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Research Article

A single nucleotide polymorphism in *COQ9* affects mitochondrial and ovarian function and fertility in Holstein cows†

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Abstract

A single missense mutation at position 159 of coenzyme Q9 (*COQ9*) (G→A; rs109301586) has been associated with genetic variation in fertility in Holstein cattle, with the A allele associated with higher fertility. COQ9 is involved in the synthesis of coenzyme COQ10, a component of the electron transport system of the mitochondria. Here we tested whether reproductive phenotype is associated with the mutation and evaluated functional consequences for cellular oxygen metabolism, body weight changes, and ovarian function. The mutation in *COQ9* modifies predicted tertiary protein structure and affected mitochondrial respiration of peripheral blood mononuclear cells. The A allele was associated with low resting oxygen consumption and high electron transport system capacity. Phenotypic measurements for fertility were evaluated for up to five lactations in a population of 2273 Holstein cows. There were additive effects of the mutation ($P < 0.05$) in favor of the A allele for pregnancy rate, interval from calving to conception, and services per conception. There was no association of genotype with milk production or body weight changes postpartum. The mutation in *COQ9* affected ovarian function; the A allele was associated with increased mitochondrial DNA copy number in oocytes, and there were overdominance effects for *COQ9* expression in oocytes, follicle number, and antimullerian hormone concentrations. Overall, results show how a gene involved in mitochondrial function is associated with overall fertility, possibly in part by affecting oocyte quality.

Summary Sentence

A SNP in *COQ9* was described that changes predicted protein structure, is associated with altered mitochondrial afunction, and that modulates reproductive function in dairy cattle, possibly by regulating oocyte quality.

Key words: COQ9, genetic variation, cattle, oocyte, mitochondria.

Introduction

Despite the low heritability of fertility traits [1, 2], reproductive function is determined in part by genetic variation. Indeed, lines of animals differing in reproductive capacity have been described for many species including dairy cattle [3, 4], sheep [5, 6], mice [7, 8], and pigs [9]. Identification of the specific alleles responsible for genetic variation in reproduction would increase ability to select animals genetically for reproductive function. In addition, new opportunities to regulate reproduction in domestic animals and humans could result from an understanding of the physiological consequences of inheritance of specific alleles controlling reproduction.

Many of the identified mutations affecting fertility to date are loss-of-function alleles that are often lethal to embryos, such as mutations in transducin like enhancer of split 6 (*TLE6*) [10] and thrombospondin type 1 domain containing 1 (*THSD1*) [11] in humans and CWC15 spliceosome associated protein homolog (*CWC15*) [12] and fanconi anemia complementation-group I (*FANCI*) in cattle [13]. Another example is a missense mutation in phospholipase C zeta 1 (*PLCZ1*) in humans that leads to the absence of the 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1 in sperm resulting in failure of oocyte activation after sperm–oocyte fusion [14]. Other mutations cause altered gene expression patterns. In sheep, the FecL haplotype in the Lacaune breed has been identified as a mutation in the galactosyl transferase *B4GALNT2* that leads to ectopic expression in granulosa cells and glycosylation of proteins important for follicular growth, such as inhibin alpha (INHA), and inhibin beta A (INHBA)[15]. In cattle, a deletion mutation in the promoter region of heat shock protein family A member 1 like (*HSPA1L*), which encodes for a member of the heat shock protein 70 family, results in increased expression of the protein in response to heat shock [16] and is associated with increased calf crop in Brahman cattle [17] and with resistance to elevated temperature in embryos [18]. Other mutations cause a change in amino acid sequence. Examples include a mutation in bone morphogenic protein 15 (*BMP15*) in sheep that alters signaling activity of the encoded protein [19] and a mutation in protamine 1 (*PRM1*) in the human of one of the conserved arginine clusters necessary for DNA binding that is associated with infertility in males [20].

Putative mutations affecting reproduction have been identified by genome-wide association studies in species such as humans, sheep, and cattle [21–24] and by relating reproductive function to specific mutations in candidate genes [25–29]. The single nucleotide polymorphism (SNP) explaining the greatest genetic variation in Holstein cattle for reproductive traits identified by Ortega et al. [29] was rs109301586, located in bovine coenzyme Q9 (*COQ9*) on chromosome 18 (18:25527339). The missense mutation causes a change from G → A which induces an amino acid change from aspartic acid to asparagine at position 53 of the protein. The A allele, which has a frequency of 49.1% in Holsteins [25, 29], is associated with higher genetic merit for daughter pregnancy rate (DPR; a trait related to interval between calving and conception) and cow conception rate and is not associated with milk production traits [25, 29]. The mutation explained 3% of genetic variation in DPR [29].

COQ9, along with other COQ proteins (COQ2-COQ8), is involved in the biosynthesis of COQ10 [30, 31], which is a component of the mitochondrial electron transport system and which is required for mitochondrial adenosine triphosphate synthesis. COQ9 is therefore critical for cellular energy metabolism. In mice, a mutation in the coding region of *Coq9* that encodes for a truncated protein reduced the amount of Coq7, Coq9, and Coq10 in several tissues and resulted in mitochondrial encephalomyopathy [32].

There were two main goals of the present series of experiments. The first was to verify that the mutation in *COQ9* in cattle related to DPR is also associated with phenotypic changes in reproductive function. The second goal was to determine how the mutation could act physiologically to change reproductive function. Being a gene involved in energy metabolism of the cell, it was hypothesized that the *COQ9* mutation modulates reproduction through regulation of energy metabolism in the mitochondria, which in turn affects energy balance postpartum.

Materials and methods

Animal care

Procedures for use of animals were approved by the University of Florida Institutional Care and Use Committee.

Effect of the missense mutation in *COQ9* on predicted protein structure

Amino acid sequence for bovine COQ9 was retrieved from the NCBI database (NP001039767.1). Predicted protein structure for both allelic variants of *COQ9* (rs109301586) was determined using the I-Tasser v4.4 software program [33]. The program pipeline consists of four general phases: threading template identification through a nonredundant structure library to identify templates, iterative structure assembly simulation, model selection and refinement, and structure-based function annotation [33–35]. The three-dimensional models for each variant of the protein were superimposed and aligned to identify potential changes in structure. Visualization of the models, location of the amino acid substitution, and alignment of the models was performed using the software PyMOL Molecular Graphics System Version 1.8 (Schrödinger, Cambridge, MA, USA).

Phylogenetic analysis

Coding sequences for *COQ9* for the bovine and other mammalian organisms were retrieved from the NCBI nucleotide database [36] using the BLAST tool. Sequences were aligned using the software CLUSTAL omega [37]. Aligned sequences were used for phylogenetic and molecular evolutionary analyses conducted using MEGA version 6 [38]. The evolutionary history was inferred using a maximum likelihood approach based on the Kimura two-parameter model [39, 40]. Bootstrap values were calculated based on 1,000 replicates to assess the level of confidence of each branch pattern [41]. The initial trees from each heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and the topology with largest log-likelihood value was selected. A discrete gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.5809)]. All position containing gaps and missing data were eliminated [38]. The nucleotide at the SNP in the bovine *COQ9* was then identified for each species and mapped to the phylogenetic trees.

