ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

doi:10.1111/j.1365-2052.2010.02148.x

Exploration of relationships between production and fertility traits in dairy cattle via association studies of SNPs within candidate genes derived by expression profiling

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Summary

The objective of this work was to integrate findings from functional genomics studies with genome-wide association studies for fertility and production traits in dairy cattle. Association analyses of production and fertility traits with SNPs located within or close to 170 candidate genes derived from two gene expression studies and from the literature were performed. Data from 2294 Holstein bulls genotyped for 39557 SNPs were used. A total of 111 SNPs were located on chromosomal segments covered by a candidate gene. Allele substitution effects for each SNP were estimated using a mixed model with a fixed effect of marker and a random polygenic effect. Assumed covariance was derived either from marker or from pedigree information. Results from the analysis with the kinship matrix built from marker genotypes were more conservative than from the analysis with the pedigree-derived relationship matrix. From sixteen SNPs with significant effects on both classes of traits, ten provided evidence of an antagonistic relationship between productivity and fertility. However, we found four SNPs with favourable effects on fertility and on yield traits, one SNP with favourable effects on fertility and percentage traits, and one SNP with antagonistic effects on two fertility traits. While most quantitative genetic studies have proven genetic antagonisms between yield and functional traits, improvements in both production and functionality may be possible when focusing on a few relevant SNPs. Investigations combining input from quantitative genetics and functional genomics with association analysis may be applied for the identification of such SNPs.

Keywords association analysis, genetic marker, reproduction.

Introduction

Selection strategies focusing on milk yield in dairy cattle have generally led to a decline in fertility (Pryce *et al.* 2004). This fact is due to an antagonistic relationship between production and reproduction, which has been proven via the estimation of genetic correlations in quantitative genetic studies using 305-day lactation milk yield (e.g. Holtsmark *et al.* 2008), or using test day observations (e.g. König *et al.* 2009). There is also evidence from selection experiments that increasing genetic merit for production traits is associated with a decline in fertility on the phenotypic scale (Pryce & Veerkamp 2001). Fertility traits used in quantitative genetic analyses to infer relationships between production and reproduction have usually been non-return rates, days open or the interval from calving to first service. However, the biological background of fertility is based on much more complexity, as pointed out by Darwash *et al.*

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Accepted for publication 17 August 2010

(1997). These authors defined fertility of a dairy cow as 'the ability of the animal to conceive and maintain pregnancy if served at the appropriate time in relation to the ovulation'. Hence, successful pregnancy on a biological scale includes oestrous detection, ovulation, appropriate patterns of ovarian cyclicity and the prevention of embryo or foetal loss (Royal *et al.* 2000).

To gain a deeper insight into fertility mechanisms and for a detailed explanation of relationships between fertility and production, modern geneticists focus on two main approaches. The first approach uses 'new' traits such as progesterone profiles to estimate genetic parameters for the interval to commencement of luteal activity post-partum, for the length of the first luteal phase post-partum or for the occurrence of persistent corpus luteum type I (Royal *et al.* 2002). In Royal *et al.* (2002), genetic correlations between the predicted 56-d milk yield and endocrine parameters were high and significant, thus providing a clearer evidence of antagonistic genetic relationships compared to studies using 'traditional' fertility traits.

The clear negative genetic association between production and reproduction at the gene level may be explained by pleiotropic gene effects, linkage, or further complex physiological associations (Veerkamp *et al.* 2003). Hence, the second approach to clarify interaction between fertility and production is based on molecular genetic markers. DNA markers such as microsatellites, RFLPs or SNPs have been used in several studies for the successful identification of quantitative trait loci (QTL; e.g. Ashwell *et al.* 2004). The practical application of identified QTL for animal breeding is limited, because associations between markers and QTL might only exist in some families and may also erode over time (Dekkers 2004). At present, SNP markers are more relevant for genome-wide association studies (Meuwissen *et al.* 2001) rather than for QTL detection. On the other hand, the candidate gene approach uses physiological findings to identify variations in genes that are associated with the phenotype of interest. 'Omics' technologies facilitate a deeper insight into the molecular pathways involved in key steps of reproduction (Hiendleder *et al.* 2005). Additional knowledge of genes and their products will make a substantial contribution to understanding and eventually improving fertility in dairy cattle (e.g. Dawson 2006).

The objective of this work was to integrate findings from two previous gene expression studies (Bauersachs *et al.* 2005; Mitko *et al.* 2008) with genome-wide association studies for fertility and production traits in dairy cattle. This study can thus make an additional contribution to the evidence for antagonistic relationships between production and reproduction, as found in most quantitative genetic studies, based on a novel concept. Detection of beneficial SNP genotypes for both production and reproduction within candidate genes would have the combined effect of improving production without decreasing fertility of animals, as outlined by Moe *et al.* (2009) for breeding objectives in swine.

Materials and methods

Candidate genes

A set of 151 genes that were found to be upregulated in the bovine endometrium during the luteal phase compared to the ovulatory phase (Bauersachs *et al.* 2005; Mitko *et al.* 2008) were used as candidate genes. Fifty-two genes were derived from the first study and 135 genes from the second. Of these genes, 36 were found in both studies. In the first study, a combination of subtracted cDNA libraries and cDNA arrays was used to identify differentially expressed genes between the ovulatory and the luteal phase. Endometrial tissue samples were collected at oestrus (day 0) and at dioestrus (day 12) from three animals per group. Tissue samples were taken from seven locations, three from each horn and one from the corpus. All samples were analysed by hybridization to cDNA arrays produced from cDNA clones of two cDNA libraries that resulted from subtraction of cDNA samples from oestrus vs. dioestrus (for details see Bauersachs *et al.* 2005). In the second study (Mitko *et al.* 2008), endometrial tissue samples were collected from four time points, at days 0, 3.5, 12 and 18 of the oestrous cycle. Samples from four heifers per time point were hybridized to a custom cDNA array (Bauersachs *et al.* 2007) to identify genes differentially expressed during the oestrous cycle. The upregulation during the luteal phase suggests a potential role of these genes for the proper development of the early embryo and for successful pregnancy establishment and maintenance, because the steroid hormone progesterone (P4) plays a key role in these reproductive events in the endometrium (Clemente *et al.* 2009; reviewed in Bauersachs *et al.* 2008). In addition, 19 genes were included that have proposed conserved roles in pregnancy in different mammalian species.

