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Single Nucleotide Polymorphisms in Candidate Genes Associated with Fertilizing Ability of Sperm and Subsequent Embryonic Development in Cattle[1]

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ABSTRACT

Fertilization and development of the preimplantation embryo is under genetic control. The present goal was to test 434 single nucleotide polymorphisms (SNPs) for association with genetic variation in fertilization and early embryonic development. The approach was to produce embryos from 93 bulls using *in vitro* procedures (n=3-6 replicates per bull) and relate cleavage rate (CR) and development of cleaved embryos to the blastocyst stage (BDRC) with the genotype for each SNP. Bulls were selected to have either high or low estimates for predicted transmitted ability for daughter pregnancy rate (DPR), an estimate of female fertility. The repeatability was 0.84 for CR and 0.55 for BDRC. Semen extender affected CR, with lower results for milk extender than yolk extender. There was no significant correlation between DPR and either CR or BDRC. A total of 100 SNP had a minor allele frequency sufficiently high (>5%) to allow association analysis. There were 9 genes with SNPs associated with CR (*AVP, DEPP, EPAS1, HSD17B6, NT5E, SERPINE2, SLC18A2, TBC1D24*, and a non-characterized gene)and 12 genes SNPs associated with BDRC ((*C1QB, FAM5C, HSPA1A, IRF9, MON1B, PARM1, PCCB, PMM2, SLC18A2, TBC1D24, TTLL3*, and *WBP1*). Results demonstrate that *in vitro* fertilization and blastocyst development are under genetic control and point out the potential importance of some previously-unknown genes in these processes. Selection of cattle based on the genotype at one or more of these 19 loci may prove useful in conjunction with other genetic markers for improving genetic ability for fertility. .

Key words: *Fertilization, preimplantation development, genetics, SNP*

INTRODUCTION

Fertilization and development of the preimplantation embryo is under genetic control. In cattle, several single nucleotide polymorphisms (SNP) have been associated with these traits. For example SNPs in *FGF2* [1], *STAT3* [2], *STAT5A* [3-5], *GHR* [4], *SPP* [4], *DNAJC27* [6] and *PGR* [7] have been associated with *in vitro* fertilization. Furthermore, SNPs in *STAT5A* [3,4,6], *STAT3* [3], *FGF2* [1, 8], *PGR* [7], *GHR* [4], *PRLR* [4], *SERPINA14* [4], *SPP* [4] and *DNAJC15* [6] have been associated with *in vitro* development of embryos to the blastocyst stage.

The existence of genes controlling fertilization and embryonic development is indicative that some of the historical decline in fertility in dairy cattle [9, 10] may be the result of increased frequency of alleles that inhibit development. There are SNPs in cattle where the beneficial allele for milk production is the less desirable allele for reproduction [11,12]. Therefore, selection for production traits may have reduced the frequency of alleles that benefit fertility. Identification of genes controlling embryonic development could lead to new methods for genetic selection to improve fertility in dairy cattle, as well as increase the understanding of genes whose function is critical for the processes of fertilization and early development.

In a previous study, SNPs in 434 genes related to reproductive processes were evaluated for their relationship to fertility and reproduction traits in dairy cattle [12]; a total of 40 genes significantly related to fertility were identified. The goal of the current study was to test whether any of the 434 SNPs previously studied are associated with genetic variation in fertilization and early embryonic development. The approach was to produce embryos from 93 bulls using *in vitro* procedures and relate cleavage rate and development to the blastocyst stage with the genotype at each SNP.

MATERIALS AND METHODS

Selection of Bulls

Straws of semen were obtained from 93 Holstein bulls born between 1973 and 2008. These bulls, which represent a subset of bulls from a previous study [12], were chosen based on predicted transmitting ability (PTA) for daughter pregnancy rate (DPR). Predicted transmitting ability is the average genetic value for a trait that an animal transmits to its offspring and DPR is an estimate of the percent of a bull's daughters eligible to become pregnant in a 21-d period that actually become pregnant. Bulls in the high DPR group (n=47) had PTA for DPR that ranged from 2.0 to 5.3 (average = 3.0), and bulls in the low DPR group (n=46) had PTA for DPR that ranged from -4.6 to -2.0 (average = -3.1). Reliability of estimates of PTA for DPR ranged from 0.64-0.99 (average = 0.82). Semen samples were obtained from the USDA National Animal Germplasm Program (NAGP; Fort Collins, CO; 23 bulls), Alta Genetics (Watertown, WI, USA; 16 bulls), Taurus-Service Inc. (Mehoopany, PA, USA; 16 bulls), Select Sires Inc. (Plain City, OH, USA; 13 bulls), American Breeders Service Global Inc. (DeForest, WI, USA; 11 bulls), Genex Cooperative Inc. (Shawano, WI, USA; 5 bulls), Semex (Madison, WI, USA; 5 bulls), and Accelerated Genetics (Baraboo, WI, USA; 4 bulls). Semen extender type was classified based on appearance: white and milky was classified as milk, yellow was classified as egg yolk, and extenders of other colors were classified as unknown.

SNP Genotyping

Bulls were genotyped for 434 SNPs located in genes involved in reproductive processes as described elsewhere [12]. Genes were chosen based on one or more of the following criteria: well-known to be involved in reproductive processes, physically close to SNPs related to reproductive traits in other studies, contain SNPs previously linked to fertility, and were differentially regulated between physiological conditions in a variety of reproductive tissues. SNPs were also chosen based on the likelihood that a change in protein function would result (nonsense mutations, frameshifts, and non-synonomous mutations that were predicted to cause a large change in protein function). Only SNPs that had a call rate > 70% in the larger study as well as had a minor allele frequency (MAF) > 0.05 among the

93 bulls in the current study were analyzed. A list of the SNPs and associated MAF is presented in Supplemental Table S1.

Evaluation of Fertilizing Ability and Competence of Embryos to Develop to the Blastocyst Stage

Each bull was evaluated for ability of sperm to fertilize matured oocytes, as determined by percent of oocytes exposed to sperm that underwent cleavage (cleavage rate; CR) and on the ability of cleaved oocytes (i.e., newly-formed embryos) to become a blastocyst at d 7 after insemination (blastocyst development rate of cleaved embryos; BDRC). For each bull, traits were evaluated in a total of 3 to 6 separate *in vitro* fertilization (IVF) and embryo culture procedures.

