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How SNP chips will advance our knowledge of factors controlling puberty and aid in selecting replacement beef females[1,2,3]

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ABSTRACT: The promise of genomic selection is accurate prediction of animals' genetic potential from their genotypes. Simple DNA tests might replace low accuracy predictions for expensive or lowly heritable measures of puberty and fertility based on performance and pedigree. Knowing which DNA variants (i.e., SNP) affect puberty and fertility with some certainty is the best way to fulfill the promise. Several SNP from the BovineSNP50 assay have tentatively been associated with reproductive traits including age at puberty, antral follicle count, and pregnancy observed on different sets of heifers. However, sample sizes are too small and SNP density is too sparse to definitively determine genomic regions harboring causal variants affecting reproductive success. Additionally, associations between individual SNP and similar phenotypes are inconsistent across data sets, and genomic predictions do not appear globally applicable to cattle of different breeds. Discrepancies may be a result of different QTL segregating in the sampled populations, differences in linkage disequilibrium (LD) patterns such that the same SNP are not correlated with the same QTL, and spurious correlations with phenotype. A number of approaches can be used independently, or in combination, to improve detection of genomic factors affecting heifer puberty and fertility. Larger samples and denser SNP will increase power to detect real associations with SNP having more consistent LD with underlying QTL. Meta-analysis combining results from different studies can also be used to effectively increase sample size. High-density genotyping with heifers pooled by pregnancy status, or early and late puberty can be a cost-effective means to sample large numbers. Networks of genes, implicated by associations with multiple traits correlated with puberty and fertility, could provide insight into the complex nature of these traits, especially if corroborated by functional annotation, established gene interaction pathways, and transcript expression. Example analyses are provided to demonstrate how integrating information about gene function and regulation with statistical associations from whole-genome SNP genotyping assays might enhance knowledge of genomic mechanisms affecting puberty and fertility, enabling reliable DNA tests to guide heifer selection decisions.

Key words: beef cattle, fertility, genomics, puberty

INTRODUCTION

The ideal beef cow has been defined as a cow who first calves at 2 yr of age, maintains a 365-d calving interval, and weans a marketable calf every year. During her extended productive lifespan, she never needs human intervention to assist calving or nursing her calf; remains structurally sound, able to graze the entire area available to her; and is tolerant of weather, disease, parasites and other stressors of her environment (Hohenboken, 1988). Consistent with impact of reproduction on cow herd profitability (Melton, 1995), fertility - raising a calf every year - is paramount to this ideal, with the caveat that the calf be marketable. Remaining criteria address factors associated with keeping her fertile at a minimal cost until she has raised a replacement daughter and her calves have generated sufficient revenue to pay for her development and maintenance.

To approach this ideal, replacement heifers must reach puberty, conceive and calve early as 2-yr-olds, subsequently increasing their lifetime productivity compared to contemporaries who calve later (Lesmeister et al., 1973; Garcia Poloma et al., 1992). Older, heavier heifers in adequate body condition are more likely to attain puberty and conceive early in their first breeding season, thus, age, weaning weight, and body condition scores are convenient and commonly suggested indicators of heifers most likely to calve early as 2-yr-olds and remain in the herd as productive cows (Bolze and Corah, 1993; Merck, 2005; Engelken, 2008). Prior to breeding as yearlings, examination of the reproductive tract (Anderson et al., 1991; Rosenkrans and Hardin, 2003; Cushman et al., 2008) may provide phenotypic means to more accurately determine potential fertility and eliminate heifers least likely to become pregnant as yearlings and stay as productive, revenue-generating cows. Genomic indicators could also allow earlier screening to lessen costs of developing subfertile heifers as potential replacements. The complexity of reproductive traits, affected by genetic and environmental factors (Martin et al., 1992; Patterson et al., 1992; Cammack et al., 2009), implies development of genomic tests for reproduction will be challenging. This paper examines challenges facing development of cost-effective DNA tests, and provides examples of analytical approaches to address those challenges and opportunities to gain insight into genomic mechanisms affecting heifer puberty and fertility.

CHALLENGES

Obtaining Data to Enable Heifer Selection with SNP Chips

Genomic selection (Meuwissen et al., 2001) facilitated by the BovineSNP50 BeadChip (50K; Illumina Inc., San Diego, CA), with over 54,000 SNP located throughout the genome (Matukumalli et al., 2009), appears to increase accuracy of breeding values predicted for complex traits on young animals with no or few progeny (Van Raden et al., 2009; MacNeil et al., 2010; Snelling et al., 2011). Accuracy of genomic selection is affected by heritability of the trait, number of recorded individuals with genotypes, and effective population size. For example, equations describing accuracy of genomic selection (Goddard, 2009) show traits with heritabilities of 0.05 to 0.10 need 9 to 19 times as many records as a trait with heritability of 0.50 to achieve the same level of accuracy (**Figure 1**). Within-herd genomic evaluation, using 1,000 phenotyped and genotyped animals representing a small population ($N_e < 100$), may be adequate to explain at least one-half the additive genetic variation for moderately heritable ($h^2 > 0.30$) traits, but less than one-fourth the additive variation for traits with low heritability ($h^2 < 0.10$). As a sample of a broader population ($N_e = 1,000$), the 1,000 records may explain about a one-fourth of the variation in moderately heritable traits, and less than 5% of additive genetic variation in lowly heritable traits.

Age at puberty has been reported to be at least moderately heritable, with most estimates greater than 0.25 (Martin et al., 1992; Morris et al., 2000; Johnston et al., 2009), while other measures of reproduction such as pregnancy rate, calving interval, and calving day (i.e., the difference between calving date and the earliest calving date of contemporary females) have a low heritability; estimates are often less than 0.10 (Cammack et al., 2009; Minick Borman and Wilson, 2010). With individual breeds having effective population sizes between 64 and 445 (Cleveland et al., 2005; McParland et al., 2007; Marquez et al., 2010), several hundred to a few thousand observations may be adequate for within-breed whole-genome prediction of age at puberty, but tens of thousands of records are needed for lower heritability traits, such as first service AI pregnancy rate and breeding season pregnancy rate. The number of records required may increase by an order of magnitude for whole-genome predictions applicable to heifers representing a wide variety of genetic backgrounds, or breeds with larger effective population sizes (Bouquet et al., 2011). With this need for a large number of records, and a

price of approximately $100 per 50K SNP assay, the cost of individually genotyping females to enable accurate prediction of fertility traits remains prohibitive.