To further examine occurrence of the allele in the *Bos* genus, DNA sequence of *COQ9* from a single *B. primigenius* animal was obtained from Park et al. [42]. In addition, DNA sequences from three *B. indicus* bulls (two Gyr and one Guzerat) were examined to

identify the allele present for the SNP in *COQ9*. Data consisted of whole-genome sequences generated using an Illumina HiSeq 2000 sequencer (Illumina Inc., San Diego, CA, USA), and mapped to the UMD 3.1 bovine genome reference assembly [43]. The number of mapped reads was 368,087,825 and 412,887,976 for the two Gyr bulls, and 302,400,730 for the Guzerat bull.

Genotyping

The genotype of the SNP in *COQ9* of Holstein animals was determined using the Sequenom MassARRAY system (iPLEX GOLD; Sequenom, San Diego, CA, USA) according to the manufacturer's instructions (see Supplemental File S1 for more details) [44].

Genotyping for the *COQ9* SNP in ovarian tissues was performed using a KASP by design assay (LGC Genomics, Middlesex, UK). Details are available in Supplemental File S1. The assay is a polymerase-chain reaction (PCR)-based technique involving a common reverse primer and two allele-specific forward primers, with one bound to fluorescein amidite and the second to hexachlorofluorescein to produce genotype-specific PCR products. A total of 43 samples (11 AA, 20 AG, and 12 GG) were used in the experiment. Genotypes of DNA samples from ovarian tissues were also confirmed by high-resolution melting analysis (details in Supplemental File S1).

Mitochondrial respiration

Mitochondrial respiration was determined in peripheral blood mononuclear cells (PBMC) from lactating Holstein cows at the Dairy Unit of the University of Florida that had been genotyped for the SNP in *COQ9* using the Sequenom MassARRAY system. Cows ranged between 200 and 253 days of lactation at the time of the experiment. PBMC from a total of 12 cows per genotype (AA, AG, and GG) were assessed for mitochondrial respiration.

The PBMC were isolated from 10 ml of whole blood using Ficoll-Plaque gradient, following a previously established procedure [45]. Isolated PBMC were suspended in 3–4 ml of Roswell Park Memorial Institute (RPMI) 1640 medium (ThermoFisher Scientific, Waltham, MA, USA). Concentration and viability of the PBMC stock solution was determined using the TC20 automated cell counter (BioRad, Hercules, CA, USA). Only samples with viability above 90% were used for respirometry. Samples were diluted to 3×10^6 viable cells/ml using RPMI medium, and maintained at 38.5°C and 5% (v/v) CO_2 until analysis of oxygen consumption, within 2 h after isolation.

The Oroboros O2k high-resolution respirometer system (OROBOROS Instruments, Innsbruck, Austria) was used to determine mitochondrial respiration of isolated PBMCs according to methods previously described [46]. A total of 6×10^6 viable cells suspended in 2.0 ml of RPMI-1640 were placed in the respirometer chamber and maintained at a constant temperature of 37°C throughout the measurement. All samples were run in duplicate.

Oxygen consumption (O_2 flux; pmol/sec/million cells) of PBMCs was measured using the following protocol: routine respiration, considered basal oxygen consumption in quiescent cells; leak respiration after addition of oligomycin, an inhibitor of the ATP synthase (Sigma-Aldrich, St. Louis, MO, USA; 2.5 μM final concentration), corresponding to O_2 flux not associated with ATP synthesis; and maximum noncoupled respiration after addition of the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (Sigma-Aldrich; 0.5 μl steps of a 0.1-mM stock solution, to a final concentration between 1.5 and 2.0 μM). Finally, residual oxygen consumption from oxidative side reactions independent of mitochondrial O_2 flux was determined by inhibiting electron transport to oxygen with the addition of 1 μl 5 mM antimycin A (Sigma-Aldrich),

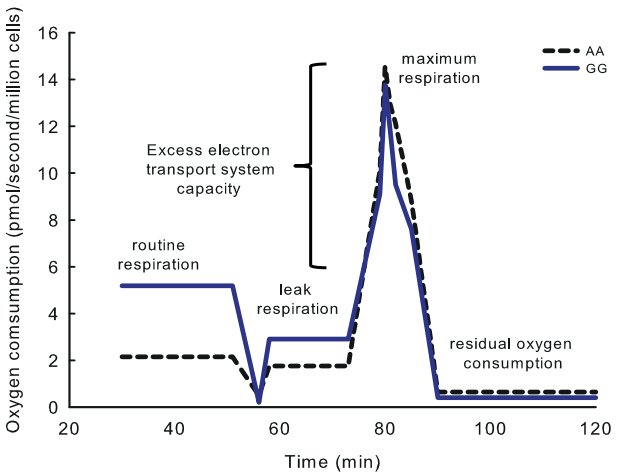

Figure 1. Representative example of the pattern of oxygen consumption for assessment of mitochondrial function. The dashed line represents values from a cow of the AA genotype, and the blue line the values for a cow of the GG genotype. Excess electron transport system capacity was calculated as the ratio of maximum respiration and routine respiration.

an inhibitor of complex III of the electron transport system. A representative example of the pattern of O_2 flux is shown in Figure 1.

Routine respiration, leak state, and maximum respiration were corrected by subtracting the value for residual oxygen consumption from each measurement. Excess electron transport system capacity was considered as the ratio of maximum respiration and routine respiration. Data were analyzed by analysis of variance using the GLM procedure of SAS v 9.4 (SAS Institute Inc., Cary, North Carolina, USA). The model included effect of genotype; additive and dominance values were estimated by orthogonal contrasts.

Single nucleotide polymorphism effects on phenotypic measurements of fertility and milk production

Holstein cows were selected based on having a high ($\geq$1.5; n = 1,053) or low ($\leq$ –1.0; n = 1,220) predicted transmitting ability for DPR (see ref. 29). Cows were located on six dairies in Florida and five in California. Records for up to five lactations were retrieved from each farm computer and combined with records from the national dairy database maintained by the Council on Dairy Cattle Breeding (Bowie, MD). Data for days from calving to conception, services per conception, pregnancy rate at first service, and 305-day milk, fat, and protein yields were evaluated.

The association of each genetic variant with phenotypic traits was performed by analysis of variance using the Statistical Analysis System v 9.4 (SAS Institute Inc., Cary, North Carolina). In all analyses, genotype was considered a categorical variable. The full model was as follows:

$$Y_{ijkl} = \mu + a_i + g_j + l_k + f_l + e_{ijkl},$$

where Y_{ijkl} is the value of the trait of interest for the ith cow (i = 1, 2, . . . , n), a_i is the random polygenic effect (including all available pedigree information) for the ith cow, g_j is the fixed effect of SNP genotype (j = 1,. . . ,3 such that g_1 is the genotypic value of *AA* homozygotes, g_2 is the genotypic value of *AB* heterozygotes, and g_3 is the genotypic value of *BB* homozygotes), l_k is the fixed effect of lactation number (k = 1,. . . ,5), f_l is the fixed effect of farm (l = 1,. . . ,6), and e_{ijkl} is the random residual effect. We assume that random polygenic effects $a \sim N(0, \mathbf{A}\sigma_a^2)$ and residuals $e \sim N(0, \sigma_e^2)$, where **A** is the numerator relationship matrix, σ_a^2 is the

additive genetic variance of the trait of interest, and σ_e^2 is the residual error variance. All of the available pedigree information for each cow was used to generate **A**, which models the covariance among the polygenic effects. Following Falconer et al. [48], additive and dominance parameters were estimated for each locus as $(g_3 - g_1)/2$, and $g_2 - (g_1 + g_2)/2$, respectively.