Positions of markers from the dense panel of SNPs were checked to identify the ones that were within or close to the physical location of candidate genes.

Samples

Genotypic, phenotypic and pedigree information was collected from a set of 2294 Holstein-Friesian bulls born between 1981 and 2003. The bulls were sons of 362 bull sires and 1858 bull dams. Number of offspring per bull sire ranged from 1 to 80, with an average of 6.37, and number of offspring per bull dam ranged from 1 to 8, with an average of 1.24. Average numerator relationship among the bulls was 0.09, and almost all of them were inbred. Average inbreeding coefficient was 0.04.

Genomic DNA was purified from either full blood or semen samples, applying a modified protocol according to Miller *et al.* (1988). Leucocyte pellets were prepared from the full blood samples and subjected to cell lysis in a solution containing SDS and proteinase K. Protein precipitation was

performed with ammonium acetate, and genomic DNA was precipitated with isopropanol and ethanol. The semen samples were initially treated with a lysis solution containing proteinase K and dithiothreitol; proteins were salted out using sodium chloride; and DNA was precipitated with ethanol. Subsequently, the DNA was resolved in 100 μl Tris–EDTA buffer, and the concentration was measured with a NanoDrop1000 UV-spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). The DNA was adjusted to a concentration of 70 ng/μl, and quality control was performed by visual inspection of the genomic DNA solution and a duplexed test PCR run on a 0.8% agarose gel.

Phenotypic data

A total of twelve traits related to fertility and production and typically included in national dairy cattle genetic evaluations were considered in this study. From the six fertility traits, two were heifer traits: non-return rate to 56 days (NRh) and interval from first to successful insemination (FLh); and four were cow traits: interval from calving to first insemination (CFc), non-return rate to 56 days (NRc), interval from first to successful insemination (FLc) and days open (DOc). Production traits were milk yield (Mkg), fat yield (Fkg), protein yield (Pkg), fat percentage (Fpr) and protein percentage (Ppr). Another functional trait considered here was somatic cell score (SCS). Estimated breeding values (EBV) were computed for production traits using a random regression test day model and for fertility traits using a multiple-trait animal model. EBVs for SCS and the fertility traits were standardized to a mean of 100 and a standard deviation of 12 points, with higher EBVs being in the desired direction of selection. Detailed information about the traits and the breeding value estimation can be found in Liu *et al.* (2001, 2008). Basic descriptive statistics for EBVs of the traits and corresponding accuracies are presented in Tables 1 and 2, respectively. Accuracy statistics of EBVs for Fkg, Pkg, Fpr and Ppr are not shown in Table 2, as they were the same as the ones of EBVs for Mkg. Pairwise correlations among EBVs for all production and fertility traits are presented in Table 3.

Genotypic data

The genotyping was carried out at the German Helmholtz Research Center for Environmental Health in Munich. The 2294 samples were successfully (at most 3% of missing calls) genotyped for the Illumina BovineSNP50 BeadChip. The genotyping was performed according to the manufacturer's instructions using the ILLUMINA BEADSTUDIO® software to create genotypes from the raw data, applying the settings recommended by Illumina. The Illumina BovineSNP50 BeadChip assay contains 54001 SNP markers (Matukumalli *et al.* 2009), from which 52255 were mapped to the BTAU4.0 assembly (ftp://ftp.hgsc.bcm.tmc.edu/pub/data/Btaurus/). Average space between mapped markers in the chip was 51.5 kb, and median spacing was 37.32 kb. Besides the 1746 SNPs located in unassigned contigs (unknown chromosome and/or position in the BTAU4.0 assembly), some of the markers were also excluded from the data set due to more than 5% of calls being missing or due to minor marker allele frequency being lower than 0.05. Criteria for filtering samples and markers from the data set were based on results from the study of The Welcome Trust Case Control Consortium (2007). The final number of markers kept in the data set after quality control was 39557. Given the density of the panel and the low rate of missing calls after the filtering process, imputation of missing genotypes using linkage disequilibrium (LD) information is expected to be very accurate. Imputation was performed using the software FASTPHASE 1.3.0c (Scheet & Stephens 2006).

Statistical analyses

For every trait, association analyses via regression on individual SNP genotypes were performed using the following mixed linear model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zu} + \mathbf{e}$$

Table 1 Descriptive statistics of estimated breeding values for traits used in the association analyses.

Trait	Abbreviation	Mean	SD	Min	Max
Non-return rate to 56 days (heifer)	NRh	99.53	8.96	69.0	129.0
Interval from first to successful insemination (heifer)	FLh	99.86	8.38	72.0	125.0
Interval from calving to first insemination	CFc	98.73	8.57	67.0	129.0
Non-return rate to 56 days (cow)	NRc	99.94	9.72	67.0	134.0
Interval from first to successful insemination (cow)	FLc	99.52	8.82	69.0	128.0
Days open	DOc	99.04	8.94	68.0	132.0
Milk yield (kg)	Mkg	731.04	611.32	−1362.0	2892.0
Fat yield (kg)	Fkg	20.43	23.64	−59.0	115.0
Protein yield (kg)	Pkg	22.13	18.33	−45.0	79.0
Fat percentage	Fpr	−0.097	0.29	−1.01	1.05
Protein percentage	Ppr	−0.028	0.12	−0.47	0.50
Somatic cell score	SCS	100.37	12.08	58.0	139.0

Table 2 Descriptive statistics of accuracies (in %) of the estimated breeding values used in the association analyses.

