Genome-wide mapping of 10 calving and fertility traits in Holstein dairy cattle with special regard to chromosome 18

M.-P. Müller,*† S. Rothammer,† D. Seichter,* I. Russ,* D. Hinrichs,‡¹ J. Tetens,‡² G. Thaller,*‡ and I. Medjugorac†³

*Tierzuchtforchung e.V. München, Senator-Gerauer-Str. 23, 85586 Poing, Germany

†Chair of Animal Genetics and Husbandry, Ludwig-Maximilians-Universität Munich, Veterinärstr. 13, 80539 Munich, Germany

‡Institute of Animal Breeding and Husbandry, Christian-Albrechts-University, Olshausenstr. 40, 24098 Kiel, Germany

ABSTRACT

Over the last decades, a dramatic decrease in reproductive performance has been observed in Holstein cattle and fertility problems have become the most common reason for a cow to leave the herd. The premature removal of animals with high breeding values results in both economic and breeding losses. For efficient future Holstein breeding, the identification of loci associated with low fertility is of major interest and thus constitutes the aim of this study. To reach this aim, a genome-wide combined linkage disequilibrium and linkage analysis (cLDLA) was conducted using data on the following 10 calving and fertility traits in the form of estimated breeding values: days from first service to conception of heifers and cows, nonreturn rate on d 56 of heifers and cows, days from calving to first insemination, days open, paternal and maternal calving ease, paternal and maternal stillbirth. The animal data set contained 2,527 daughter-proven Holstein bulls from Germany that were genotyped with Illumina's BovineSNP50 BeadChip (Illumina Inc., San Diego, CA). For the cLDLA, 41,635 sliding windows of 40 adjacent single nucleotide polymorphisms (SNP) were used. At each window midpoint, a variance component analysis was executed using ASReml. The underlying mixed linear model included random quantitative trait locus (QTL) and polygenic effects. We identified 50 genome-wide significant QTL. The most significant peak was detected for direct calving ease

at 59,179,424 bp on chromosome 18 (BTA18). Next, a mixed-linear model association (MLMA) analysis was conducted. A comparison of the cLDLA and MLMA results with special regard to BTA18 showed that the genome-wide most significant SNP from the MLMA was associated with the same trait and located on the same chromosome at 57,589,121 bp (i.e., about 1.5 Mb apart from the cLDLA peak). The results of 5 different cLDLA and 2 MLMA models, which included the fixed effects of either SNP or haplotypes, suggested that the cLDLA method outperformed the MLMA in accuracy and precision. The haplotype-based cLDLA method allowed for a more precise mapping and the definition of ancestral and derived QTL alleles, both of which are essential for the detection of underlying quantitative trait nucleotides.

Key words: cattle, fertility, calving traits, quantitative trait locus mapping

INTRODUCTION

Over the last few decades, an association between increasing milk yield and decreasing fertility has been observed in Holstein cattle (Hoekstra et al., 1994; Walsh et al., 2011). In 2013, German Holstein-Friesians reached on average only 2.7 lactations before slaughter; Red Holsteins showed a comparable number (2.8 lactations). In general, about 21.5% of all dairy cows were slaughtered due to infertility (ADR, 2014). Infertility thus represented the most common reason for culling of dairy cows. Moreover, Dematawena and Berger (1997) estimated costs per cow at around \$380 (~£230 in 1996) for dystocia grade 5 (out of 5), and McGuirk et al. (2007) reported £395 (~€568, \$825 in 2007) for serious calving difficulties, both of which require veterinary assistance. However, dystocia not only causes economic costs, but is also a very serious problem in terms of animal welfare. According to Huxley and Whay (2006), dystocia is one of the most painful conditions in cows (pain score 7 out of 10).

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¹Current address: Department of Crop and Animal Sciences, Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt University of Berlin, Invalidenstr. 42, 10115 Berlin, Germany.

²Current address: Department of Animal Sciences, Division of Functional Breeding, Georg-August-University, Burckhardtweg 2, 37077 Göttingen, Germany.

³Corresponding author: Ivica.Medjugorac@gen.vetmed.uni-muenchen.de

To identify loci with a major effect on fertility and thus find a solution to this serious problem, a lot of research has already been conducted (Kühn et al., 2003; Thomasen et al., 2008; Cole et al., 2009, 2014b; Seidenspinner et al., 2009; Sahana et al., 2011). These studies covered various Holstein subpopulations [German Holsteins: Kühn et al. (2003), Seidenspinner et al. (2009), Cole et al. (2014b); US Holsteins: Cole et al. (2009), Danish Holsteins: Sahana et al. (2011), Thomasen et al. (2008); Swedish Holsteins: Sahana et al. (2011)] and were based on genome-wide microsatellite (Kühn et al., 2003; Thomasen et al., 2008; Seidenspinner et al., 2009) or SNP markers (Cole et al., 2009; Sahana et al., 2011; Cole et al., 2014b). They consistently identified a very prominent QTL for various fertility traits on chromosome 18 (BTA18) in a region roughly located between 50 and 60 Mb. However, the underlying mutation has not been identified yet (Cole et al., 2014a,b).

