Differentially Expressed Genes in Endometrium and Corpus Luteum of Holstein Cows Selected for High and Low Fertility Are Enriched for Sequence Variants Associated with Fertility[1]

Stephen G. Moore,[3,4,5] Jennie E. Pryce,[6] Ben J. Hayes,[6] Amanda J. Chamberlain,[6] Kathryn E. Kemper,[7] Donagh P. Berry,[4] Matt McCabe,[8] Paul Cormican,[8] Pat Lonergan,[5] Trudee Fair,[5] and Stephen T. Butler[2,4]

[4]Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, County Cork, Ireland
[5]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin, Ireland
[6]Department of Economic Development, Jobs, Transport and Resources & Dairy Futures Cooperative Research Centre, Agribio, La Trobe University, Bundoora, Australia
[7]Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Australia
[8]Animal and Grassland Research and Innovation Centre, Teagasc, Grange, Dunsany, County Meath, Ireland

ABSTRACT

Despite the importance of fertility in humans and livestock, there has been little success dissecting the genetic basis of fertility. Our hypothesis was that genes differentially expressed in the endometrium and corpus luteum on Day 13 of the estrous cycle between cows with either good or poor genetic merit for fertility would be enriched for genetic variants associated with fertility. We combined a unique genetic model of fertility (cattle that have been selected for high and low fertility and show substantial difference in fertility) with gene expression data from these cattle and genome-wide association study (GWAS) results in ~20 000 cattle to identify quantitative trait loci (QTL) regions and sequence variants associated with genetic variation in fertility. Two hundred and forty-five QTL regions and 17 sequence variants associated primarily with prostaglandin $F_{2\alpha}$, steroidogenesis, mRNA processing, energy status, and immune-related processes were identified. Ninety-three of the QTL regions were validated by two independent GWAS, with signals for fertility detected primarily on chromosomes 18, 5, 7, 8, and 29. Plausible causative mutations were identified, including one missense variant significantly associated with fertility and predicted to affect the protein function of *EIF4EBP3*. The results of this study enhance our understanding of 1) the contribution of the endometrium and corpus luteum transcriptome to phenotypic fertility differences and 2) the genetic architecture of fertility in dairy cattle. Including these variants in predictions of genomic breeding values may improve the rate of genetic gain for this critical trait.

corpus luteum, differentially expressed genes, endometrium, fertility, genetic merit, genome-wide association, transcriptomics, variants

INTRODUCTION

The genetic basis of variation in fertility between individuals is of great interest in mammals, particularly humans and livestock. While a number of studies have identified genetic variants affecting male fertility in humans and cattle (e.g., Fries et al. [1] and Kosova et al. [2]), female fertility is more challenging to dissect, as the trait has a low heritability and collection of phenotypes is difficult. One of the first genome-wide association studies (GWAS) for human female fertility was recently published [3].

Dairy cattle are a potential model for dissecting the genetic basis of fertility in mammals that have one (or occasionally two or more) offspring per parturition and gestation times of approximately 9 mo—fertility phenotypes are routinely recorded in large volumes in several countries, and whole genome sequence data are available for the key ancestors of modern dairy cattle populations [4]. Dairy cattle fertility also has a high economic value in its own right, with evidence that fertility in dairy cattle has declined significantly in recent decades [5, 6]. The causes of this decline are multifactorial [5, 6] and include negative pleiotropic effects with variants improving milk production [7]. More recently, greater selection intensity for fertility traits [8] and improved reproductive management [9, 10] have halted the downward trend in female fertility in the Holstein-Friesian breed, and in some populations fertility has improved [11]. More rapid improvement, however, is necessary to return the fertility of the Holstein-Friesian breed to previous levels and to improve the economic viability of dairy farming.

The establishment and maintenance of pregnancy involves a complex interplay between the endometrium, the embryo, and the corpus luteum (CL) [12, 13]. The endometrium, a mucosal membrane lining the lumen of the uterus, promotes embryo development via secretions in the histotroph [14–16], and is also involved in the regulation of the estrous cycle [17]. After ovulation, cellular reorganization and angiogenesis of the ovulatory follicle are essential to create a highly vascularized CL capable of producing a rapid rise in progesterone (P4)
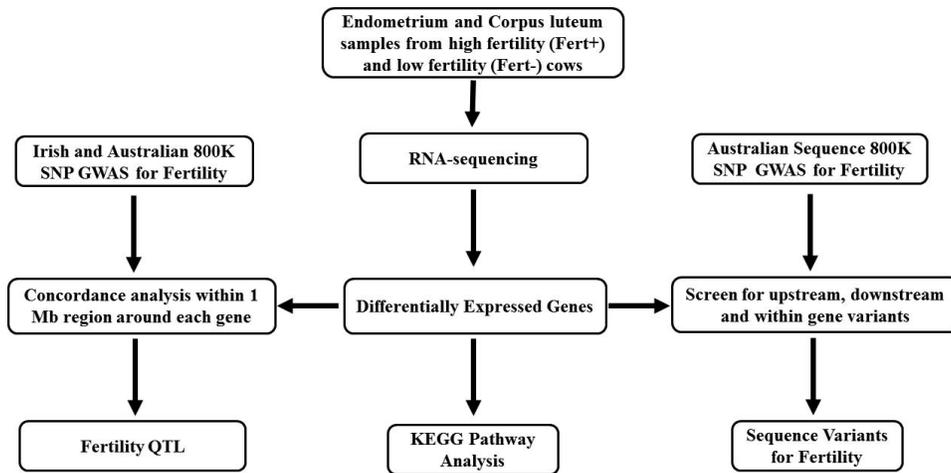
FIG. 1. Outline of the experimental design.

concentrations [18]. The endometrium and CL are obvious targets for gene expression studies to detect differentially expressed genes (DEG), for example, between high-fertility and low-fertility cattle [19]. Another method used to identify genomic variation involved in a trait is a GWAS. A number of GWAS have been conducted for fertility traits in cattle [20–25] and in humans [3]. In cattle, genomic variation associated with fertility traits was detected on BTA 1, 5, 13, 16, and 18 [26]; however, to date, there has been little agreement between studies. This is partly due to the complex nature of fertility traits but also due to insufficient power, inconsistencies in the fertility traits used, and the high significance threshold required to avoid detecting false positives [26]. An alternative approach is to use prior information from candidate gene or functional pathway studies to focus on specific genomic regions that are likely to harbor variants directly affecting biological processes. The advantage of this approach is that less stringent significance thresholds can be applied than with a traditional GWAS since the false discovery rate is reduced [27–29].

In this study, we used global gene expression profiles from endometrium and CL and GWAS and imputed sequence data to identify variants associated with dairy cow fertility. The experimental design is illustrated in Figure 1. Differentially expressed genes in the CL and endometrium affecting fertility were identified using a unique resource herd of cows with similar genetic merit for milk production traits but either good (Fert+) or poor (Fert−) genetic merit for fertility [30, 31]. The results of this study enhance our understanding of both the contribution of the endometrium and CL transcriptome to phenotypic reproductive performance and the genetic architecture affecting fertility in a higher mammal that has a small number of offspring per parturition and also enables us to identify genetic variants that could be used to accelerate genomic selection for improved fertility in dairy cattle.