Cost and value of selecting heifers with SNP chips

If perfectly accurate 50K genomic predictions of fertility were available, the $100 per 50K cost to screen candidate replacement heifers is also prohibitive. Assuming an 85% heifer pregnancy rate and that 10% of phenotypic variation in pregnancy rate is genetic, the top one-half of heifers screened by a test that completely explains genetic variation in heifer pregnancy rate are expected to have a 2% greater pregnancy rate than a randomly-selected half. The breakeven cost of $4.30 per cow to increase heifer pregnancy rate by 1%, estimated by net present value analysis of a Nebraska Sandhills ranch (Meek et al., 1999) and by bio-economic simulation to determine economic weights for a selection index for Simmental cattle (M. D. MacNeil, personal communication), provides a return of $8.60 per heifer saved, or $4.30 per heifer screened with the perfectly accurate DNA test. Applied to candidate sires, the same genomic test may have much greater value, considering greater selection differential for males and the number of daughters they might sire. Simulated genomic selection with a multi-trait index including reproductive traits (Van Eenennaam et al., 2011a, b) indicated that breakeven costs werre between $3.63 and $6.53 to individually genotype heifers, whereas breakeven costs to genotype bull calves were over $200.

Reproductive tract scores (**RTS**), obtained in prebreeding examination to phenotypically screen heifers, may be at least as effective as an accurate genomic test for heifer pregnancy. Reproductive tract scores may substitute for intense observation of estrous behavior to identify which heifers are mature enough to reach puberty, exhibit multiple estruses, and become pregnant early in the upcoming breeding season, thereby offering a predictor of initial pregnancy and potential lifetime productivity, given the favorable relationships between early pregnancy, age at first calving, and lifetime production (Lesmeister et al., 1973; Garcia Poloma et al., 1992). Heifers with RTS of at least 3 on 5 point scale (1 = immature, 5 = corpus luteum present) tend to become pregnant earlier and consistently have greater breeding season pregnancy rates than lower RTS contemporaries (Anderson et al., 1991; Martin et al., 1992; Pence et al., 1999). An examination of 271 heifers 1

d before the start of a 50-d AI breeding season found that those with RTS ≥ 3 had a 5% greater than average breeding season pregnancy rate (Holm et al., 2009). When heifers were scored earlier, 30 to 70 d before breeding, the advantage for heifers with RTS ≥ 3 was closer to 1% for the breeding season (Randle, 2002; Patterson and Bullock, 1995), leading Geary (2000) to question the value of routine RTS. The high scoring heifers, however, were pregnant earlier in the breeding season, and would subsequently calve earlier and wean older, heavier calves, ultimately resulting in greater lifetime productivity relative to low RTS heifers who became pregnant late in their first breeding season.

The $3 to $5/heifer cost of RTS (K. G. Odde and R. L. Weaber, personal communication) provides a useful target price for genomic tests to screen heifers. This price range is consistent with the potential economic value of a genomic test for heifer pregnancy, without considering the impact of screening for early puberty and associated effects on productivity. A major challenge for an effective genomic test for heifer selection is to keep cost of the test below its potential value, competitive with the price of phenotypic evaluations. Costs of genotyping the large numbers needed to develop an accurate whole-genome test, as well as to screen heifers with whole-genome SNP chips will be substantially more than costs incurred to select heifers according to weight, age, body condition, and RTS.

OPPORTUNITIES

Low-Cost Genotyping

To reduce genotyping costs, the number of phenotypes represented by each genotyping assay can be increased or the number of markers genotyped by an assay can be reduced. The number of phenotypes per assay can be also be increased by genotyping progeny-tested parents and replacing individual phenotypes with progeny means (Goddard and Hayes, 2009) or deregressed estimated breeding values (Garrick et al., 2009) to estimate SNP effects and calibrate genomic predictions. The number of phenotypes represented by each genotyping assay can also be increased by DNA pooling, where each SNP assay represents mixed DNA sampled from several individuals. Pools can be constructed according to phenotype, so phenotypes are similar within pools and distinctly different between pools. Differences in allele frequencies between the pools allow

genome-wide association studies (**GWAS**) to identify SNP associated with the trait separating the pools (MacGregor et al., 2006, 2008; Hunag et al., 2010). Huang et al. (2010) pooled DNA from mature Holstein cow ovaries to identify SNP from the 50K having significant effects on *in vitro* fertilization and subsequent development to the blastocyst stage. In this study, ovaries of 589 cows were obtained from an abattoir. The mature oocytes aspirated from these ovaries were exposed to bull semen, and then the number of fertilized oocytes and number of fertilized oocytes that developed into blastocysts after 7 d in culture were counted. Samples of DNA from ovaries producing these oocytes were pooled by fertilization rate (i.e., embryos per exposed oocyte) and blastocyst rate (i.e., blastocysts per fertilized oocyte). Eight pools, 2 high and 2 low for each of the 2 traits, were genotyped. Each pool contained mixed DNA from 42 to 49 ovaries. The study required 1.4% of the genotyping assays that would have been needed to individually genotype each of the ovaries sampled.

Pooling DNA and parental genotyping enable estimation of genome-wide SNP effects at a fraction of the cost of individual genotyping, but individual genotypes are still required for selection. Using existing technologies, targeted panels with a small number of informative markers are less expensive than whole-genome arrays, and techniques that are under development to utilize next generation sequencing with barcoded DNA may further reduce genotyping costs (Elshire et al., 2011; Davey et al., 2011). These technologies will allow inexpensive genotyping of targeted regions or low-coverage whole-genome genotyping, with costs around $20/sample projected to decrease below $5/sample (Elshire et al., 2011; Davey et al., 2011).

Two basic strategies to build small, low-cost panels for individual genotyping are to include markers informative for imputation, allowing genotypes for denser markers to be inferred; or to include markers targeting a particular trait or suite of traits. Genotypes for the 50K chip can be imputed from the lower-density Bovine3K BeadChip (3K, Illumina Inc., San Diego, CA) containing 2,900 SNP. Likewise, 50K genotypes can be imputed to higher density, more expensive 777,962 SNP BovineHD BeadChip (Illumina Inc) and the 648,855 SNP Axiom Genome-Wide BOS 1 Array (Affymetrix, Santa Clara, CA). In dairy cattle, 92% to 97% of 50K genotypes were correctly imputed from 3K genotypes (Daetwyler et al., 2011; Dassonneville et al., 2011; Sargolzaei et al., 2011; Van Raden et al., 2011).

Imputation from 50K to higher densities may enable SNP that are consistently associated with underlying QTL across cattle populations to be identified. Spacing of the 50K is sufficient to locate SNP that are in linkage disequilibrium (**LD**) with underlying QTL within cattle breeds, but inconsistent LD patterns among cattle breeds (Gautier et al., 2007; Bovine HapMap Consortium, 2009) indicate that across breeds, the same SNP from the 50K will not consistently be associated with the same underlying QTL. A comparison of 50K SNP effects on feed intake and efficiency estimated in Australian and U.S. cattle showed individual SNP effects were inconsistent but identified 1-Mb intervals containing SNP associated with the traits in each population (Bolormaa et al., 2011; Pollak et al., 2012). Higher marker density within these intervals might identify SNP having more consistent associations across populations of cattle, due to stronger, more consistent LD between QTL and SNP with closer spacing between SNP. These high-density assays satisfy the estimated need of 300,000 SNP to maintain consistent phase between SNP and QTL across breeds of cattle (de Roos et al., 2008; Goddard and Hayes, 2009).