Days open and pregnancy rate were analyzed using the model described above with the MIXED procedure of SAS. Days open were log transformed before analysis to establish normality. Pregnancy rate was considered a linear response because convergence could not be achieved when data were analyzed with the GLIMMIX procedure with binary distribution specified. To achieve convergence, pregnancy rate was also analyzed using GLIMMIX without random polygenic effects in the model. The number of services per conception was analyzed with the GLIMMIX procedure using a negative binomial distribution for the responses and a logarithmic link function to account for the statistical properties of count data [47], and the full model described above.

Body weight changes after calving

Body weights for the first 24 weeks after calving were recorded for 415 lactating Holstein cows at the University of Florida Dairy Unit that had been genotyped for the *COQ9* SNP by Sequenom. Distribution of cows for each genotype was 86 AA, 223 AG, and 106 GG. Body weights were recorded twice daily as cows left the milking parlor after each milking using the AfiWeigh automatic walk-over weighing system (Afimilk, Kibbutz Afikim, Israel). Body weights were obtained for up to two lactations. Weights were averaged by week and effects of genotype and week after calving on daily average body weights were analyzed by analysis of variance using the MIXED procedure of SAS. The model included genotype, cow nested within genotype, week postpartum, the interaction between genotype and week postpartum, and with body weight at calving as a covariate. Cow was considered random and other main effects were considered fixed.

Maximum body weight loss was estimated for each cow by determining the largest change in daily body weight within the first 45 days after calving as compared to body weight at calving. Data for maximum body weight loss and the day postpartum when maximum weight loss occurred were analyzed for genotype effects. Weight loss values were log transformed before analysis to improve normality. Data were analyzed by analysis of variance using the GLIMMIX procedure of SAS, with genotype as fixed effect and cow as a random effect. Means and SEM were back transformed for data presentation.

Expression of *COQ9* in the reproductive tract

Expression pattern of *COQ9* was determined during the first 7 days after ovulation in the endometrium of nonlactating Holstein cows. On day –18, cows were given 25 mg of prostaglandin $F_{2\alpha}$, i.m., (PGF, Lutalyse, Zoetis, Florham Park, NJ, USA), followed by 100 μg gonadotropin-releasing hormone, i.m., (GnRH; Cystorelin, Merial Inc., Duluth, GA, USA) 2 days later. A second injection of 100 μg GnRH was given on day –9 in addition to intravaginal insertion of controlled internal drug release device (CIDR, Zoetis), followed by 25 mg PGF, i.m., five days later when the intravaginal device was removed. The PGF injection was repeated 24 h later and 100 μg of GnRH given one day later. The expected day of ovulation was day 0. Cows were slaughtered at days 0, 3, 5, and 7 relative to predicted ovulation (n = 3–4 per day). Reproductive tracts were obtained and intercaruncular regions of endometrial tissue ipsilateral to the corpus luteum were harvested for gene expression.

Details of RNA expression and RT-PCR are in Supplemental File S1. The Δ cycle threshold (Δ CT) was determined by subtracting the average CT value of the sample from the geometric mean of the CT for three housekeeping genes, *SDHA*, *GAPDH*, and *YWHAZ*, using previously described primers [49]. Fold change was calculated relative to the housekeeping genes ($2^{\Delta CT}$). Data were analyzed by analysis of variance using the GLM procedure of SAS with day included as fixed effect, and cow as a random effect.

Expression of *COQ9* in the matured oocyte and preimplantation embryo

Matured oocytes and embryos of various breeds were produced in vitro as described in Supplemental File S1. Pools of 30 matured oocytes or embryos were collected. Matured oocytes were collected after 21 h of maturation. Embryos were collected at the 2 cell [27–31 h post insemination (hpi)], 3–4 cell (46–52 hpi), 5–8 cell (49–59 hpi), 9–16 cell (72–75 hpi), morula (120–123 hpi), and blastocyst (168–171 hpi) stages. A total of four pools (replicates) were analyzed for each of the seven stages. For collection, pools of embryos were treated with 0.1% (w/v) proteinase from *Streptococcus griseus* to remove the zona pellucida, washed three times in 50 μl droplets of Dulbecco phosphate buffered saline (DPBS) containing 1% (w/v) polyvinylpyrrolidone (PVP) (DPBS-PVP), placed in 100 μl extraction buffer from the PicoPure RNA isolation kit (Applied Biosystems, Carlsbad, CA, USA), and kept at –80°C so that processing of each stage could be performed at one time.

Total RNA was isolated using the PicoPure RNA isolation kit following manufacturer's instructions. RNA was treated with 1 μl (2 U) of DNAse (New England Biolabs, Ipswich, MA, USA) per sample. Reverse transcription and PCR to determine *COQ9* expression was performed as described in Supplemental File S1. Stage of development effects on gene expression was analyzed by least-squares analysis of variance of the ΔCT values using the GLM procedure of SAS. Stage was included as main effects in the model, and replicate as a random effect.

Effect of *COQ9* genotype on oocyte numbers, expression of *COQ9* in cumulus cells, and expression of *COQ9*, *BMP15*, and *GDF9* in matured oocytes

Ovaries of cattle of various breeds collected from the abattoir were processed individually to obtain oocytes and cumulus cells from cows of specific *COQ9* genotypes. The surface of each ovary was sliced with a scalpel to harvest immature cumulus-oocyte complexes (COC) into 20 ml of oocyte-washing medium (MOFA, Verona, WI, USA). The COC were washed and placed in groups of 10 in 50 μl droplets of oocyte maturation medium covered with mineral oil and cultured for 22 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO_2. The total number of COC retrieved per ovary was recorded. For each ovary from which COC were retrieved, a separate population of cumulus cells was harvested from the oocyte collection medium after removal of COC for genotyping by KASP assay while the COC were in maturation. At the end of maturation, COC from each ovary were denuded from the surrounding cumulus cells by vortexing for 5 min in 600 μl of DPBS. Cumulus and oocytes were collected separately. Cumulus cell suspensions were washed by centrifugation three times (each for 3 min at 4000 g). After each centrifugation, the supernatant was removed by aspiration, and the pellet was suspended in 500 μl of fresh DPBS. Lastly, the pellet of cells was

snap frozen and stored at –80°C until analysis. For oocytes, pools ranging from 18 to 23 oocytes were washed in DPBS-PVP, the zona pellucida was removed as detailed before, and oocytes were snap frozen in liquid nitrogen and stored at –80°C until analysis.

RNA was isolated from cumulus cells using the RNeasy micro kit from QIAGEN (Valencia, CA, USA); RNA and DNA were isolated from the oocyte samples using the AllPrep DNA/RNA Mini Kit from QIAGEN following manufacturer's instructions.