MATERIALS AND METHODS

Lactating Holstein Cow Genetic Model of Fertility

A lactating cow genetic model of fertility was established in Teagasc Moorepark, Ireland, to elucidate the mechanisms responsible for suboptimal fertility in lactating Holstein dairy cows [30]. Briefly, heifers of >75% Holstein ancestry with either extreme positive (i.e., poor fertility; Fert−) or negative (i.e., high fertility; Fert+) estimated breeding value (EBV) for calving interval were selected from the Irish national dairy cattle database. Genetic evaluations for calving interval are undertaken three times annually in a multitrait genetic evaluation model that includes the first five parity records for calving interval

and other reproductive traits. Within the Irish national herd, the selected heifers represented the top 25% in genetic merit for milk production. Fert− heifers represented the bottom 5% in genetic merit for calving interval, whereas Fert+ heifers represented the top 20% in genetic merit for calving interval. In subsequent years, herd replacements were generated by selecting suitable artificial insemination (AI) sires to maintain the difference in genetic merit for calving interval. The selection criteria for candidate sires were >200 kg predicted transmitting ability (PTA) for milk production, positive PTA for milk fat and protein concentration, and possessing >75% Holstein genetic ancestry. Sires with >5 days (mean = 6.50, SD = 1.54) PTA for calving interval were selected for mating with Fert− cows, and sires with <5 days (mean = −5.47, SD = 1.12) PTA for calving interval were selected for mating with Fert+ cows.

Fourteen cows were enrolled in an ovulation synchronization protocol, 8 Fert+ and 6 Fert−. The EBVs of the cows from both genotypes are summarized in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org). Fert+ and Fert− cows were sired by five and six sires, respectively. The experimental procedures involving animals were licensed by the Department of Health, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EEC. The management of the Fert+ and Fert− cows has been described in detail elsewhere [32]. Mean calving dates were 19 February (SD ± 22.3 days) and 20 February (SD ± 16.8 days) for the Fert+ and Fert− cows, respectively.

Ovulation Synchronization

Cows were enrolled in an ovulation synchronization protocol (CIDR_TAI) described previously [33] to facilitate collection of tissue samples on fixed calendar dates. Mean days postpartum (±SD) when cows were enrolled in the protocol were 56 ± 5.4 (range: 47–63) and 56 ± 3.6 (range: 50–61) for the Fert+ and Fert− cows, respectively. On Day −10 of the protocol, each cow was administered an i.m. injection of a gonadotropin-releasing hormone (GnRH) agonist containing 10 µg of buserelin (Receptal; Intervet Ireland), and a controlled internal drug release device containing 1.38 g of P4 (CIDR; Pfizer Ireland) was inserted per vaginum. On Day −3, each cow was administered an i.m. injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) containing 25 mg of dinoprost tromethamine (Lutalyse; Pfizer Ireland). On Day −2, the CIDR device was removed, and 36 h later, each cow was administered a second i.m. injection of GnRH agonist.

Tissue Biopsies

On Day 13 of the estrous cycle, endometrium and CL biopsies were collected from each cow as described previously [34, 35]. Briefly, cows were sedated with intravenous xylazine (1 mg/100 kg body weight), and caudal epidural anesthesia was induced using 4 ml of 2% lidocaine to prevent abdominal straining. The vulva and perineal area were sanitized with antiseptic solution and dried. The luteal biopsy was performed using a tissue biopsy needle (16 gauge, 48 cm, trocar tip; SABD-1648-15-T; US Biopsy) placed in the needle guide of an ovum pickup probe (7.5-MHz convex transducer; Esaote Pie Medical Equipment B.V.). The endometrial biopsy was collected from a site in the uterine horn ipsilateral to the CL with an endometrial biopsy tool (Kruuse) approximately 3 cm past the uterine bifurcation. Tissue samples were rinsed with saline, blotted dry, and trimmed of any connective or myometrial

tissue. Biopsy samples were immediately snap frozen in liquid nitrogen and stored at −80°C.

RNA Extraction

Total RNA was extracted from endometrium (eight Fert+ and six Fert−) and CL (seven Fert+ and five Fert−) tissue samples using a Trizol-based method [36]. Total RNA was purified using the RNeasy Plus Mini kit (Qiagen) removing RNAs <200 nucleotides and any genomic DNA contamination. The RNA quality and concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC) and a 2100 Bioanalyzer system (Agilent Technologies UK) using the RNA Nano Chip. The 260:280-nm absorbance ratio ranged from 1.85 to 2.13 for all samples. The RNA integrity number and 28S:18S ratio ranged from 7.0 to 9.4 and from 1.2 to 2.2, respectively.

cDNA Library Preparation and Sequencing

The mRNA samples were converted to cDNA libraries for sequencing following the protocol of the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina). RNAseq libraries were amplified by 11 cycles of PCR. Library concentration was determined by Qubit (Invitrogen) and quality was determined using DNA-1000 chips on a Bioanalyser 2100 (Agilent Technologies). A random-block design was used to reduce the risk of technical bias in the experimental design. Each sample was sequenced on a single lane over a total of two flow cells on the Illumina HiSeq 2500 platform to generate 40 million 75-base paired-end reads, and FASTQ files were created using CASAVA v1.9 (Illumina).

mRNA Sequence Quality and Alignment

FASTQC v0.10.0 was used to perform basic quality control checks on raw sequence data. Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) was used to remove adaptor sequences and low-quality bases from the 3′ end of the sequence reads; reads less than 20 bases in length were then discarded. The remaining reads were aligned to the bovine genome (UMD3.1 assembly) [37] using STAR v2.3.0 [38], allowing two mismatches to account for sequencing errors and single nucleotide polymorphisms (SNP). The variation in gene body coverage by the RNA-Seq reads from 5′ to 3′ ends was assessed using RSeQC [39]. Only uniquely mapped reads were retained for downstream analysis; featureCounts [40] was used to assign uniquely aligned reads to Ensembl (v73) annotated exons. Reads mapping to multiple features or overlapping genes were discarded. Read counts for all samples were amalgamated into two matrices for endometrium and CL, respectively, for subsequent differential expression analysis.

Differential Analysis of Gene Expression

Differential expression analysis of the endometrium and CL count data was performed separately using the Bioconductor software package edgeR [19] with the R statistical programming language. Genes with <1 count per million in only six endometrial samples or five CL samples (the lowest level of replication) were removed from the data set. Library size was normalized by the trimmed mean of M-values. The edgeR package assumes that RNA-seq data have a negative binomial distribution. A fixed effects model was fitted to the read counts (expressed as counts per million) for each gene, with genotype (Fert+, Fert−), parity (2, 3, 4), and sample date (n = 4) all included as fixed effects. DEG were identified based on the likelihood ratio test. P-values were adjusted using the Benjamini and Hochberg [41] method with a false discovery rate of 0.05 to correct for multiple testing.