Small, inexpensive panels targeting important traits can be constructed from SNP associated with the traits of interest, although effectiveness of these panels may be low if the selected SNP are not consistently correlated with underlying QTL. Including additional SNP that surround associated SNP may accommodate variable LD patterns, increasing the probability of the panel having SNP that are correlated with the QTL in different populations. Information from multiple sources can also be leveraged by using gene set and network analysis to integrate SNP identified by GWAS with gene expression, functional annotation, regulatory pathways, and other evidence to develop panels likely to contain biologically relevant SNP (Medina et al., 2009; Zhong et al., 2010; Wang et al., 2011). Fortes et al. (2010) described a systems biology approach to construct an association weight matrix (**AWM**) from GWAS of several traits, with support from pathway and transcription factor networks to develop gene networks associated with complex traits. The AWM approach was applied to GWAS of age of observation of the first corpus luteum and 21 additional measures of heifer puberty, weight, growth, and body composition taken on separate populations of *Bos indicus* and *Bos taurus* × *Bos indicus* composite females. A network of 1,272 genes predicted to interact and affect puberty was defined by this approach (Fortes et al., 2011).

Integrating GWAS with functional characterization of sequence variation and genome features may enable development of relatively small, inexpensive marker sets that are sufficiently robust to describe phenotypic variation in puberty and fertility in heifers having diverse genetic backgrounds. Panels consisting of several SNP within and surrounding genes initially identified by functional gene set and regulatory network analysis may facilitate genotyping the large numbers needed to support accurate genomic testing, and the process of developing and refining these panels may contribute to greater understanding of genomic factors affecting heifer puberty and fertility (Luna-Nevarez et al., 2011).

Evaluation of Large and Reduced SNP Sets from the BovineHD BeadChip

Background. To provide examples of analyses for development of small marker sets for screening heifers, subsets of SNP from the BovineHD chip were evaluated for effects on age at puberty, antral follicle count and yearling pregnancy status of heifers in Cycle VII of the U.S. Meat Animal Research Center Germplasm Evaluation (**GPE**) project (Wheeler et al., 2005). Age at puberty, determined by observed estrus behavior, and yearling pregnancy are established measures for beef heifers (Short and Bellows, 1971; Laster et al., 1979; Martin et al., 1992). Antral follicle count, ascertained by rectal ultrasound, is not commonly measured but may contribute to more complete assessment of female fertility. Antral follicle count is indicative of ovarian primordial follicle reserve in cattle (Cushman et al., 1999; Ireland et al., 2008), and association between depletion of the ovarian reserve and reproductive senescence in mammals suggests antral follicle count may be indicative of reproductive longevity (Cushman et al., 2009). Further, relationships between antral follicle count, failure to conceive in consecutive breeding seasons, and calving interval have been reported (Maurer and Echternkamp, 1985; Oliveira et al., 2002).

The observed heifers were F_1 and F_1^2 ($F_1 \times F_1$), with the F_1 generation resulting from mating 151 AI sires of 7 popular breeds (i.e., Angus, Charolais, Gelbvieh, Hereford, Limousin, Red Angus, and Simmental) to Angus, Hereford and MARCIII composite cows (Gregory et al., 1991). The F_1^2 generation was produced by naturally mating F_1 bulls and females (Snelling et al., 2010). Genotypes for 735,239 autosomal SNP were imputed with findhap version 2 (Van Raden et al., 2011) from 50K genotypes of 4,525 individuals in a 10,899

animal pedigree, and a BovineHD reference of 326 individuals including the 150 AI sires, 51 F_1 sires, and 122 dams that had not been genotyped with the 50K panel. Nine hundred seventy-eight records of age at puberty, 452 antral follicle counts, and 1,386 pregnancy observations were available for these analyses.

Process. Procedures for partial-genome analysis, initially developed to assess heritability due to SNP selected according to associations with feedlot intake and efficiency (Snelling et al., 2011), were employed to evaluate subsets of BovineHD SNP. Genotypic relationship matrices (**M**) for each BovineHD subset were computed as $M = SS'/[2\Sigma p_i(1-p_i)]$ (Van Raden, 2008), where p_i was the B allele frequency for the i^{th} SNP in the set, and S is a matrix of differences between individual genotypes (i.e., 0, 1, or 2 copies of the B allele) and the mean genotype ($2p_i$). To avoid singularity, a scaled matrix M* was computed as $M^* = 0.99M + 0.01A$, where A is the pedigree relationship matrix. The inverse of M* replaced A^{-1} in mixed model equations to estimate heritability and predict genomic breeding values. Fixed effects in the analyses included year-season contemporary groups, covariates for breed composition, and genomic inbreeding, taken from the diagonal of M (Van Raden, 2008). The genomic inbreeding coefficients are analogous to pedigree inbreeding coefficients from the diagonal of A (Wright, 1922).

Individual SNP effects were estimated by solving $\hat{g}=S'[SS']^{-1}\hat{u}$, where $\hat{g}$ is a vector of additive allele B effects for all SNP in the set, and $\hat{u}$ is the solution vector from BLUP of individual genomic breeding values (Stranden and Garrick, 2009). Means and SD for each set of estimates were computed, and large BovineHD subsets were reduced by identifying SNP whose mean deviation was greater than 2 SD from the mean effect on age at puberty, antral follicle count and yearling pregnancy rate. Effects on yearling pregnancy of GPE Cycle VII heifers, estimated from large and reduced BovineHD subsets, were then applied to pooling allele frequencies estimated from pooled DNA to predict genomic differences between groups of pregnant and non-pregnant *Bos indicus* × *Bos taurus* composite heifers from a commercial ranch in Central Florida. Large BovineHD subsets contained between 12,000 and 76,000 SNP, derived from 50K GWAS and AWM gene network analysis of Brangus seedstock heifers (Thomas et al., 2012); multivariate 50K and gene set analysis of GPE Cycle VII heifers; analysis of BovineHD pooling allele frequencies with female DNA pooled by

pregnancy status (McDaneld et al., 2011); and analysis combining the top BovineHD SNP from each autosome. The following sections summarize data and results from each source utilized to select the SNP sets.

Brangus GWAS-association weight matrix. Pedigree and 50K genotypes of approximately 800 Brangus seedstock heifers from New Mexico State University and a Central Texas producer were analyzed (Peters et al., 2010; Thomas et al., 2012). In addition to first service AI pregnancy and breeding season pregnancy status, phenotypes measured in these studies included weaning and yearling weights and hip heights; and ultrasound backfat, LM area and intramuscular fat taken the same time as yearling weight and height. Heifers were estrous synchronized with a progestin treatment (i.e., melengestrol acetate or a progesterone-releasing device) for first service at approximately 15 mo of age. Timed AI was used, so all heifers in a synchronized group were first inseminated during the synchronized breeding. After the first AI, heifers were exposed to natural service or additional AI for up to 3 estrous cycles. Pregnancy was assessed via ultrasound following breeding. First service pregnancy was based on fetal development at pregnancy testing, and confirmed with records on non-return to estrus after initial AI and subsequent calving dates. Age at puberty was not recorded on these heifers because the synchronization treatment prevented natural estrus from being observed on heifers not cycling before treatment started.