Reverse transcription and real-time PCR were performed as described for *COQ9* in Supplemental File S1. In addition to *COQ9*, expression of *BMP15* and growth differentiation factor 9 (*GDF9*) was quantified in the oocyte samples. Primer sequences were retrieved from the literature and generated by Integrated DNA Technologies. The primers for *BMP15* were forward 5′-AGCCTTCCTGTGTCCCTTAT-3′ and reverse 5′-GGGCAATCATACCCTCATACTC-3′ [50]. The primers for *GDF9* were forward 5′-AGCGCCCTCACTGCTTCTATAT-3′ and reverse 5′-TTCCTTTTAGGGTGGAGGGAA-3′ [51]. Amplicon sizes were 99 bp for *BMP15* and 80 bp for *GDF9*. Both set of primers were validated as described in Supplemental File S1; slopes were between –3.0 and –3.3, and primer efficiencies were 98.1 and 107% for *BMP15* and *GDF9*, respectively. The ΔCT was determined using the same housekeeping genes as stated before.

Genotype effect on gene expression was analyzed by least-squares analysis of variance of the ΔCT values using the GLM procedure of SAS. The number of COC collected per genotype was analyzed by the GLIMMIX procedure of SAS, using a Poisson distribution for responses and a logarithmic link function. Genotype was considered the main effect and each ovary a replicate. Data were back transformed for presentation.

Mitochondria DNA copy number

To determine mitochondrial DNA content, 43 DNA samples from the same pools of matured oocytes described above (11 AA, 20 AG, and 12 GG) were used. The mitochondrial gene cytochrome c oxidase subunit I (*COX1*) was used as reference to amplify mitochondrial DNA. Primers previously described [52] were used: forward 5′-AAATAATATAAGCTTCTGACTCC-3′, and reverse 5′-TCCTAAAATTGAGCAAACTCC-3′. The PCR product was a 190-bp DNA fragment, primers were validated as described in Supplemental File S1; the slope of the regression was –3.3 and the efficiency of the primers was 98.2%. A standard curve of 6 points in a 10-fold serial dilution was generated using the synthetic gene fragment gBlocks (IDT Technologies) which contained the sequence amplified by *COX1* primers. Dilution ranged from 1.2×10^6 to 1.2 copies of the DNA fragment. Numbers of transcript copies in the sample were determined by comparing the CT values of each sample to the CT values of the standard curve. PCR conditions were as described to determine gene expression (Supplemental File S1). Samples and standards were run in duplicate. Genotype effect on mitochondrial copy number was analyzed by least-squares analysis of variance by the GLIMMIX procedure of SAS, using a Poisson distribution for responses and a logarithmic link function. Genotype was considered the main effect.

Determination of circulating concentrations of antimullerian hormone

Total antimullerian hormone (AMH) concentration in plasma obtained from saphenous venipuncture was determined using a commercial enzyme-linked immunosorbent assay (MOFA, Verona, WI, USA) according to manufacturer's instructions. Plasma from 34 Holstein cows previously genotyped for the SNP in *COQ9* (12 AA, 9 AG, and 13 GG) were analyzed in duplicate. Differences in AMH concentration between genotypes was determined by analysis of variance

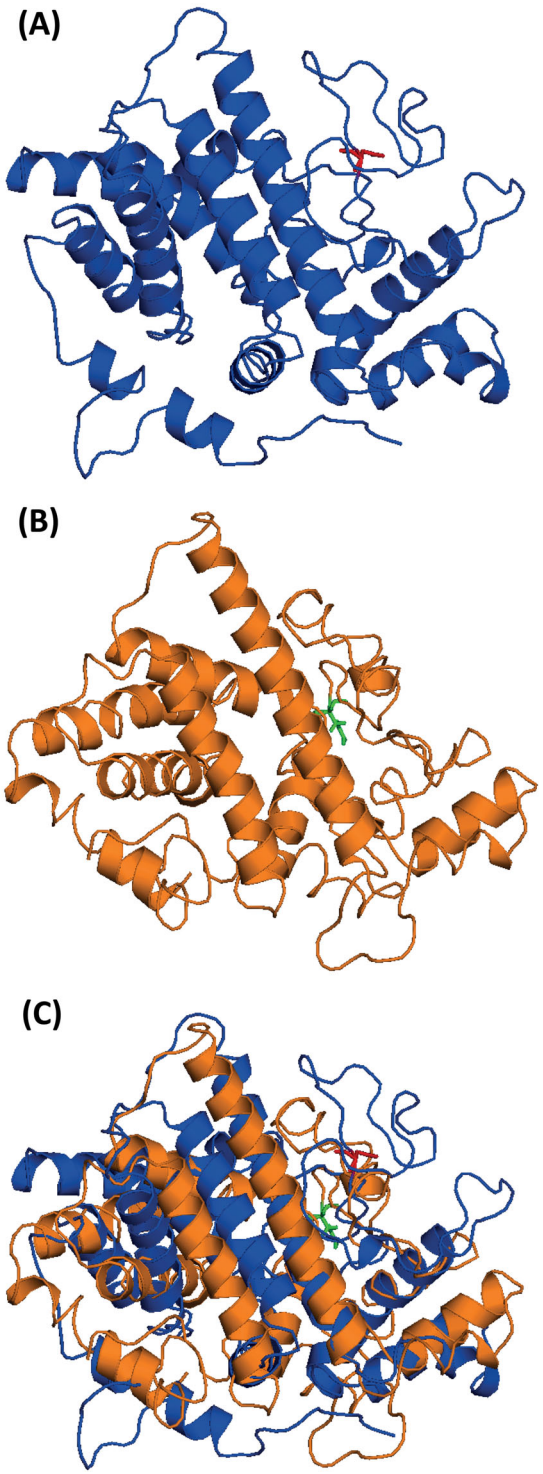

Figure 2. Models of the three-dimensional structure of COQ9. (A) Variant G, corresponding to the major allele; (B) variant A corresponding to the minor allele; (C) superimposed models of both isoforms of the protein. The residue affected by the SNP is shown in red in panel A and in green in panels B and C.