Ensembl Biomart (http://www.ensembl.org/biomart) was used to search the UMD 3.1 database for descriptions of the DEG. Attempts were made to annotate genes described as uncharacterized proteins by analyzing their protein coding sequence with the NCBI Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A summary of the endometrium and CL RNA-seq data processing steps is shown in Supplemental Table S2.

Pathway Analysis of Differentially Expressed Genes

Pathway analysis of the DEG was conducted by overrepresentation analysis using GOSeq (v1.16.2) in the R statistical programming language [42]. GOSeq accounts for gene length bias. The KEGG database (release 71.0) was used to define pathways that contain significantly more DEG than would be expected by chance given the background set of all genes found expressed in the tissue [43].

GWAS Using High-Density Genotypes

Illumina (http://www.illumina.com) high-density (BovineHD) genotypes (777 962 SNP) were available for 719 Holstein-Friesian AI bulls from Ireland; all animals had a call rate of >95%. Illumina BovineHD genotypes were available for 1620 Holstein bulls and cows and 125 Jersey bulls from Australia. The genotypes were edited using the genotype quality control processes described previously for the Irish genotypes [44] and for the Australian genotypes [45]. Briefly, for the Australian genotypes, quality criteria included the Illumina GenCall score, which describes the performance of genotyping each SNP in each individual. Genotype calls with GenTrain score (GenCall) <0.6 were excluded, leaving 650 934 SNP genotyped at GenCall >0.6. Furthermore, 343 mitochondrial SNP, 1124 Y chromosome SNP, and 1735 unmapped SNP were excluded. Some 55 SNP with duplicate map positions were removed, as were SNPs that were mismapped [45]. Individuals with fewer than 90% of SNP genotyped at GenCall <0.6 were removed, as were animals with excess heterozygosity (>0.4), as this is a good indicator of sample contamination. Five animals were identified with heterozygosity above this threshold; however, all of these had already been removed in the step above (i.e., >90% of SNP genotyped). For the Irish genotypes, only unique autosomal SNP with a known position, <0.5% Mendelian inconsistencies between parent-progeny pairs, a minor allele frequency >0.02. and not deviating from Hardy-Weinberg equilibrium ($P < 0.1 \times 10^{-7}$) were retained. Following quality control, 630 337 and 616 350 segregating autosomal SNP remained in the Irish and Australian data sets, respectively. A total of 4682 AI bulls in Ireland and 15 190 bulls and cows in Australia (10 644 Holstein and 4546 Jersey, with 2806 Holstein bulls, 716 Jersey bulls, and the remainder cows in each breed) had Illumina BovineSNP50 bead-chip genotypes (54 001 SNP); after applying similar quality control edits as for the HD genotypes, all lower-density genotypes were used to impute, within country, BovineHD genotypes using Beagle [46].

The fertility trait evaluated was calving interval, in the case of bulls, the average of their daughters' calving interval. Calving interval is the length in time in days from one calving to the next. In the absence of adequate amounts of mating and pregnancy data, genetic evaluations are often based on calving interval data, which is the case in both Australia and Ireland. In Australia, 72% of cows have calving interval data, while only 15% have mating data, and 5% have pregnancy test results [47]. Predicted transmitting ability for calving interval for each of the Irish genotyped bulls was available from the December 2013 Irish genetic evaluations. Calving interval in Ireland is evaluated in a multitrait model (with calving to first service interval, number of services, and survival), treating calving interval in each of the first five parities as separate traits. The single PTA value per animal is the average of each of the individual parity PTAs. Predicted transmitting ability values were deregressed using the full pedigree as described previously [48]. Only animals with a PTA reliability >40% were retained for the GWAS. The final data set for the Irish GWAS consisted of 2660 sires.

Calving interval trait deviations (for cows) and daughter trait deviations (for bulls) for the genotyped animals were available from the Australian Dairy Herd Improvement Scheme official April 2013 evaluation for the genotyped animals in Australia. Trait deviations were calculated within breed and were corrected for fixed effects, including contemporary group, age, permanent environment, and heterosis. Daughter trait deviations were the average of trait deviations for a bull's daughters.

The GWAS in both countries were undertaken separately in WOMBAT [49] using a series of univariate animal linear mixed models, where each SNP was fitted one at a time as a continuous fixed effect (i.e., number of copies of an allele) in the model. In the Australian GWAS, additional fixed effects for breed, gender, and gender nested within breed were also included in the statistical model. The direct additive genetic effect of the animal was included as a random effect linked to the pedigree file. Phenotypes were weighted by a function of the information contributing to that phenotypic record as outlined previously [50]. Test statistics were obtained for each SNP separately.

Concordance Analysis

We investigated if the DEG from the endometrium and CL identified above were enriched for significant SNP from the GWAS. The genomic position of each DEG was identified using the *Bos taurus* genes (UMD3.1) data set downloaded from Ensembl BioMart (v73) database (http://www.ensembl.org/biomart). A region 500 kb flanking either side of the center of each DEG was calculated. The DEG were considered validated by the GWAS results if the number of SNP significant at $P < 1 \times 10^{-3}$ in a 1-Mb interval encompassing the DEG was greater than expected by chance. The false discovery threshold was calculated as mP/n, where m is the total number of SNP tested, P is the P-value, and n is the number of variants that were actually significant. If the number of SNP associated with fertility in a 1-Mb interval was greater than

TABLE 1. Endometrial genes determined to be differentially expressed between Fert+ and Fert− cows on Day 13 of the estrous cycle.

| | | | | | Gene | | Concordance | | | |
| | | | | | | | Australia[c] | | Ireland[d] | |
Ensembl gene ID	Gene name	LogFC[a]	P-value[b]	Chr	Start	Stop	Sig SNP[e]	Validated[f]	Sig SNP[e]	Validated[f]
ENSBTAG00000019547	LILRA6*	−1.91	0.05	18	63 146 729	63 154 412	43	Yes	31	Yes
ENSBTAG00000020406	GPC3[#]	1.61	0.03	X	17 305 874	17 770 661	—	—	—	—
ENSBTAG00000005260	SPP1	1.76	0.03	6	38 120 578	38 127 577	0	No	0	No
ENSBTAG00000011985	FLVCR2	−1.76	0.03	10	87 879 330	87 907 703	0	No	0	No
ENSBTAG00000013492	PRKAG3[AU]	1.63	0.03	2	107 509 452	107 516 981	1	Yes	0	No
ENSBTAG00000022396	SAA3*	1.80	0.02	29	26 668 047	26 671 801	1	Yes	2	Yes
ENSBTAG00000011873	KCNE3[IE]	2.30	0.004	15	54 587 990	54 588 289	0	No	10	Yes
ENSBTAG00000022570	PGFS2*	2.01	0.004	13	44 064 647	44 085 039	1	Yes	2	Yes
ENSBTAG00000047383	ABCC4[AU]	3.09	< 0.0001	12	71 822 642	71 987 841	19	Yes	0	No