Estimated genetic and phenotypic correlations among the weights and heights were strong, and weak to moderate between observations of conception and body measurements (**Table 1;** Thomas et al., 2012). Genetic correlations were positive between first service conception, body weights and heights, but pregnancy was negatively correlated with weights and heights. Ultrasound backfat thickness and LM area were positively correlated with both first service and breeding season pregnancy; the strongest of all genetic correlations with first service conception was between first service conception and backfat thickness. Each of the 50K SNP were tested for association with each trait with a univariate model including a covariate for SNP effects (i.e., 0, 1, or 2 copies of allele B) and a polygenic random animal effect with known pedigree relationships, using procedures initially developed for 50K GWAS of GPE (Snelling et al., 2010; 2011).

Following methodology described by Fortes et al. (2010), minimally associated SNP ($P < 0.05$) and their effects estimated for the 10 traits provided the basis for an AWM and underlying gene network related to

first service conception (Thomas et al., 2012). An initial network of 1,555 genes indicated by univariate GWAS was filtered for genes expressed in the hypothalamus of pre- and post-pubertal half-sib heifers, resulting in a network of 1,096 genes supported by GWAS and hypothalamic expression. Pathway and gene ontology (**GO**) term enrichment analysis (Dennis et al 2003; Maere et al., 2005; Eden et al., 2009; Huang et al., 2009) revealed that the network was enriched with genes involved with axon guidance. Axon guidance is a pathway that can affect pulsatile GnRH release, which is essential for pubertal development and fertility (Clarkson and Herbison, 2006; Ojeda et al., 2010a, b). Five transcription factors: *ZMAT3, RFX4, NR6A1, STAT6* and *PLAGL1,* were highly connected to genes in the network and predicted to be molecular regulators of growth and developmental processes affecting when a heifer attains puberty and becomes pregnant. The UMD3.1 bovine assembly and annotation (Zimin et al., 2009) and BovineHD positions (Illumina Inc., 2010) were used to locate 75,560 SNP within 50 Kbp of the genes in this AWM-expression network. This subset of BovineHD SNP was subsequently evaluated in the GPE Cycle VII heifers.

GPE Cycle VII GWAS - Gene Set Analysis. Single-trait GWAS of age at puberty determined by observed estrus behavior, pre-breeding antral follicle count and yearling pregnancy assessed 40 to 50 d after bulls were removed from breeding pastures showed fewer 50K SNP were associated with these traits in GPE Cycle VII heifers than would be expected by chance. To allow interrelated phenotypes to inform SNP estimates, multivariate analysis including genomic relationships among the Cycle VII heifers and ancestors with 50K genotypes were conducted. Phenotypic and genomic correlations between yearling weight, postweaning gain, body condition score at pregnancy check following breeding, age at puberty, antral follicle count, and pregnancy were estimated (**Table 2**). Near-zero phenotypic correlations between yearling pregnancy and yearling weight or postweaning gain indicate weight and growth rate prior to breeding are ineffective indicators of reproductive success in these heifers. Moderate genomic correlations among the measures of body weight, growth, fatness, puberty and pregnancy indicate that each trait contributes information but none of the traits is a strong indicator of heifer fertility. Genomic selection considering a combination of these traits may be more effective than focusing solely on puberty, pregnancy rate or a single indicator trait.

Genomic breeding values from the 6-trait analysis were used to solve 50K SNP effects, and SNP with

strong effects (> 2 SD from mean) on each trait were scrutinized. Hypergeometric tests of GO terms and pathways revealed significant ($P < 0.01$) overrepresentation of genes involved with olfactory and G-protein coupled receptor functions near the SNP affecting puberty, follicle count and pregnancy (**Table 3**). The olfactory system is high in G-protein coupled receptors, and G-protein coupled receptors regulate the hypothalamic-pituitary-gonadal axis, affecting reproduction and sex hormone-dependent diseases (Heitman and Ijzerman, 2008). The set of genes involved with the overrepresented olfactory and G-protein receptor functions were used to identify a set of 56,730 BovineHD SNP for further evaluation in Cycle VII heifers.

Pregnancy GWAS with pooled DNA. A set of 12,869 autosomal BovineHD SNP was identified from DNA of 3,270 Braford, Brangus, and Simbrah females from a Central Florida ranch, pooled by breed and pregnancy status (i.e., pregnant or non-pregnant) after exposure to fertile bulls as yearlings and 2-yr-olds (McDaneld et al., 2011). These females passed a reproductive tract examination as yearlings and were exposed for 2 seasons; heifers who did not conceive as yearlings remained in the herd and were exposed as 2-yr-olds. The selected SNP were determined to have significantly different ($P < 0.05$) pooling allele frequencies between replicated pools containing females failing to conceive in both breeding seasons (10%), failing as yearlings then conceiving as 2-yr-olds (28%), conceiving as yearlings then failing as 2-yr-olds (16%), or conceiving as in both seasons (47%).

Smaller SNP sets, selected from the Central Florida pooling GWAS study to meet stricter criteria to account for multiple testing, failed to explain variation in age at puberty, antral follicle counts or pregnancy of the GPE Cycle VII heifers, and there was no overlap of these SNP with SNP meeting similarly strict criteria in 3 sets of pools from other locations (USMARC, a Western Nebraska ranch, and females from 7 herds in 6 states). Across sets of pools, regions of *Bos taurus* chromosomes (**BTA**) 1, 5, and 17 were identified with different SNP by at least 2 of the 4 pool sets. The SNP on BTA 1 SNP were located within a previously described conception rate QTL (Boichard et al., 2003) and the SNP on BTA 5 were between two ovulation rate QTL (Kirkpatrick et al., 2000; Allan et al., 2009).