Trait	Mean	SD	Min	Max
Non-return rate to 56 days (heifer)	49.63	12.07	26.0	99.0
Interval from first to successful insemination (heifer)	48.43	11.49	26.0	99.0
Interval from calving to first insemination	66.75	9.64	44.0	99.0
Non-return rate to 56 days (cow)	56.29	10.60	33.0	99.0
Interval from first to successful insemination (cow)	49.48	10.12	26.0	99.0
Days open	68.20	9.41	45.0	99.0
Milk yield (kg)	94.24	2.12	88.0	99.0
Somatic cell score	87.98	4.32	76.0	99.0

where **y** is the vector of observations (the EBV for the corresponding trait); **b** is the vector of fixed effects, including an overall mean and the marker genotype; **u** is the vector of random polygenic effects and **e** is a random residual term. Matrices **X** and **Z** relate observations to fixed and random effects, respectively. The covariate defining marker effects was set up as the number of copies of one of the alleles observed at the given marker locus. Assumptions regarding the distributions of random polygenic effects and error term were: $u \sim N(0, A\sigma_a^2)$ and $e \sim N(0, R\sigma_e^2)$, where **A** is a symmetric relationship matrix which accounts for population structure via relatedness among individuals, **R** is a diagonal matrix of residual variances which were assumed to be inversely proportional to the accuracies of the corresponding EBV, σ_a^2 is the additive genetic variance, and σ_e^2 is the residual variance. Estimated heritabilities from Liu *et al.* (2001, 2008) were assumed to compute the ratio of variances used in the mixed model equations. Matrix **A** was derived in two ways: (i) using pedigree information to calculate numerator relationship coefficients and (ii) using genotype information for the full set of 39557 markers to compute kinship coefficients, following the similarity index calculation described in Eding & Meuwissen (2001) and applied by Hayes & Goddard (2008). In the former case, the inverse of **A** was computed using the software CFC v1.0 (Sargolzaei *et al.* 2005), including all relevant animals in the pedigree (21 646 animals), tracing back to 1906.

For every analysis of association between one of the markers and a given trait, the null hypothesis of a null marker effect was tested with a Student *t*-test, contrasting a full model including the marker effect with a reduced model with just the polygenic term. The problem of testing multiple hypotheses was addressed by deriving a significance threshold considering the false discovery rate concept (FDR, Benjamini & Hochberg 1995), to achieve a compromise between control of type I error and the need for increased power. The maximum expected proportion of false discoveries was set to 0.05, for which critical values were calculated using the software QVALUE v1.0 (Storey *et al.* 2004). The significance threshold for each trait was a genome-wise 5% FDR threshold, i.e., it was computed considering a set of *P*-values resulting from a whole-genome scan using all 39 557 markers (results from the whole-genome scan are not shown).

Results

From the total number of markers that passed the filtering process, 73 were located within and 38 were located in the vicinity of (5 kb upstream and downstream of the genes) chromosomal segments that were covered by genes in the list of candidates.

Kinship coefficients estimated from marker data were plotted against the relationship coefficients estimated from pedigree data (Fig. 1). As in the study by Hayes & Goddard (2008), marker- and pedigree-derived relationship coefficients were highly associated (correlation of 0.78). The two ways of computing the matrix **A** provided two different sets

Table 3 Pearson correlation coefficients between estimated breeding values for all production and fertility traits*.

	FLh	CFc	NRc	FLc	DOc	Mkg	Fkg	Pkg	Fpr	Ppr	SCS
NRh	0.63	0.06	0.73	0.48	0.29	−0.22	−0.20	−0.26	0.06	−0.02	0.03
FLh		0.40	0.55	0.75	0.63	−0.35	−0.31	−0.37	0.12	0.07	0.06
CFc			−0.06	0.65	0.91	−0.53	−0.43	−0.50	0.20	0.19	0.17
NRc				0.58	0.30	−0.27	−0.24	−0.31	0.09	NS	0.04
FLc					0.90	−0.51	−0.41	−0.50	0.19	0.15	0.13
DOc						−0.58	−0.47	−0.56	0.22	0.19	0.16
Mkg							0.63	0.91	−0.58	−0.45	−0.08
Fkg								0.76	0.23	0.11	−0.05
Pkg									−0.35	−0.06	−0.11
Fpr										0.70	0.05
Ppr											−0.04

*Mkg, milk yield; Fkg, fat yield; Pkg, protein yield; Fpr, fat percentage; Ppr, protein percentage; SCS, somatic cell score; NRh, non-return rate to 56 days (heifer); FLh, interval from first to successful insemination (heifer); CFc, interval from calving to first insemination; NRc, non-return rate to 56 days (cow); FLc, interval from first to successful insemination (cow); DOc, days open; NS, not significant.

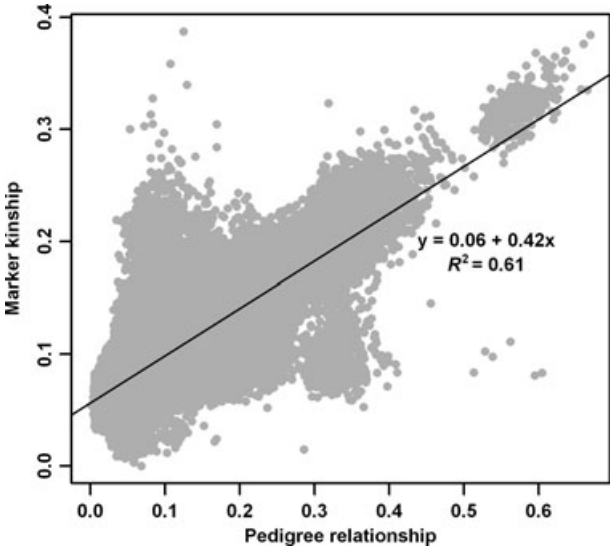

Figure 1 Kinship coefficients estimated from marker data against relationship coefficients estimated from pedigree data.