Techniques for fertilization of matured oocytes and culture of the resultant embryos to the blastocyst stage were as follows. Bovine ovaries were collected from a local abattoir and transported to the laboratory within 5 h after slaughter. Follicles 2 to 10 mm in diameter were lacerated, and cumulus oocyte complexes (COCs) were collected in oocyte collection medium [tissue culture medium-199 with Hanks' salts (Sigma-Aldrich; St. Louis, MO, USA) supplemented with 350 mg/L sodium bicarbonate (Sigma-Aldrich), 2.38 g/L HEPES (Sigma-Aldrich), 2% (vol/vol) bovine steer serum (Pel-Freez; Rogers AR) containing 2 U/ml heparin (Sigma-Aldrich), 100 U/ml penicillin-G (Caisson Labs; North Logan, UT, USA), 100 mg/ml streptomycin (Caisson Labs; North Logan, UT, USA), and 1 mM Glutamax (Life Technologies; Grand Island, NY, USA)]. The COCs were incubated in groups of 10 in 50 µl of maturation medium [tissue culture medium-199 with Earle's salts (Life Technologies) supplemented with 10% (vol/vol) bovine steer serum, 0.22 g/µl sodium pyruvate (Sigma-Aldrich), 2 µg/ml estradiol 17-β (Sigma-Aldrich), 25 µg/ml bovine follicle stimulating hormone (Folltropin-V; AgTech Inc.; Manhattan, KS, USA), 22 µg/ml sodium bicarbonate, 50 µg/ml gentamicin sulfate (Life Technologies), and 1 mM Glutamax] overlaid with mineral oil for 22 h at 38.5° C in an atmosphere of 5% (vol/vol) CO_2 in humidified air.

On the day of fertilization (designated d 0), matured COCs were washed in HEPES- Tyrodes albumin lactate pyruvate (Hepes-TALP) [13] supplemented with 3 mg/ml bovine serum albumin (Sigma-Aldrich), 22 µg/ml sodium pyruvate, and 15 µg/mL gentamicin, divided into groups of about 100, transferred to a fertilization medium consisting of 1.7 ml of SOF-IVF fertilization medium [14] and 80 µl of PHE [0.5 mM penicillamine (Sigma-Aldrich), 0.25 mM hypotaurine (Sigma-Aldrich), and 25 mM epinephrine (Sigma-Aldrich) in 0.9% (w/v) NaCl]. Fertilization was initiated by addition of 120 µl Percoll-purified sperm suspended in HEPES-TALP from a single bull to achieve a final concentration of 1.1×10^6 sperm/ml. The COCs were incubated with sperm for 12 h at 38.5°C in an atmosphere of 5% (vol/vol) CO_2 in humidified air. Cumulus cells were then removed by vortexing COCs for 4 min in 1429 U/ml hyaluronidase (Sigma-Aldrich) in HEPES-TALP. Then, putative zygotes (i.e., oocytes exposed to sperm) were cultured in groups of ~30 in 50 µl drops of SOF-BE1 [15] overlaid with mineral oil. Embryos were incubated at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO_2 and 5% (vol/vol) O_2 with the balance N_2 until d 7 after insemination. The CR was determined on d 3, and the BDRC was determined on d 7.

A total of 308 IVF procedures were performed. For each procedure, 2 to 6 bulls were evaluated, with the number depending on the number of oocytes obtained. Each day, bulls were randomly chosen from the low and high DPR groups so that at least 1 low and 1 high DPR bull were used on each fertilization day. After all bulls were tested once, the experiment was repeated an additional two times for all bulls, with bulls again being selected at random within DPR group for each series. A few bulls were used in a total of 4 (n = 27), 5 (n = 3) or 6 (n = 1) IVF procedures because earlier results were considered unreliable due to results that deviated widely from expectations. Unreliable procedures were most often ascribed to a defective medium used for the procedure.

Statistical Analysis

Minor allele frequency (MAF) was determined using the FREQ procedure of SAS (SAS Institute, Inc., Cary, NC, ver. 9.2). Repeatability of CR and BDRC was determined by calculating the ratio of the between-animal variance to the sum of the between-animal and the residual variances. The association

between SNP genetic variants and each trait was evaluated using the GLM procedure of SAS. The full model included effects of extender, semen company, birth year, daughter pregnancy rate class (high or low), and SNP. All nonsignificant variables were removed and the final model for CR included:

$$Y_i = \text{extender}_j + \text{company}_l + \beta SNP_k + \varepsilon_i$$

and the final model for BDRC included:

$$Y_i = \beta SNP_k + \varepsilon_i$$

where Y_i is the trait of interest (CR or BDRC, averaged for all runs for an individual bull) for the i^{th} bull (i = 1, 2, …, 93), extender_j is the effect of the j^{th} type of extender (j = milk, egg yolk, unknown) used on the i^{th} bull, company_l is the effect of the l^{th} semen company, β is the linear regression coefficient for the k^{th} SNP, SNP_k is the number of copies (k=0, 1, or 2) of the major allele, and ε_i is the random residual effect.

Allele substitution effects were computed using two mixed model analyses that included random animal effects [16] and either fixed or continuous SNP terms. In the first, genotype was considered a continuous variable to determine the allele substitution effect (the linear effect of the number of copies of the major allele). In the second, genotype was considered a class variable, and orthogonal contrasts were used to estimate dominant effects [(AA + aa)/2 vs Aa].

Correlations were calculated between average values of CR and BDRC with each other as well as with the standard deviation of these traits and with DPR using the PROC CORR procedure of SAS.

Averages are reported as least-squares means ± SEM as derived from PROC GLM except for results for individual bulls (Fig. 1 and 2), in which case data represent arithmetic means ± SEM.

RESULTS

Minor Allele Frequencies

Of the 434 SNPs, 405 previously passed the call rate requirements in a previous study [12]. Of those 405 SNPs, 100 had MAF > 0.05 in the bulls tested in the current study (Supplemental Table S1) and were used for further analyses.