The set of autosomal SNP derived from the Central Florida pools did not include SNP located on chromosome Y, which were consistently identified by the 4 sets of pools created to identify regions of the

genome associated with reproductive success. Presence of SNP mapped to chromosome Y in pools of non-pregnant or low fertility females was an unexpected finding from the pooling studies (McDaneld et al., 2011). Presence of Y SNP in pools of pregnant females was negligible. Individually genotyping females with a PCR test using Y-specific primers developed to sex embryos (Park et al., 2001), showed 21% to 29% of the Central Florida females who failed to conceive as both yearlings and 2-yr-olds carried at least a portion of the Y chromosome. These studies also found chromosome Y markers were also present in blood of USMARC heifers born twin to a bull, indicating chimerism from blood shared by male and female fetuses. While heifers with a known bull co-twin are usually excluded from breeding, undetected single-born freemartins may explain some Y-positive females (Padula, 2005). Heifers born twin to a bull were not recorded under the extensive management of the Central Florida ranch, but the pre-breeding examination should have detected freemartins without a palpable reproductive tract. Other possible explanations of anatomical females carrying Y DNA include X/Y recombination resulting in crossover of Y material to X during gametogenisis, chromosome abnormalities (Swartz and Vogt, 1983), mutations in the sex determining region Y (*SRY*) gene, and autosomal mutations affecting expression of *SRY* and other genes affecting gonadal development (Biason-Lauber et al., 2009; Paliwal et al., 2011). While specific causes of Y DNA in non-pregnant females have not been determined, screening for Y may eliminate a small percentage of heifers likely to be infertile. The expected increase in heifer pregnancy rate corresponding to elimination of infertile Y-positive heifers is approximately the pregnancy rate (including Y-positive heifers) times the incidence of Y-positive heifers. With an 85% heifer pregnancy rate and the previously described $4.30 per cow breakeven to increase pregnancy by 1% (Meek et al., 1999), Y-positive incidence of 2% to 3% is high enough to justify spending $3 to $5 to test heifers for chromosome Y DNA.

Combined chromosomes. Analyses considering all 735,239 autosomal SNP were conducted for comparison to BovineHD subsets selected with external information, using the Brangus AWM-hypothalamus network, multi-trait GWAS-gene set analysis, or pooled DNA GWAS. Each chromosome was evaluated independently in Cycle VII heifers, using between 12,931 (BTA 25) and 46,492 (BTA 1) SNP, and markers having strong within-chromosome effects on puberty, follicle count and pregnancy, averaging >2 SD from the

mean effect for the 3 traits, were combined in a set of 46,695 SNP containing markers from each of the 29 autosomes (703 to 2,996 SNP per chromosome).

Genetic and Genomic Heritabilities

Heritabilities (± SE) estimated from univariate analyses using pedigree relationships among the GPE Cycle VII heifers and ancestors were 0.17 (± 0.07) for age at puberty, 0.73 (± 0.18) for antral follicle count, and 0.25 (± 0.08) for yearling pregnancy. The estimates for age at puberty observed on 978 heifers, and yearling pregnancy from 1,386 heifers are within the ranges summarized by Cammack et al. (2009), although the age at puberty estimate is less than often-reported values of >0.40, and the pregnancy estimate is at the upper end of the reported range. Breed differences, between antral follicle counts of Brahman, Senepol and Angus cows have been detected (Alvarez et al., 2000), but reports providing estimates of heritability of antral follicle count in cattle were not found in literature. Strong relationships between follicle counts and age at menopause in women (Broekmans et al., 2004; Giacobbe et al., 2004) coupled with heritability estimates of age at menopause near 0.50 (van Asselt et al., 2004; Murabito et al., 2005) indicate that much variation in antral follicle count may be inherited.

Genomic heritabilities, indicating the proportion of phenotypic variance explained by genomic relationships using 4 large and 5 reduced subsets of the BovineHD SNP (**Table 4**), were also count and yearling pregnancy varied by subset of the BovineHD (**Figure 2**). Estimates using the estimated from Cycle VII heifers. The heritabilities (h^2) of age at puberty, antral follicle 46,695 SNP selected from combined chromosome analysis (**HDA**), which evaluated all BovineHD SNP, were consistently higher than estimates from pedigree relationships. Estimated h^2 for antral follicle count from pedigree and HDA were within SE, but the HDA estimates for age at puberty and heifer pregnancy were severely inflated, 3 to 4 times greater than pedigree h^2. The large SNP sets selected from Central Florida pooling studies, Brangus association weight matrix and hypothalamus expression network, and multiple-trait GWAS and gene set analysis of Cycle VII heifers generally yielded similar h^2 estimates. These estimates were not different than pedigree h^2 for puberty, and approximately one-half of the pedigree h^2 for follicle count. Estimated h^2 for yearling pregnancy from pooling-

derived SNP was greater than those from the Brangus network and GPE gene sets, SE of the latter 2 estimates included zero.

Reduced sets, selected according to mean effect on age at puberty, antral follicle count, and yearling pregnancy (average >2 SD from mean of each trait), usually appeared to explain as much or more variation than the large subsets of BovineHD SNP. For puberty, h^2 from HDA and the reduced set of 890 SNP (**HDAr**) with strong effects were similar, as were estimates from pooling-derived and the corresponding reduced set of 100 SNP. Estimates using 511 SNP selected from the Brangus network, and 350 SNP from GPE gene sets were somewhat greater than the corresponding large-set heritabilities. The amount of variation explained by each of the reduced gene-oriented sets, the Brangus network, and GPE gene sets was similar. Combining these two sets into a set of 814 unique SNP (i.e., 47 SNP shared by the Brangus and GPE sets) resulted in a slightly larger estimate for age at puberty, but less than that estimated from HDAr, the set based solely on associations with the Cycle VII heifer data. The pattern of variance explained by reduced sets was consistent for the three heifer fertility measures. The highest h^2 was estimated from HDAr, followed by h^2 from the set combining both of the reduced gene-oriented sets. Estimates from either reduced gene-oriented set were somewhat lower than the combined set, and the numerically lowest h^2 estimates were from the set reduced from SNP identified by pooling studies.

Heterosis and Homozygosity

Earlier reports indicate favorable relationships between heterosis and measures of puberty and fertility (Wiltbank et al., 1966; Laster et al., 1976; Gregory et al., 1991), and heterosis is indicative of hetero- and homozygosis. In the Cycle VII heifers, however, effects of heterosis on the three measures of fertility were not detected, perhaps due to limited variation in heterosis of these crossbred heifers, and a lack of contrasts with contemporary purebreds. Genomic inbreeding coefficients, reflecting homozygosis based on SNP genotypes, were generally not associated with antral follicle count, age at puberty, or yearling pregnancy, although there was a tendency for yearling pregnancy to decrease with increased homozygosis. Among the SNP sets evaluated, regressions of genomic inbreeding on pregnancy were significant ($P < 0.05$) for BTA 11, 17, and 22,

as well as the HDA and HDAr sets derived from the complete set of 735,239 autosomal SNP on the BovineHD chip (**Figure 3**). More thorough investigation of homozygosis, leading to possible incorporation of non-additive SNP effects into screening tests for heifer puberty and pregnancy, appears warranted.