of results, with one of the sets almost contained in the other. Almost all of the SNP effect estimates that were significant in the analysis with the kinship matrix built from markers were also significant in the analysis with the relationship matrix built from pedigree data. Decision about which results should be taken as the final ones and considered for discussion was made by putting more emphasis on the minimization of Type I error, i.e., picking the results from the seemingly more stringent method. Furthermore, as pointed out by Schork (2001), elements of the relationship matrix built from pedigree contain the expected genome sharing for two individuals, whereas the marker-based estimates of whole-genome allele sharing are calculated by summing up allele sharing estimates at many loci in the genome. Hence, the marker approach accommodates variation in kinship among similarly related animals and thus more adequately characterizes the genome sharing of two animals than could be achieved through the use of expectations about such sharing. Therefore, in the following section, only the results from the analysis performed with the kinship matrix built from marker information (more conservative) are presented.

From the 111 SNPs located within or close to candidate genes, 16 SNPs were found to be significantly associated (at a genome-wise 5% FDR) with at least one of the fertility plus one of the production traits. In addition, 15 SNPs were significantly associated with Mkg, Fpr and Ppr but to none of the fertility traits. The interpretation of the control of a FDR at the level of 0.05 is that 5% of the significant tests are expected to be false positives (Storey *et al.* 2004). A summary of these SNPs with the corresponding genes, alleles and minor allele frequencies is presented in Table 4. As all the SNPs considered here had an adenine as one of the alleles, this base was taken as the reference allele for all loci. Therefore, coefficients in **X** for marker effects were defined as the count of A alleles at the given locus (i.e., 2 for genotype AA, 1 for AG and 0 for GG). Estimated allele substitution effects (substitution of a guanine or a cytosine by an adenine) and corresponding standard errors of these sets of 15 and 16 SNPs are presented in Tables 5 and 6 respectively. The only exception with respect to the type of base was marker ss105262426 (close to *CNOT1*), which had a cytosine as the reference and a guanine as the other allele.

From the sixteen SNPs with significant effects on both classes of traits, ten provided evidence of an antagonistic relationship between production and fertility. However, we found four SNPs with favourable effects on fertility and on yield traits (milk, fat and protein), one SNP with favourable effects on fertility and percentage traits and one SNP with antagonistic effects on two fertility traits.

Discussion

Association between production traits

The antagonistic relationship between yield traits (Mkg, Pkg, and Fkg) and percentage traits (Ppr and Fpr) in a quantitative genetic approach is depicted via correlations among EBVs (Table 3). The correlation between Mkg and Fpr was −0.58, and −0.45 between Mkg and Ppr. However, correlations between breeding values are not identical to genetic correlations unless accuracies of estimated breeding values are close to one. Therefore, results should only be interpreted as general trends, keeping in mind that correlations between breeding values are always an underestimation of genetic correlations (Calo *et al.* 1973).

Investigations into relationships between yield traits and percentage traits were beyond the initial scope of the study, but SNPs within or close to some of the chosen candidate genes for fertility showed significant (mostly antagonistic) allele substitution effects for yield and percentage traits (Table 5). The only SNP with significant effects for yield and percentage on the same direction was the one close to *MTMR3*. The SNPs showing clearest antagonistic effects between Mkg and Fpr were located within the *GABARAPL1* gene (allele substitution effect of −93.19 for Mkg and +0.035 for Fpr), and within the *MGP* gene (allele substitution effect of +52.05 for Mkg, and −0.063 for Fpr). Some of those estimated allele substitution effects account for more than 10% of the standard deviation for the respective trait. *GABARAPL1* (gamma-aminobutyric acid receptor-associated protein-like 1) is a known protein-coding gene, the position of which is conserved in human, dog, mouse, rat and cattle. GABARAP (GABA-receptor-associated protein) belongs to a large family of proteins that mediate intracellular membrane trafficking and/or fusion. Kolbehdari *et al.* (2008) reported a significant effect of a SNP linked to *GABARAPL1* on dairy strength in Canadian Holstein

Table 4 SNPs with reported significant effects, and their corresponding genes.

SNP	Chromosome	Position (bp)	Alleles	MAF*	Gene symbol	Gene start (bp)	Gene end (bp)
ss86335092	1	97100732	(A)/C	0.335	*TNFSF10*	97102014	97117887
rs43293161	2	35323344	(A)/G	0.267	*IFIH1*	35282354	35337702
ss117963620	2	101655571	A/(G)	0.440	*IDH1*	101647000	101668021
rs41584659	2	108823151	A/(G)	0.122	*IGFBP2*	108810787	108839273
rs43315150	2	111158169	(A)/G	0.288	*CYP27A1*	111142261	111184549
rs29027468	3	55442801	A/(G)	0.053	*HFM1*	55417764	55526104
rs42737927	3	120598358	A/(G)	0.388	*UGT1A6*	120598637	120780533
rs41573170	4	69056690	(A)/G	0.250	*SCRN1*	69060818	69123400
rs41631261	4	80892532	A/(C)	0.439	*MRPL32*	80894195	80899293
rs29013402	4	107155361	(A)/G	0.252	*PARP12*	107155611	107200983
rs41591907	5	91479479	(A)/G	0.074	*BCAT1*	91481325	91599017
rs41256890	5	101979581	(A)/G	0.441	*MGP*	101976185	101979729
ss117963826	5	107086995	(A)/G	0.396	*GABARAPL1*	107086033	107090484
rs43709090	5	119734178	(A)/G	0.094	*ACO2*	119723315	119772376
ss86324560	6	75063475	(A)/G	0.291	*IGFBP7*	75049708	75130502
ss86297639	8	47895369	(A)/G	0.308	*APBA1*	47897901	47932556
ss86297966	10	21266129	(A)/G	0.174	*IRF9*	21266332	21270932
ss86324438	13	67597675	A/(G)	0.229	*TGM2*	67596832	67631318
ss86337595	17	60557021	A/(G)	0.427	*KSR2*	60551030	60745671
rs29022475	17	64563142	(A)/C	0.274	*OAS1*	64548726	64582484
ss86301005	17	72467388	(A)/G	0.398	*MTMR3*	72469842	72601406
ss86313004	18	26293754	(A)/G	0.397	*NDRG4*	26283377	26296569
ss105262426	18	26352342	(C)/G	0.276	*CNOT1*	26302881	26350050
ss86333273	19	53072293	A/(G)	0.172	*BAIAP2*	53066543	53130128
rs41934711	20	11110602	(A)/C	0.154	*CCNB1*	11106664	11116387
rs41641704	21	35969288	(A)/G	0.240	*LGMN*	35951338	35965956
ss86317929	21	59390649	(A)/G	0.090	*SERPINA14*	59392348	59400967
ss86297596	23	16318674	A/(G)	0.156	*CCND3*	16321582	16328397
ss86287169	24	34159594	(A)/G	0.093	*NPC1*	34152320	34199392
ss86327124	26	11355933	(A)/G	0.187	*LIPA*	11348494	11390918
ss117974311	28	16827333	(A)/G	0.454	*ARID5B*	16818385	17005336

