Genome-Wide Association Study of age at puberty and its (co)variances with fertility and stature in growing and lactating Holstein-Friesian dairy cattle.

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ABSTRACT

Reproductive performance is a key determinant of cow longevity in a pasture-based, seasonal dairy system. Unfortunately, direct fertility phenotypes such as intercalving interval or pregnancy rate tend to have low heritabilities and occur relatively late in an animal’s life. In contrast, age at puberty (AGEP) is a moderately heritable, early-in-life trait, that may be estimated using an animal’s age at first measured elevation in blood plasma progesterone (AGEP4) concentrations. Understanding the genetic architecture of AGEP4 in addition to genetic relationships between AGEP4 and fertility traits in lactating cows is important, as is its relationship with body size in the growing animal. Thus, the objectives of this research were 3-fold. First, to estimate the genetic and phenotypic (co)variances between AGEP4 and subsequent fertility during first and second lactations. Second, to quantify the associations between AGEP4 and height, length, and body weight (BW) measured when animals were around 11 mo old (SD = 0.5). Third, to identify genomic regions that are likely to be associated with variation in AGEP4. We measured AGEP4, height, length, and BW in around 5,000 Holstein-Friesian or Holstein-Friesian x Jersey crossbred yearling heifers, across 54 pasture-based herds managed in seasonal calving farm systems. We also obtained calving rate (CR42: success or failure to calve within the first 42 d of the seasonal calving period), breeding rate (PB21: success or failure to be presented for breeding within the first 21 d of the seasonal breeding period) and pregnancy rate (PR42: success or failure to become pregnant within the first 42 d of the seasonal breeding period) phenotypes from their first and second lactations. The animals were genotyped using the Weatherby’s Versa 50K SNP array (Illumina, USA). The estimated heritabilities of AGEP4, height, length, and BW were 0.34 (0.30, 0.37), 0.28 (0.25, 0.31), 0.21 (0.18, 0.23), and 0.33 (0.30, 0.36), respectively. In contrast, the heritabilities of CR42, PB21 and PR42 were all < 0.05 in both first and second lactations. The genetic correlations between AGEP4 and these fertility traits were generally moderate ranging from 0.11 to 0.60, whereas genetic correlations between AGEP4 and yearling body conformation traits ranged from 0.02 to 0.28. Our genome wide association study (GWAS) highlighted a genomic window on chromosome 5 that was strongly associated with variation in AGEP4. We also identified 4 regions, located on chromosomes 14, 6, 1 and 11 (in order of decreasing importance), that exhibited suggestive associations with AGEP4. Our results show that AGEP4 is a reasonable predictor of estimated breeding values (EBVs) for fertility traits in lactating cows. While the GWAS provided insights into genetic mechanisms underpinning AGEP4, further work is required to test genomic predictions of fertility that use this information.

Key words: MCMC, BayesC, Heifer, Gibbs

INTRODUCTION

The profitability of seasonal, pasture-based dairy systems is substantially reduced by poor reproductive performance. Dairy producers desire a timely and condensed calving pattern as a strategy to align the feed demands of the herd to the seasonal feed supply from pasture grazed in situ (Macdonald and Roche, 2023). Holstein-Friesian and Jersey cattle have gestation lengths around 280 d (Norman et al., 2009); therefore, to maintain an annual calving pattern, each cow must resume normal estrous cycles and initiate a pregnancy within 85 d after calving. Cows that extend that interval are more likely to be culled from the herd, truncat-
Stephen et al.: Genome-Wide Association…

Many traits that are of interest to dairy farmers are highly heritable, such as milk production and fertility. Despite the high heritability, progress in improving these traits has been limited by the low heritability of reproduction phenotypes (Berry et al., 2014). This is because the heritability of reproduction is low, which reduces the accuracy of genetic evaluation.

One approach for overcoming the constraints of low heritability on selection progress is to increase the number of phenotypes contributing to an analysis. Genomic evaluation can be used to leverage phenotypes measured on animals that do not necessarily share close pedigree relationships with selection candidates. That technology can provide high reliability EBVs for selection candidates despite them having relatively few direct or daughter phenotypes measured. Employing genomic evaluation might reduce the differential between the accuracy of EBVs for fertility, relative to other priority traits like milk production, provided there is a large reference population of genotyped animals with fertility phenotypes.

Another strategy for improving the rate of genetic gain in fertility is to use a correlated predictor trait that either responds more readily to selection, or is expressed earlier in life, or both. Age at puberty (AGEP) is a candidate trait that may meet these requirements. In Bos taurus, the onset of puberty occurs when animals are around 1 yr of age (Hickson et al., 2011; Meier et al., 2021), and literature indicates that AGEP has a moderate heritability of around 0.30 (Meier et al., 2021). Several authors have reported moderate genetic correlations of around 0.45 between AGEP and fertility traits measured during lactation (Morris et al., 2000; Mialon et al., 2001; Lefebvre et al., 2021). Furthermore, a predictor trait with higher heritability than the target trait, such as AGEP, can also add value in the context of genomic selection, as more accurate EBVs can be produced from smaller reference populations (Gonzalez-Recio et al., 2014). The improved accuracy of genomic EBVs for higher heritability traits provides evidence of improved estimation of marker effects, which may also extend to better quantitative trait loci (QTL) detection when using GWAS analysis. If 2 genetically correlated traits share QTL in common, a GWAS analysis of a higher heritability predictor trait may provide insight into the genetic architecture of a low heritability target trait, that would otherwise require a much larger population of phenotyped animals. Subsequent enrichment of a SNP chip in QTL regions can improve the accuracy of genomic prediction (Xiang et al., 2021), and, therefore, GWAS of a suitable predictor trait may contribute toward improving the accuracy of genomic EBVs for fertility.

A key limitation of AGEP as a predictor trait for fertility is the cost of measuring phenotypes, as a precise definition of onset of puberty might require confirmation of 3 events. First, behavioral estrus, second, ovulation and the formation of a corpus luteum (CL), and third, normal luteal function (Moran et al., 1989). That said, researchers who aim to measure AGEP at scale tend to simplify the definition of puberty, reducing the frequency of observations, and focusing on just one or 2 of the components of puberty. For example, Meier et al. (2021) focused on ovulation and the length of the luteal phase. They characterized AGEP as the age of first measured elevation in blood plasma progesterone (AGEP4). Johnston et al. (2009) simplified the AGEP criteria further by only requiring evidence of ovulation to categorize animals as post-pubertal. They used rectal ultrasound scanning at intervals of up to 6 weeks to detect the presence of a CL. The phenotypes measured by both Meier et al. (2021) and Johnston et al. (2009) were subject to left-, interval- or right-censoring, as some animals were pubertal before the first observation, some attained puberty between 2 observations and some were not pubertal at the end of the observation period. This type of simplification will compromise the precision of each phenotype but it is essential for large scale field studies where detailed and frequent phenotyping is not economically and logistically feasible.