The overall objective of this study was to add new information that will help to resolve the serious fertility problem in Holstein dairy cattle. To reach this aim, a genome-wide SNP-based combined linkage disequilibrium and linkage analysis (**cLDLA**) as already described in Rothhammer et al. (2014) and Kunz et al. (2016) was chosen for QTL mapping. In contrast to most of the previous SNP-based studies that primarily performed genome-wide association analyses and identified only SNP-trait associations (Cole et al., 2009, 2011, 2014b; Sahana et al., 2011; Purfield et al., 2014), this study was optimized to identify haplotype-trait associations. Furthermore, the results of 5 different cLDLA and 2 genome-wide association study (**GWAS**) models (mixed-linear-model association, **MLMA**; Yang et al., 2014), which all included the fixed effects of SNP or haplotypes, were compared. We were thus able to draw conclusions regarding the QTL positions and the performance of the 2 mapping methods.

MATERIALS AND METHODS

Animals and Phenotypes

This study used 2,527 daughter-proven AI Holstein sires. Mapping of QTL was carried out for the following 10 calving and fertility traits: days from first service to conception of heifers (**FSTC-h**) and cows (**FSTC-c**), nonreturn rate on d 56 of heifers (**NR56-h**) and cows, days from calving to first insemination (**CTFS**), days open (**DO**), paternal calving ease (**pCE**) for heifers, maternal calving ease (**mCE**) for heifers, paternal stillbirth (**pSB**) for heifers, and maternal stillbirth (**mSB**) for heifers. Depending on the authors' preferences, varying synonyms for the same trait are found in the literature. "Calving ease," for example,

is also known under the terms "dystocia" or "calving difficulty." Furthermore, "direct" and "indirect" are synonymously used for "paternal" and "maternal." For reasons of clarity, especially when discussing the results of other studies, we used the terms "calving ease," "paternal," and "maternal" regardless of the terminology in the original publications. Pedigree records and EBV were taken from the official breeding value evaluation of daughter-proven bulls, which was performed by Vereinigte Informationsysteme Tierhaltung (**VIT**, Verden, Germany) in June 2013. The genotyped sires built a complex pedigree of 239 half-sib families. The number of sons per grandsire ranged from 2 to 95 with an average of 5.6 sons. Three families included more than 50 sons, 9 families had 30 to 49 sons, 68 families had 10 to 29, and 159 families had 2 to 9 sons. Because this study only used already existing data and DNA from previous projects (Thaller, 2011; Medugorac et al., 2012; Zeller-Adam, 2013), no specific permission or ethical approval was required. Moreover, original semen samples were collected by approved commercial AI stations as part of their regular reproduction and breeding management program.

Genotyping Data, Quality Control, and Reconstruction of Haplotypes

The DNA of all animals was genotyped for either 54,001 SNP using version 1 of the BovineSNP50 Genotyping BeadChip (Illumina Inc., San Diego, CA) or for 54,609 SNP using version 2 of this BeadChip. The SNP positions were determined according to the *Bos taurus* UMD 3.1 reference assembly. For further analysis, all markers that met one of the following criteria were excluded: (1) call rate of less than 95%, (2) frequent paternity conflicts in animals with confirmed paternity (i.e., SNP showing Mendelian error rates of more than 0.2%), (3) unknown position according to the UMD 3.1 reference assembly, (4) markers with heterozygosity below 5%, and (5) all markers located on bovine chromosomes X or Y because we concentrated only on autosomes. After this filtering process, 42,215 SNP remained in the marker set.

A hidden Markov model implemented in Beagle 3.0.4 (Browning and Browning, 2009) was used for reconstruction of haplotypes and imputation of missing genotypes. To improve haplotype reconstruction, additional pedigree information and SNP genotypes from previous projects were used. This included additional Holstein animals without appropriate phenotypes that were related to the animals in our mapping population as well as *Bos taurus* animals of European origin that otherwise had no direct relevance for this study. Overall, a total of 8,154 genotyped animals, which were

assigned to 3 cohorts (trios, pairs, and unrelated animals) according to the Beagle manual, were used for haplotyping.

Locus Identity by Descent and Diplotype Relationship Matrix

The Meuwissen and Goddard (2001) procedure for the prediction of identity by descent (**IBD**) probabilities from marker haplotypes is capable of estimating the IBD between chromosome pairs at any given position. We adopted this method to estimate the local IBD (**LocIBD**) at the median position in the 40-SNP sliding windows (i.e., the IBD between putative QTL alleles that are located between SNP 20 and 21 in each window). Following the method described in Lee and Van der Werf (2006) for additive genetic relationship matrices (**G_{RM}**), the LocIBD matrices were converted into locus diplotype relationship matrices (hereafter the **D_{RM}** matrix). A diplotype is here defined as the specific combination of two 40-SNP haplotypes.