Predicted Differences Between Pools

Additive allele effects on yearling heifer pregnancy, solved from analyzing the large and reduced BovineHD subsets with GPE Cycle VII heifer data, were applied to pooling allele frequencies from the Central Florida *Bos taurus* × *Bos indicus* females to predict differences in average genomic breeding values between the pregnant and non-pregnant pools. All predicted differences in pregnancy rate were small, the largest being 0.8% predicted by the reduced set from the Brangus AWM network. Differences exceeding 0.5% were predicted by the sets reduced from large SNP sets initially selected with *Bos indicus*-influenced data, both the Brangus network and Central Florida pools, although differences predicted by the large subsets were miniscule. The reduced set resulting from autosomal analyses of all BovineHD SNP, based solely on GPE heifer data without assistance from functional annotation or non-GPE heifers, was the only set to predict the non-pregnant pools to have higher genomic breeding values for pregnancy than the pregnant pools. Because only pooling allele frequencies (MacGregor et al., 2006; 2008), not genotype frequencies, can be obtained from BovineHD assays of pooled DNA, the predictions of pool differences did not include effects of homozygosity that could be included when screening heifers by individual genotypes.

LEARNING ABOUT PUBERTY AND PREGNANCY, AND SELECTING HEIFERS WITH SNP CHIPS

Genomic mechanisms underlying female fertility, initially expressed by attainment of puberty and initiation of estrus, followed by successful conception, gestation and parturition, and ultimately by annual repetition of the estrus-conception-gestation-parturition cycle, are largely unknown. Genomic selection using SNP dense enough to guarantee that the unknown DNA variants affecting fertility are linked to genotyped markers, could allow accurate selection for puberty and pregnancy with no knowledge of the underlying

mechanisms. However, collecting the massive number of genotypes and phenotypes to develop such a selection tool is prohibitively expensive, as would be applying high-density genotypes to select heifers in lieu of relatively inexpensive phenotypic screening. Physiology of puberty and pregnancy is known to be complex. Genomic analysis of limited data has failed to locate a specific marker or single gene with an overwhelming influence on heifer puberty and subsequent pregnancy. However, networks of interacting genes conforming to current understanding of molecular pathways involved in reproduction have been identified from associations between SNP genotypes and multiple traits correlated with puberty and pregnancy. Genotypes of SNP in and near genes in the identified networks and pathways, however, do not appear to completely describe genetic variation of fertility-related traits.

Additional data will be needed for further elucidation of genomic mechanisms affecting reproduction, but expensive individual dense genotypes and phenotypes need not be the only source of information. Genotyping DNA pooled by phenotype offers one opportunity to detect associations between genotype and individual performance with few genotyping assays. Via imputation, representative sets of high-density genotypes can be used to determine probable high-density genotypes of individuals genotyped with lower-cost, low density assays. As demonstrated in the example analyses, GWAS of multiple traits was coupled with gene expression and information about gene function and known interactions to identify high-density marker sets that appear useful for predicting some phenotypic differences.

The approaches can be combined. For example, application of association weight matrix and gene set analysis to the GWAS of pooled allele frequencies might enhance the currently identified gene sets associated with heifer fertility traits. Targeted imputation could utilize no more than a few hundred SNP to impute high-density genotypes covering networks associated with puberty and pregnancy. Given complexity of reproductive processes and the number of genes and regulatory factors involved, small marker sets focusing on one or a few candidate genes may be inadequate to detect meaningful variation; candidate gene sets, considering functional annotation and established pathways not necessarily supported by existing genotype-phenotype associations, may be more descriptive. Emerging high-throughput genome and transcriptome sequencing technologies can be employed to refine and expand understanding of pathways affecting reproduction, providing information to

characterize DNA variants that may regulate gene expression and interaction (Saccone et al., 2008; Xu and Taylor, 2009), and develop more cost-effective genotyping that could be used to test heifers.

Before implementing genomic screening, cost and effectiveness of the test should be weighed against cost and effectiveness of phenotypic screening and management strategies addressing reproduction. Pre-breeding reproductive tract examination may eliminate heifers least likely to conceive (Anderson et al., 1991; Rosenkrans and Hardin, 2003). Dietary manipulation to delay weight gain until late in the development period, or develop heifers to less than the recommended 60% of mature weight can be implemented to reduce costs with minimal effect on age at puberty and pregnancy rates (Funston et al., 2011; Perry, 2011). Cow herd nutrition, particularly protein supplementation in late gestation, has been shown to increase pregnancy of subsequently born heifer progeny (Martin et al., 2007; Funston et al., 2010). Historic prices indicate that delaying heifer selection until pregnancy diagnosis, and selling cull yearlings can be profitable if development costs are not excessive (Clark et al., 2005), although more profit might be extracted from the probable culls if they can be identified at weaning or earlier, and managed as market animals.

SUMMARY AND CONCLUSIONS

Genomic selection of heifers with 50K or denser SNP chips will be costly, both to genotype large numbers needed to calibrate predictions and to genotype heifers for selection. Test panels with few markers focusing on genes involved in reproductive processes may be more cost effective. The network and pathways identified here provide a starting point for a heifer-screening test. A small marker set, allowing imputation of high-density genotypes in and around the genes identified, including those involved in axon guidance and G-protein coupled receptor pathways, may be developed to predict differences between heifers. Additionally, screening for Y-specific markers could eliminate a small percentage likely to be infertile. Some consideration may be given to non-additive heterozygosis effects, rather than evaluating heifers only for additive allele effects. Further investigation, including incorporation of pooled DNA representing large numbers of phenotyped individuals, and experimentation to identify and quantify gene expression and regulation can contribute to better understanding of factors affecting puberty and pregnancy, and enable more accurate

genomic tests for heifer selection.

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Table 1. Estimated heritability and correlations among measures of growth, carcass characteristics and pregnancy status of Brangus heifers[1,2]

Trait[3]	WWT	WH	YWT	YH	PWG	BFT	IMF	REA	FSC	HPR
WWT	**0.48**	0.80	0.86	0.76	0.05	0.45	-0.11	0.72	0.19	-0.28
WH	0.76	**0.55**	0.70	0.89	0.02	0.60	0.16	0.59	0.23	-0.39
YWT	0.76	0.64	**0.48**	0.71	0.54	0.64	-0.09	0.84	0.21	-0.14
YH	0.62	0.76	0.71	**0.52**	0.17	0.57	0.05	0.55	0.21	-0.23
PWG	-0.13	0.01	0.55	0.29	**0.27**	0.49	-0.02	0.46	0.07	0.20
BFT	0.43	0.29	0.52	0.26	0.25	**0.30**	-0.08	0.67	0.71	0.27
IMF	-0.05	-0.06	-0.04	-0.06	0.00	0.20	**0.42**	0.01	0.10	0.11
REA	0.58	0.41	0.71	0.42	0.33	0.54	0.01	**0.63**	0.31	0.17
FSC	0.03	0.04	0.08	0.06	0.08	0.14	0.01	0.14	**0.06**	0.66
HPR	0.00	-0.01	0.03	-0.04	0.05	0.10	0.00	0.08	0.58	**0.07**

[1] Heritability on diagonal, genetic correlations above and phenotypic correlations below diagonal.

[2] Adapted from Thomas et al. (2012).