Characteristics of In Vitro *Fertilization and Development*

The average CR ± SEM for each bull is plotted in Figure 1. The CR did not differ between bulls of high (73.0 ± 2.1%) and low (73.7 ± 2.0%) DPR (P = 0.76). . The repeatability of CR was 0.84. Semen extender affected CR (P = 0.004). Semen that was extended in milk extender had the lowest CR (63.9 ± 2.7%), followed by semen in egg extender (73.8 ± 2.3%), and semen in unknown extender (82.3 ± 4.0%). Furthermore, semen company of the bull had an effect on CR (P = 0.01), with differences between companies varying from a low of 64.1 ± 3.2% to a high of 85.4 ± 8.1%.

The average ± SEM BDRC for each bull is plotted in Figure 2. The BDRC did not differ between bulls of high (40.8 ± 1.5%) and low (41.7 ± 1.4%) DPR(P = 0.58) . The repeatability of BDRC was 0.55. Neither extender (P = 0.45) nor semen company (P = 0.67) affected BDRC.

Correlations among *in vitro* development measurements, DPR, and within-bull standard deviation (SD) of *in vitro* development measurements are shown in Table 1. Daughter pregnancy rate was not correlated with any *in vitro* measurements that were assessed in the current study. Cleavage rate was positively correlated with BDRC (0.23; P = 0.02). Cleavage rate was negatively correlated with the SD of CR (-.59; P < 0.0001); therefore, bulls with greater cleavage rates had less variability in percent cleavage than bulls with lower cleavage rates. There was no correlation between BDRC and SD of BDRC (-0.02).

SNP Effects

Each of the 100 SNPs with MAF > 0.05 were analyzed for effects on *in vitro* embryo development. Two types of analyses were performed: a regression analysis to determine the linear allele

substitution effect of each SNP (0, 1, or 2, copies of the major allele) and orthogonal contrasts (heterozygote vs. average of the two homozygotes) to determine the dominance effect.

Cleavage rate. Results for SNPs significantly associated with CR are shown in Table 2. Allele substitution effects were significantly different from 0 for 5 genes [*C28H10orf10* (predicted to encode for DEPP)*, HSD17B6, NT5E, SERPINE2*, and *TBC1D24*). The largest magnitude for SNP effects was for *NT5E*, where the difference in CR between homogygotes was 21.2%. For this gene, only 2 bulls were homozygous for the deleterious allele. The other large SNP effects were seen for *SERPINE2*, where homozygotes differed by 12.8%, and *SLC18A2*, where homozygotes differed by 10.1%.

Two SNPs with significant substitution effects, *NT5E* and *SERPINE2*, also exhibited dominance. In both cases, the heterozygote had a phenotype similar to that of the desirable homozygote. Four other genes exhibited dominance. In two cases, for *C7H19orf60,* and *SLC18A2*, the heterozygote had a phenotype similar to that of the desirable homozygote. For the other two cases, for *AVP* and *EPAS1*, the heterozygote was lower than either homozygote.

Development of cleaved embryos to the blastocyst stage. Results are presented in Table 3. Allele substitution effects were significant for 9 genes (*FAM5C, HSPA1A, IRF9, MON1B, PCCB, PMM2, SLC18A2, TTLL3*, and *WBP1*). Of these, *SLC18A2* was also significant for CR but the desirable allele was different. There were large SNP effects for *MON1B* and *WBP1*, but the number of observation for the deleterious homozygote was only 1-2. The greatest SNP effect for the remaining 7 genes was for *IRF9*, in which the difference between homozygotes was 6.3%, and *HSPA1A*, in which the difference was 6.1%.

There were no dominant effects for genes in which allele substitution effects were significant but there were significant dominance effects for an additional three genes (*C1QB, PARM1*, and *TBC1D24*). For *C1QB*, the heterozygote had a phenotype similar to that of the desirable homozygote but the fact that there was only one bull that was homozygous for the deleterious allele adds uncertainty to the conclusion regarding dominance. For *PARM1* and *TBC1D24*, the heterozygote had higher BDRC than either homozygote.

Relationship Between SNP Effects and SNPs Previously Associated with In Vivo Measurements of Fertility

The SNPs studied here were also examined by Cochran et al. [12] for association with three measures of fertility in vivo: DPR, heifer conception rate (HCR) [17], and cow conception rate (CCR) [17]. We evaluated whether any of the 20 SNP that were associated with CR or BDRC in the current study were the same as SNPs related to DPR, HCR, or CCR. A total of 4 individual SNPs that were associated with CR (*C7H19orf60, SERPINE2, SLC18A2,*and *TBC1D24*) and 8 SNP that were associated with BDRC (*C1QB, MON1B, PARM1, PCCB, PMM2, SLC18A2, TBC1D24* and *WBP1*) had earlier been shown to be related to DPR, HCR or CCR. The majority of those associations were in the opposite direction for CR or BDRC as compared to effects on DPR, HCR, or CCR. In only 4 cases was the desirable allele the same *in vitro* and *in vivo*: *C7H19orf60* for CR and *C1QB, PARM1*, and *SLC18A2* for BDRC. Note that the desirable allele for *SLC18A2* was different for CR than it was for BDRC.

Five SNPs examined in the current study had previously been associated with fertility in dairy or beef cattle. These were SNPs in *FGF2* [18]*, HSPA1A* [19], *PAPPA2* [20]*, PGR* [21], and *STAT5A* [18]. One of these, *PAPPA2*, was not analyzed due to low MAF. Of the other four, none were significantly related to CR and only one (*HSPA1A*) was significantly associated with BDRC. However, the favorable genotype for BDRC (-/-) was the opposite as the favorable genotype (C/C) reported earlier for calving rate and Julian calving date [19] (Table 3).

Relationship between SNP Effects and SNPs Previously Associated with In Vitro Fertilization and Development

Two SNPs examined (*PGR* and *STAT5A*) had been associated with CR in earlier studies [5,7]. There were no significant associations of either SNP with CR in the current study although the favorable allele in the earlier study for PGR [7] (G) was associated with numerically-greater CR in the current study. The CR for *PGR* was 74.22 ± 4.03, 74.66 ± 2.05, and 71.18 ± 2.16 % for G/G, G/C, and C/C, respectively. However, the favorable allele for *STAT5A* in a previous study was C for CR, [5] and the CR

for *STAT5A* was 74.60 ± 2.99, 73.23 ± 2.08, and 71.91 ± 2.66% for G/G, G/C, and C/C, respectively (least-squares means ± SEM). Three SNPs examined here had previously been related to blastocyst development *in vitro*, including *FGF2* [1], *PGR* [7], and *STAT5A* [3]. None of these three SNPs were significantly related to BDRC in the current study. The least-squares means for BDRC for each of these genes was as follows: *FGF2*, 42.46 ± 2.23, 39.92 ± 1.29, and 40.69 ± 1.07% for A/A, A/G, and G/G, respectively; *PGR*, 40.67 ± 2.52, 40.82± 1.10, and 40.75 ± 1.26% for G/G, G/C, and C/C, respectively; and *STAT5A*, 40.73 ± 1.85, 39.90 ± 1.10, and 41.85 ± 1.35% for G/G, G/C, and C/C respectively (least-squares means ± SEM).