The objectives for this study were 3-fold. First, to estimate the (co)variance parameters of AGEP4 and fertility traits expressed during lactation in a population of Holstein-Friesian and Holstein-Friesian x Jersey crossbred dairy cattle managed in seasonal, pasture-
based systems in NZ. Second, to quantify the associations between AGEP4 and height, length, and body weight (BW) measured when animals were around 1 yr of age. Third, to identify genomic regions that are likely to be associated with variation in AGEP4. We hypothesized that AGEP4 would exhibit a nonzero genetic correlation with reproductive performance during lactation, and that our GWAS analysis would detect genetic regions associated with variance in AGEP.

**METHODS**

**Animals**

The Ruakura Animal Ethics Committee (Hamilton, NZ) approved this study and all manipulations (AE applications: 14448 and 15004). Fifty-four herds were enrolled from a purposive selection of seasonal calving, pasture-based herds located in one of 3 regions (n = 35, Waikato; n = 15, Taranaki; and n = 4, Otago) of NZ, as described by Stephen et al. (2023). The herds were selected based on breed composition (herds with higher proportion of Holstein-Friesian animals were preferred), the quality of existing herd records, and regional location. The study focused on 2018-born cows, comprising 5,010 yearling heifers present when the AGEP phenotypes were measured. Some 322 of those animals were either not genotyped, had missing heifer phenotypes or had incomplete parentage records, leaving 4,688 animals representing 257 sires. Among those sires, 56 had at least 10 daughters that were spread over at least 3 herds. Binary fertility phenotypes were measured during first and second lactation, denoting an animal’s success or failure to calve within the first 42 d of the herd’s seasonal calving period (CR42_first, n = 4,327; CR42_second, n = 3,575), to be presented for breeding within the first 21 d of the seasonal breeding period (PB21_first, n = 4,111; PB21_second, n = 3,507), and to become pregnant within the first 42 d of the seasonal breeding period (PR42_first, n = 3,939; PR42_second, n = 3,353). Most animals enrolled in this study were Holstein-Friesian (>90% Holstein-Friesian; n = 2,340) or Holstein-Friesian x Jersey crossbreds (Holstein-Friesian or Jersey breed proportions are each < 90%, but together sum to > 90%; n = 2,276). The remaining animals were Jersey (>90% Jersey; n = 24) or other breeds (all breeds other than Holstein-Friesian, Jersey or Holstein-Friesian x Jersey crossbred; n = 48). This reference population is hereafter referred to as the Puberty at Scale (PS) population (Figure 1). The PS animals were genotyped using the Weatherbys Versa 50K SNP array (Illumina, USA). The SNPs were mapped to Bos taurus 3.1 genome build (UMD 3.1, College Park, MD). Some 47,000 SNP were included in our analysis following the removal of unmapped or X-chromosome SNP (n = 806), SNP with low call rate, and any SNP with minor allele frequency <1% (n = 2,120). We used find-hap (VanRaden et al., 2013) software to impute the small proportion of missing SNP genotypes.

**Age at puberty phenotypes**

We used the trait AGEP4 as a practical proxy for age at puberty (AGEP), with animals phenotyped as described by Stephen et al. (2023). Briefly, animals were blood tested on 3 occasions when each herd cohort was approximately 10-, 11- and 12-mo of age, between May and August 2019. Blood was collected from the coccygeal vein into lithium heparin evacuated tubes (BD Vacutainers, BD New Zealand, Auckland, NZ), and immediately stored on ice. Samples were then centrifuged (at 4°C, 1,900 × g for 12 min) within 24 h of collection. Plasma was harvested and stored frozen at −20°C until a progesterone assay could be carried out using a commercial radioimmune assay kit (ImmuChem Progesterone Double Antibody RIA, MP Biomedicals LLC, Irvine, CA) as described by Meier et al. (2021). Animals were categorized as having elevated blood plasma progesterone (BP4) once concentrations exceeded 1 ng/mL indicating that ovulation was likely to have occurred (Meier et al., 2021). The AGEP4 phenotype for each animal was comprised of 2 ages which were the known lower and upper bounds of their AGEP4, representing left-, interval- and right-censoring. The type of phenotype censoring present in our study is described elsewhere (Stephen et al., 2022a). Briefly, animals whose BP4 was already elevated in the first blood sample collected have left-censored phenotypes, whereby the lower bound of the phenotype is not known (and is set to 250 d in our analysis, Dennis et al., 2018), and the upper bound comprises their age on the day of their first blood test. Animals who had their first elevation in BP4 measured at visit 2 or visit 3 had interval-censored phenotypes. Their first BP4 elevation occurred at some stage between 2 blood test days, but the exact day is not known. The upper bound of their interval-censored phenotype is their age on the blood test day when their BP4 was first observed as > 1 ng/mL. The lower bound is their age on the previous blood test day, approximately 30 d earlier. Animals that had not been measured with BP4 elevation before the final blood test had right-censored phenotypes. The lower bound of their phenotype is their age on the last blood test day and the upper bound is not known but is defined as 600 d in our analysis; we chose 600 d as we would expect all animals in this study to have reached puberty (and therefore have exhibited BP4 elevation) by that age, based on a phenotypic mean and standard
deviation of 342 d and 30 d derived from a previous study (Dennis et al., 2018).

**Height, Length, and BW phenotypes**

We measured 3 yearling body conformation traits when the animals were approximately 1 year old (mean = 11 mo, SD = 0.5). These included height (distance in cm from the ground to the top of the shoulder), length (distance in cm from the base of the neck to the base of the tail) and BW (stationary weight in kg).

**Calving and Breeding phenotypes**

Animals were seasonally bred for the first time at around 15 mo old between September and December 2019, as per routine farm management. During first and second lactations, animals were assigned binary CR42 and PB21 phenotypes, denoting whether they calved within the first 42 d of their herd’s seasonal calving period (score of 1) or not (score of 2), and whether they presented for breeding in the first 21 d of their herd’s seasonal breeding period (score of 1) or not (score of 2), respectively. Therefore, a higher score for calving and breeding rate represented poorer performance. The start dates of the calving period and the breeding period in each herd were calculated as the mean calving date and the mean breeding date of the first 10% of the animals in the herd to calve or be bred, respectively (Bowley et al., 2015). The animals in this study calved at an average age of 24 mo (SD = 0.75; between June and November 2020). Breeding in first lactation occurred between October and December 2020, using a combination of artificial insemination (AI) followed by natural breeding. The AI occurred over a period of 61 to 100 d (mean ± SD: 77 ± 9 d), as per routine farm management. Second calving occurred when the animals were an average age of 36 mo (SD = 0.80; between June and November 2021). Animals that had a calving recorded in the first lactation but failed to become pregnant again were culled before a second lactation (n = 409). These missing animals were assigned a CR42 phenotype of 2 in second lactation, because if they had not been culled this would likely have been their phenotype. Breeding in second lactation occurred between October and December 2021, using a combination of AI and natural breeding. The AI occurred over a period of 28 to 95 d (mean ± SD: 61 ± 19 d). Calving and breeding data were recorded by farmers using commercial software, as is routine on NZ dairy farms. These data were accessed from the national Dairy Industry Good Animal Database (DIGAD) at the end of each lactation.