*MAF is the sample frequency of the minor allele, indicated within parentheses.

cattle. In the study by Mitko *et al.* (2008), it was found to be upregulated in bovine endometrium during the luteal phase.

Allele substitution effects (Tables 5 and 6) for production traits were lower compared to substitution effects found in studies focusing directly on candidate genes for production traits, especially for *DGAT1* variants (Grisart *et al.* 2002; Thaller *et al.* 2003). Antagonistic genetic relationships between allele substitution effects on Mkg and Fpr, and on Mkg and Ppr, were also found in these previous studies. Thaller *et al.* (2003) also reported such antagonism via negative correlations between Mkg and content traits when comparing correlations of daughter yield deviation residuals.

For the sake of a combined breeding goal for production in dairy cattle on the national as well as on the international scale, which includes milk yield as well as percentage traits (e.g. König *et al.* 2007), the practical application of detected genes or markers having antagonistic effects is limited. The existence of nearly intermediate allele frequencies and no tendency towards fixation for SNP alleles (as one example: see allele A located within the *GABARAPL1* gene, Table 4) or for the gene variant itself (e.g. Thaller *et al.* 2003) within a pure dairy cattle population like German Holsteins support this latter statement. Thaller *et al.* (2003) concluded that benefits for breeding programs are expected if special markets demand more of a single component of milk. Demands formulated by the World Health Organization related to unhealthy nutrition (World Health Organization 2006) encourage a differentiation of breeding goals and the execution of genetic studies focusing on further milk components (e.g. Soyeurt *et al.* 2008). Faster progress for future breeding goals based on relatively new phenotypes is possible by using information obtained in molecular genetic studies, i.e., the gene itself or a genetic marker being in close association with the functional mutation.

Significant allele substitution effects for SCS are also summarized in Table 5, despite the fact that SCS belongs to the group of functional traits. However, EBVs for SCS and

Table 5 Estimated effects (standard errors) of SNPs that were significantly associated (at a 5% false discovery rate) with milk yield and fat and protein percentage but with none of the fertility traits.

		Trait*					
Chromosome	Gene symbol	Mkg	Fkg	Pkg	Fpr	Ppr	SCS
2	*CYP27A1*	52.025 ± 9.146	NS	1.101 ± 0.268	−0.020 ± 0.003	−0.007 ± 0.001	0.878 ± 0.251
2	*IDH1*	−37.881 ± 7.664	−0.763 ± 0.280	−1.046 ± 0.225	0.009 ± 0.002	0.002 ± 0.001	NS
3	*UGT1A6*	−18.877 ± 7.776	0.870 ± 0.284	NS	0.019 ± 0.003	0.006 ± 0.001	NS
4	*MRPL32*	21.533 ± 8.376	NS	NS	−0.012 ± 0.003	−0.009 ± 0.001	NS
5	*ACO2*	−38.976 ± 11.708	NS	NS	0.023 ± 0.004	0.008 ± 0.002	NS
5	*BCAT1*	65.064 ± 13.326	NS	1.062 ± 0.390	−0.038 ± 0.004	−0.011 ± 0.002	NS
5	*GABARAPL1*	−93.189 ± 7.865	−0.796 ± 0.288	−1.848 ± 0.231	0.035 ± 0.003	0.015 ± 0.001	NS
5	*MGP*	52.051 ± 7.484	−3.505 ± 0.273	NS	−0.063 ± 0.002	−0.016 ± 0.001	1.241 ± 0.208
13	*TGM2*	−29.947 ± 10.124	NS	−0.712 ± 0.297	0.016 ± 0.003	0.004 ± 0.001	NS
17	*MTMR3*	−18.565 ± 7.617	−1.815 ± 0.278	−1.050 ± 0.223	−0.013 ± 0.002	−0.005 ± 0.001	NS
17	*OAS1*	−51.017 ± 7.985	NS	−1.121 ± 0.234	0.019 ± 0.003	0.007 ± 0.001	NS
21	*LGMN*	−37.049 ± 8.808	NS	NS	0.019 ± 0.003	0.008 ± 0.001	NS
21	*SERPINA14*	30.829 ± 10.672	NS	NS	−0.014 ± 0.003	−0.006 ± 0.001	NS
26	*LIPA*	35.818 ± 8.723	1.046 ± 0.319	NS	−0.007 ± 0.003	−0.010 ± 0.001	NS
28	*ARID5B*	−65.214 ± 7.848	NS	−1.641 ± 0.230	0.023 ± 0.003	0.006 ± 0.001	NS