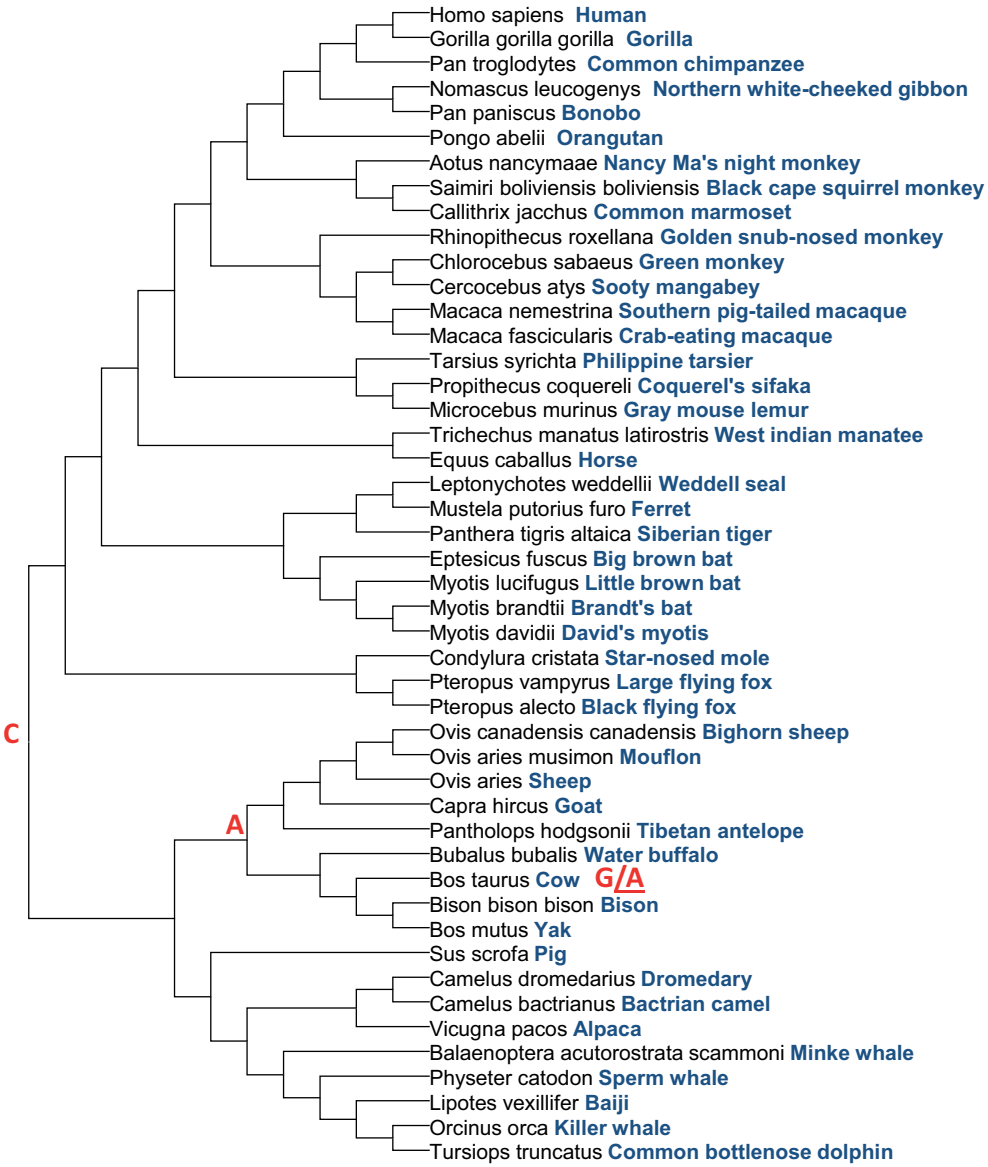

Figure 3. Phylogeny of the mutation in *COQ9*. The reference allele is indicated in red. All species distal to the common ancestor had the same reference allele unless indicated by placement of a distinct letter. For *B. taurus*, both alleles are presented (major/minor). The allele underlined is the one associated with superior fertility.

with the GLIMMIX procedure of SAS, using a normal distribution. Antimullerian hormone concentration was the dependent variable and genotype was considered a fixed effect.

Results

Characteristics of the missense mutation in *COQ9*

Examination of predicted protein structures for the G (Figure 2A) and A (Figure 2B) variants indicates that the mutation caused a change in protein structure and is reflected in rearrangement of several alpha helixes (see superimposed structures in Figure 2C). When both protein models are superimposed, the average distance between the atoms (root-mean-square deviation of atomic positions) was 19.85, which indicate that the two models cannot be properly aligned.

Phylogenetic analysis was conducted to determine whether the A mutation occurred recently in cattle or related species. Sequences of *COQ9* from 47 species retrieved from the NCBI database [36] were aligned, and the nucleotide at the SNP location in the bovine compared to the reference nucleotide in the other mammals. The constructed phylogenetic tree is shown in Figure 3. The reference nucleotide for the majority of species was C but became A in ruminants except for the cow (G). Thus, it appears that the ancestral A was mutated to G in cattle and that the G mutation accumulated over time so that it is now represented in just over half of Holstein *COQ9* genes. In the only sample available from *B. primigenius*, the allele was A. Of three indicine cattle, one animal (a Gyr) was heterozygous and the other two (a Gyr and Guzerat) were homozygous for A. Thus, appearance of the G allele preceded divergence of *B. taurus* and *B. indicus*.

Table 1. Association of *COQ9* genotype with mitochondrial respiration in PBMC.

		Oxygen consumption (pmol/s/million cells)[1]			
Genotype	n	Routine respiration	Leak respiration	Maximum respiration	Excess electron transport system capacity
GG	12	4.9 (0.3)	3.0 (0.3)	10.3 (0.7)	2.1 (0.2)
AG	12	4.7 (0.3)	2.7 (0.3)	11.0 (0.7)	2.5 (0.2)
AA	12	3.4 (0.3)	1.9 (0.3)	11.4 (0.7)	3.4 (0.2)
P-value	Additive	0.0021	0.0109	0.2573	0.0008
	Dominance	0.1581	0.4144	0.8138	0.3489

[1]Values are least-squares means (SEM).

Table 2. Association of *COQ9* genotype with phenotypic measurements of fertility and production after adjustment for random polygenic effects.

	Genotype[1]						Additive effect	Additive *P*-value
	n	GG	n	AG	n	AA		
Pregnancy rate (%)	588	33.0 (0.01)	1028	34.0 (0.01)	629	36.0 (0.01)	1.00	<0.001
Services per conception (n)	597	2.8 (0.06)	1039	2.7 (0.05)	638	2.6 (0.05)	−0.03	0.014
Days open (d)	597	146 (2.9)	1039	145 (2.3)	638	138 (2.8)	−4.21	0.037
Milk yield (kg)	581	11,406 (59)	1019	11,416 (49)	519	11,395 (58)	−5.87	0.860
Fat yield (kg)	581	422 (2.0)	1019	421 (1.7)	519	422 (2.0)	−0.22	0.880
Protein yield (kg)	581	351(1.6)	1019	348 (1.3)	519	348 (1.6)	−0.23	0.880

[1]Values are least-squares means (SEM). Dominance effects were not significant.

Mitochondrial respiration

Results of mitochondrial respiration are summarized in Table 1. There were additive effects of *COQ9* genotype on routine respiration ($P = 0.002$) with the A allele being associated with lower oxygen consumption. Similarly, there was an additive effect on leak respiration ($P = 0.010$), with the A allele again being associated with lower oxygen consumption. There was no effect of genotype on maximum respiration (additive effect; $P = 0.257$). Overall, excess electron transport system capacity was affected by the additive actions of genotype with the A allele being associated with higher capacity ($P < 0.001$) than the G allele.

Phenotypic measurements of reproduction and milk production

Results for phenotypic measurements for reproductive and milk production characteristics as analyzed after adjustments for random polygenic effects are summarized in Table 2. Cows of the AA genotype, previously associated with higher genetic merit for fertility [25,29], had higher pregnancy rate, required fewer services per conception and showed a reduced interval from calving to conception as compared to cows of the GG genotype. The effects on reproduction were additive, and animals of the heterozygous genotype (AG) showed intermediate values for each trait. Specifically, there were significant additive effects of genotype on pregnancy rate ($P < 0.001$), services per conception ($P = 0.014$), and days from calving to conception ($P = 0.037$). Pregnancy rate at first service was also analyzed as a binomial variable without adjusting for random polygenic effects. In this analysis, there was an additive effect for the trait ($P = 0.022$); pregnancy rate [% (SEM)] was 37 (0.01), 35 (0.01), and 33 (0.01) for the AA, AG, and GG genotypes, respectively.