Genome-Wide QTL Mapping

The genome-wide cDLA was performed by a procedure equivalent to that described by Meuwissen et al. (2002). Linkage information was exploited during the reconstruction of haplotypes based on the available relationships among the genotyped animals, whereas linkage disequilibrium was accounted for by LocIBD estimation. The mixed linear model used for QTL mapping considered random QTL effects based on the **D_{RM}** and 110 principal components (**PC**) correcting for population stratification and family relationships, i.e., for polygenic effects. These PC of the unified additive relationships matrix among all animals (Powell et al., 2010) were estimated using R (R Core Team, 2013). Only the top PC that needed to remain in the mixed linear model to explain at least 90% of the genetic variance [determined using the R package *PARAN* (Dinno, 2007)] were included in the following model:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}\mathbf{q} + \mathbf{e},$$

where **y** = vector of 2,527 phenotypes of the investigated trait, **X** and **Z** = incidence matrices for fixed and random effects, respectively, **β** = vector of fixed effects (including overall mean μ and the 110 PC), **q** = vector of random additive genetic effects due to the QTL with $\mathbf{q} \sim N(0, \mathbf{D}_{\text{RMp}}\sigma_q^2)$, where **D_{RMp}** is the diplotype relationship matrix at the location p (p = 1 to 41,635, i.e., the number of investigated positions) of the putative QTL, **e** = vector of random residual effects with

$\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where **I** is an identity matrix. The random effects **q** and **e** were assumed to be uncorrelated and normally distributed. The variances σ_q^2 and σ_e^2 were simultaneously estimated. This basic cDLA mapping model will hereafter be referred to as model 1.

Variance component analyses for each of the 41,635 positions (10 marker intervals at both the start and the end of each of the 29 autosomes were skipped) and each of the 10 traits were performed using the ASREML package (Gilmour et al., 2009). The logarithms of the likelihood that ASREML estimated for the model with ($\log L_P$) and without QTL effects ($\log L_0$; corresponds to null hypothesis) were then used to calculate the likelihood ratio test statistic [**LRT** = $-2(\log L_0 - \log L_P)$], which is known to be χ^2 -distributed with one degree of freedom (Olsen et al., 2004). To keep the false discovery rate low, a significance threshold of $\text{LRT} = 19.511$, which resembles a *P*-value of less than 10^{-5} , was chosen. This choice of threshold corresponds to a Bonferroni-corrected testing of 1,000 independent hypotheses (*m*) at a significance level of $\alpha = 0.01$ ($\alpha/m = 10^{-5}$). The 40-SNP windows that were used for estimation of all 41,635 LRT values were moved SNP by SNP along the genome. Because directly neighboring windows only differ by one SNP, the LRT values of neighboring positions contain identical information at 39 out of 40 SNP (i.e., they are not independent of each other). The LRT values distanced by more than 40 SNP (~ 2.5 Mb) are based on different markers but can still depend on each other due to long haplotypes [i.e., the so-called carry-over effect (Meuwissen and Goddard, 2004; Olsen et al., 2004)]. Therefore, the Bonferroni correction applied here considers 1,000 ($41,635/40 = 1,041$) independent tests.

The LRT curves were created by plotting LRT values against the corresponding base pairs (**LRT_{max}**). All local maxima with LRT values > 19.511 ($P < 10^{-5}$) were recognized as significant. Confidence intervals for the QTL positions were defined as **2-LOD** (log of odds) support intervals surrounding **LRT_{max}** and included all LRT values exceeding **LRT_{max}** [$4 \times \ln(10)$] (van Ooijen, 1992; Visscher and Goddard, 2004; Kim et al., 2009). The 2-LOD intervals thus correspond to **LRT_{max}** - 2 LOD with 1 LOD = $2 \times \ln(10) = 4.605$. To assess if any **LRT_{max}** were mainly caused by the same animals, Spearman's correlations of the animals ranked by diplotype effect were calculated between all peaks on each chromosome. Closely located LRT peaks with a high correlation ($r_{[q1,q2]} > 0.7$) were then assumed to belong to the same QTL.

Summing up, LRT peaks were assigned to QTL according to the following criteria:

- Only peaks with LRT values >19.511 were considered.
- The LRT peaks for the same trait that were located in the same 2-LOD support interval or in intersecting intervals were assumed to be the same QTL.
- Estimates of LRT statistics at directly neighboring intervals are dependent and reflect the similarity in haplotype architecture. Although 2 LRT values separated by one window length (40 SNP) could partly be due to similar haplotype architecture, they are the result of 2 nonoverlapping variance-covariance analyses. We thus postulated that 2 LRT peaks for the same trait had to be at least one window length (40 SNP) apart to be assigned to independent QTL.
- Peaks for different traits were combined into one QTL when Spearman’s correlation of diplotype effects exceeded 0.7, thus indicating strong genetic correlation between the traits at the specific locus (Dancey and Reidy, 2007; Leyer and Wesche, 2007).

For each QTL, a literature search was performed to identify associations that have already been reported for dairy cattle.