[3] WWT = weaning BW; WH = hip height at weaning; YWT = yearling BW; YH = hip height at yearling; PWG = postweaning gain to yearling; BFT = ultrasound backfat; IFT = ultrasound % intramuscular fat; REA = ultrasound LM area; FSC = first service conception; HPR = yearling pregnancy rate.

Table 2. Estimated genomic heritabilities and correlations among measures of growth, body condition, puberty and pregnancy of crossbred heifers representing 7 popular beef breeds[1,2]

Trait[3]	YW	PWG	AFC	AAP	BCS	HPR
YW	**0.54**	0.83	-0.16	0.30	0.73	-0.17
PWG	0.82	**0.46**	-0.26	0.26	0.52	-0.04
AFC	0.08	0.06	**0.44**	0.37	-0.63	-0.55
AAP	-0.01	0.06	0.02	**0.14**	0.15	-0.33
BCS	0.28	0.22	0.03	0.02	**0.09**	-0.07
HPR	0.04	0.05	0.00	0.00	0.12	**0.11**

[1] Parameters estimated from genomic relationship matrix using BovineSNP50 genotypes. Heritability on diagonal, genomic correlations above and phenotypic correlations below diagonal.

[2] 2-, 3- and 4-breed crosses of Angus, Hereford, Charolais, Gelbvieh, Limousin, Red Angus and Simmental in Cycle VII, USMARC Germplasm Evaluation Project.

[3] YW = yearling BW; PWG = postweaning gain to yearling; AFC = antral follicle count; AAP = age at puberty, BCS = body condition score (1 to 9) following breeding; HPR = yearling heifer pregnancy rate.

Table 3. Overrepresented gene ontology terms and KEGG pathways identified by multitrait BovineSNP50 associations and gene set analysis of crossbred heifers[1,2]

Gene set	Source[3]	Function
bta04740	KEGG	Olfactory transduction
GO:0004984	GO	molecular function: olfactory receptor activity
GO:0007186	GO	biological process: G-protein coupled receptor protein signaling pathway

[1] 2-, 3- and 4-breed crosses of Angus, Hereford, Charolais, Gelbvieh, Limousin, Red Angus and Simmental in Cycle VII, USMARC Germplasm Evaluation Project.

[2] Gene sets overrepresented ($P < 0.01$) by associations with antral follicle count, age at puberty and yearling heifer pregnancy.

[3] KEGG = pathways described by Kyoto encyclopedia of genes and genomes; GO = Gene ontology.

Table 4. Selected large and reduced subsets of BovineHD SNP evaluated in crossbred heifers of 7 popular beef breeds[1,2]

Set type	SNP set designation	# SNP	SNP selection criteria
Large	HDA	46,695	AFC, AAP and HPR effects from 29 single-chromosome analyses. (average > 2 SD from mean within-chromosome effect). All 735,239 autosomal BovineHD SNP were evaluated.
Large	CFP	12,869	Pooling allele frequencies different ($P < 0.05$) among Central Florida *Bos taurus* x *Bos indicus* females pooled by pregnancy status as yearlings and 2-yr-olds (McDaneld et al., 2011).
Large	BN	75,560	In or near genes in Brangus 10-trait association weight matrix – hypothalmus expression network (Thomas et al., 2012). < 50 kbp from genes in network
Large	GS	56,730	In or near genes involved with overrepresented gene ontology terms and KEGG pathways from multitrait BovineSNP50 – gene set analysis of Cycle VII heifers. < 50 kbp from genes in overrepresented sets
Reduced	HDAr	890	AFC, AAP and HPR effects from HDA 3-trait average > 2 SD from mean HDA effect
Reduced	CFPr	100	AFC, AAP and HPR effects from CFP 3-trait average > 2 SD from mean CFP effect
Reduced	BNr		AFC, AAP and HPR effects from BN 3-trait average > 2 SD from mean BN effect
Reduced	GSr	350	AFC, AAP and HPR effects from GS 3-trait average > 2 SD from mean GS effect
Reduced	CBG	814	SNP in BNr or GSr (47 SNP in both)

[1] 2-, 3- and 4-breed crosses of Angus, Hereford, Charolais, Gelbvieh, Limousin, Red Angus and Simmental in Cycle VII, USMARC Germplasm Evaluation Project.

[2] AFC = antral follicle count; AAP = age at puberty; HPR = yearling heifer pregnancy rate.

FIGURE CAPTIONS

Figure 1. Approximate number of phenotypes needed to realize genomic selection accuracies (r^2) of 0.50 and 0.80, with heritabilities between 0.05 and 0.50 for effective population sizes (N_e) of 100 and 1,000. Equations from Goddard (2009).

Figure 2. Heritabilities of age at puberty (AAP), antral follicle count (AFC) and yearling heifer pregnancy (HPR) in crossbred heifers, estimated with pedigree relationships and genomic relationships from large and reduced subsets of BovineHD SNP. Large subsets were selected from analysis of the BovineHD SNP on each autosome (HDA), SNP with different pooling allele frequencies among Central Florida females pooled by pregnancy status following breeding as yearlings and 2-yr-olds (CFP), SNP near genes implicated by an association weight matrix network from associations with growth, carcass and pregnancy observations and genes expressed in hypothalmus of Brangus heifers (BN), and SNP near genes involved in pathways identified from multiple-trait evaluation and gene set analysis of the crossbred heifers (GS). Reduced sets selected from the corresponding large sets were 890 of the HDA, 100 of the CFP, 511 of the BN and 350 of the GS SNP. Sets reduced from BN and GS were combined into a set of 814 SNP derived from the large sets supported by gene expression and functional annotation.

Figure 3. Effects of heterosis and genomic inbreeding on pregnancy of crossbred yearling heifers. Heterosis was estimated from pedigree-based breed composition, and genomic inbreeding from diagonals of genomic relationship matrices computed from large and reduced subsets of BovineHD SNP. Large subsets were selected from analysis of the BovineHD SNP on each autosome (HDA), SNP with different pooling allele frequencies among Central Florida females pooled by pregnancy status following breeding as yearlings and 2-yr-olds (CFP), SNP near genes implicated by an association weight matrix derived from associations with growth, carcass and pregnancy observations and genes expressed in hypothalmus of Brangus heifers (BN), and SNP near genes involved in pathways identified from multiple-trait evaluation of the crossbred heifers (GS). Reduced sets selected from the corresponding large sets were 890 of the HDA, 100 of the CFP, 511 of the BN and 350 of the GS SNP. Sets reduced from BN and GS were combined into a set of 814 SNP derived from the large sets supported by gene expression and functional annotation.