There were no significant effects of the *COQ9* genotype on milk yield ($P = 0.861$), fat yield ($P = 0.880$), or protein yield ($P = 0.882$).

Body weight changes after calving

Results are shown in Figure 4. Body weight during first lactation was affected by the interaction of genotype and week of lactation for both first and second lactation ($P < 0.001$). However, patterns of change in body weight over time were generally similar for each genotype. Moreover, maximum body weight loss during lactation was similar among genotypes for the first ($P = 0.251$) and second ($P = 0.591$) lactation (Table 3). Similarly, there was no effect of genotype on the day cows reached their maximum body weight loss in the first ($P = 0.802$) or second ($P = 0.292$) lactation (Table 3).

COQ9 expression in the endometrium and preimplantation embryo

Transcripts for *COQ9* in the endometrium were detected at day 0, 3, 5, and 7 of the estrous cycle but there were no significant changes in *COQ9* expression over this period of the estrous cycle ($P = 0.410$) (Figure 5A). Expression of *COQ9* in the matured oocyte and preimplantation embryo was affected by stage of development ($P < 0.001$). Expression was high in the oocyte and the two-cell embryo and declined thereafter to a nadir at the blastocyst stage (Figure 5B).

Genotype effects on *COQ9* expression in cumulus cells and oocytes

Genotype had no effect ($P = 0.138$) on *COQ9* expression in cumulus cells (Figure 6A) but did affect expression in oocytes ($P < 0.001$) (Figure 6B). Transcript abundance was higher for heterozygotes (AG) compared to either homozygote condition. There was no difference in expression between the two homozygous genotypes. Expression of two-oocyte-associated genes, *BMP15* and *GDF9*, were also measured in oocytes (Figure 7A). Genotype did not affect expression of either *BMP15* ($P = 0.779$) or *GDF9* ($P = 0.685$).

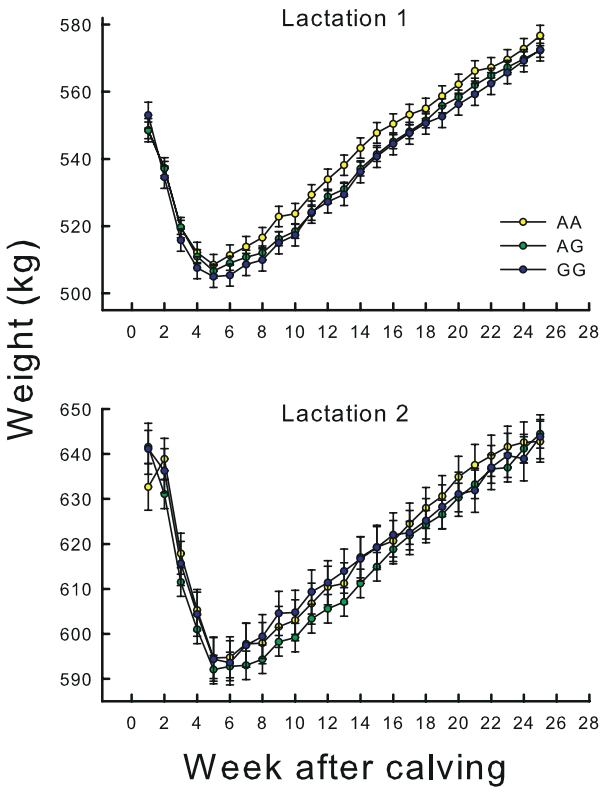

Figure 4. Association of *COQ9* genotype with body weight changes after calving during the first and second lactation of Holstein cows. Values are least-squares means ± SEM of daily body weights averaged for each week. The number of cows for each genotype was 86 AA, 223 AG, and 106 GG. Body weight was affected by the interaction between genotype and week after calving ($P < 0.001$) for both lactations.

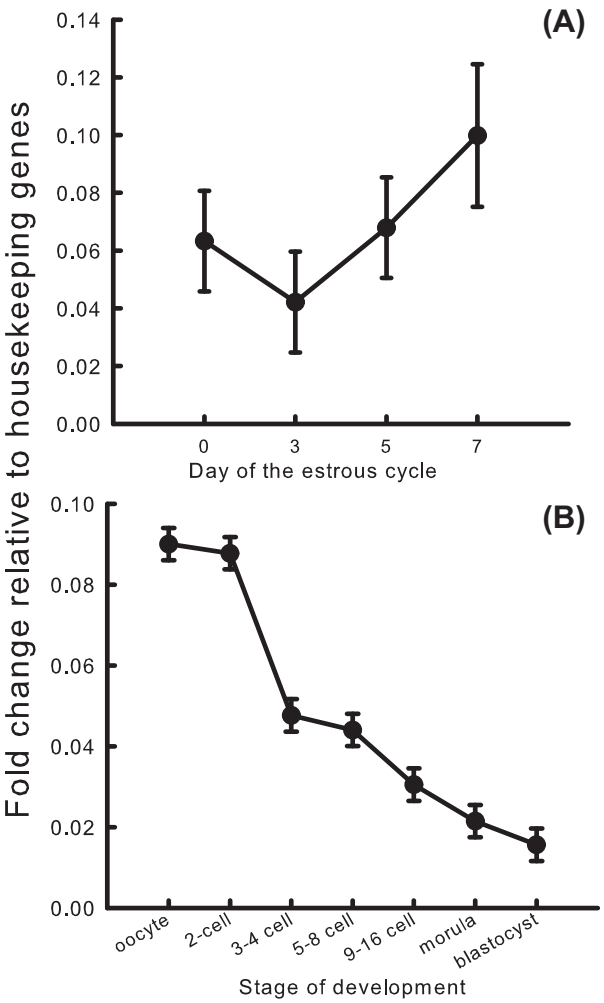

Figure 5. Expression of *COQ9* in the endometrium, oocyte, and preimplantation embryo. All values are least-squares means ± SEM of the fold change of expression relative to the geometric mean of three housekeeping genes. (A) Changes in *COQ9* expression in the endometrium during the first 7 days of the estrous cycle. Expression of *COQ9* was measured for samples from 3 to 4 cows per day. There was no significant effect of day of the estrous cycle ($P = 0.412$). (B) *COQ9* expression in the matured oocyte and early embryo. A total of four pools of 30 oocytes or embryos per pool were assessed for each stage. *COQ9* expression was higher for oocytes and two-cell embryos than for embryos at other stages ($P < 0.001$).