MLMA: Genome-Wide Association Study

To be able to compare the haplotype-based cLDLA results with SNP-based GWAS results, we used the GCTA software and performed a genome-wide association study using the LOCO option (Yang et al., 2014). Since Yang et al. (2014) had shown that in contrast to the MLM_i approach (candidate marker included), the MLM_e approach (candidate marker excluded as in GCTA-LOCO) increased power compared with linear regression, the LOCO option was the option of choice. PLINK was used for GCTA-LOCO input file creation (Purcell et al., 2007). This basic association analysis

model will hereafter be called **MLMA#1**, and it was performed on a marker set of 41,787 SNP with MAF >2.5%. For each SNP, the program estimated a *P*-value representing the magnitude of the SNP-phenotype association. For an easy visual comparison of the GWAS results with the cLDLA results, the negative logarithms of the *P*-values and the LRT values were displayed in combined plots for each chromosome.

Additional cLDLA and MLMA Run on BTA18 for Validation of Results

To validate the results of the cLDLA in general and the results on BTA18 in particular, we compared 5 different cLDLA and 2 MLMA models. Table 1 gives an overview of the differences between the basic (model 1 and MLMA#1) and the alternative models (model 2, model 3, model 4, model 5, and MLMA#2). For paternal calving ease, which is the trait with the genome-wide most significant LRT value (LRT = 160.92, BTA18), we analyzed all haplotypes based on the distribution of diplotype effects. This approach clearly implied covariance between a single discrete haplotype with the most negative diplotype effects (hereafter called Q1) and a large set of various haplotypes excluding Q1 in the remaining part of the diplotype effects distribution (hereafter called q1; see results of model 3). Depending on the presence of the Q1 haplotype, the animals were divided into 3 groups: Q1/Q1, Q1/q1, and q1/q1; q1 represents all possible haplotypes but Q1 (Q1 ∉ q1). These 3 groups were included as fixed effect in model 3 and as artificial SNP in MLMA#2 (Table 1).

The SNP rs109478645 was the most significant SNP for 4 calving traits (body depth, rump width, stature, and strength) in Cole et al. (2009). It was also detected by MLMA#1 as the most significantly associated SNP with paternal calving ease. We therefore evaluated rs109478645 in 2 models (excluded in model 2 and included as fixed effect in model 4).

Table 1. Description of alternative combined linkage disequilibrium and linkage analysis (cLDLA) and mixed-linear-model association (MLMA) models¹

Model	Differences from basic model	Results
Model 2	rs109478645 at 57,589,121 bp on BTA18 excluded	Figure 2
Model 3	Vector β included fixed effects of the artificial SNP with genotypes Q1/Q1, Q1/q1, and q1/q1 corresponding to the most probable QTL allele on BTA18	Figure 4
Model 4	Vector β included fixed effects of 3 rs109478645 genotypes	Figure 5, 6
Model 5	rs43072554 at 58,696,066 bp on BTA18 excluded	Figure 7
MLMA#2	Artificial SNP with genotypes Q1/Q1, Q1/q1, and q1/q1 corresponding to the most probable QTL allele on BTA18 included. MLMA#2 is equivalent to cLDLA model 3.	Results of MLMA#2

¹Only the differences to the basic models (model 1 and MLMA#1) that are described in the main text are given. The Results column points out the most important results of a given model.

The SNP rs43072554, which was the most significant SNP for paternal stillbirth in Cole et al. (2011), was identical with SNP 20 of the most significant 40-SNP window in the cLDLA for paternal calving ease. Therefore, SNP rs43072554 was excluded in model 5.

RESULTS

Genome-Wide QTL Mapping

In total, 90 significant ($P < 10^{-5}$) LRT peaks were identified for all 10 traits of interest. According to the 4 criteria described in Materials and Methods, these 90 LRT peaks represent 50 QTL (Table 2). The most significant QTL of all chromosomes and traits is located on BTA18 and affects pCE and pSB. Figure 1 shows that although significant associations were found for all traits, the number of peaks varied considerably from 4 (FSTC-h) to 14 (pCE). Table 2 shows the distribution of the significant LRT peaks per trait and chromosome and the final number of QTL per chromosome. Table 3 offers more detailed information (trait composition, chromosome, physical position, and LRT values) on the 50 significant QTL.

A literature search showed that in many cases the used *Bos taurus* reference genome had meanwhile become obsolete. Therefore, time-consuming procedures

were necessary to check the actual UMD 3.1 marker positions in *ensembl* (cow release 77, October 2014, accessed November 17–18, 2014) or *Arkdb* (accessed November 17–18, 2014). In cases where the actual UMD3.1 marker positions were not given, marker sequences were blasted using *ensembl*. To facilitate similar comparisons for future authors, Supplemental Table S1 (<https://doi.org/10.3168/jds.2016-11506>) lists all QTL that have been reported for dairy cattle at comparable physical positions and for related traits.