DISCUSSION

There are a few reports, with smaller numbers of bulls than examined here, that the success of *in vitro* fertilization and embryonic development varies with sire [22-24]. In the current study, there was a large range among bulls in average cleavage rates (23.8 to 85.2) and blastocyst development rates (17.6 to 57.3). Much of this variation is probably due to environmental differences among bulls in conditions for spermatogenesis, semen collection and processing, and semen storage. Present results indicate there is also a genetic component to this variation as well. Using a candidate gene approach, a total of 19 genes were identified that were significantly associated with cleavage rate (a measure of fertilization) and embryonic development to the blastocyst stage. Many of these genes are likely to encode for proteins that play an important role in the process whereby sperm fertilize an oocyte and the resultant embryo develops to the blastocyst stage. Moreover, it is possible that changes in allele frequency for some of these genes contributed to changes in fertility of dairy cattle.

Identification of an association between a SNP in a candidate gene and a phenotype does not necessarily mean the SNP is causative. Associations could also be due to proximity of the genotyped SNP to the actual causative SNP. Nonetheless, many of the genes that were associated with CR and BDRC have functions consistent with a role in these processes. Genes affecting CR could do so by participating in sperm formation and maturation in the male reproductive tract, sperm survival after ejaculation and cryopreservation, or sperm function in fertilization. Nine genes were significantly associated with CR. *HSD17B6* encodes for an enzyme with oxidoreductase and epimerase activity towards androgens and estrogens [25, 26] and likely regulates steroid action in the male reproductive tract. Another gene, *SLC18A2,* is a neurotransmitter transporter for monoamines [27] and monoaminergic nerves may be involved in regulation of blood flow in the testis [28]. Other genes associated with CR have functions suggesting they play a role in survival of sperm *in vivo* to environmental insults or *in vitro* following semen processing and cryopreservation. For example, *AVP* is anti-apoptotic [29,30] and *EPAS1* encodes for a transcription factor induced by hypoxia [31] . Furthermore, *SERPINE2* can prevent capacitation [32] and may act to prevent premature capacitation. Another gene associated with CR, *NT5E,* encodes for an enzyme which converts adenosine monophosphate to adenosine [33], which could conceivably affect energy availability or signal transduction. Also, TBC1D24 interacts with ARF6, a member of the RAS superfamily that is involved in vesicular trafficking, and remodeling of membrane lipids [34]. Perhaps *TBC1D24* is involved in the membrane reorganization occurring during capacitation and the acrosome reaction [35]. The gene might also play a role in recovery of sperm from freezing since membrane architecture is disrupted by that process [35]. It is unclear what roles *C28H10orf10* or *C7H19orf60* might play in CR. The protein encoded by *C28H10orf10* is orthologous to DEPP, which is expressed in several tissues and which is involved in activation of the Elk1 transcription factor [36]. The protein encoded by *C7H19orf60* is not known.

The 12 genes associated with development to the blastocyst stage are involved in a wide variety of functions that could be important for the preimplantation embryo. At least four of the genes may be involved in signaling systems that control embryonic development. One, *IRF9,* is a transcription factor involved in the IFNA signaling pathway [37]. The bovine blastocyst produces the type I interferon, IFNT2 [38], and perhaps IFNT signaling in the embryo is important for regulation of embryonic development. The *FAM5C* gene encodes for a secreted protein that promotes differentiation of osteoblasts [39]. As mentioned previously, *SLC18A2* is a neurotransmitter transporter for monoamines [27] and

monoamines can participate in regulation of preimplantation development [40]. Another gene associated with blastocyst development, *WBP1*, interacts with the kinase gene *YAP* to positively or negatively regulate cell growth, differentiation, and migration [41].

Based on upregulation of gene expression in the trophectoderm [42], outer cells of the preimplantation embryo are very active in endocytosis, lipid biosynthesis and transmembrane transport. Three genes associated with development to the blastocyst stage are involved in these processes. The gene *TBC1D24* interacts with *ARF6* to regulate vesicular trafficking and remodeling of membrane lipids [34], *MON1B* regulates autophagy and processing of ingested cells [43,44], and *TTLL3* is a tubulin glycine ligase that participates in cilia assembly [45].

Three other genes related to embryonic development are involved in protection from cellular stress. *HSPA1A* protects cells from heat shock, in large part by blocking apoptosis [46] and is upregulated in preimplantation bovine embryos in response to heat shock [14,47,48]. Addition of an antibody against HSPA1A to culture medium reduced the proportion of bovine embryos developing to the blastocyst stage [49]. *PARM1* is antiapoptotic [50] and *MON1B* is required for autophagy [43].

A SNP in *PMM2* was also associated with blastocyst development. It may be involved in cell-cell interactions in the embryo because it encodes for an enzyme that isomerizes mannose 6-phosphate into mannose 1-phosphate [51], which in turn is used to make fucosylated glycans [52]. It is likely that *PCCB* is involved in embryo energy metabolism because it encodes for an enzyme that converts proponyl CoA to methylmalonyl CoA during gluconeogenesis [53]. The possible role of the complement gene *C1QB* in embryonic development is less clear. There was only one homoyzygote of the minor allele, and the conclusion regarding association with BDRC is tentative.

When testing for dominance, three types were identified: 1) dominance where inheritance of one copy of the dominant allele resulted in the same phenotype as having two copies of the dominant allele (*C1QB, NT5E, SERPINE2*, and *SLC18A2*), 2) heterozygote advantage (*C7H19orf60, PARM1,* and *TBC1D24*), where the heterozygote was better than either homozygote, and 3) heterozygote disadvantage (*AVP* and *EPAS1*). Heterozygote advantage could theoretically result if the optimal level of gene expression is intermediate between that for the two homozygotes, if the desirable allele is different in different cell types, or if heterozygosity increases ability of proteins to interaction with other molecules, for example binding of MHC class I to peptide antigens [54]. It is unknown why heterozygote disadvantage could occur.