[a] Log fold change of DEG for Fert− cows relative to Fert+ cows. Positive values indicate greater expression in Fert− cows.
[b] Significance level after controlling for multiple testing (Benjamini & Hochberg [41]).
[c] Concordance between DEG in the endometrium of Fert+ cows and Fert− cows on Day 13 of the estrous cycle and the Australian fertility GWAS.
[d] Concordance between DEG in the endometrium of Fert+ cows and Fert− cows on Day 13 of the estrous cycle and the Irish fertility GWAS.
[e] The number of single nucleotide polymorphisms significantly associated with calving interval in the 1-Mb interval surrounding the gene.
[f] A gene was validated if the number of Sig SNP in the 1-Mb interval surrounding the gene was greater than expected by chance at a false discovery rate of 10^{-3}.
* Indicates DEG validated by both Irish and Australian fertility GWAS.
[#] It was not possible to determine the concordance of GPC3 with both GWAS because SNP from chromosome 30 were not included.

expected by chance, then the region was deemed to have been validated (referred to as a QTL region). Concordance of DEG located on the X chromosome was not considered because SNP on the X chromosome were not included in the Australian and Irish GWAS.

GWAS Using Whole Genome Sequence

In an attempt to identify possible sequence variants that underlie the significant associations in the DEG regions, we imputed whole genome sequence variant genotypes in the gene region and 2 kb upstream and downstream of gene start and gene stop, respectively, into all animals used in the Australian GWAS, using the 1000-bull genomes sequence data reference set Run2.0 [4]. Beagle [46] was used for imputation. In Daetwyler et al. [4], the accuracy of imputation was assessed using a cross-validation approach where a subset of sequenced animals was dropped out, their genotypes taken down to the 777K HD genotypes subset; then their genotypes were imputed back to sequence using the remainder of the sequenced animals as a reference. Imputed genotypes were then correlated with the real sequence genotypes for the subset of animals. Accuracy of imputation was 0.9 for common variants but lower for variants with low minor allele frequency [4].

The model fitted to the sequence variants was as described for the Australian GWAS. Subsequently, the number of significant SNP ($P < 1 \times 10^{-5}$ and $P < 1 \times 10^{-8}$) within each DEG and 2 kb upstream and downstream of the gene start and stop position was counted. The false discovery threshold was calculated for each significance threshold as mP/n, where m is the total number of variants tested per threshold, P is the P-value of the threshold, and n is the number of variants that were actually significant at that threshold.

Availability of Supporting Data

The RNA-seq data are available through the NCBI gene expression omnibus, RNA-seq project accession code GSE74076. The 1000-bull genomes data are available through the NCBI sequence read archive, 1000-bull genomes project accession code SRP039339, run accessions SRR1293227, SRR1262614–SRR1262659, SRR1188706, SRR1262533, SRR1262536, SRR1262538, SRR1262539, and SRR1262660–SRR1262788.

RESULTS

Gene Expression in Endometrium and Corpus Luteum of High-Fertility and Low-Fertility Cows and Concordance with High-Density GWAS

Following stringent quality control, 19 066 636 and 19 632 540 read pairs per cow on average were obtained from sequencing the endometrium and CL libraries, respectively. Of these, ~65% and ~71% of the endometrium and CL sequence reads, respectively, were uniquely mapped to the bovine reference genome (UMD3.1) and overlapped with protein-coding genes. Gene body plots of the libraries indicated no bias in read coverage from 5′ to 3′ ends (Supplemental Figure S1). Of the 24 616 genes in the bovine genome, ~57% and ~51% (endometrium and CL, respectively) had sufficient coverage for differential expression following the quality control. Nine endometrial genes and 560 CL genes were differentially expressed between Fert+ and Fert− cows ($P \le 0.05$ corrected for multiple testing; Table 1 and Supplemental Table S3).

The GWAS for fertility with genome-wide SNP in the Australian and Irish populations identified a number of genome regions with significant SNP associated with fertility (Fig. 2). In the Australian GWAS, 293 SNP were significantly associated with fertility ($P < 1 \times 10^{-5}$), of which 30 were highly significant ($P < 1 \times 10^{-8}$). In the Irish GWAS, 568 SNP were significantly associated with fertility ($P < 1 \times 10^{-5}$), of which 15 were highly significant ($P < 1 \times 10^{-8}$). False discovery rates calculated at each threshold (Supplemental Table S4), and Q-Q plots (Supplemental Figure S2) indicated a low level of false-positive signals. Excluding DEG on the X chromosome, 547 individual DEG were identified across the endometrium and CL data sets, of which 203 (37%; indicated with AU) were deemed to be in QTL regions by the Australian GWAS and 245 (45%; indicated with IE) were deemed to be in QTL regions by the Irish GWAS (Fig. 2, Table 1, and Supplemental Table S3). Three hundred and fifty-five of the 547 DEG (65%) were deemed to be in QTL regions by either the Irish GWAS or the Australian GWAS. Ninety-three of the 547 DEG (17%) were deemed to be in QTL regions by both the Irish GWAS and the Australian GWAS (indicated with *). Of these 93 DEG, over half (54%) were located on the following chromosomes: BTA 18 (23%), 5 (9%), 7 (8%), 8 (8%), and 29 (6%).

Differences in Endometrial and Corpus Luteum Function Between Fert+ and Fert− Cows