**Pregnancy**

Animals were assigned a binary PR42 phenotype in both their first and second lactations denoting whether they became pregnant within the first 42 d of their herd’s seasonal breeding period (score of 1) or not (score of 2). Therefore, as with the calving and breeding phenotypes, a higher score represented poorer performance.

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![Figure 1](image-url)  
**Figure 1.** Phenotypes measured in the ‘Puberty at Scale’ (PS) population (n = 4,688) were used to undertake a marker effects analysis and a GWAS of ‘Age at first elevation in blood plasma progesterone’ (AGEP4). The marker effects and GWAS results were subsequently validated in the independent ‘Fertility Research Herd’ (FRH) population (n = 501).
reproductive performance. We used transrectal ultrasonography 11 to 14 weeks after the start of the herd’s breeding period to diagnose pregnancy and determine conception date based on the size of the fetus, relative to the insemination records available for each cow. Ultrasonography and interpretation were carried out by qualified veterinary professionals. Pregnancy data were supplied by one or other of the herd record providers (LIC, Hamilton, NZ; CRV, Hamilton, NZ).

Analysis

We used a combination of univariate, bivariate and trivariate marker effects models, depending on the parameters we were estimating, and the traits involved. All variances (genetic, residual, and phenotypic) were first estimated using univariate analyses. We estimated covariances between AGEP4 and each of the yearling body conformation traits using pair-wise bivariate analyses. Finally, we estimated covariances between AGEP4 and fertility traits recorded during first and second lactation using trivariate analyses, where AGEP4 was analyzed alongside both the first and second lactation phenotype for CR42, PB21 or PR42. In all cases, the marker effects models can be represented by the following model equation:

\[ y = Xb + Ma + e \]  Equation 1

In Equation 1, \( y \) was a vector of phenotypes. The vector \( b \) represented fixed effects, which comprised a herd effect for every analysis, and a measurement age in days effect for the analysis of yearling body conformation traits. Breed proportions were not included as fixed effects in our analyses, as the Bayesian multiple-regression analyses that we have implemented here have been shown to be robust to population structure (Toosi et al., 2018). The vector \( a \) represented additive marker effects and the vector \( e \) represented residual effects relating to each phenotype record. Phenotypes were related to each fixed effect using the incidence matrix \( X \), and each marker effect using \( M \), which was a matrix of genotype covariates (coded as 0,1,2). The number of rows in \( M \) was equal to the number of animals with both a genotype and a phenotype, and the number of columns of \( M \) was equal to the number of SNP markers included in the analysis. The vectors \( b \) and \( a \) were unknowns, as were the genetic and residual (co)variances. In addition, analyses that involve AGEP4 had another unknown, where the vector \( y \) that corresponds to AGEP4 phenotypes was itself unknown, except for upper and lower bounds. We applied BayesC methodology to estimate marker effects. The value of \( \Pi \), which was a scalar representing the proportion of markers that had no effect on the phenotypes, was fixed as 0.99 in univariate analyses, but sampled as an unknown parameter in our bivariate and trivariate analyses (Habier et al., 2011).

Software and solver

We generated phenotype files in the relevant formats required for analyses using command line bash scripts. We used the JWAS package (Cheng et al., 2018) implemented in Julia (Bezanson et al., 2017) to complete the genetic analyses, applying a Markov Chain Monte Carlo (MCMC) technique, using a single site Gibbs sampler to obtain samples of the unknown parameters. The first 20,000 MCMC samples were disregarded from each run as a burn in. A literature search to establish prior variance parameter values for each univariate analysis was undertaken (see supplementary materials). For example, our prior values for the genetic and residual variance for AGEP4 were 649 d² and 1202 d², respectively, and these were derived from a phenotypic variance of 1849 d² (Mialon et al., 2001) and a heritability of 0.35 (Mialon et al., 2000; Dennis et al., 2018). The posterior mean of variance parameters from univariate analyses were used as priors for the bivariate and trivariate analyses. Prior values for covariances were established from the literature (see supplementary materials).

We used 2 approaches to test for non-convergence of the Markov chain. First, we applied the method described by Geweke (1992). Second, we inspected box plots and trace plots of relevant statistics, such as heritabilities, to visually assess the MCMC chains for evidence of non-convergence of the posterior means and credibility intervals of the parameter estimated. To construct the box plots, we grouped the MCMC chains into 4 groups according to sample number. For example, if the chain length was 100,000 samples: group 1 comprised samples 1 to 25,000, group 2 comprised samples 25,001 to 50,000, etc.). A parameter was considered converged if the 25th and 75th percentile of each group overlapped. We increased the chain lengths in models that were estimating more parameters, with chain lengths of 100,000, 300,000 and 600,000 for the univariate, bivariate and trivariate analyses. We selected these chain lengths by completing convergence testing on a series of test analyses with varied chain lengths. Longer chains were required to achieve convergence in the models that included larger numbers of (co)variance parameters. The results for each parameter are presented as the mean of the MCMC samples, with credibility intervals representing the 5th and 95th percentiles of the MCMC samples (90% CRI).
GWAS analysis

In a post-processing step, the ‘JWAS’ software was used to produce a WPPA (Window Posterior Probability of association) for the set of markers that were located within predefined genomic windows. Each genomic window included approximately 20 contiguous SNPs, spanning an average of 1 cM. The WPPA represents the proportion of MCMC samples where a given window was associated with at least 1% of the variance in AGEP4 (Fernando et al., 2017). We used a WPPA threshold of 0.95 to identify regions that were associated with genetic variance in AGEP4. At that threshold, we would expect the proportion of false positives to be 0.05 (Fernando et al., 2017). We used the lower WPPA threshold of 0.70 to identify genomic windows that shared a suggestive association with AGEP4. We then validated each of the genomic regions with WPPA > 0.70 using phenotypes measured in an independent validation population from a prior experiment (Meier et al., 2021). We used Ensembl (http://ensembl.org/) to investigate protein coding-genes within genomic regions of interest using the University of Maryland assembly of the Bos taurus 3.1 genome build (UMD 3.1, College Park, MD).