There was no association between CR or BDRC and *in vivo* fertility as determined by a lack of difference between bulls of high and low DPR as well as non-significant correlations of CR or BDRC with DPR. Moreover, there were only 3 SNPs (*C1QB, PARM1*, and *SLC18A2*) in which the allele associated with improved BDRC was also associated with higher fertility in vivo [12]. In one case, *HSPA1A*, the favorable allele for BDRC was the unfavorable allele for fertility in beef cattle [19]. It was not expected that CR would be related to DPR because CR is determined by the bull whereas DPR is a measure of female fertility. In contrast, it was hypothesized that BDRC would be related to DPR because competence of the embryo to develop to the blastocyst stage is one of the determinants of whether a female reproduces successfully. The lack of a relationship could be due to inaccuracies in estimates of *in vitro* development (repeatability for BRRC was only 0.55), as well as the fact that early embryonic development is only one of many physiological events that determine *in vivo* fertility. In addition, some genes that affect embryonic development *in vitro* may do so by controlling processes that would not be important for development *in vivo* (for example, genes that help embryos survive stressful culture conditions). One implication of the lack of relationship between CR and BDRC with DPR and other estimates of fertility *in vivo* is that it is unlikely that bull differences in daughter fertility can be identified by screening procedures based on *in vitro* fertilization.

Three SNPs examined in the current study had been previously been associated with in vitro fertilization and development. These were *PGR* [7] and *STAT5A* [5] for CR, *FGF2* [1,8], *PGR* [7], and *STAT5A*, [3,5] for BDRC . None of these relationships were significant in the current study although the trend was as expected for *PGR* and CR. Some of the lack of repeatability could be due to different measurements of development *in vitro* as well as the fact that only sire was genotyped in the current study

vs. genotyping of the dam [1,7,8], embryo [5] or sire and dam [3] in the other studies. However, inability to repeat SNP effects on biological traits is a common phenomenon [55] and confidence in the genetic relationship between SNPs and phenotype will be increased with independent replication.

Semen extender had an effect on the fertilizing ability of the semen with slightly-superior results for semen in egg yolk-based extenders as compared to semen in milk extenders. This result is in contrast to what is seen in the goat, where semen extended in milk resulted in better *in vitro* fertilization rates compared to semen extended in egg yolk [56], and the human, where there was no difference in fertilization rates between semen extended in milk or egg yolk [57]. More research is required to determine whether the interaction between extender and species is real and, if so, the mechanism responsible. The enhanced fertilizing ability of semen extended in yolk extender in the current study may be due to *in vitro* conditions because there was no consistent difference between milk and egg-yolk extended semen for cows subjected to artificial insemination [58,59].

In conclusion, the current study showed variation in *in vitro* fertilization and blastocyst development among different bulls. Moreover, this variation was due, at least in part, to genetic variation. Single nucleotide polymorphisms were identified in a total of 19 genes that were associated with *in vitro* fertilization and embryo development. Selection of cattle based on the genotype at one or more of these 19 loci may prove useful in conjunction with other genetic markers for improving genetic ability for fertility. In addition, these results point out the potential importance of some previously-unknown genes for control of the processes leading to fertilization and embryonic development.

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REFERENCES

[1] Wang X, Schutzkus V, Huang W, Rosa GJM, Khatib H. Analysis of segregation distortion and association of the bovine *FGF2* with fertilization rate and early embryonic survival. Anim Genet 2009;40: 722-728.

[2] Khatib H, Huang W, Mikheil D, Schutzkus V, Monson RL. Effects of signal transducer and activator of transcription (STAT) genes *STAT1* and *STAT3* genotypic combinations on fertilization and embryonic survival rates in Holstein cattle. J Dairy Sci 2009;92:6186-6191.

[3] Khatib H, Monson RL, Schutzkus V, Kohl DM, Rosa GJM, Rutledge JJ. Mutations in the *STAT5A* gene are associated with embryonic survival and milk composition in cattle. J Dairy Sci 2008;91:784-793.

[4] Khatib H, Huang W, Wang X, Tran AH, Bindrim AB, Schutzkus V, Monson RL, Yandell BS. Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle. J Dairy Sci 2009;92:2238-2247.

[5] Khatib H, Maltecca C, Monson RL, Schutzkus V, Rutledge JJ. Monallelic maternal expression of *STAT5A* affects embryonic survival in cattle. BMC Genet 2009;10:13-22.

[6] Zhang B, Peñagaricano F, Driver A, Chen H, Khatib H. Differential expression of heat shock protein genes and their splice variants in bovine preimplantation embryos. J Dairy Sci 2011;94:4174-4182.

[7] Driver M, Huang W, Gajic S, Monson RL, Rosa GJM, Khatib H. Short communication: Effects of the progesterone receptor variants on fertility traits in cattle. J Dairy Sci 2009;92: 4082-4085.

[8] Khatib H, Maltecca C, Monson RL, Schutzkus V, Wang X, Rutledge JJ. The fibroblast growth factor 2 gene is associated with embryonic mortality in cattle. J Anim Sci 2008; 86:2063-2067.

[9] Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 2001;84:1277-1293.

[10] Hansen PJ. Challenges to fertility in dairy cattle: From ovulation to the fetal stage of pregnancy. Rev Brasil Reprod Anim 2011; 35:229-238.

[11] Pimentel EC, Bauersachs S, Tietze M, Simianer H, Tetens J, Thaller G, Reinhardt F, Wolf E, König S. Exploration of relationships between production and fertility traits in dairy cattle via association studies of SNPs within candidate genes derived by expression profiling. Anim Genet 2010; 42:251-262.

[12] Cochran SD, Cole JB, Null DJ, Hansen PJ. Discovery of single nucleotide polymoprhims in candidate genes associated with fertility and production traits in Holstein cattle. BMC Genet 2103; 14:49.

[13] Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986; 25:591-600.