Major Results on BTA18: Results of Model 1

The genome-wide most significant QTL is located on BTA18 at a position close to what has already been reported by various authors and affects pCE and pSB. Quantitative trait loci with a major effect on fertility traits on BTA18 that were found in this or previous studies are listed in Table 3 and Supplemental Table S1 (<https://doi.org/10.3168/jds.2016-11506>). The here applied design and criteria suggested 7 different QTL on BTA18. The first QTL (18.1) was mapped between 44,543,348 and 46,396,728 bp and affects CTFS. The second QTL (18.2) between 52,035,136 and 53,042,160 bp affects mCE. The third QTL (18.3) between 54,040,400 and 54,481,864 bp was mapped for mSB. The fourth and most significant QTL (18.4) was

Table 2. Summary of combined linkage disequilibrium and linkage analysis (cLDLA) results (model 1)¹

Chr	Trait ²										nLRT _{max}	nQTL
	CTFS	DO	FSTC-h	NR56-h	FSTC-c	NR56-c	mCE	mSB	pCE	pSB		
3	—	—	—	—	—	—	—	1	—	—	1	1
4	2	1	—	—	2	4	—	—	—	—	9	4
5	2	1	—	—	—	—	—	—	3	3	9	4
6	1	3	—	—	—	—	—	3	—	—	7	4
7	—	—	—	—	—	—	—	—	4	1	5	1
9	—	—	—	—	2	—	—	—	—	—	2	1
10	2	2	—	—	1	—	—	—	—	—	5	1
11	—	—	—	3	1	—	1	2	—	—	7	6
12	1	—	—	—	—	—	1	—	—	—	2	2
14	—	—	2	—	1	—	—	—	—	—	3	2
18	1	1	—	—	1	—	2	1	6	4	16	7
19	—	—	—	2	—	—	—	—	1	—	3	2
21	1	—	—	—	2	—	—	—	—	—	3	1
22	—	—	—	—	1	—	—	—	—	—	1	2
23	1	1	2	2	—	1	—	—	—	—	7	4
25	—	—	—	—	—	—	5	—	—	—	5	3
26	—	1	—	—	1	—	—	—	—	—	2	2
27	—	1	—	—	—	1	—	—	—	—	2	2
29	—	1	—	—	—	—	—	—	—	—	1	1
Sum	11	12	4	7	12	6	9	7	14	8	90	50

¹Number of significant QTL with likelihood ratio test statistic (LRT) values >19.511 per trait and chromosome (Chr) as well as the sum of distinct significant LRT_{max} (nLRT_{max}) and QTL (nQTL) are shown.

²Days from first service to conception of heifers (FSTC-h) and cows (FSTC-c), nonreturn rate on d 56 of heifers (NR56-h) and cows (NR56-c), days from calving to first insemination (CTFS), days open (DO), paternal (direct) calving ease (pCE), maternal (indirect) calving ease (mCE), paternal stillbirth (pSB), and maternal stillbirth (mSB).

Figure 1. Manhattan plot of QTL mapping results. Likelihood ratio test statistic (LRT) values for all traits are shown on the y-axes, the 29 autosomes (BTA) are shown on the x-axes. The gray dashed line represents the significance threshold of LRT = 19.511. For each phenotype, a separate chart was plotted. DO = days open.

identified for pCE and pSB and mapped to an interval between 55,282,968 and 60,119,636 bp. The fifth QTL (18.5) between 57,206,512 and 59,732,548 bp affects DO and FSTC-c and the sixth QTL (18.6) between 57,959,312 and 60,119,636 bp affects mCE. The seventh QTL (18.7) between 62,624,960 and 64,521,568 bp was mapped for pCE and pSB.

Table 4 presents heritabilities and genome-wide genetic correlations that were provided by VIT for the 10 traits investigated in this study. For reasons of comparison, locus-specific genetic correlations between the same traits at the most significant position that are based on diplotype effects are also presented in Table 4 (estimated with Spearman's test of rank correlations). The most important aspect is the highly positive locus-specific genetic correlation between maternal and paternal traits ($r_{[mCE,pCE]}$, $r_{[mCE,pSB]}$, $r_{[mSB,pSB]}$) compared with the effectively nonexistent genome-wide correlations ($r_{[mCE,pCE]}$, $r_{[mCE,pSB]}$, $r_{[mSB,pSB]}$). Furthermore, FSTC-h is not associated with maternal and paternal stillbirth and calving ease on a genome-wide level ($r_{[FSTC-h,pCE]}$, $r_{[FSTC-h,mCE]}$, $r_{[FSTC-h,pSB]}$, $r_{[FSTC-h,mSB]}$) but shows a strong positive correlation at the most significant QTL ($r_{[FSTC-h,pCE]}$, $r_{[FSTC-h,mCE]}$, $r_{[FSTC-h,pSB]}$, $r_{[FSTC-h,mSB]}$).