*Mkg, milk yield; Fkg, fat yield; Pkg, protein yield; Fpr, fat percentage; Ppr, protein percentage; SCS, somatic cell score; NS, not significant.

the remaining production traits are based on the same daughters and obtained from the same milk recording scheme, and they are also estimated by applying a random regression test day model (Liu *et al.* 2001). Only two SNPs were significantly associated with Mkg, Fpr, Ppr and SCS (Table 5). For both SNPs, the relationships between the estimated effects on Mkg and SCS were not antagonistic. The estimated effect of the SNP located within *CYP27A1* was 52.03 for Mkg and 0.88 for SCS. Hence, a positive effect on Mkg (and also on Pkg) was associated with a favourable increase in the relative breeding value for SCS. The higher the EBV for SCS, the lower the expected somatic cell count of the daughters of a bull. The physiological background of *CYP27A1* has been extensively investigated in humans (Prosser *et al.* 2006). These authors found that human *CYP27A1* plays an important role in the biological activation of vitamin D_3 in the liver. Leaflet (2009) found that the inflamed mammary tissue of cows infected with *Streptococcus uberis* had higher concentrations of 1α-hydroxylase than the control group, resulting in increased production of 1,25-dihydroxvitamin D_3. Thus, vitamin D_3 may play an important role in the resolution of mastitis in cattle, which can also explain the relatively high allele substitution effect for the SNP marker located in the physical position of *CYP27A1*, found in the present study. The upregulation in bovine endometrium at dioestrus (Mitko *et al.* 2008) and differential mRNA expression of *CYP27A1* when comparing the endometrium response of cloned vs. fertilized embryos (Bauersachs *et al.* 2009) also suggests a role in the context of reproductive function.

Significant allele substitution effects for Mkg, Fpr and SCS were found for the SNP located within the *MGP* (*matrix gla protein*) gene (Table 5). The same allele is positive for Mkg and SCS, but has an unfavourable effect on Fpr. The MGP protein has been described as an inhibitor of bone and vascular mineralization (Gopalakrishnan *et al.* 2005). Furthermore, a role in endothelial cell function by altering the response to the TGF-beta superfamily growth factors has also been shown (Boström *et al.* 2004). The matrix GLA protein stimulates vascular endothelial growth factor expression through increased transforming growth factor-β1 activity in endothelial cells. The upregulation of *MGP* in bovine endometrium during the luteal phase could also be associated with the modulation of the TGF-beta signalling pathway (Bauersachs *et al.* 2005).

Estimated effects of SNPs for production traits located within genes derived from gene expression studies confirmed results from quantitative genetics for the association between Mkg and percentage traits (comparison of results in Table 3 with those in Tables 5 and 6). Unfavourable correlations and unfavourable allele substitution effects between Mkg and Fpr, and between Mkg and Ppr, make selection for improving both antagonistic production traits simultaneously extremely challenging. However, some interesting results were found with respect to allele substitution effects of some SNPs on both Mkg and SCS, or on both Pkg and SCS. Target orientated selection on favourable alleles, especially within *CYP27A1*, can be used to improve both Mkg and SCS, despite the unfavourable associations estimated in most of the quantitative genetic studies (e.g., Koenig *et al.* 2005).

Association between production and fertility traits

Significant allele substitution effects for at least one fertility trait plus one production trait are given in Table 6. The

Table 6 Estimated effects (standard errors) of SNPs that were significantly associated (at a 5% false discovery rate) with at least one fertility plus one production trait.

Chromosome	Gene symbol	Trait*											
		Mkg	Fkg	Pkg	Fpr	Ppr	SCS	NRh	FLh	CFc	NRc	FLc	DOc
1	*TNFSF10*	NS	1.725	0.650	0.011	NS	NS	NS	NS	NS	NS	0.669	NS
		NS	(0.294)	(0.235)	(0.003)	NS	NS	NS	NS	NS	NS	(0.211)	NS
2	*IFIH1*	−36.228	NS	−0.667	0.016	0.007	NS	NS	NS	NS	NS	−0.963	NS
		(9.122)	NS	(0.267)	(0.003)	(0.001)	NS	NS	NS	NS	NS	(0.236)	NS
2	*IGFBP2*	27.402	NS	NS	−0.013	−0.006	NS	NS	NS	NS	NS	−0.918	−0.959
		(9.990)	NS	NS	(0.003)	(0.001)	NS	NS	NS	NS	NS	(0.294)	(0.285)
3	*HFM1*	NS	NS	NS	NS	0.006	NS	NS	NS	NS	NS	−1.329	NS
		NS	NS	NS	NS	(0.002)	NS	NS	NS	NS	NS	(0.450)	NS
4	*PARP12*	−43.339	NS	−0.915	0.027	0.006	NS	NS	NS	NS	NS	0.673	NS
		(8.589)	NS	(0.252)	(0.003)	(0.001)	NS	NS	NS	NS	NS	(0.230)	NS
4	*SCRN1*	NS	−1.464	−0.952	−0.015	−0.009	NS	NS	NS	NS	−1.013	NS	NS
		NS	(0.330)	(0.265)	(0.003)	(0.001)	NS	NS	NS	NS	(0.282)	NS	NS
6	*IGFBP7*	37.902	0.836	NS	−0.009	−0.009	NS	0.958	0.932	NS	NS	NS	NS
		(9.425)	(0.345)	NS	(0.003)	(0.001)	NS	(0.276)	(0.250)	NS	NS	NS	NS
8	*APBA1*	NS	NS	NS	−0.016	−0.004	NS	NS	NS	NS	NS	−0.700	NS
		NS	NS	NS	(0.003)	(0.001)	NS	NS	NS	NS	NS	(0.230)	NS
10	*IRF9*	56.879	1.022	1.569	−0.016	−0.005	NS	NS	NS	NS	NS	−0.824	NS
		(10.518)	(0.385)	(0.308)	(0.003)	(0.001)	NS	NS	NS	NS	NS	(0.273)	NS
17	*KSR2*	NS	0.999	0.634	0.007	0.003	NS	NS	−0.794	NS	NS	NS	NS
		NS	(0.281)	(0.225)	(0.003)	(0.001)	NS	NS	(0.215)	NS	NS	NS	NS
18	*CNOT1*	NS	−0.810	NS	NS	NS	NS	NS	NS	NS	NS	0.786	NS
		NS	(0.325)	NS	NS	NS	NS	NS	NS	NS	NS	(0.233)	NS
18	*NDRG4*	NS	−0.831	−0.747	NS	NS	NS	NS	NS	0.945	NS	NS	0.867
		NS	(0.298)	(0.239)	NS	NS	NS	NS	NS	(0.212)	NS	NS	(0.214)
19	*BAIAP2*	31.820	NS	NS	−0.011	−0.007	NS	−1.369	NS	NS	NS	NS	NS
		(9.286)	NS	NS	(0.003)	(0.001)	NS	(0.309)	NS	NS	NS	NS	NS
20	*CCNB1*	−34.435	NS	−1.240	0.013	NS	NS	NS	NS	−1.065	1.156	NS	NS
		(10.958)	NS	(0.321)	(0.004)	NS	NS	NS	NS	(0.289)	(0.343)	NS	NS
23	*CCND3*	NS	NS	−0.673	NS	−0.003	NS	1.348	1.136	NS	NS	NS	NS
		NS	NS	(0.265)	NS	(0.001)	NS	(0.321)	(0.289)	NS	NS	NS	NS
24	*NPC1*	NS	−1.807	−1.046	−0.016	−0.006	NS	NS	NS	NS	1.302	NS	NS
		NS	(0.413)	(0.331)	(0.004)	(0.002)	NS	NS	NS	NS	(0.395)	NS	NS