[14] Sakatani M, Alvarez NV, Takahashi M, Hansen PJ. Consequences of physiological heat shock beginning at the zygote stage on embryonic development and expression of stress response genes in cattle. J Dairy Sci 2012;95: 3080-3091.

[15] Fields SD, Hansen PJ, Ealy AD. Fibroblast growth factor requirements for in vitro development of bovine embryos. Theriogenology 2011;75:1466-1475.

[16] Tempelman RJ, Rosa GJM. Empirical Bayes approaches to mixed model inference in quantitative genetics. In: Genetic Analysis of Complex Traits Using SAS. Saxton A. (Editor). Cary, NC: SAS Institute Inc, 2004.
[17] Norman, HD, Hutchison JL, VanRaden PM. Evaluations for service-sire conception rate for heifer and cow inseminations with conventional and sexed semen. J Dairy Sci 2011; 94: 6135–6142.
[18] Khatib H, Monson RL, Huang W, Khatib R, Schutzkus V, Khateeb H, Parrish JJ. Short communication: Validation of in vitro fertility genes in a Holstein bull population. J Dairy Sci 2010: 93: 2244-2249.
[19] Rosenkrans, C Jr, Banksa A, Reitera S, Looperb M. Calving traits of crossbred Brahman cows are associated with heat shock protein 70 genetic polymorphisms. Anim Reprod Sci 2010; 119: 178-182.
[20] Luna-Nevarez P, Rincon G, Medrano JF, Riley DG, Chase CC Jr, Coleman SW, Vanleeuwen DM, DeAtley KL, Islas-Trejo A, Silver GA, Thomas MG. Single nucleotide polymorphisms in the growth hormone-insulin-like growth factor axis in straightbred and crossbred Angus, Brahman, and Romosinuano heifers: population genetic analyses and association of genotypes with reproductive phenotypes. J Anim Sci 2011; 89:926-934.
[21] Tang KQ, Yang WC, Pai B, Li SJ, Chen L, Yang LG. Effects of *PGR* and *ESRa* genotypes on the pregnancy rates after embryo transfer in Luxi cattle. Mol Biol Rep 2013; 40: 579-584.
[22] Palma GA, Sinowatz F. Male and female effects on the in vitro production of bovine embryos. Anat Histol Embryol 2004; 33: 257-262.
[23] Wilson RD, Weigel KA, Fricke PM, Rutledge JJ, Leibfried-Rutledge ML, Matthews DL, Schutzkus VR. In vitro production of Holstein embryos using sex-sorted sperm and oocytes from selected cull cows. J Dairy Sci 2005; 88: 776-782.
[24] Xu J, Guo Z, Su L, Nedambale TL, Zhang J, Schenk J, Moreno JF, Dinnyés A, Ji W, Tian XC, Yang X, Du F. Developmental potential of vitrified Holstein cattle embryos fertilized in vitro with sex-sorted sperm. J Dairy Sci 2006; 89: 2510-2518.
[25] Fomitcheva J, Baker ME, Anderson E, Lee GY, Aziz N. Characterization of Ke 6, a new 17β-hydroxysteroid dehydrogenase, and its expression in gonadal tissues. J Biol Chem 1998; 273:22664-22671.
[26] Huang XF, Luu-The V. Molecular characterization of a first human 3(α→β)-hydroxysteroid epimerase. J Biol Chem 2000; 275:29452-29457.
[27] Lawal HO, Krantz DE. SLC18: Vesicular neurotransmitter transporters for monoamines and acetylcholine. Mol Aspects Med 2013; 34:360-372.
[28] Zhu BC, Chiocchio SR, Suburo AM, Tramezzani JH. Monoaminergic and peptidergic contributions of the superior and the inferior spermatic nerves to the innervation of the testis in the rat. J Androl 1995; 16:248-258.
[29] Higashiyama M, Ishikawa S, Saito T, Nakamura T, Kusaka I, Nagasaka S, Honda K, Saito T. Arginine vasopressin inhibits apoptosis of rat glomerular mesangial cells via V1a receptors. Life Sci 2001;16;68:1485-1493.
[30] Chen J, Liu Y, Soh JW, Aguilera G. Antiapoptotic effects of vasopressin in the neuronal cell line H32 involve protein kinase Cα and β. J Neurochem 2009; 110:1310-1320.
[31] Conrad PW, Conforti L, Kobayashi S, Beitner-Johnson D, Rust RT, Yuan Y, Kim HW, Kim RH, Seta K, Millhorn DE. The molecular basis of O_2-sensing and hypoxia tolerance in pheochromocytoma cells. Comp Biochem Physiol B Biochem Mol Biol 2001;128:187-204.
[32] Lu CH, Lee RK, Hwu YM, Chu SL, Chen YJ, Chang WC, Lin SP, Li SH. SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor. Biol Reprod 2011; 84:514-525.
[33] Martín-Satué M, Lavoie EG, Fausther M, Lecka J, Aliagas E, Kukulski F, Sévigny J. High expression and activity of ecto-5'-nucleotidase/CD73 in the male murine reproductive tract. Histochem Cell Biol 2010; 133:659-668.
[34] Schweitzer JK, Sedgwick AE, D'Souza-Schorey C. ARF6-mediated endocytic recycling impacts cell movement, cell division and lipid homeostasis. Semin Cell Dev Biol 2011; 22:39-47.
[35] Tapia JA, Macias-Garcia B, Miro-Moran A, Ortega-Ferrusola C, Salido GM, Peña FJ, Aparicio IM. The membrane of the mammalian spermatozoa: much more than an inert envelope. Reprod Domest Anim 2012;47 Suppl 3:65-75.
[36] Watanabe H, Nonoguchi K, Sakurai T, Masuda T, Itoh K, Fujita J. A novel protein Depp, which is induced by progesterone in human endometrial stromal cells activates Elk-1 transcription factor. Mol Hum Reprod 2005;11:471-476.
[37] Mamane Y, Heylbroeck C, Génin P, Algarté M, Servant MJ, LePage C, DeLuca C, Kwon H, Lin R, Hiscott J. Interferon regulatory factors: the next generation. Gene 1999; 237:1-14.
[38] Kubisch HM, Rasmussen TA, Johnson KM. Interferon-τ in bovine blastocysts following parthenogenetic activation of oocytes: pattern of secretion and polymorphism in expressed mRNA sequences. Mol Reprod Dev 2003;64:79-85.
[39] Tanaka K, Matsumoto E, Higashimaki Y, Sugimoto T, Seino S, Kaji H. FAM5C is a soluble osteoblast differentiation factor linking muscle to bone. Biochem Biophys Res Commun 2012;418:134-139.
[40] Cikos S, Fabian D, Makarevich AV, Chrenek P, Koppel J. Biogenic monoamines in preimplantation development. Hum Reprod 2011;26:2296-2305.
[41] Zhang X, Milton CC, Humbert PO, Harvey KF. Transcriptional output of the Salvador/warts/hippo pathway is controlled in distinct fashions in *Drosophila melanogaster* and mammalian cell lines. Cancer Res. 2009; 69:6033-6041.
[42] Ozawa M, Sakatani M, Yao J, Shanker S, Yu F, Yamashita R, Wakabayashi S, Nakai K, Dobbs KB, Sudano MJ, Farmerie WG, Hansen PJ. Global gene expression of the inner cell mass and trophectoderm of the bovine blastocyst. BMC Dev Biol 2012;12:33.