Validation method

We validated the estimated marker effects from the analysis of the PS population using an independent validation population from a prior experiment (Meier et al., 2021), consisting of 522 Holstein-Friesian cows, born in 2015. That herd (hereafter referred to as the Fertility Research Herd; FRH) was comprised of animals with extremely divergent parent average fertility EBVs based on re-calving rate within 42 d during second lactation (positive line: POS +5% fertility EBV; negative line: NEG −5% fertility EBV). That divergence in fertility EBVs was associated with phenotypic divergence in AGEP4, where POS animals were an average of 28 d younger than NEG animals when an elevation in BP4 concentrations was first detected (Meier et al., 2021). There were 21 animals in the validation population that were half-siblings to one or more PS animal; these were removed, leaving 501 animals (n = 260 POS; n = 241 NEG) contributing to our validation (Figure 1). There were 21 animals in the validation population that were half-siblings to one or more PS animal; these were removed, leaving 501 animals (n = 260 POS; n = 241 NEG) contributing to our validation (Meier et al., 2021). In this smaller population, animals were blood tested more frequently (weekly intervals) and for a longer period of time compared with the approach taken in the PS population. The phenotyping method in the FRH population is described in detail by Meier et al., (2021). Briefly, animals were blood tested weekly from when they were approximately 190 kg BW (around 240 d old), through until 3 weeks after the start of their first seasonal breeding period (around 440-d old). An animal was deemed to have reached puberty once 2 of 3 consecutive weekly blood tests had elevated BP4 concentrations. Animals that failed to reach puberty by the end of the study were assigned an AGEP4 of their age on the final blood test, with the addition of a 7-d penalty.

All FRH animals were genotyped using the GeneSeek GGP Bovine 150K SNP Illumina array as described by Grala et al. (2021). Some 30K SNP on this array were common to SNP on the Weatherbys Versa 50K SNP array. We imputed approximately 17,000 SNPs missing GGP array that were present on the Weatherbys array using find-hap software, VanRaden et al., 2013, which enabled us to validate our marker effects directly derived from analysis of the PS population. The SNPs that were not present on the Weatherbys Versa 50K SNP array were not used in validation.

To produce validation EBVs for the FRH animals, we multiplied the vector of AGEP4 marker effects (number of SNP x 1) from the analysis of the phenotypes measured in the PS population by the matrix of observed and imputed SNP genotypes (number of animals x number of SNP) for the FRH animals. The multiplication yielded a vector of validation EBVs (number of animals x 1) for the FRH animals. The accuracy of the validation EBVs were represented as the correlation between the observed AGEP4 phenotypes (adjusted for fixed herd effects) and the EBVs obtained without the use of those phenotypes. We generated the mean and 90% CRI for these correlations using a bootstrap (with replacement) method (Zhu, 1997) with a total of 1,000 bootstrap samples. We also used the FRH to validate the SNP effects within individual windows. To do this, we produced window EBVs where all SNP not located in the window of interest were disregarded from the EBV calculation.

RESULTS

Heritabilities

We estimated moderate single-trait heritabilities of 0.34 (0.30, 0.37), 0.28 (0.25, 0.31), 0.21 (0.18, 0.23) and 0.33 (0.30, 0.36) for AGEP4, height, length, and BW, respectively (Table 1; see supplementary materials for estimated genetic and phenotypic variances). Estimated heritabilities of the 6 fertility traits measured during lactation (CR42, PB21 and PR42 in both first and second lactations) were much lower, ranging from 0.01 to 0.04 (Table 2).
**Correlations among age at puberty and fertility traits**

Overall, the genetic correlations (Table 2) between AGEP4 and fertility traits were positive and moderate. These correlations tended to be highest for PB21 (0.53, 0.60 in first and second lactations, respectively), and lowest for PR42 (0.34, 0.11 in first and second lactations respectively). The genetic correlations between AGEP4 and CR42 in first and second lactations were 0.45 and 0.58, respectively. Phenotypic correlations between AGEP4 and fertility traits were all near zero, ranging from 0.02 to 0.03.

**Correlations among age at puberty and yearling body traits**

The estimates of the genetic correlations between AGEP4 and the 3 body conformation traits are presented in Table 1. Height exhibited the highest genetic correlation with AGEP4 of 0.28, whereas length and BW exhibited near zero genetic correlations of 0.02 and 0.04, respectively. Phenotypic correlations between AGEP4 and yearling body traits were −0.04, −0.14 and −0.24 (height), −0.16 (length) and −0.26 (BW). Genetic correlations among height, length and BW were indicative of strong associations, ranging from 0.63 to 0.82, whereas phenotypic correlations were moderate, ranging from 0.32 to 0.48.

**GWAS of age at puberty**

Using a GWAS for AGEP4 in our reference PS population, we identified 1 window with WPPA values greater than 0.95 (Figure 2). This window was located on chromosome 5 (UMD: 105,337,527 bp to 106,432,283 bp; WPPA: 1.00). Four other windows had WPPA greater than 0.70. These windows were located on chromosome 14 (UMD: 24,482,969 bp to 25,731,992 bp; WPPA: 0.93), chromosome 6 (UMD: 22,273,911 bp to 23,352,794 bp; WPPA: 0.84), chromosome 1 (UMD: 79,755,519 bp to 80,661,482 bp; WPPA: 0.75) and chromosome 11 (UMD: 39,708,839 bp to 41,789,973 bp; WPPA: 0.71). Collectively, these windows on chromosomes 5, 14, 6 and 11 harbored between 2 and 16 protein-coding genes each, listed in Table 3.

**Validation of marker effects**

The mean AGEP4 phenotypes for the 2 fertility subgroups of the FRH population were 356 d (SD = 42.8) and 384 d (SD = 39.8) for POS and NEG, respectively (Meier et al., 2021). The mean gEBVs (Figure 3) for NEG and POS were 5.3 d (SD = 12.1) and −3.9 d (SD = 13.3) respectively. Therefore, the marker effects derived from the current study accounted for 9.2 d of the total 28-d phenotypic difference in AGEP4 between the 2 subgroups of our validation FRH population. The correlation between gEBVs and AGEP4 phenotypes in the FRH population was 0.41 (Table 4). The correlation between gEBVs and adjusted AGEP4 phenotypes within the POS and NEG fertility subgroups of the FRH population were 0.38 and 0.30, respectively. The correlations between each of the 5 window EBVs and the validation AGEP4 phenotypes from the FRH are reported in Table 4. The posterior mean of all 5 correlations were positive, although the 90% CRI for the correlations involving the windows on chromosome 14 (24,482,969 bp to 25,731,992 bp) and chromosome 11 (39,708,839 bp to 41,789,973 bp) overlapped zero. The highest correlation between a window EBV and the validation phenotypes was observed for the genomic window on chromosome 5 (105,337,527 bp to 106,432,283 bp). This window on chromosome 5 also had the highest WPPA in our GWAS analysis of the PS population (Figure 2). The correlations between the 5 window EBVs were low, ranging from −0.05 to 0.11 (Table 4).

The minor allele frequencies of the largest effect SNP within high (>0.70) WPPA windows differed by between 0 and 0.16 (Table 5) between the PS and the FRH populations. The commonality across the Weath-
erby’s Versa 50k SNP array (Illumina, USA) and the GeneSeek GGP Bovine 150K SNP Illumina array (Illumina, USA) was high across most of the high WPPA windows, ranging from 65 to 100%.

DISCUSSION

In this study, we measured AGEP4 in a population of predominantly Holstein-Friesian and Holstein-Friesian x Jersey crossbred dairy cattle to investigate its suitability as a predictor for genetic merit for fertility in a seasonal management system. We determined a moderate heritability for AGEP4 and detected moderate genetic associations with key fertility traits that represent reproductive success during lactation. Conversely, genetic associations between AGEP4 and body conformation traits were low. Our results indicated that incorporating AGEP4 as a predictor of fertility EBVs will result in an accelerated rate of genetic gain in fertility.