Results of Additional cLDLA Models and Methods Applied to BTA18

Using the cLDLA approach, the genome-wide most significant peak was found for pCE at 59,179,424 bp, which is located between markers rs43072554 and rs42550320. To compare our haplotype-based cLDLA method with a commonly used SNP-based GWAS approach, we additionally conducted a MLMA. MLMA#1 identified rs109478645 at 57,589,121 bp on BTA18 as the genome-wide most significant SNP for pCE. In this MLMA model, all SNP with a P -value below 10^{-5} were declared to be significant. In total, MLMA#1 revealed 27 SNP for pCE, 23 SNP for pSB, 7 SNP for mCE, 4 SNP for DO, 3 SNP for CTFS, and 1 SNP for mSB on BTA18. The exact positions of all significant SNP for pCE and pSB and the overlap with cLDLA results are presented in Table 5. Twenty out of the 27 SNP found for pCE and 18 out of the 23 SNP found for pSB on BTA18 were located within the confidence interval of a QTL for the same trait.

Results of Model 2—Excluding rs109478645 from cLDLA

For model 2, the most significant SNP detected by MLMA#1 was excluded from the analyses, whereas the mixed linear model did not differ from model 1. As can

be seen in Figure 2, both model 1 and model 2 show highly significant LRT values.

Results of Model 3—Including the Perfectly Associated Haplotype as Fixed Effect

Analyses of QTL 18.4, which was the most significant QTL on BTA18, revealed a high diversity of haplotypes at the positive tail of the distribution and a low diversity at the negative tail. All 55 animals with the most negative estimated diplotype effects (<-7.3) were homozygous for the same haplotype (Q1) that consisted of 24 SNP from 58,157,353 to 60,978,019 bp. An additional 562 animals with estimated diplotype effects between -7.3 and -2.0 were heterozygous with one copy of Q1 and diverse other haplotypes encompassed by q1. The remaining 1,910 animals with estimated diplotype effects above -2.0 were homozygous or heterozygous for haplotypes different from Q1. These 55 Q1/Q1 homozygotes and the 562 Q1/q1 heterozygotes presented 13.30% of all haplotypes (672 out of 5,054). Figure 3 presents the frequencies of the Q1 and q1 haplotypes in subsequent bins of estimated diplotype effects and clearly demonstrates that Q1 indeed causes the observed effect in model 1. From the haplotype distribution, it can also be seen that a single haplotype (Q1) represents QTL allele 1 and that the entire spectrum of all other remaining haplotypes represents QTL allele 2. These QTL alleles cause average diplotype effects for pCE of -8.78 (± 1.33), -4.12 (± 2.12), and 0.61 (± 3.79) for Q1/Q1, Q1/q1, and q1/q1 animals, respectively. Model 3 considers Q1/Q1, Q1/q1, and q1/q1 as fixed effects in the mixed linear model and demonstrates a perfect correlation between Q1 and the putative QTL from model 1. In contrast to model 2, the original LRT peak of the most significant trait for BTA18, pCE, disappeared (see Figure 4, LRT: 2.16 in model 3).

All previously significant calving traits lost their significance along the entire region of BTA18, whereas the LRT statistic for CTFS (QTL 18.1), FSTC-c, and DO (QTL 18.5) changed only marginally and remained significant. For the trait DO, the maximum LRT value at 58,232,128 bp dropped by around 6 units from 30.2 to 24.761. For FSTC-c, the LRT value at 58,378,336 bp dropped by around 10 units from 30.82 to 20.04.

Results of Model 4—Including rs109478645 as Fixed Effect in the cLDLA

In model 4, a fixed effect based on the rs109478645 genotypes was added to the initial mixed linear model. Compared with model 3, 117 animals were classified differently. As can be seen for pCE and pSB in Figure 5

Table 3. QTL identified by combined linkage disequilibrium and linkage analysis (cLDLA; model 1), associated phenotypes, position, and $LRT_{\max}$ (peak) with the 2-LOD (log of odds) support intervals and the overall CI for all traits from the lowest $-2LOD$ to the highest $+2LOD$ are shown¹

