin receptor (*PRLR*), known to be associated with production traits and SCS (Meredith et al., 2012), was found to be down-regulated in bovine mammary epithelial cells infected with *Staphylococcus aureus* (Lara-Zárate et al., 2011), as well as in bovine hepatic tissue following intramammary injection of *Escherichia coli* LPS to simulate mastitis infection (Jiang et al., 2008). Prolactin was found to be significantly increased in udder quarters with high SCS and chronic mastitis (Boutet et al., 2007). In addition, *PRLR* has been suggested to be associated with mammary gland epithelium development and mammary gland alveolus development and milk production traits (Lü et al., 2011; Viale et al., 2017; Marete et al., 2018). The *COL4A3* and *TRAPPC9* genes were suggested as candidate genes with increasing effects over DIM in the first lactation, and decreasing effects over DIM in the second and third lactations. Wang et al. (2015b) identified that *TRAPPC9* was associated with mastitis susceptibility in Chinese Holstein cattle.

CONCLUSIONS

Our results suggest evidence of differential sets of candidate genes underlying the phenotypic variation of the analyzed longitudinal traits across breeds. Moreover, the suggested pattern of phenotypic effect for some of the candidate genes changed among lactations and breeds. Well-known candidate genes with major effects on milk production traits showed different suggested patterns of possible phenotypic effect across breeds, traits, and lactations in which they were identified. This information about candidate genes affecting more or less strongly the analyzed traits at specific stages of lactation may contribute to increase the genetic gain in

breeding programs, by taking into account the shape of the lactation curves. In addition, the candidate genes identified in this study can be used as target genes in gene expression studies, which are necessary to validate our findings.

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