The endometrial expression profile identified 1) more severe uterine inflammation in Fert− cows indicated by greater expression of serum amyloid A3 (SAA3*) and secreted phosphoprotein 1 (SPP1); 2) suboptimal energy status in Fert−
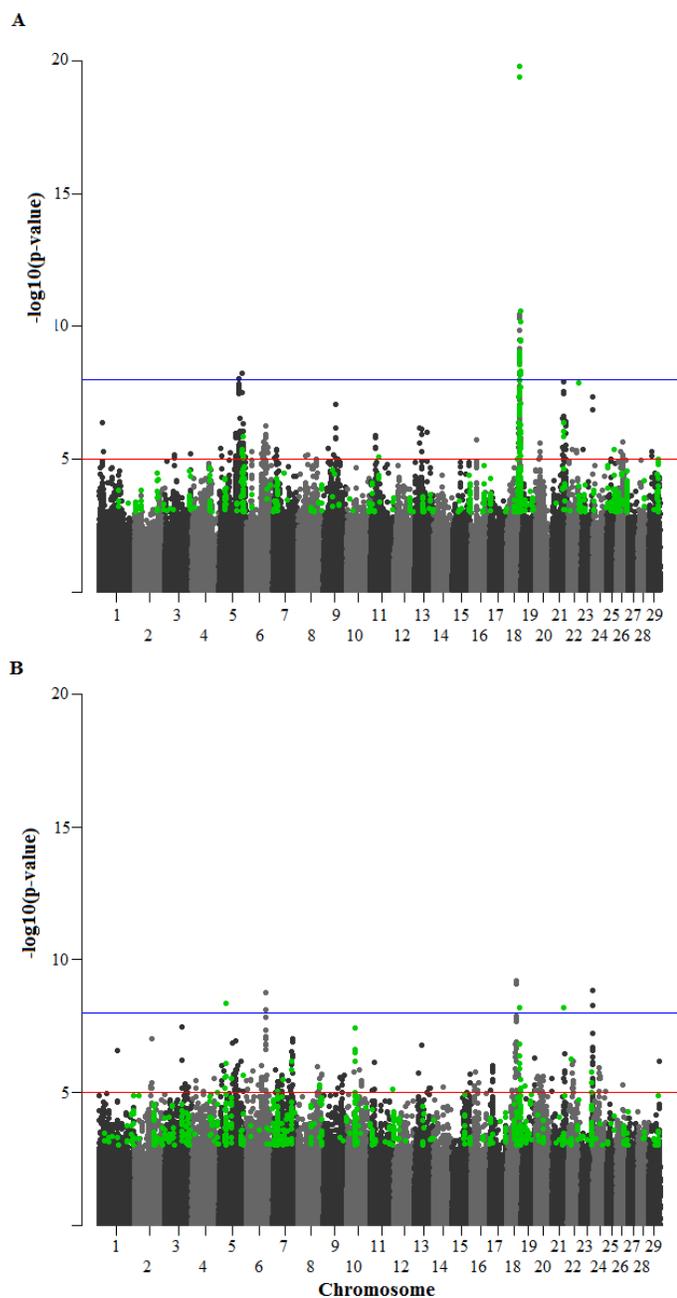
A

B

FIG. 2. Manhattan plots for calving interval in Australian (**A**) and Irish (**B**) dairy populations. The x-axis is the position of each SNP on the bovine chromosomes, and the y-axis is the $-log_{10}(P\text{-value})$. The SNP highlighted green are concordant with the differentially expressed genes. The red line represents the moderate significance threshold of $P < 1 \times 10^{-5}$. The blue line represents the high significance threshold of $P < 1 \times 10^{-8}$.

cows indicated by greater expression of protein kinase, AMP-activated, gamma 3 noncatalytic subunit (*PRKAG3*[AU]); and 3) greater PGF$_{2\alpha}$ synthesis and secretion in Fert− cows indicated by greater expression of prostaglandin F synthetase II-like (*PGFS2**) and ATP-binding cassette subfamily C member 4 (*ABCC4*[AU]) compared with Fert+ cows.

The luteal expression profile identified greater PGF$_{2\alpha}$ response in Fert− cows compared with Fert+ cows indicated by lesser expression of two homologs of ADAMTS-like 5 (*ADAMTSL5*[IE]), ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 (*ATP2A1*[AU]), and nuclear receptor subfamily 5, group A, member 1 (*NR5A1*), and greater expression of

crystallin, alpha B (CRYAB), inhibin, beta A (*INHBA**), interleukin 4 receptor (*IL4R*), serpin peptidase inhibitor, clade B (ovalbumin), member 2 (*SERPINB2*[IE]), thrombospondin 1 (*THBS1*[IE]), and tissue factor pathway inhibitor 2 (*TFPI2*[AU]). Reduced steroidogenesis in Fert− cows compared with Fert+ cows was indicated by lesser expression of *NR5A1*, two homologs of StAR-related lipid transfer (START) domain containing 9 (*STARD9*[IE]), patatin-like phospholipase domain containing 3 (*PNPLA3*[AU]), phospholipase A2, group IVB (cytosolic) (*PLA2G4B*[IE]), and patatin-like phospholipase domain containing 7 (*PNPLA7*) and greater expression of cytochrome P450, subfamily IIIA, polypeptide 4 (*CYP3A4*). Genes involved in the cytoskeleton, extracellular matrix (ECM), mRNA replication, zinc finger motifs, the cell cycle, DNA repair, and apoptosis were also differentially expressed between genotypes, with their expression primarily down-regulated in Fert− cows (Table 2). KEGG pathway analysis of DEG in the CL revealed overrepresentation of genes involved in the spliceosome pathway (*P*-value = 0.06). Three genes—leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 6 (*LILRA6**); *SAA3**; and feline leukemia virus subgroup C cellular receptor family, member 2 (*FLVCR2*)—were differentially expressed in both the endometrium and the CL, with the same direction of expression between genotypes in both tissues.

Sequence Variants GWAS for Fertility

When the imputed variants were tested for association with calving interval, 17 variants were significantly associated with fertility ($P < 1 \times 10^{-5}$) in the Australian dairy cattle population (Table 3), of which nine were highly significant ($P < 1 \times 10^{-8}$). On BTA 21 and BTA 18, one upstream variant of pre-mRNA processing factor 39 (*PRPF39**) and one upstream variant, one intron variant, and six downstream variants of ribosomal protein S9 (*RPS9**) had the strongest associations with fertility ($P < 1 \times 10^{-10}$). In addition to the variants flanking RPS9*, one upstream variant and one 3′UTR variant of leukocyte receptor cluster (LRC) member 8 (*LENG8**; both $P < 1 \times 10^{-8}$) and one downstream variant of *LILRA6** ($P < 1 \times 10^{-5}$) significantly associated with fertility were located within a 280-kb region at 63 Mb. Also on BTA 18, one upstream variant of lysine (K)-specific methyltransferase 2B (*KMT2B*) was associated with fertility. On BTA 7, one missense variant of eukaryotic translation initiation factor 4E binding protein 3 (*EIF4EBP3*) was significantly associated with fertility ($P < 1 \times 10^{-5}$). The missense variant of *EIF4EBP3* had a SIFT [51] value of 0.01, indicating that the amino acid substitution was predicted to affect protein function. On BTA 19, one 3′UTR variant and one missense variant of RecQ protein-like 5 (*RECQL5*[AU]) were associated with fertility ($P < 1 \times 10^{-5}$). The missense variant of *RECQL5*[AU] had a SIFT value of 0.51, indicating that the amino acid substitution was predicted to not affect protein function.

DISCUSSION

The results of this study indicate that the combination of transcriptomic data from key reproductive tissues with GWAS data and imputed whole genome sequence provided a novel and useful approach to elucidate the mechanisms contributing to suboptimal reproductive performance in lactating dairy cows. A differential expression analysis between high-fertility and low-fertility cows revealed nine DEG in the endometrium and 560 DEG in the CL. The DEG in the endometrium were involved primarily in processes associated with uterine inflammation, energy status, and PGF$_{2\alpha}$ synthesis and

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TABLE 2. Categories of differentially expressed genes in the corpus luteum between Fert+ and Fert− cows on Day 13 of the estrous cycle.