*Mkg, milk yield; Fkg, fat yield; Pkg, protein yield; Fpr, fat percentage; Ppr, protein percentage; SCS, somatic cell score; NRh, non-return rate to 56 days (heifer); FLh, interval from first to successful insemination (heifer); CFc, interval from calving to first insemination; NRc, non-return rate to 56 days (cow); FLc, interval from first to successful insemination (cow); DOc, days open; NS, not significant.

fertility traits can be distinguished as those being relevant for the start of the first cycle after calving (CFc), those being relevant for conception (NRh, NRc, FLh, and FLc), and those combining both aspects (DOc). A detailed description of the analysed fertility traits on a time axis is depicted in Fig. 2.

We found several SNP alleles having favourable effects on yield traits (Mkg, Fkg, and Pkg), as well as on at least one fertility trait (Table 6). For the SNP within *TNFSF10*, the A allele has a positive effect on Fkg, Pkg, Fpr and FLc. The tumour necrosis factor (ligand) superfamily, member 10 (*TNFSF10*, TRAIL) mRNA has been shown to be upregulated in human endometrium during the window of implantation (Riesewijk *et al.* 2003), in equine endometrium at day 12 of pregnancy (Merkl *et al.* 2010), and in bovine endometrium at day 18 of pregnancy (Bauersachs *et al.* 2006). Furthermore, a role of *TNFSF10* in the modulation of the cytokine milieu at the implantation site has been suggested based on the differential regulation of

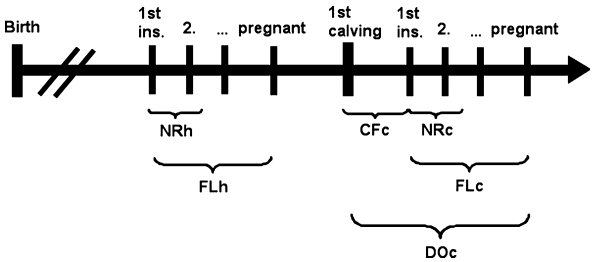

Figure 2 Representation of fertility traits used in this study on a time axis. NRh, non-return rate to 56 days (heifer); FLh, interval from first to successful insemination (heifer); CFc, interval from calving to first insemination; NRc, non-return rate to 56 days (cow); FLc, interval from first to successful insemination (cow); DOc, days open.

cytokines and chemokines in human endometrial stromal cells by TNFSF10 (Fluhr *et al.* 2009). The favourable SNP allele located within *IFIH1* increased Mkg and also increased the EBV for FLc, which means a reduction in the interval from first to successful insemination. *IFIH1* is a RNA helicase known to be involved in cellular recognition of RNA viruses (Wilkins & Gayle 2010). Song *et al.* (2007) reported that *IFIH1* is involved in the establishment of uterine receptivity to the conceptus during implantation. Such role was also discussed by Spencer *et al.* (2008).

Antagonistic relationships for allele substitution effects between NRh and Mkg, as well as for the reciprocal effect between Mkg and NRc, were found for the SNP located within *BAIAP2* [encoding actin-crosslinking protein involved in the generation of actin bundles and promotion of filopodial protrusions, as described by Disanza *et al.* (2006)] and the SNP located within *CCNB1* (encoding cyclin B1), respectively. These effects provide evidence that high milk yield in the first lactation is genetically negatively associated with non-return rates in heifers and in cows, which is also suggested by the negative correlations depicted in Table 3. Effects of the SNP located within the *insulin-like growth factor binding protein 7* gene (*IGFBP7*) on Mkg, NRh and FLh follow the same direction. Regarding the expression of *IGFBP7*, Merkl *et al.* (2010) reported higher mRNA levels in equine endometrial samples derived from day 12 of pregnancy compared to non-pregnant samples. Furthermore, abundant expression of *IGFBP7* has been found in human glandular epithelial cells during the secretory phase, and an *in vitro* knock-down revealed a role of IGFBP7 protein in differentiation of these cells (Kutsukake *et al.* 2010). In a study of human endometrium during the menstrual cycle, an increase in expression during the receptive phase compared with the pre-receptive phase was found to be followed by a sharp increase in the late luteal phase, suggesting an implication of *IGFBP7* in endometrial physiology and receptivity (Domínguez *et al.* 2003). The unfavourable relationship between NRc and CFc in terms of allele substitution effects for the SNP within *CCNB1* is consistent with the weak negative correlation of −0.06 presented in Table 3. A negative genetic correlation close to zero between traits describing the start of the first cycle after calving and those being relevant for conception was also found in König *et al.* (2009).