Heritability

We estimated the phenotypic standard deviation of AGEP4 to be around 43d, with a heritability of approximately 0.34. This heritability provides an indication of how readily it might respond to direct selection, and heritability is an important factor when considering the value of AGEP4 in the context of a predictor of fertility EBVs. In particular, if the heritability of a target trait (for example, fertility during lactation) is very low, it would be advantageous to select for a higher heritability trait that exhibits a moderate to high genetic correlation with the target trait, if such a trait exists. In general, fertility traits measured during lactation have heritabilities of < 0.10 as determined in the current study (where CR42, PB21, and PR42 traits had heritabilities between 0.01 and 0.04) and reported previously (Brotherstone et al., 2002; Harris et al., 2006; Bailey et al., 2018; Dorr et al., 2021; Stephen et al., 2021). The heritability of a trait can be estimated directly using statistical methods such as the BLUP method or by using marker-assisted selection methods that involve the use of genomic information.

In our study, the phenotypes for AGEP4 were subject to left, interval and right censoring. This censorship was the result of our strategic trade-off between precision to left, interval and right censoring. This censorship was the result of our strategic trade-off between precision and practicality in our phenotyping strategy.

Table 2. Heritabilities (diagonal), phenotypic (below) and genetic (above) correlations for age at puberty (AGEP4: defined as age at first measured elevation in blood plasma progesterone) and fertility phenotypes (CR42: calving within 42 d; PB21: breeding within 21 d; PR42: pregnant within 42 d) from first and second lactation. Heritabilities were estimated using univariate analysis of each phenotype, while correlations were estimated from trivariate analyses including AGEP4 and both first and second lactation phenotypes for each of the calving, mating, and pregnancy traits. Credibility intervals representing the 5th and 95th percentiles of the MCMC samples are shown in parentheses. Fertility phenotypes are represented by binary scores, whereby a higher score indicates poorer fertility performance.

<table>
<thead>
<tr>
<th>Trait</th>
<th>AGEP4</th>
<th>CR42_first</th>
<th>PB21_first</th>
<th>PR42_first</th>
<th>CR42_second</th>
<th>PB21_second</th>
<th>PR42_second</th>
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<tbody>
<tr>
<td>AGEP4</td>
<td>0.34 (0.30, 0.37)</td>
<td>0.45 (0.09, 0.73)</td>
<td>0.53 (0.27, 0.75)</td>
<td>0.34 (0.14, 0.53)</td>
<td>0.58 (0.29, 0.81)</td>
<td>0.60 (0.38, 0.79)</td>
<td>0.11 (−0.10, 0.36)</td>
</tr>
<tr>
<td>CR42_first</td>
<td>0.02 (−0.01, 0.05)</td>
<td>0.01 (0.00, 0.02)</td>
<td>0.78 (0.52, 0.94)</td>
<td>0.82 (0.56, 0.94)</td>
<td>0.78 (0.47, 0.94)</td>
<td>0.64 (0.01, 0.92)</td>
<td>0.71 (0.35, 0.92)</td>
</tr>
<tr>
<td>PB21_first</td>
<td>0.02 (−0.00, 0.05)</td>
<td>0.03 (0.01, 0.04)</td>
<td>0.73 (0.39, 0.92)</td>
<td>0.77 (0.40, 0.95)</td>
<td>0.84 (0.68, 0.94)</td>
<td>0.82 (0.56, 0.94)</td>
<td>0.88 (0.75, 0.97)</td>
</tr>
<tr>
<td>PR42_first</td>
<td>0.01 (−0.02, 0.04)</td>
<td>0.12 (0.09, 0.14)</td>
<td>0.19 (0.16, 0.21)</td>
<td>0.04 (0.02, 0.06)</td>
<td>0.91 (0.84, 0.96)</td>
<td>0.82 (0.55, 0.94)</td>
<td>0.69 (0.39, 0.86)</td>
</tr>
<tr>
<td>CR42_second</td>
<td>0.03 (0.00, 0.07)</td>
<td>0.05 (0.02, 0.08)</td>
<td>0.14 (0.11, 0.17)</td>
<td>0.27 (0.23, 0.30)</td>
<td>0.26 (0.23, 0.29)</td>
<td>0.03 (0.01, 0.05)</td>
<td>0.48 (0.01, 0.77)</td>
</tr>
<tr>
<td>PB21_second</td>
<td>0.03 (−0.01, 0.06)</td>
<td>0.08 (0.05, 0.11)</td>
<td>0.00 (0.06, 0.12)</td>
<td>0.20 (0.16, 0.23)</td>
<td>0.20 (0.16, 0.23)</td>
<td>0.21 (0.18, 0.24)</td>
<td>0.02 (0.01, 0.05)</td>
</tr>
<tr>
<td>PR42_second</td>
<td>0.03 (−0.01, 0.06)</td>
<td>0.11 (0.08, 0.14)</td>
<td>0.19 (0.17, 0.22)</td>
<td>0.85 (0.84, 0.86)</td>
<td>0.01 (0.00, 0.03)</td>
<td>0.36 (−0.16, 0.71)</td>
<td>0.69 (0.39, 0.86)</td>
</tr>
</tbody>
</table>

Note: A positive correlation between an AGEP trait and a fertility trait (CR42, PB21, PR42) indicates that selection for younger AGEP would result in improved fertility outcomes.
of the AGEP4 phenotypes and the number of animals we could practically and cost-effectively include in the study. It was a priority for us to maximize the number of animals involved in the study as this was a key parameter that would dictate the stability of our model solutions for marker effects and (co)variance parameters. That is, we would expect the credibility intervals surrounding our parameter estimates to decrease as animal numbers with phenotypes increase. Importantly, an analysis of low heritability traits like CR42, PB21 or PR42 requires more phenotypes to achieve the same level of certainty (that is, width of credibility intervals) of estimated parameters when compared with higher heritability traits like AGEP4. Therefore, we decided to compromise the precision of the AGEP4 phenotypes so that we could measure a larger number of animals and, therefore, improve the certainty of our (co)variance parameters that involve calving, breeding, and pregnancy phenotypes. The phenotyping strategy that we applied to AGEP4 was supported by previous studies. Stephen et al., (2022a) used simulated AGEP phenotypes to demonstrate that EBV and variance parameter estimation are robust to left-, right- and interval-phenotype censoring. Furthermore, Stephen et al., (2022b) used real AGEP4 phenotypes measured in a population of some 500 Holstein-Friesians heifers (i.e., the FRH) to demonstrate the robustness of AGEP4 variance parameter estimation to phenotype censoring.

If AGEP phenotypes were measured routinely in commercial dairy herds, it is likely that further compromise in phenotype precision would be required. Stephen et al. (2023a) demonstrated that one blood test per animal may provide sufficient information for genetic analysis of AGEP, although the timing of that blood test may be important. Correlation between age at puberty and fertility traits measured during lactation.