QTL	Phenotype	Position (bp)	Peak	$-2LOD$ (bp)	$+2LOD$ (bp)	CI start (bp)	CI end (bp)
3.1	mSB	17,500,312	20.58	16,229,866	18,418,688	16,229,866	18,418,688
4.1	DO	64,138,408	25.50	63,402,100	64,566,792	63,294,560	66,956,696
	CTFS	64,255,608	24.42	63,332,592	65,323,432		
	CTFS	66,571,280	20.90	63,294,560	66,956,696		
4.2	NR56-c	79,095,824	21.62	78,598,160	79,810,568	78,570,840	83,064,144
	NR56-c	80,476,304	24.24	79,963,024	83,016,680		
	NR56-c	81,276,128	20.92	78,570,840	83,064,144		
4.3	FSTC-c	82,980,528	19.52	81,977,056	85,995,704	81,977,056	85,995,704
	FSTC-c	83,977,648	23.00	82,061,696	85,317,040		
4.4	NR56-c	86,667,432	21.72	85,559,104	86,793,608	85,559,104	86,793,608
5.1	DO	17,115,856	20.26	16,967,248	18,008,814	16,967,248	18,008,814
	CTFS	17,115,856	20.08	16,967,248	18,008,814		
5.2	pCE	59,152,132	19.88	55,978,176	60,278,480	55,978,176	63,580,096 ²
	pSB	59,152,132	21.48	57,990,432	60,278,480		
	pSB	62,115,292	19.80	61,344,408	68,424,352		
5.3	pCE	65,954,352	22.86	63,708,608	68,424,352	63,708,608	68,424,352
	pSB	67,925,480	24.08	64,260,632	68,375,120		
	pCE	67,994,072	22.96	63,708,608	68,424,352		
5.4	CTFS	89,823,168	19.82	88,927,400	93,978,248	88,927,400	93,978,248
6.1	DO	45,489,692	19.94	43,933,680	47,877,736	43,933,680	47,877,736
6.2	DO	89,119,008	24.72	88,464,144	89,937,544	87,658,304	92,843,432
	DO	91,250,160	20.76	87,658,304	92,843,432		
	CTFS	91,362,376	22.74	90,756,816	91,672,568		
6.3	mSB	112,222,152	22.92	111,401,704	113,840,648	111,356,448	113,840,648
	mSB	113,061,480	23.26	111,356,448	113,840,648		
6.4	mSB	114,958,192	24.44	114,591,224	115,072,400	114,591,224	115,072,400
7.1	pCE	7,260,348	22.66	6,338,948	8,346,253	6,338,948	15,907,424
	pCE	10,617,091	22.50	8,909,124	13,447,101		
	pCE	11,860,695	28.74	9,101,074	13,213,260		
	pCE	15,447,476	28.00	14,465,366	15,907,424		
	pSB	11,860,695	21.84	9,101,074	12,416,950		
9.1	FSTC-c	55,602,800	26.06	53,484,176	57,398,820	53,484,176	59,583,728
	FSTC-c	58,557,872	23.58	57,637,600	59,583,728		
10.1	DO	34,806,460	24.06	33,403,448	38,487,140	33,403,448	38,487,140
	DO	36,646,012	27.84	35,794,236	37,625,424		
	CTFS	34,277,184	20.16	33,539,836	36,821,600		
	CTFS	34,481,592	20.26	33,678,048	36,783,148		
	FSTC-c	36,646,012	25.42	36,044,728	37,575,456		
11.1	NR56-h	30,188,228	20.76	29,392,336	31,507,764	29,392,336	31,507,764
11.2	NR56-h	60,139,480	25.72	59,572,488	60,289,840	59,173,476	61,913,288
	NR56-h	61,215,016	20.04	59,173,476	61,913,288		
11.3	mCE	78,398,688	24.78	77,923,336	78,742,032	77,923,336	78,742,032
11.4	FSTC-c	82,877,192	19.76	82,707,456	83,116,088	82,707,456	83,116,088
11.5	mSB	92,271,528	22.18	92,108,024	92,710,616	92,108,024	92,710,616
11.6	mSB	96,255,512	20.22	95,485,248	98,346,368	95,485,248	98,346,368
12.1	CTFS	6,085,616	20.14	5,240,630	6,891,264	5,240,630	6,891,264
12.2	mCE	25,922,100	26.28	25,295,396	26,583,152	25,295,396	26,583,152
14.1	FSTC-h	59,849,356	22.06	58,568,400	62,390,948	58,426,288	62,390,948
	FSTC-h	61,369,572	21.22	58,426,288	62,390,948		
14.2	FSTC-c	72,977,472	20.60	70,548,176	73,908,416	70,548,176	73,908,416
18.1	CTFS	44,963,112	27.48	44,543,384	46,396,728	44,543,384	46,396,728
18.2	mCE	52,503,488	19.74	52,035,136	53,042,160	52,035,136	53,042,160
18.3	mSB	54,331,572	19.92	54,040,400	54,481,864	54,040,400	54,481,864
18.4	pCE	55,849,872	54.92	55,282,968	56,209,120	55,282,968	60,119,636
	pCE	57,688,764	139.80	57,577,264	57,879,104		
	pCE	59,179,424	160.92	58,681,172	59,732,548		
	pSB	55,924,624	43.64	55,282,968	56,259,620		
	pSB	57,688,764	85.48	57,449,184	57,822,060		
	pSB	59,179,424	102.08	58,681,172	60,119,636		
18.5	DO	58,320,692	31.20	57,206,512	58,608,792	57,206,512	59,732,548
	FSTC-c	58,378,336	30.82	58,112,328	59,732,548		
18.6	mCE	59,179,424	31.24	57,959,312	60,119,636	57,959,312	60,119,636

Continued

Table 3 (Continued). QTL identified by combined linkage disequilibrium and linkage analysis (cLDLA; model 1), associated phenotypes, position, and $LRT_{\max}$ (peak) with the 2-LOD (log of odds) support intervals and the overall CI for all traits from the lowest $-2LOD$ to the highest $+2LOD$ are shown¹