[43] Wang CW, Stromhaug PE, Kauffman EJ, Weisman LS, Klionsky DJ. Yeast homotypic vacuole fusion requires the Ccz1-Mon1 complex during the tethering/docking stage. J Cell Biol 2003; 163: 973–985.
[44] Kinchen JM, Ravichandran KS. Identification of two evolutionarily conserved genes regulating processing of engulfed apoptotic cells. Nature 2010; 464:778-782.
[45] Wloga D, Webster DM, Rogowski K, Bré MH, Levilliers N, Jerka-Dziadosz M, Janke C, Dougan ST, Gaertig J. TTLL3 Is a tubulin glycine ligase that regulates the assembly of cilia. Dev Cell 2009;16:867-876.
[46] Arya R, Mallik M, Lakhotia SC. Heat shock genes - integrating cell survival and death. J Biosci 2007;32:595-610.
[47] Edwards JL, Ealy AD, Monterroso VH, Hansen PJ. Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos. Mol Reprod Dev 1997; 48:25–33.
[48] Sakatani M, Bonilla L, Dobbs KB, Block J, Ozawa M, Shanker S, Yao J, Hansen PJ. Changes in the transcriptome of morula-stage bovine embryos caused by heat shock: relationship to developmental acquisition of thermotolerance. Reprod Biol Endocrinol 2013; 11:3.
[49] Al-Katanani YM, Hansen PJ. Induced thermotolerance in bovine two-cell embryos and the role of heat shock protein 70 in embryonic development. Mol Reprod Dev 2002; 62:174-180.
[50] Isodono K, Takahashi T, Imoto H, Nakanishi N, Ogata T, Asada S, Adachi A, Ueyama T, Oh H, Matsubara H. PARM-1 is an endoplasmic reticulum molecule involved in endoplasmic reticulum stress-induced apoptosis in rat cardiac myocytes. PLoS One 2010;5:e9746.
[51] Guha SK, Rose ZB. The synthesis of mannose 1-phosphate in brain. Arch Biochem Biophys 1985; 243:168-173.
[52] Becker DJ, Lowe JB. Fucose: biosynthesis and biological function in mammals. Glycobiology 2003; 13:41R-53R.
[53] Aschenbach JR, Kristensen NB, Donkin SS, Hammon HM, Penner GB. Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. IUBMB Life 2010; 62:869-677.
[54] Spurgin LG, Richardson DS. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. Proc Biol Sci 2010; 277:979-988.
[55] Ioannidis JPA. An epidemic of false claims. Competition and conflicts of interest distort too many medical findings. Sci Am 2011;304:16.
[56] Keskintepe L, Simplicio AA, Brackett BG. Caprine blastocyst development after in vitro fertilization with spermatozoa frozen in different extenders. Theriogenology 1998; 49:1265-1274.
[57] Barisić D, Suchanek E, Jeyendran RS. Milk versus TEST-yolk—preincubated sperm; in vitro fertilization outcome. Eur J Obstet Gynecol Reprod Biol 1995;62:231-233.
[58] Perkins JR, Carpenter MC, Seath DM. A comparison of the fertility of bull semen diluted in egg yolk–citrate and homogenized milk. J Dairy Sci 1955; 38: 155-158.
[59] DeJarnette JM, Marshall CE. Straw-thawing method interacts with sire and extender to influence sperm motility and conception rates of dairy cows. J Dairy Sci 2005;88: 3868-3875.

TABLE 1. Correlation of in vitro measurements of fertilization and embryonic development with Daughter Pregnancy Rate.[a,b]

	DPR	CR	SD of CR	SD of BDRC
CR	-0.06		-0.59	
	(0.54)		(<0.0001)	
BDRC	-0.12	0.23		-0.02
	(0.25)	(0.02)		(0.85)

[a]The *P* value of each correlation is in parentheses.
[b]Abbreviations are as follows: BDRC, blastocyst development rate from cleaved embryos; CR, percent cleavage; DPR, daughter pregnancy rate; SD, standard deviation.

TABLE 2. SNPs associated with percent of oocytes that cleaved.[a]

		Least-squares means ± SEM			Linear		Dominance
SNP	Gene	0	1	2	Effect	*P* value	*P* value
rs43114141	*AVP*	76.92 ± 2.82 (n = 16)	70.22 ± 2.20 (n = 31)	74.56 ± 2.16 (n = 34)	-0.82	0.6075	0.0350
rs134150850	*C28H10orf10*	69.51 ± 2.77 (n = 16)	73.11 ± 1.93 (n = 45)	77.70 ± 2.41 (n = 31)	4.18	0.0105	0.8229
rs109332658	*C7H19orf60*	66.17 ± 3.60 (n = 9)	76.79 ± 2.29 (n = 32)	72.86 ± 1.97 (n = 50)	1.07	0.5530	0.0086
rs43676052	*EPAS1*	80.24 ± 5.03 (n = 5)	70.78 ± 2.28 (n = 34)	74.50 ± 1.87 (n = 53)	0.64	0.7512	0.0316
rs109769865	*HSD17B6*	78.50 ± 5.94 (n = 4)	77.64 ± 2.59 (n = 26)	71.51 ± 1.85 (n = 61)	-5.06	0.0285	0.4571
rs42508588	*NT5E*	53.25 ± 7.50 (n = 2)	72.51 ± 2.34 (n = 32)	74.47 ± 1.83 (n = 58)	4.44	0.0485	0.0464
rs43321188	*SERPINE2*	62.33 ± 3.68 (n = 9)	74.20 ± 2.24 (n = 31)	75.08 ± 1.83 (n = 52)	4.30	0.0126	0.0381
rs110365063	*SLC18A2*	63.32 ± 5.38 (n = 4)	76.46 ± 2.59 (n = 24)	73.40 ± 1.82 (n = 63)	0.75	0.7217	0.0251
rs110660625	*TBC1D24*	68.76 ± 3.29 (n = 14)	71.41 ± 2.12 (n = 39)	76.12 ± 2.19 (n = 35)	3.88	0.0229	0.6724

[a]Single nucleotide polymorphism represented as the rs number given by the National Center for Biotechnology Information data base SNP.