We detected favorable genetic correlations between AGEP4 and calving, breeding, and pregnancy phenotypes, indicating that earlier onset of puberty is genetically associated with earlier calving, breeding, and pregnancy dates. This is an important result, as it confirms the value of AGEP4 as a predictor trait for fertility EBVs representing reproductive success during lactation. Although the genetic correlations between AGEP4 and calving, breeding and pregnancy phenotypes were generally moderate, there was some suggestion that the strength of the association depended on the specific fertility phenotype of interest. For example, AGEP4 tended to exhibit the strongest genetic correlation with PB21, and that tendency was evident in both first and second lactations. Our results align well with several existing studies, which have reported genetic correlations between AGEP and fertility dur-

![Figure 2. GWAS analysis of age at puberty. Five windows had a WPPA (Window Posterior Probability of Association) of 0.70 or greater. We used the UMD assembly to annotate our results, and these 5 windows were located on chromosomes 5 (105,337,527 bp to 106,432,283 bp), 14 (24,482,969 bp to 25,731,992 bp), 6 (22,273,911 bp to 23,352,794 bp), 1 (79,755,519 bp to 80,661,482 bp) and 11 (39,708,839 bp to 41,789,973 bp).]
ing lactation ranging from 0.36 to 0.58 in both beef (Mialon et al., 1999; Morris et al., 2000) and dairy cattle populations (Lefebvre et al., 2021). Lefebvre et al., (2021) and Mialon et al., (2001) used postpartum anestrus interval (PPAI) to represent fertility during lactation. That phenotype would be most similar to our PB21 phenotype, as cows with shorter PPAI would be more likely to present for breeding earlier. Morris et al., (2000) used calving date (continuous) and pregnancy rate (binary) traits to represent fertility during lactation, and those traits are very comparable to our CR42 and PR42 phenotypes. In contrast to these 3 studies, Patterson et al., (1992) reported low genetic correlations between AGEP and PPAI of 0.05 (Angus x Hereford, n = 148) and 0.12 (Brahman x Hereford, n = 148). However, their very small population sizes will have limited their ability to detect an association.

We have reported phenotypic correlations of near zero between AGEP4 and fertility traits measured during lactation. These results indicate that an animal’s own phenotype for AGEP does not provide any real insight into her reproductive performance later in life once the herd effect is removed. Our results align with those published by Lefebvre et al., (2021) who reported a phenotypic association of 0.08 between AGEP and PPAI.

In our study population, animals that failed to become pregnant during the seasonal breeding period in first lactation were removed from the herd before the next season, and so do not have calving, breeding and pregnancy phenotypes recorded for a second lactation. Culling on reproductive failure is common practice in

**Figure 3.** Distribution of EBVs for age at puberty (AGEP4: defined as age at first measured elevation in blood plasma progesterone) in the validation population (Fertility Research Herd; FRH). The EBVs were derived using SNP effects from the analysis of the animals in the PS (Puberty at Scale) population. The FRH animals are grouped according to the 2 sub-groups (positive line; POS +5% calving rate EBV, negative line; NEG −5% calving rate EBV).

**Table 3.** Candidate protein-coding genes located within genomic windows that had a WPPA > 0.7 in our GWAS of age at puberty defined as age at first measured elevation in blood plasma progesterone using Ensembl UMD3.1. Top SNP refers to the SNP with the strongest effect within the genomic window.

<table>
<thead>
<tr>
<th>BTA (WPPA)</th>
<th>Window Position</th>
<th>Top SNP (position)</th>
<th>Genes within region</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (1.00)</td>
<td>105,357,327 bp</td>
<td>ARS-BFGL-NGS-54006 (106,780,255 bp)</td>
<td>KCNA5, KCNA6, ENSBTAG00000026522, GALNT8, NDUFA9, AKAP3, DYRK4, ENSBTAG00000047347, ENSBTAG00000048162, RAD51AP1, C5H12orf4, FGF23, TIGAR, CCN2</td>
</tr>
<tr>
<td>11 (0.93)</td>
<td>24,482,969 bp</td>
<td>BOVINEHD1400007259 (25,015,640 bp)</td>
<td>XKR4, TMEM68, TGS1, LYN, RPS2O, RF01277, RF00003, MOS, PLAG1, CHCHD7, IMPAD1</td>
</tr>
<tr>
<td>6 (0.84)</td>
<td>22,373,311 bp</td>
<td>HAPMAP1252-BTA-11558 (22,967,467 bp)</td>
<td>BCL2L15, RFTD2, STT, RFTP4, MASP1</td>
</tr>
<tr>
<td>1 (0.75)</td>
<td>79,755,519 bp</td>
<td>BOVINEHD1400007259 (25,015,640 bp)</td>
<td>BCL2L15, RFTD2, STT, RFTP4, MASP1</td>
</tr>
<tr>
<td>11 (0.71)</td>
<td>39,708,839 bp</td>
<td>ARS-BFGL-NGS-38675 (41,379,246 bp)</td>
<td>VRK2, FANCL</td>
</tr>
</tbody>
</table>

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seasonal calving, pasture-based systems; however, this fertility-driven preselection could have been a source of bias in our estimation of (co)variance parameters as the removed animals represent the poorest performing group from first lactation. These animals would be likely to perform poorly in second lactation, but this was not captured in the second lactation phenotype measurements, and so can never be known with certainty.

We mitigated the effects of this preselection by analyzing each of the 3 fertility traits in a trivariate context, with each analysis including AGEP4, the first lactation phenotype and the second lactation phenotype for each animal. This approach ensured that the missing animals were accounted when estimating the relationship between AGEP4 and second lactation traits. In addition, we were also able to take a second approach to mitigate the effects of fertility-driven preselection on the calving rate trait, as we could identify the animals that were culled due to late pregnancies or failure to attain pregnancy in first lactation, and accurately assign them a penalty calving phenotype in second lactation. The genetic relationships between AGEP4 and both the CR42 and the PB21 traits were stable across first and second lactations, suggesting that analyzing them in a trivariate context sufficiently mitigated the effects of fertility-driven preselection on (co)variance parameter estimation. Conversely, the PR42 trait was not as consistent, and the positive association between AGEP4 and PR42 in first lactation (older AGEP genetically associated with later pregnancy) was not apparent in second lactation. Given our approach to account for fertility-driven preselection, it is possible that this difference reflects a real interaction of parity on the genetic association between AGEP4 and PR42, but further work is required to confirm this premise.