Genotype effects on oocyte recovery, antimullerian hormone concentration in plasma, and mitochondrial DNA copy number per oocyte

The number of COC retrieved per ovary was affected by genotype (Table 4), with heterozygotes having more COC than either homozygous genotype (dominance effect; $P < 0.001$), and the GG homozygous having more COC per ovary than the AA genotype (additive effect $P = 0.041$). Similarly, cows with the AG genotype had signif-

Table 3. Association of *COQ9* genotype with maximum body weight loss and days to the maximum (max.) body weight loss for first and second lactation.[1]

	Genotype[2]			
	GG	AG	AA	*P*-value[3]
Lactation 1				
n	106	223	86	
Max. body weight loss (kg)	62.74 (3.09)	59.73 (1.99)	55.65 (3.03)	0.103
Days to max. body weight loss	21.10 (0.67)	21.61(0.43)	21.58 (0.66)	0.606
Lactation 2				
n	69	161	78	
Max. body weight loss (kg)	69.26 (4.43)	74.35 (3.02)	71.76 (4.23)	0.684
Days to max. body weight loss	28.97 (1.49)	31.05 (1.01)	28.65 (1.36)	0.872

[1]Abbreviation: max., maximum.
[2]Values are least-squares means (SEM).
[3]*P*-values are for additive effects. Dominance effects were not significant.

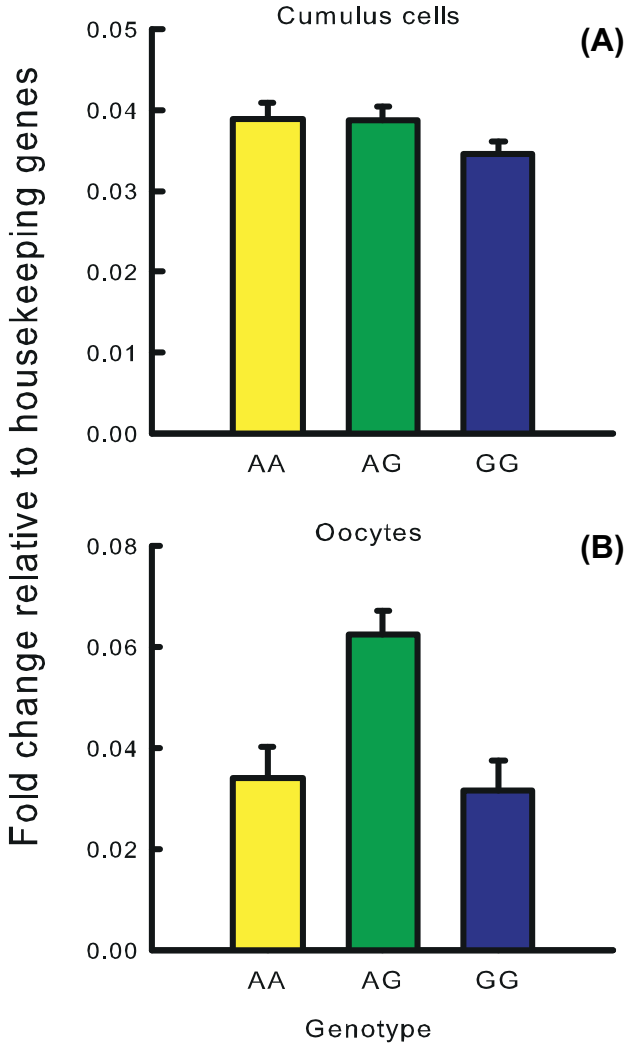

Figure 6. Expression of *COQ9* in genotype-identified cumulus cells (A) and oocytes (B). Values are least-squares means ± SEM. The number of pools of oocytes or cumulus cells evaluated was 11 for AA, 20 for AG, and 12 for GG. Expression in cumulus cells was not affected by genotype ($P = 0.1370$), but expression in oocytes was affected by genotype ($P < 0.001$), with oocytes of the AG genotype having greater expression than either homozygote.

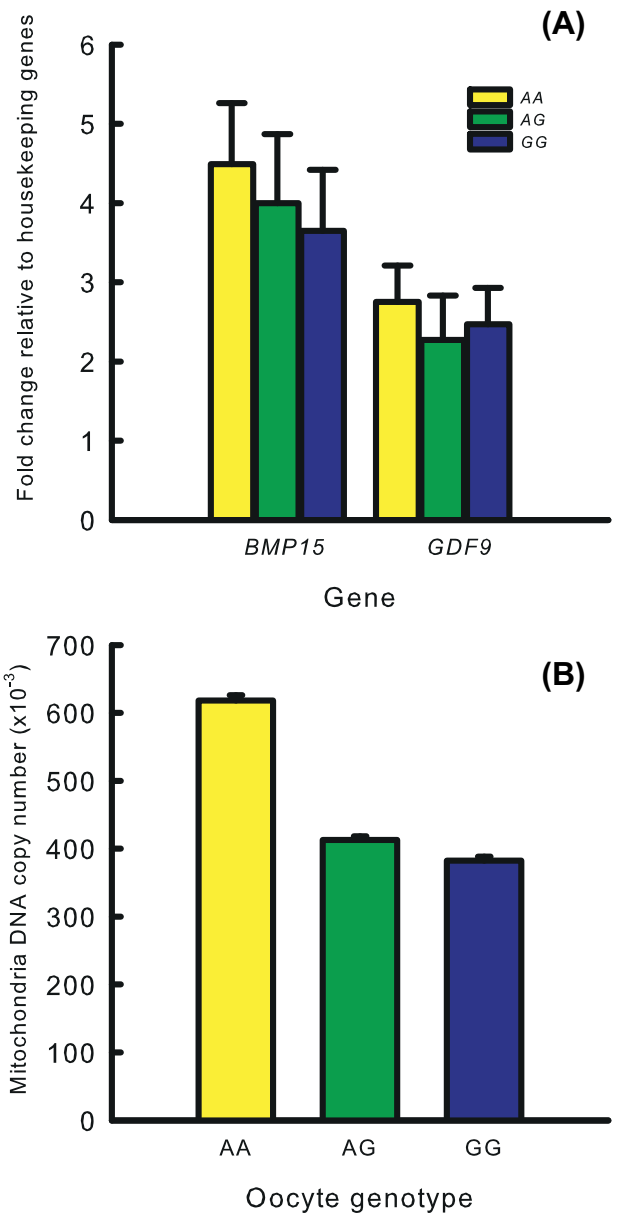

Figure 7. Association of *COQ9* genotype with markers of oocyte quality. (A) Expression of *BMP15* and *GDF9* in oocytes of the three genotypes for *COQ9*. Values are least-squares means ± SEM. There were 11 pools of oocytes for AA, 20 for AG, and 12 for GG. There was no effect of genotype on expression of *BMP15* ($P = 0.779$) or *GDF9* ($P = 0.685$). (B) Mitochondrial copy number in oocytes of the three genotypes for *COQ9*. Values are least-squares means ± SEM. There were 11 pools of oocytes for 11 AA, 20 for AG, and 12 for GG. There was an additive effect of genotype on mitochondrial copy number in favor of the A allele ($P < 0.0001$) and a dominance effect ($P < 0.0001$).

icantly higher AMH concentrations than cows of the AA and GG genotype (dominance effect; $P = 0.038$).

As shown in Figure 7B, total mitochondrial content in matured oocytes, indicated by mitochondrial DNA copy number, was affected by *COQ9* genotype. Numbers were greatest in AA, intermediate in AG, and least in GG (additive effect; $P < 0.0001$; dominance effect, $P < 0.0001$).