Gene category	Lesser expression in Fert− cows	Greater expression in Fert− cows
Cytoskeleton	ABLIM1*, ABTB1[AU], ANKRD11, ANKRD23, ANKRD32[IE], ANKS1A, ASB3[AU], ATAT1[IE], CCDC64, CCDC141, CCNL1, CCNL2*, CEP95[AU], CEP250[AU], CNTRL, CNTROB, CSPP1, DNAH1*, DST, KIFC2[AU], KIF7[AU], KLC2[AU], LOC522322, MACF1[IE], MAPT, PCNT, SHANK3, SPTBN2[AU], SPTBN5[IE], SYNE1[IE], SYNE2, TTLL3[IE], TUBGCP5, TUBGCP6, UBR4[IE], ZMYM3	ACTA2[AU], ANKRD1, TUBB6, CCDC80[IE]
Extracellular matrix	ABLIM1*, ATAT1[IE], DST, LAMC1[AU], TENC1*	ACTA2[AU], DPT, EFEMP1, FNI[AU], SPARC*, TAGLN[AU], TGFBI*, THBS1[AU], TNC*
mRNA replication	ACIN1, AKAP17A, CLASRP*, DDX5[AU], DDX39B, DDX46, EIF4EBP3, LOC618220[IE], LUC7L, PABPN1, PCF11, PRPF3*, PRPF38B[IE], PRPF39*, PRPF40B*, RBM5[AU], RBM25[IE], RPL12, RPL17, RPL36[IE], RPL36AL[IE], RPL39, RPS9*, RPS23[IE], RPS25, SF3B1, SFRS4[IE], SFRS5, SFRS11[IE], SFRS18[AU], SFSWAP, SREK1[IE], SUGP2, TCERG1[IE], U2SURP[IE]	RPS6KL1
Zinc finger	FLJ20531[IE], LOC528802*, MSS51*, ZBTB40[AU], ZC3H13[IE], ZDHHC1[AU], ZMYM3, ZMYND15[IE], ZNF34, ZNF76, ZNF192, ZNF236[AU], ZNF311, ZNF318[AU], ZNF454[AU], ZNF462*, ZNF500, ZNF512B, ZNF598*, ZNF605, ZNF784*, ZSCAN26, ZSWIM8*	
Cell cycle	CCAR2[IE], CDAN1[IE], CLK1[IE], CLK2[IE], CLK4[IE], two homologs of CCAR1[IE], PPP6R2, RTEL1, SFI1[IE], SPICE1, TRRAP[AU]	FBL*
DNA repair	FANCA, FANCG*, FANCP[IE], MDC1, PARP3*, PARP4, PARP6, RECQL5[AU]	
Apoptosis	ACIN1, CCAR2[IE], DDX17[AU], MKS1[AU], RBBP6, TNFRS10D[IE]	ISG12b*, CRYAB
Spliceosome	ACIN1, PRPF38B[IE], U2SURP[IE], DDX39B, DDX46, PRPF3[IE], PRPF40B*, RBM25[IE], TCERG1[IE], SFRS4[IE], SFRS5, SF3B1	

* DEG validated by both the Australian and the Irish GWAS.

secretion. The DEG in the CL were involved primarily in processes associated with the $PGF_{2\alpha}$ response, steroidogenesis, and mRNA processing. Many of the DEG overlapped with QTL regions associated with fertility in the Australian and Irish dairy populations or proximal to SNP previously associated with dairy cow fertility [20–23, 25, 28]. Additionally, imputed sequence variants strongly associated with fertility were identified in and within 2 kb upstream and downstream of many DEG.

Concordance Between Differentially Expressed Genes and Fertility GWAS

Although genomic regions associated with dairy cattle fertility have previously been identified, they have extended megabases in length due to their long-range linkage disequilibrium with SNP markers, making it difficult to identify the causative mutation. Furthermore, there has been little agreement between studies, likely due to limited power for this low heritability trait [26]. Here, RNA sequencing was used to determine the global gene expression profile in the endometrium and the CL (both key reproductive tissues) of high-fertility (Fert+) and low-fertility (Fert−) cows, allowing us to identify a strong list of candidates genes affecting fertility prior to undertaking the GWAS. Importantly, tissue samples were collected on Day 13 of the estrous cycle, a critical time point for embryo development in cattle, coinciding with the initiation of conceptus elongation and secretion of interferon-τ in preparation for maternal recognition of pregnancy. This period also coincides with the majority of pregnancy loss in cattle [52]. The differential expression analysis allowed us to reduce the number of SNP tested, to some extent reducing multiple testing.

Although the importance of the DEG to female fertility in other dairy cattle populations is unknown, identifying QTL regions that were validated in both dairy populations further reduced the likelihood of false discoveries. We identified 355 QTL regions from the concordance analysis with either the Australian GWAS or Irish GWAS. Of the 355 QTL regions, 93 were validated in both populations, primarily on BTA 18, 5, 7,

TABLE 3. Sequence variants associated with fertility in the Australian dairy cattle population.

Ensembl gene ID	Gene name	BTA	Position	$-\log_{10}$ (P-value)	Annotation	SIFT
ENSBTAG00000014742	LRWD1[AU]	25	35 098 555	5.37	Intron variant	
ENSBTAG00000002603	PRPF39*	21	55 288 491	11.83	Upstream gene variant	
ENSBTAG00000011715	RECQL5[AU]	19	56 579 964	5.58	3′ UTR variant	
ENSBTAG00000011715	RECQL5[AU]	19	56 578 201	5.18	Missense variant	0.51
ENSBTAG00000006487	RPS9*	18	63 381 402	10.58	Downstream gene variant	
ENSBTAG00000006487	RPS9*	18	63 383 118	10.58	Intron variant	
ENSBTAG00000006487	RPS9*	18	63 389 258	10.58	Upstream gene variant	
ENSBTAG00000006487	RPS9*	18	63 379 597	10.39	Downstream gene variant	
ENSBTAG00000006487	RPS9*	18	63 381 172	10.37	Downstream gene variant	
ENSBTAG00000006487	RPS9*	18	63 379 604	10.36	Downstream gene variant	
ENSBTAG00000006487	RPS9*	18	63 379 694	10.35	Downstream gene variant	
ENSBTAG00000006487	RPS9*	18	63 379 514	10.34	Downstream gene variant	
ENSBTAG00000011689	LENG8*	18	63 114 910	7.78	3′ UTR variant	
ENSBTAG00000002763	KMT2B	18	46 620 241	7.65	Upstream gene variant	
ENSBTAG00000011689	LENG8*	18	63 107 476	7.51	Upstream gene variant	
ENSBTAG00000019547	LILRA6*	18	63 155 939	5.7	Downstream gene variant	
ENSBTAG00000010871	EIF4EBP3	7	53 334 235	5.97	Missense variant	0.01