**Correlation between age at puberty and yearling body conformation traits**

Our results indicate that, in this population, AGEP4 was not genetically associated with yearling length or BW. Conversely, there was a positive moderate genetic association between AGEP4 and yearling height, whereby taller height was associated with older AGEP4. Our result of a near zero genetic correlation between AGEP4 and BW is consistent with the null to moderate correlations reported in the existing literature, which range from −0.30 and 0 (Smith et al., 1989; Gregory et al., 1995; Mialon et al., 2001; Wolcott et al., 2014). The genetic associations between AGEP4 and height reported in existing literature vary widely from −0.24 to 0.36 (Gregory et al., 1995; Wolcott et al., 2014), and so our result of 0.28 is consistent with
this range. However, to the best of our knowledge, the genetic association between AGEP4 and length has not been previously reported. We also determined that AGEP4 exhibited negative phenotypic correlations with yearling height, length, and BW. That is, larger animals at yearling age tended to attain puberty at a younger age. These results aligned well with existing literature, wherein negative phenotypic correlations have been reported between AGEP and yearling height (Gregory et al., 1995) and AGEP and yearling BW (Martin et al., 1992). It is well established that the onset of puberty is linked to an animal’s nutrition, and faster growing animals are likely to reach puberty before their slower growing contemporaries (Patterson et al., 1992b). The effects of different feeding levels across herds were accounted for in our analysis (through inclusion of herd as a fixed effect in the model) and should not be reflected in our phenotypic correlations. That said, feeding levels within herd can also vary, and even in a grazing context, the more dominant animals may consume more feed. Therefore, differences in feed intake may be a driver of the phenotypic association between AGEP and yearling body traits. We tested the effect of breed on our analyses by fitting proportion jersey as a fixed covariate. Including this covariate did not have a meaningful impact on estimated (co)variance parameters between age at puberty and body conformation traits.

Validation of genomic EBVs for age at puberty

We validated our gEBVs for AGEP4 (derived from analysis of the PS population) in an independent FRH population, which consisted of 2 subgroups with divergent calving rate EBVs and significantly different phenotypic expression of AGEP4 (Meier et al., 2021). Our gEBVs predicted a mean difference of 9.2 d in AGEP4 between the POS and NEG groups in the FRH population, which corresponds to approximately 33% of the observed 28-d phenotypic difference. This result indicates that the marker effects estimated using the PS population are relevant to other populations; however, the effects may be underestimated, perhaps because the PS population exhibited less extreme phenotypes and, therefore, had proportionally fewer genotypes from very low and very high fertility animals than the divergently selected FRH population. Another explanation could be the number of phenotypes contributing to the marker effects analysis. The estimated marker effects may have exhibited less shrinkage with a larger reference population, perhaps resulting in a larger mean difference between the validation gEBVs for the POS and NEG groups.

The mean reliability of gEBVs in the PS population was 0.59, indicating that the expected correlation between those gEBVs and true BVs in the PS population was 0.77. The true BVs of the FRH animals used in our validation are unknown, and so we assessed the prediction accuracy of the gEBVs in relation to phenotypes on FRH animals adjusted for herd effects. The correlation of 0.41 between the gEBVs and FRH adjusted phenotypes (Table 4) is a promising validation, indicating that about 17% of the phenotypic variation in that population was being explained.

Daetwyler et al. (2012) analyzed traits with similar heritabilities to our AGEP4 trait and they reported slightly lower correlations between gEBVs and validation phenotypes; however, their reference populations were smaller than ours, ranging from 500 to 2,500 animals. The accuracy of gEBVs increases relative to the size of the reference population (Daetwyler et al., 2012). Hence, the higher accuracy in our analysis relative to the study by Daetwyler et al. (2012) is not unexpected.

Furthermore, the accuracy of our gEBVs for AGEP4 would likely have been higher if our reference population had included more animals. Nevertheless, both of our approaches for validation indicate that the results of our gEBVs have predictive value outside of our reference population.

Table 5: Minor allele frequencies (MAF) for selected SNP in the Puberty at Scale (PS) population compared with the Fertility Research Herd (FRH) used for independent validation. The selected SNP are the largest effect SNP within high (>0.70) WPPA windows in the analysis of age at puberty in the PS population. SNP array commonality describes the proportion of SNP within the given genomic window that were common across the two arrays used to genotype the PS population (Weatherby’s Versa 50K SNP array; Illumina, USA) and the FRH population (GeneSeek GGP Bovine 150K SNP Illumina array [Illumina, USA]). The SNPs that were on the PS array, but not the FRH array were imputed using find-hap software (VanRaden et al., 2013)

<table>
<thead>
<tr>
<th>Chromosome (window position)</th>
<th>SNP ID (UMD 3.1 location)</th>
<th>MAF</th>
<th>SNP array commonality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PS/FRH</td>
<td>(percent commonality)</td>
</tr>
<tr>
<td>5 (105,337,527 bp – 106,432,283 bp)</td>
<td>ARS-BFGL-NGS-54496 (106,178,425 bp)</td>
<td>0.35/0.37</td>
<td>Yes (72%)</td>
</tr>
<tr>
<td>14 (24,182,969 bp – 25,731,992 bp)</td>
<td>BOVINEHD1400007259 (25,015,640 bp)</td>
<td>0.29/0.13</td>
<td>Yes (100%)</td>
</tr>
<tr>
<td>6 (22,273,911 bp – 23,352,794 bp)</td>
<td>HAPMAP1572-BTA-11558 (22,967,387 bp)</td>
<td>0.40/0.40</td>
<td>Yes (72%)</td>
</tr>
<tr>
<td>1 (70,755,519 bp – 80,661,482 bp)</td>
<td>HAPMAP11259-BTA-38429 (80,140,397 bp)</td>
<td>0.18/0.26</td>
<td>Yes (82%)</td>
</tr>
<tr>
<td>11 (39,708,839 bp – 41,789,973 bp)</td>
<td>ARS-BFGL-NGS-36765 (41,379,246 bp)</td>
<td>0.37/0.21</td>
<td>No (65%)</td>
</tr>
</tbody>
</table>
Validation of genomic windows for age at puberty

Several genomic windows of interest were identified in our initial GWAS of AGEP4 in the PS reference population, and window gEBVs produced for these genomic windows were then compared with AGEP4 phenotypes in our validation FRH population. The window located on chromosome 5, spanning 105,337,527 bp - 106,432,283 bp, had the highest WPPA value in our GWAS of AGEP4 in the PS population. The WPPA was close to 1, indicates that the window explained at least 1% of the variation in AGEP4 phenotypes in almost every MCMC sample. Further validation of this region indicated that gEBVs produced using only the SNP within this window (n = 20) explained around 11% of the variance in AGEP4 phenotypes in the independent FRH validation population. The high WPPA of this genomic window on chromosome 5 along with its successful validation in an independent population provide compelling evidence that this region harbors a QTL that is associated with variation in AGEP4. This region contains 14 candidate genes (Table 3). Several of these candidate genes have been implicated in fertility. For example, the CCND2 gene is differentially expressed in the granulosa cells of bovine follicles in varying developmental stages (Shimizu et al., 2013). The expression of CCND2 has been shown to be regulated by FSH, and female mice knockouts for the CCND2 gene exhibited infertility, while their male counterparts had hypoplastic testes (Sicinski et al., 1996). Similarly, the TIGAR gene, which encodes the TP53-induced glycolysis and apoptosis regulator protein, has been associated with oocyte quality in mice (Wang et al., 2018), and both CCND2 and TIGAR genes have been implicated in BW and stature in cattle (Hardie et al., 2017; Bouwman et al., 2018).