Discussion

Here we show an example of a missense mutation that is associated with reproductive function in dairy cattle. The mutation, a nucleotide change from G→A, generates an amino acid change from aspartic acid to asparagine at position 53 of COQ9, modifies the predicted protein structure, and results in a change in oxidative phosphorylation as reflected in changes in mitochondrial respiratory function. Furthermore, the A allele, which is the ancestral allele in ruminants, is associated with improved fertility. Body weight changes do not appear to be important for the change in reproductive function. Rather, the A allele is associated with a higher oocyte mitochondrial content, which could conceivably improve oocyte competence for supporting subsequent embryonic development. An unexpected finding was an overdominance effect on follicular growth, with heterozygotes having increased follicular numbers compared to either homozygote. Overall, results indicate that a gene involved in mitochondrial function is associated with fertility.

Table 4. Association of *COQ9* genotype with number of COC per ovary and circulating AMH concentration.

	Genotype[1]				*P*-value[2]	
	GG	AG	AA	Effect	A	D
Ovaries						
n	12	20	11			
COC per ovary (n)[3]	16.6 (0.69)	19.0 (0.35)	14.4 (0.84)	2.29	0.041	<0.001
Cows						
n	13	9	12			
AMH (pg/ml)	274.1 (50.7)	426.7 (60.9)	287.4 (52.8)	–0.047	0.857	0.038

[1]Values are least-squares means (SEM). Cows used for COC numbers were different than those used for AMH determination.
[2]A: additive, D: dominance.
[3]COC, cumulus-oocyte complexes.

The effect of the SNP in *COQ9* (rs109301586) on mitochondrial function resulted in cells with the AA genotype requiring less substrate to maintain basal cellular function, and displaying a reduced leak respiration, i.e., respiration that is not associated with energy production. These alterations in the AA genotype could affect cellular function in many reproductive tissues by improving the efficiency energy substrate utilization and, because of the reduced proton leak, could reduce production of reactive oxygen species [53, 54]. *COQ9* was found to be expressed in the endometrium, cumulus cell, oocyte, and preimplantation embryo. Indeed, data compiled for human tissues at ProteomicDB (https://www.proteomicsdb.org/), indicate, as expected, near ubiquitous presence of the protein.

The oocyte was one cell type in which the SNP in *COQ9* was associated with change in function. Mitochondrial DNA content was highest in the matured oocytes of the AA genotype. Mitochondrial DNA copy number is associated with oocyte ATP production and successful oocyte maturation and fertilization [55–57]. Thus, one reason for differences in fertility among *COQ9* genotypes could reside in competence of the oocyte due to higher mitochondrial content. Another example of the SNP in *COQ9* being associated with ovarian function was for numbers of antral follicles. Such an effect was observed for the number of COC recovered from ovaries at slaughter and, in a separate population, the concentrations of AMH in blood. Antimullerian hormone is produced by granulosa cells [58] and is a marker of the number of growing follicles in several species including cattle [59, 60]. Concentrations of AMH in blood of cattle have been negatively associated with pregnancy rate to timed artificial insemination but positively associated with pregnancy rate in cows inseminated at detected estrus and with reduced pregnancy loss [61].

In the case of follicular growth, the *COQ9* SNP exhibited overdominance because heterozygotes were superior to either homozygote. The explanation for the heterozygote advantage is not clear. However, follicular growth is a complex process involving, among other events, maintenance of the primordial follicle pool, initiation and maintenance of follicular growth, and regulation of apoptosis. Perhaps, the allele of *COQ9* favorable for one phase of follicular growth is different than the allele favorable for another phase so that overall follicular growth is maximized when both alleles are present. The fact that fertility of the AG genotype was intermediate between AA and GG genotypes may reflect differential consequences of size of the follicular pool for establishment and maintenance of pregnancy [61]. That transcript abundance for *COQ9* was also greater for AG than other genotypes, despite this genotype being intermediate in fertility, is evidence that fertility is not determined to a large extent by amount of mRNA for *COQ9* in the oocyte.

There are only a limited number of examples of overdominance affecting reproductive function. One is for the class II major histocompatibility complex in male rhesus monkeys [62]. Another example is for effects of a mutation in the X-linked gene *BMP15* on ovulation rate in sheep. Heterozygotes for the Inverdale mutation have increased ovulation rate as compared to wild-type animals, whereas those carrying two copies of the mutation do not develop functional ovaries [63].

It was hypothesized that actions of the SNP on mitochondrial function would result in cows of the AA genotype to experience less severe negative energy balance during early lactation. The degree of negative energy balance in lactating cows is related to resumption of estrous cycles postpartum and resultant fertility [64, 65]. However, differences in reproductive function between *COQ9* genotypes appear to be not related to whole body energetics. The *COQ9* mutation did not have a large effect on whole animal energetic efficiency because of the lack of association of the *COQ9* mutation with maximum body weight loss during early lactation. Additionally, there was no association of the SNP in *COQ9* with milk yield.

Analysis of the phylogeny of the mutation in *COQ9* indicates that the A allele is ancestral in ruminant species, and the G allele, which is associated with lower fertility, has been selected for in cattle. The G allele was found to be present in *B. indicus*, which indicates that the mutation predates divergence of taurine and indicine species more than 200,000 years ago [66]. Further research is needed to determine whether frequency of the G allele increased in accompaniment with artificial selection in one or more cattle breeds.

One caveat with results of the current study is that they are based on association of the mutation in *COQ9* with traits related to reproduction. It is possible that the SNP in *COQ9* is in linkage disequilibrium with a causative mutation located elsewhere in *COQ9* or in other nearby genes. Further research is required to verify that the SNP in *COQ9* described here is the causal variant.

In conclusion, the SNP in *COQ9* is an example of a mutation that changes predicted protein structure, is associated with altered mitochondrial function, and that modulates reproductive function in dairy cattle, possibly by regulating oocyte quality. The observation that the SNP was not associated with changes in milk production indicates its potential use to select for fertility without negatively impact productive efficiency.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental File S1. Materials and Methods; Additional Details

Supplemental Table S1. Composition of media composition used for in vitro production of embryos. [a]Abbreviations are as follows: OCM, oocyte collection medium; OMM, oocyte maturation medium; HEPES-SOF, HEPES-buffered synthetic oviduct fluid; SOF-FERT, synthetic oviduct fluid for fertilization; PHE, penicillamine-hypotaurine-epinephrine solution; SOF-BE2, synthetic oviduct fluid-bovine embryo 2. [b]All other ingredients were added to 1 L of tissue culture medium 199. The final concentrations listed in the table account for the dilution resulting from addition of the ingredients. [c]Prepare as fresh solutions primary stocks of 1 mM hypotaurine, 2 mM penicillamine, and 250 μM epinephrine (prepared by dissolving in a lactate-metabisulfite solution). The PHE is prepared by combining 10 ml 1 mM hypotaurine, 10 ml 2 mM penicillamine, 4 ml of 250 μM epinephrine, and 16 ml of 0.9% (W/v) NaCl. Note that the lactate-metabisulfite solution is prepared by adding 77 μl of 98% (w/w) sodium lactate syrup and 50 mg sodium metabisulfite to 50 ml water.

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