Days open combines both aspects of the start of a cycle after calving and conception and can be considered as an ultimate breeding goal for fertility (Pasman *et al.* 2006). Genetic correlations between milk yield in first lactation and days open in first lactation were found to be moderately high and unfavourable in most of the quantitative genetic studies previously conducted (e.g. Strandberg & Danell 1989). Estimated effects of SNPs that were significantly associated with at least one yield trait (Mkg, Fkg, or Pkg) plus DOc were unfavourable for practical breeding in dairy cattle. These SNPs were located within *IGFBP2* and *NDRG family member 4* (*NDRG4*). Upregulation of *IGFBP2* mRNA was found in different microarray studies in endometrium during early pregnancy, such as in porcine endometrium at day 14 of pregnancy (Østrup *et al.* 2010), in equine endometrium at day 12 of pregnancy (Merkl *et al.* 2010) and in bovine endometrium at day 18 of pregnancy (Klein *et al.* 2006). *IGFBP2* expression has also been shown to be regulated by estradiol and progesterone in human endometrial stromal cells (Giudice *et al.* 1991). As a member of the IGF system, *IGFBP2* also has an important role in lactation and nutrition, and may be implicated in the interaction between lactation and nutrition and in the establishment of pregnancy (Rhoads *et al.* 2008).

Pronounced antagonistic relationships between Mkg and percentage traits (Fpr and Ppr) were found on the quantitative genetic scale as well as when using significant SNPs located in the physical region of candidate genes derived from gene expression studies. Hence, breeding strategies to improve both yield and percentage traits simultaneously remain a challenge for animal breeding programs. Special breeding goals with focus either on yield or on percentage traits require genetically different groups of sires.

While most quantitative genetic studies have proven genetic antagonism between yield traits and functional traits (in our study fertility and SCS), improvement in both production and functionality may be possible when focusing on a few relevant SNPs. For instance, the same SNP allele located within *IGFBP7* leads to an increase in Mkg without a further undesired increase in NRh and FLh. A similar strategy was discussed by Moe *et al.* (2009) for the simultaneous improvement of antagonistic fertility traits in swine.

As shown by König *et al.* (2009), genome-wide selection increases the risk of widening the gap between production and functionality. Sophisticated approaches as suggested in the present study, i.e., combining knowledge from quantitative genetics, genome-wide association studies and gene expression studies, have the potential to overcome some of these obstacles. Other studies that focus on SNPs located in specific regions of the genome have been carried out, but

most have applied whole-genome scans for QTL mapping. Targeting relevant SNPs within genes derived from gene expression studies is a novel concept in the context of animal breeding strategies. This strategy has been successfully applied in human genetics for functional selection of SNPs associated with prostate cancer (Xu & Taylor 2009).

As candidates for this study, genes with higher mRNA expression levels in the bovine endometrium during the luteal phase were selected from two microarray studies where gene expression differences between oestrus and dioestrus (Bauersachs *et al.* 2005) and during the oestrous cycle (Mitko *et al.* 2008) were analysed. Increased expression levels in the luteal phase suggest regulation by progesterone (P4), which is the key hormone for preparation of the endometrium for embryo implantation (Bazer *et al.* 2008). A positive influence of P4 on conceptus growth and development has been shown (Clemente *et al.* 2009). Genes with lower levels at dioestrus compared to oestrus were not considered, because these genes are upregulated at oestrus by estradiol (E2) rather than being directly down-regulated by P4. Furthermore, we intentionally did not select genes upregulated during early pregnancy, as many of these genes are induced by interferon tau, the ruminant pregnancy recognition signal (Roberts 1989), which is a type I interferon that induces many genes and is also known to be involved in immune response to viral infections. For many of these genes there is probably no direct role in the context of fertility, but certainly a role in the context of immune response, which would complicate the interpretation of the results of this study. We also did not select candidate genes from human or mouse studies, because reproductive biology is quite different between these species and cattle regarding the time and the type of implantation. In addition to the genes with elevated expression during the luteal phase, 19 candidate genes, which probably have conserved roles in pregnancy in different mammalian species, were selected.

The candidate genes considered here do not cover all possible candidates to be considered, but are rather involved in part of the complexity inherent in fertility. Further research including other genes should be performed to extend the knowledge about the molecular basis underlying the relationships between production and reproduction. The density of the marker panel used in this study only permitted the identification of one marker per segment covered by a given candidate gene. The rate of development in SNP genotyping technology will allow the use of much denser marker panels and even whole sequences. Future investigations in the line of this study, but with much denser marker information, may allow the identification of multiple markers per segment. With the availability of such data, further studies may also be conducted that include the use of marker haplotypes in the association analyses.

Acknowledgements

This study is part of the projects FUGATO-plus GenoTrack and COMPENDIUM and was financially supported by the German Ministry of Education and Research, BMBF; the Förderverein Biotechnologieforschung e.V. (FBF), Bonn; and Lohmann Tierzucht GmbH, Cuxhaven. The transcriptome studies revealing candidate genes for fertility were performed with financial support from the Deutsche Forschungsgemeinschaft (Research Unit 'Mechanisms of embryo-maternal communication') and from the BMBF (FUGATO Fertilink).

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