The window on chromosome 6, spanning 22,273,911 bp - 23,352,794 bp, was also validated in the FRH. This window harbors 7 candidate genes (Table 3). A particularly promising candidate within this genomic window is TACR3, which encodes for the neurokinin B-neurokinin receptor (NK3R). Neurokinin and NK3R are upstream regulators of kisspeptin, a hypothalamic neuropeptide that stimulates GnRH secretion initiating the pulsatile LH surge that precede the ovulation of a dominant follicle. Nakamura et al. (2017) reported dose-dependent responses in mean LH concentrations, the amplitude and frequency of pulsatile LH, and the timing of first postpartum ovulation when a NK3R selective agonist was administered to lactating cattle. Cattle administered the highest dose of the NK3R agonist had the shortest interval from calving to ovulation, indicating that loss of function in the TACR3 gene would likely compromise fertility performance. The AGEP trait and postpartum resumption of cyclicity share a moderate genetic correlation (Lefebvre et al., 2021), and so it is possible that variants in the TACR3 gene could also affect the onset of puberty. Moreover, Clarke et al., (2022) directly investigated the expression of the kisspeptin gene (Kiss1) and the neurokinin B gene (TAC3) in a subset of the FRH animals. They reported greater Kiss1 and TAC3 expression in the arcuate nucleus of cows in the POS fertility group relative to NEG cows. Given that POS cows have an earlier onset of puberty (Meier et al., 2021) and greater ability to resume cycling postpartum (Meier et al., 2022), these findings indicate variants in the TACR3 gene and other genes associated with the kisspeptin signaling pathway could play a role in the regulation of these fertility traits. This premise is also supported by the direct association between loss-of-function mutations in the TAC3 or TACR3 gene that cause hypothalamic hypogonadism and pubertal failure in humans (Topaloglu et al., 2009).

Further windows of interest identified from our GWAS included genomic regions on chromosomes 1, 14, and 11. The window located on chromosome 1, spanning 79,755,519 bp - 80,661,482 bp, was validated successfully in the FRH population; however, to our knowledge, none of the 5 candidate genes (Table 3) within this window have been directly associated with cattle fertility. The region on chromosome 14 had the second highest WPPA of 0.93 in our GWAS using the PS population and harbors 16 candidate genes. The PLAG1 gene is located at 25,007,291 bp - 25,009,296 bp, which is approximately 6 kb from the highest effect SNP within this window. The PLAG1 gene is well documented to affect stature and BW in cattle (Karim et al., 2011; Littlejohn et al., 2012; Fink et al., 2017), and has previously been reported to share an association with variation in the fertility traits ‘age of first calving’ and ‘age of first corpus luteum’ (Fortes et al., 2016). Given the association between AGEP and stature, it seems likely that the PLAG1 gene was driving the association between this genomic window and our AGEP4 phenotypes in the PS data set. The final region on chromosome 11 harbors 2 candidate genes, but neither have previously been directly associated with fertility in cattle.

The EBVs corresponding to the 2 windows on chromosomes 14 and 11 were positively correlated with AGEP4 phenotypes measured in the FRH; however, in both cases, these correlations were relatively weak (<0.10) and lower bound of the 90% CRI overlapped 0 (Table 4). We cannot be confident that the association observed between SNP in these 2 regions and AGEP4 phenotypes in the PS data set will extend to other populations. However, the poor validation of these 2 windows in the FRH may be explained by differences in
the structure of the PS and FRH populations. The FRH was a single herd of 100% Holstein-Friesian cows with extreme divergence in fertility EBVs based on re-calving rate, whereas the larger PS population consisted of 54 herds that included a normal range of fertility EBVs and Holstein-Friesian and Holstein-Friesian x Jersey cows. Differences in breed composition and animal selection will result in some differences in allele frequencies, and this might explain how a genomic region could be legitimately associated with variance in AGEP4 in the PS population yet fail validation in the FRH. For example, the PLG1 gene, which is located within the genomic window identified on chromosome 14, segregates very differently between Holstein-Friesian and Jersey cows. That is, the allele associated with smaller BW and stature is almost fixed in Jersey populations (Littlejohn et al., 2012), while the alternate allele that is associated with larger stature and BW is represented at a frequency of around 85% in Holstein-Friesians (Littlejohn et al., 2012); therefore, this gene is likely to segregate to a greater extent in a mixed-breeds population, relative to a purebred population. Moreover, the selection strategy used to establish the FRH actively minimized variance in a range of traits such as BW and milk production, while maximizing variance in fertility traits. This selection process would likely result in the FRH having a unique population structure.

We gained some insight into how the population structure of the reference (PS) and validation (FRH) populations may differ by comparing the minor allele frequencies of the highest effect SNP within these windows (Table 5). These analyses showed that the top SNP within the genomic windows on chromosomes 14 and 11 were not segregating uniformly across PS and FRH populations. In both cases, the minor allele frequencies were 0.16 lower in the FRH, when compared with the PS population. Conversely, the top SNP in the other 3 windows with WPPA > 0.70 (on chromosomes 5, 6 and 1) had uniform segregation across the FRH and PS populations. The accuracy of imputation may also contribute to the poor validation of the window on chromosome 11. The commonality between the arrays used to genotype the PS and FRH population is only 65% within this window, and furthermore, the SNP with the highest effect in the analysis of the PS animals was not common across the 2 arrays. Conversely, the top SNP in each of the other 4 genomic windows were present on both SNP arrays, and the SNP commonality was at least 72%. Missing SNPs were imputed, and error association with this imputation may compromise our validation of the window on chromosome 11. Further research into the 2 regions that failed validation in this study (located on chromosome 14 and 11) is required to validate or invalidate their association with AGEP4.

**CONCLUSION**

Moderate genetic correlations between AGEP4 and breeding, calving and pregnancy rate traits indicate that selection for earlier AGEP4 will improve genetic merit for fertility during lactation. We suggest that AGEP could add value as an early predictor of fertility EBVs, especially given the large differential between the heritabilities of AGEP4 and breeding, calving and pregnancy rate traits. Genomic EBVs for AGEP4 exhibited a correlation of 0.41 with independent validation phenotypes adjusted for fixed effects. Furthermore, there was one genomic region in our GWAS analysis, located on chromosome 5 that is likely to harbor a QTL for the AGEP4 trait. Window gEBVs produced for this region on chromosome 5 explained around 11% of the variance in our validation phenotypes. Our results contribute to a growing understanding of the genetic architecture of AGEP, and may also offer insight into correlated traits, such as fertility traits measured during lactation. Further investigation of the association between our identified genomic regions and variance in key fertility traits such as calving, breeding and pregnancy performance is warranted.

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Conflict of interest: M A Stephen, C R Burke, N Steele, S Meier, and C V C Phyn are employed by DairyNZ Limited. All authors declare no other competing interests.

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