8, and 29. This is supportive of the relatively high correlation of 0.88 between countries for fertility genetic evaluations [53]. A recent meta-analysis of published GWAS indicated the importance of BTA 1, 5, 13, 16, and 18 to fertility in cattle [26]. Further supporting an important role for some of the DEG in the current study, one meta-GWAS peak for fertility identified by the meta-analysis [26] was within the QTL region around ribosomal protein L36 (*RPL36*[IE]) on BTA 5. Eight other QTL regions around mitofusin 1 (*MTFN1*) on BTA 1; around transcription factor CP2 (*TFCP2*[IE]), *KIAA1551*[AU], calcium channel, voltage-dependent, L-type alpha 1C subunit (*CACNA1C*[AU]), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (*DDX17**) on BTA 5; and around acyl-CoA oxidase 3, pristanoyl (*ACOX3*[AU]), tRNA methyltransferase 44 homolog (*S. cerevisiae*) (*TRMT44*[AU]), and carboxypeptidase Z (*CPZ*[AU]) on BTA 6 were each within 0.5 Mb of a meta-GWAS peak for fertility identified by the meta-analysis [26].

The importance of specific genes to fertility performance in dairy cattle may also vary, depending on the production environment (i.e., pasture-based systems in Ireland and Australia vs. confinement systems in North America and continental Europe). It is well documented that the reproductive performance of North American Holstein Friesians managed in pasture-based environments is severely compromised, due in part to genotype × environment interactions [54–56]. While both Australian and Irish dairy cattle populations have experienced substantial introgression of North American Holstein genes in recent decades [57, 58], there is also evidence that selection signatures vary between countries, for example, between Australia, the United States, and New Zealand, which may be associated with subtle differences in breeding goals and subsequent sire choices [59]. Despite this, 58 of the 93 QTL regions (62%) validated by both the Australian and the Irish GWAS contained SNP previously associated with female fertility traits in dairy cattle populations with confinement milk production systems (i.e., North America, Denmark, Sweden, and Finland; Supplemental Table S5) [20–23, 25, 28].

Validating SNP associations in multiple breeds represents a prudent method to exclude false-positive findings [10, 25]. Such studies have reported either no agreement [10] or some agreement [25] of significant SNP associations between breeds. Of 4474 SNP associated with fertility traits in Danish Holstein cattle, 1522 (34%) were validated in either Jersey or Nordic Red breeds also, and 152 (3%) were validated in all three breeds [25]. QTL regions identified by the current study on BTA 8 (~59 Mb) and BTA 13 (~44 Mb) contained two SNP associated with fertility in Holsteins, Jersey, and Nordic Red in the previously mentioned study (Supplemental Table S5) [25].

Although there is currently limited agreement between dairy cattle populations in the genetic control of reproduction, we have described studies where agreement appears to exist. Validation of SNP discoveries for fertility between breeds have been hampered by the size of data sets and by the low heritability of fertility traits. Our results provide new and very strong evidence that the genomic regions reported contribute to the variation in reproductive performance in multiple dairy cow populations.

PGF$_{2\alpha}$-Related QTL Regions Associated with Fertility

Greater endometrial expression of *PGFS2** and *ABCC4*[AU] in the Fert− cows indicates greater synthesis and secretion of PGF$_{2\alpha}$ in the Fert− cows [60, 61]. Greater secretion of PGF$_{2\alpha}$, the primary luteolytic agent in cattle, on Day 13 of the estrous cycle may be sufficient to compromise CL development and P4 production in the Fert− cows as previously identified [32, 62],

without inducing complete luteolysis. In support of this, nine DEG (*ADAMTSL5*[IE], *ATP2A1*[AU], *NR5A1*, *CRYAB*, *INHBA**, *IL4R*, *SERPINB2*[IE], *THBS1*[IE], *TFPI2*[AU]) were previously reported to be associated with the CL response to exogenous PGF$_{2\alpha}$ [63–65], of which six were deemed to be in QTL regions by either the Australian or the Irish GWAS.

Steroidogenesis-Related QTL Regions Associated with Fertility

Luteal genes associated with steroidogenesis were differentially expressed between Fert+ and Fert− cows, and many were validated by the Australian and Irish fertility GWAS. Greater CL expression of *CYP3A4* [66] and ECM-related genes and lesser expression of *NR5A1*, two homologs of *STARD9*[IE] [67], *PNPLA3*[AU], *PLA2G4B*[IE], *PNPLA7*, period circadian clock 1 (*PER1*), cryptochrome circadian clock 2 (*CRY2**) [68], and the majority of cytoskeleton-related genes [69] in Fert− cows compared with Fert+ cows suggested that CL development and P4 production capacity was compromised in Fert− cows. Importantly, this luteal expression profile supports the previous findings that the CL of Fert− cows have reduced steroidogenic capacity [32, 62].

NR5A1 regulates the expression of genes involved in extracellular matrix, cell proliferation, apoptosis, steroidogenesis and lipid metabolism, cytoskeleton dynamics, angiogenesis, and transcriptional regulation [70]. Conditional knockout of *NR5A1*, a gene associated with luteal P4 secretion, in Leydig cells of male mice resulted in reduced Leydig cell expression of *CYP11A* and *STAR* in males [71]. Conditional knockout of *NR5A1* in females resulted in ovaries with reduced ovarian expression of anti-Müllerian hormone, reduced gonadotropin-induced expression of aromatase and cyclin D2, reduced follicle count, and absence of ovulation [71, 72]. Lesser expression of the phospholipase genes *PNPLA3*[AU], *PLA2G4-B*[IE], and *PNPLA7* in the Fert− cows suggests reduced release of arachidonic acid, an important promoter of STAR activity [73].

Lesser expression of *PER1* and *CRY2** in Fert− cows implicates potential disruption of circadian rhythms regulating cellular processes, including steroidogenesis [74]. Disruption of the circadian clock by conditional knockout of *BMAL1* in the ovary of mice resulted in reduced steroidogenic capacity, reduced circulating P4 concentrations, and embryo implantation failure [75]. Also, mice with homozygous null genotypes for either *PER1* or *PER2* had reduced incidence of normal estrous cycles and reproductive rates compared with wild-type mice [76].