Figure 1

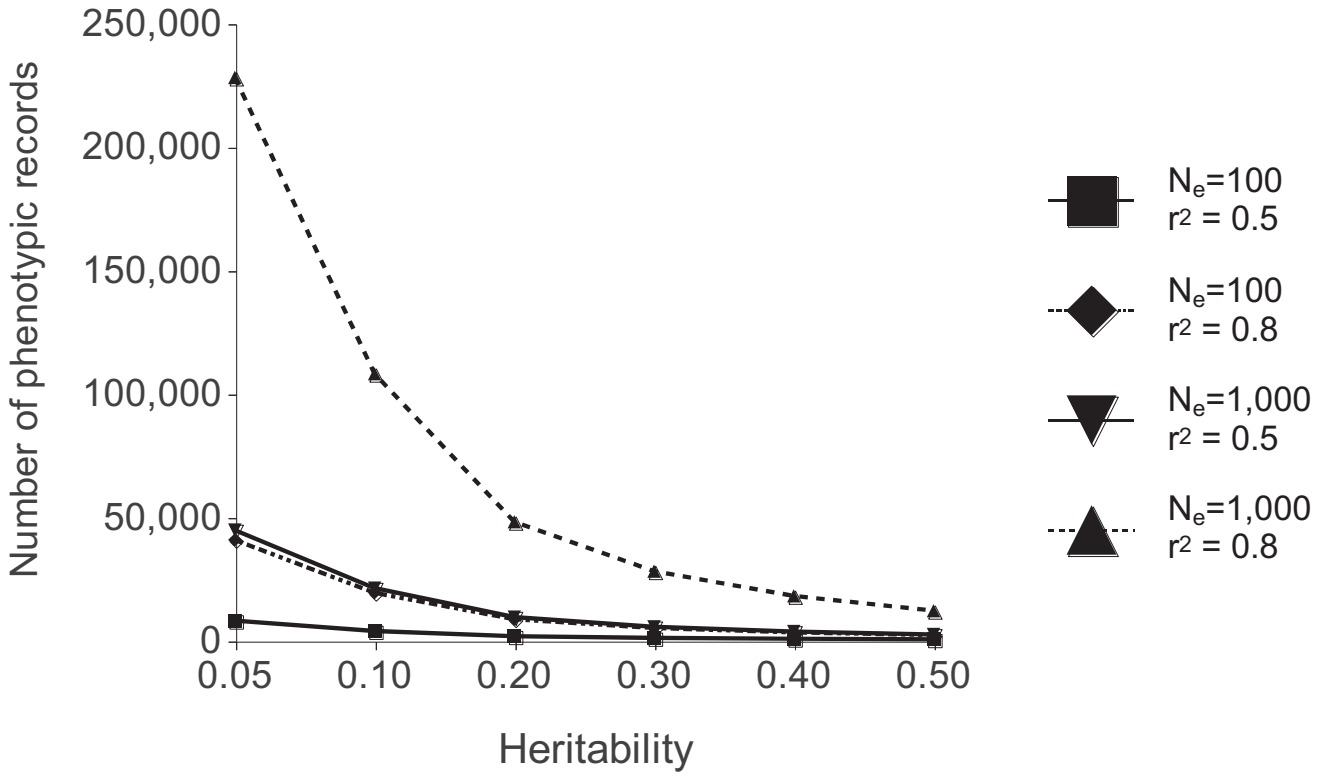

Figure 2

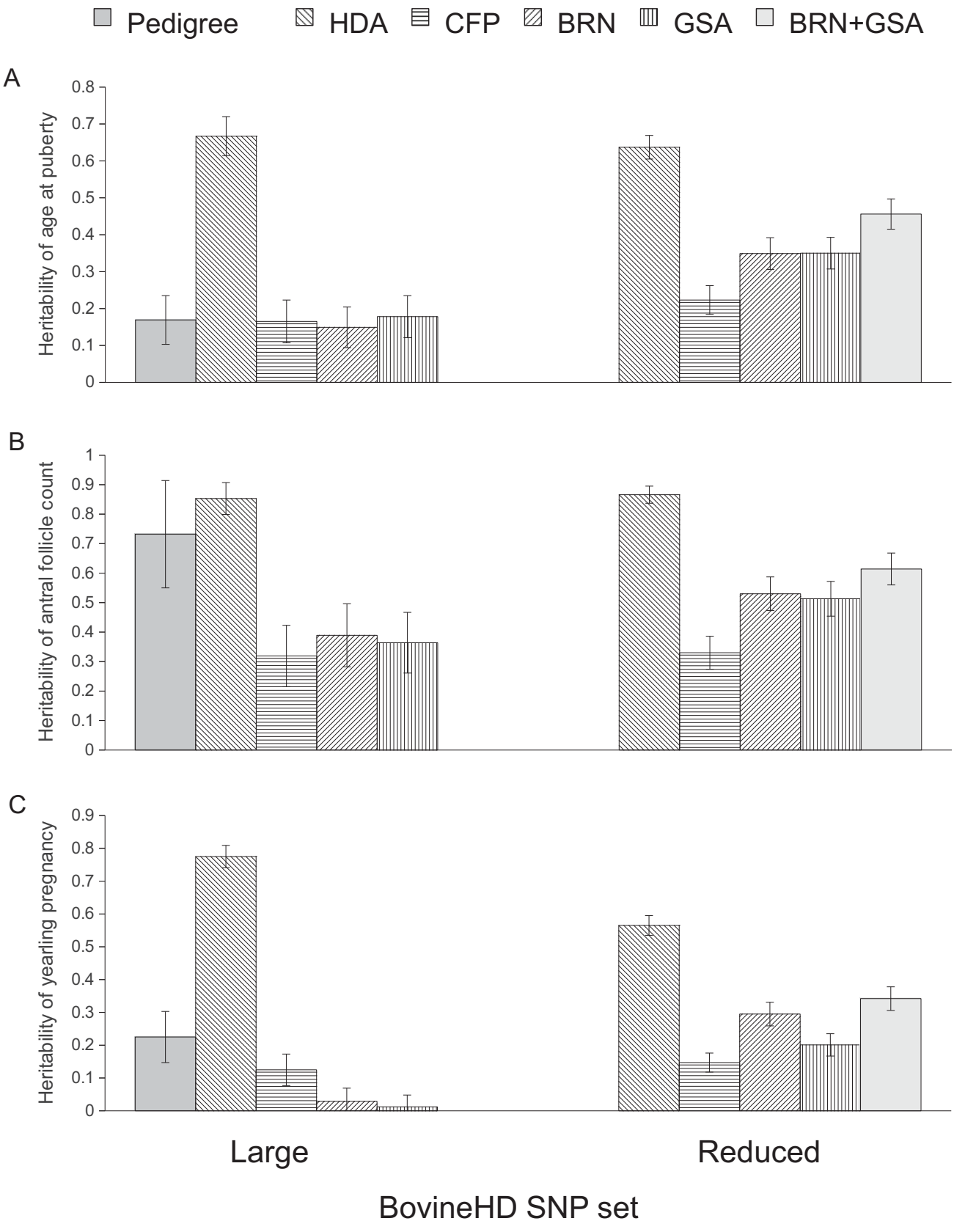

Figure 3