TABLE 3. SNPs associated with percent of cleaved embryos that became a blastocyst.[a]

		Least-squares means (SEM)			Linear		Dominance
SNP	Gene	0	1	2	Effect	*P* value	*P* value
rs135390325	*C1QB*	17.64 ± 7.07 (n = 1)	40.82 ± 1.52 (n = 22)	40.86 ± 0.75 (n = 68)	11.61	0.2140	0.0038
rs135071345	*FAM5C*	36.61 ± 5.15 (n = 2)	38.24 ± 1.52 (n = 23)	41.56 ± 0.89 (n = 67)	3.07	0.0445	0.7807
HSP70C895D	*HSPA1A*	45.27 ± 2.43 (n = 9)	41.31 ± 1.14 (n = 41)	39.29 ± 1.11 (n = 43)	-2.62	0.0259	0.5823
rs110953315	*IRF9*	46.01 ± 4.22 (n = 3)	42.53 ± 1.53 (n = 23)	39.71 ± 0.91 (n = 65)	-2.95	0.0428	0.8996
rs41859871	*MON1B*	26.51 ± 7.29 (n = 1)	39.05 ± 1.55 (n = 22)	41.50 ± 0.87 (n = 70)	3.34	0.0451	0.2091
rs111027720	*PARM1*	39.61 ± 1.64 (n = 28)	42.22 ± 1.07 (n = 44)	38.33 ± 1.35 (n = 19)	-0.95	0.3799	0.0339
rs109813896	*PCCB*	36.78 ± 2.27 (n = 10)	39.88 ± 1.08 (n = 44)	42.21 ± 1.21 (n = 35)	2.59	0.0281	0.8210
rs109629628	*PMM2*	37.77 ± 1.98 (n = 14)	40.35 ± 1.15 (n = 41)	42.33 ± 1.22 (n = 37)	2.21	0.0459	0.8533
rs110365063	*SLC18A2*	43.97 ± 3.69 (n = 4)	43.12 ± 1.51 (n = 24)	39.64 ± 0.93 (n = 63)	-2.87	0.0388	0.5892
rs110660625	*TBC1D24*	36.96 ± 1.79 (n = 14)	43.47 ± 1.07 (n = 39)	39.45 ± 1.13 (n = 35)	0.13	0.9041	0.0008
rs135236119	*TTLL3*	38.95 ± 2.91 (n = 6)	37.85 ± 1.30 (n = 30)	42.03 ± 0.97 (n = 54)	2.77	0.0255	0.1922
rs134282928	*WBP1*	50.59 ± 4.91 (n = 2)	43.75 ± 2.01 (n = 12)	39.43 ± 0.92 (n = 57)	-4.87	0.0057	0.6956

[a]Single nucleotide polymorphism represented as the rs number given by the National Center for Biotechnology Information data base SNP.

FIGURE LEGENDS

Figure 1. Variation among bulls used for in vitro fertilization in the percent of oocytes exposed to sperm that underwent cleavage. Each point on the graph represents the average percent cleavage ± SEM of an individual bull. The red diamonds represent bulls whose semen was in egg yolk extender, blue circles represents bulls with semen in milk extender, and green squares represent bulls with semen in an unknown extender.

Figure 2. Variation among bulls used for in vitro fertilization in the percent of cleaved embryos that became blastocysts. Each point on the graph represents the average percent cleavage ± SEM of an individual bull. The red diamonds represent bulls whose semen was in egg yolk extender, blue circles represents bulls with semen in milk extender, and green squares represent bulls with semen in an unknown extender.

FIGURE 1

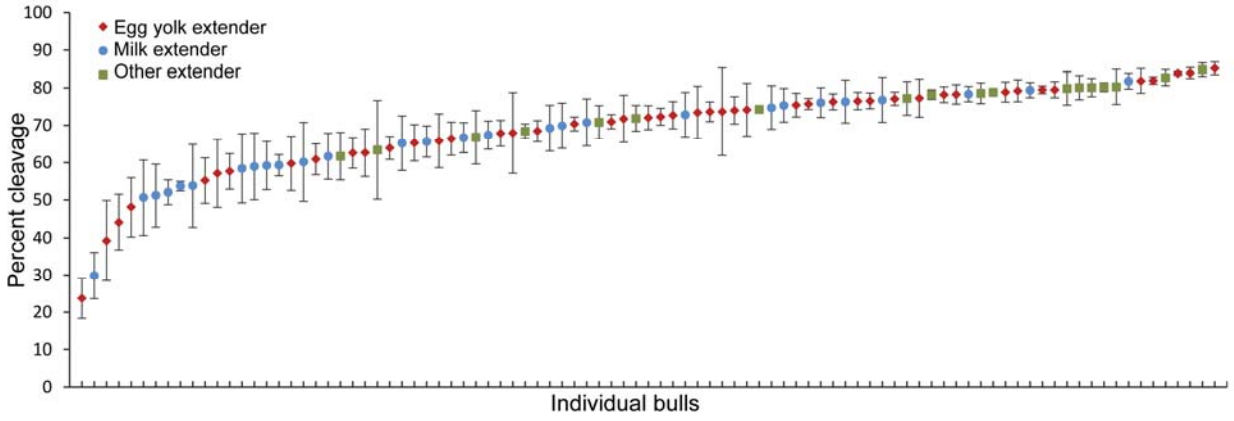

FIGURE 2