Components of the cytoskeleton are involved in the intracellular transport of substrates for steroidogenesis [69]. Cytoskeleton-related DEG had primarily lesser expression in Fert− cows compared with Fert+ cows that may compromise steroidogenesis in the luteal cells of Fert− cows. Greater CL expression of genes associated with the ECM, that is, fibronectin 1 (*FN1*[AU]), secreted protein, acidic, cysteine-rich (*SPARC**), *THBS1*[IE], and tenascin C (*TNC**) in the Fert− cows compared with Fert+ cows [77, 78], may be indicative of greater tissue remodeling and delayed CL development in Fert− cows compared with Fert+ cows [32, 62]. Interestingly, *FN1* has been identified as harboring genetic variants associated with increased risk of endometriosis in humans [79].

mRNA Processing-Related QTL Regions and Sequence Variants Associated with Fertility

Functional differences between the CL of Fert+ and Fert− cows may also be explained by the differential expression of

genes involved in mRNA replication, zinc finger motifs, the cell cycle, DNA repair, and apoptosis. In general, the majority of DEG linked to these processes had lesser expression in Fert− cows compared with Fert+ cows. In addition, there was an overrepresentation of genes involved in the spliceosome pathway. Lesser expression of *EIF4EBP3*, poly (A) binding protein, nuclear 1 (*PABPN1*), and genes encoding ribosomal proteins in the Fert− cows compared with Fert+ cows suggests differences between genotypes in translation initiation in luteal cells [80]. Many of the DEG, that is, *CEP250*[AU], CDC-like kinase 4 (*CLK4*[IE]), DEAD (Asp-Glu-Ala-Asp) box polypeptide 46 (*DDX46*), *EIF4EBP3*, *PABPN1*, *PRPF39**, *RECQL5*[AU], *RPS9**, and tumor necrosis factor receptor superfamily member 10D (*TNFRSF10D*[IE]), identified QTL regions associated with fertility.

Evidence for the importance of mRNA processing for fertility performance in dairy cows is further strengthened by sequence variants in or within 2 kb upstream and downstream of *RECQL5*[AU], *PRPF39**, *RPS9**, and *EIF4EBP3* being associated with fertility. Interestingly, the missense variant of *EIF4EBP*3 was predicted to be deleterious to protein function. Importantly, previous studies have also linked genetic variation in dairy cattle fertility with gene variants involved in events related to the spliceosome [81], ribosomal protein [82], zinc finger motifs [82, 83], apoptosis [84], DNA repair [4, 85], and cell cycle progression [86].

Immune-Related QTL Regions and Sequence Variants Associated with Fertility

A properly functioning immune system is essential to animal health and fertility. In support of this, immune-related genes were differentially expressed between the Fert+ and Fert− cows that identified QTL regions and sequence variants associated with fertility. Greater endometrial expression of *SAA3** in the Fert− cows compared with Fert+ cows is indicative of the severity of subclinical endometritis [87] and endometrial inflammation [88] in cattle. Inflammation should have been absent when the endometrial biopsies were collected [89]. Our results suggest a prolonged inflammatory response in Fert− cows, which is supported by their greater endometrial expression of *SPP1* [90, 91]. These alterations to the local immune system are likely a result of a prolonged inflammation in Fert− cows, consistent with our previous observation of more severe postpartum uterine infection in Fert− cows as assessed by vaginal mucus scores and endometrial cytology [92]. The negative effects of increased endometrial polymorphonuclear neutrophil infiltration on phenotypic fertility are well established [93].

Endometrial and luteal expression of *LILRA6** and luteal expression of *LENG8** was lesser in Fert− cows compared with Fert+ cows. Both *LILRA6** and *LENG8** are members of the leukocyte receptor cluster [94]. The QTL regions around both genes are located close to a haplotype on BTA 18 associated with calving interval in the Australian dairy cattle population [20], and sequence variants in or within 2 kb upstream and downstream of both genes were significantly associated with fertility. Luteal expression of major histocompatibility complex (MHC) NC3*50201 was greater in Fert− cows compared with Fert+ cows. In cattle, the MHC is identified as bovine leukocyte antigen and is located on BTA 23. A recent GWAS of immune response traits in Canadian Holstein cattle [95] reported that >90% of SNP associated with antibody-mediated immune response were located on BTA 23. Luteal expression of *IFITM2** and complement component 3 (*C3*[AU]) was also greater in Fert− cows compared with Fert+ cows. *IFITM2** is a member of the interferon-induced transmembrane gene family that, in pigs, is involved in antiviral activities [96]. The complement system, of which *C3*[AU] is a member, is part of the innate immune system. *C3*[AU]-deficient mice have reduced levels of mast cell granulation, TNF-α production, neutrophil infiltration, and clearance of bacteria [97].

The expression profile of the immune-related genes in the CL may be associated with premature luteal regression in the Fert− cows, as immune function is central to CL regression and influxes of macrophages, monocytes, and T lymphocytes have been described prior to the onset of luteolysis [98–100]. This interpretation further supports our summarization above and previously that CL function is compromised in Fert− cows [32, 62].

Energy Status-Related QTL Region Associated with Fertility

Previously, we reported that Fert+ cows have greater postpartum dry matter intake, adipose reserves, and circulating concentrations of the bioenergetic indicators insulin, glucose, and insulin-like growth factor-1 [30, 32, 62, 101]. *PRKAG3*[AU] encodes for the γ3-subunit of the adenosine monophosphate-activated kinase complex, the primary energy sensor in eukaryotic cells [102]. Differential expression of the γ3-subunit in the endometrium may represent a mechanism linking whole-animal bioenergetic status and the local endometrium bioenergetic status.

Implications for the Genetic Improvement and Understanding of Fertility

The study highlights the usefulness of the Fert+/Fert− lactating cow genetic model of fertility for elucidating the genetic control of reproductive performance in cattle. The validation of candidate genes using the GWAS analysis of large dairy cattle populations in two countries and sequence variant identification highlighted the value of this model. The QTL regions and sequence variants identified in the current study likely represent important genomic regions and variants underlying the genetic variation in dairy cow fertility. Opportunities exist to use this information to accelerate the genetic improvement of dairy cow fertility. At present, genomic selection exploits the strong linkage disequilibrium that exists between markers on SNP arrays and the causative mutations; its accuracy, however, declines as the relationship between the reference population and the animal to be evaluated decreases. The absence of causative mutations on SNP arrays has, consequently, limited the accuracy of genomic selection for complex traits, particularly low heritability traits such as fertility. It is therefore anticipated that the QTL regions and sequence variants, such as identified in the current study, include potential causative mutations that may enhance genomic predictions to accelerate the rate of genetic improvement in dairy cow fertility.

The generation of large volumes of high-quality phenotypes and genotypes, combined with the availability of whole genome sequence, means that dairy cattle also represent an excellent study group for understanding the genetic architecture and biology of complex quantitative traits, such as female fertility in mammals, including humans. This is the first study to examine the transcriptome of the endometrium and the CL on Day 13 of the estrous cycle in Holstein cows genetically divergent for fertility traits. Using a unique lactating cow genetic model of fertility in this study, the DEG indicate a complex dialogue between the CL and the endometrium that

influences the likelihood of pregnancy establishment via effects on circulating P4 concentrations and the uterine environment. Fert− cows had an endometrial expression profile indicative of an ongoing inflammatory response that presumably started following exposure to pathogens after parturition. Finally, it should be pointed out that by using differences in expression of genes between the Fert+ and Fert− cows as the first criteria for identifying candidate mutations affecting fertility, we have more power to discover regulatory mutations that affect the expression of genes involved in fertility rather than mutations that change the function of the encoded protein.

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