QTL	Phenotype	Position (bp)	Peak	$-2LOD$ (bp)	$+2LOD$ (bp)	CI start (bp)	CI end (bp)
18.7	pCE	62,692,824	27.74	62,624,960	62,738,976	62,624,960	64,521,568
	pCE	63,143,652	76.62	63,108,024	63,390,548		
	pCE	64,453,288	26.28	64,397,252	64,521,568		
	pSB	63,143,652	44.16	63,108,024	63,390,548		
19.1	pCE	31,288,920	21.80	31,072,674	33,523,396	31,072,674	33,523,396
19.2	NR56-h	33,960,888	21.96	32,801,004	34,759,236	32,801,004	36,654,424
	NR56-h	35,699,856	20.12	35,082,160	36,654,424		
21.1	CTFS	69,251,120	23.34	68,542,840	70,103,392	68,542,840	70,103,392
22.1	FSTC-c	54,308,968	23.06	53,962,952	54,892,716	53,962,952	54,892,716
22.2	FSTC-c	60,042,956	23.56	59,473,456	60,094,776	59,473,456	60,094,776
23.1	DO	29,704,122	19.54	28,638,672	30,068,122	28,362,210	30,068,122
	CTFS	29,704,122	20.82	28,362,210	30,068,122		
23.2	FSTC-h	45,295,144	20.78	44,735,156	45,768,732	44,735,156	45,768,732
23.3	NR56-c	47,447,552	20.52	47,162,736	48,110,124	46,490,824	49,534,016
	NR56-h	47,447,552	25.50	47,236,176	48,580,288		
	NR56-h	49,045,904	21.28	46,490,824	49,534,016		
23.4	FSTC-h	47,391,664	28.58	47,236,176	48,110,124	47,236,176	48,110,124
25.1	mCE	11,815,922	25.42	11,608,013	11,949,150	11,608,013	12,059,154
	mCE	11,902,050	23.34	11,874,838	11,949,150		
	mCE	12,002,861	21.94	11,949,150	12,059,154		
25.2	mCE	20,633,582	26.38	19,530,082	20,938,330	19,446,734	27,005,838
	mCE	21,377,844	23.98	19,446,734	22,863,676		
25.3	pSB	25,827,850	19.58	25,650,408	27,005,838	25,650,408	27,005,838
26.1	FSTC-c	7,293,437	25.42	7,038,682	8,630,166	7,038,682	8,630,166
26.2	DO	28,501,052	20.12	27,058,344	29,789,824	27,058,344	29,789,824
27.1	DO	8,815,920	20.18	7,699,264	9,295,928	7,699,264	9,295,928
27.2	NR56-c	12,656,990	21.18	11,728,440	12,806,370	11,728,440	12,806,370
29.1	DO	22,585,872	21.08	21,958,846	23,955,240	21,958,846	23,955,240

¹Days from first service to conception of heifers (FSTC-h) and cows (FSTC-c), nonreturn rate on d 56 of heifers (NR56-h) and cows (NR56-c), days from calving to first insemination (CTFS), days open (DO), paternal (direct) calving ease (pCE), maternal (indirect) calving ease (mCE), paternal stillbirth (pSB), and maternal stillbirth (mSB). LRT = likelihood ratio test statistic.

²This QTL overlaps with the neighboring QTL but does not correlate with any of its peaks. As the exact 2-LOD interval would include this noncorrelated QTL, the lowest LRT value between the 2 QTL (11.26) was chosen instead of the actual 2-LOD value ($LRT < 10.59$).

and Figure 6 for model 4 and model 3, the LRT values declined below the significance threshold ($P < 10^{-5}$). However, it is obvious that model 3 corrected more effectively than model 4.

For pCE (Figure 5) at 59,179,424 bp, model 4 still showed a LRT value of 10.979, whereas in model 3 the LRT value was reduced to 2.16. A similar result could be shown for pSB, where model 4 was able to correct the LRT value at 59,732,548 bp to 2.58 and model 3 corrected it to 0.72 (Figure 6).

Results of Model 5—Excluding rs43072554 from cLDLA

In model 5, SNP rs43072554, which was identical with SNP 20 of the most significant 40-SNP window, was excluded from the analyses. Compared with model 2, the values changed significantly and dropped by ~ 70 LRT units (from 160.92 to 93.26) for pCE and also by ~ 41 LRT units (from LRT 102.08 to 60.66) for pSB at $LRT_{\max}$ (59,179,424 bp). Figure 7 compares the results

of model 2 and model 5 between 40 and 65 Mb on BTA18. Results overlap between 40 and 53 Mb but begin to deviate from each other when the sliding windows reach the point where rs43072554 (58,696,066 bp) was excluded.

Results of MLMA#2: Additional MLMA with a Virtual SNP

The SNP created for each animal based on the diplo-type status at QTL 18.4 (Q1/Q1, Q1/q1, or q1/q1) had an even higher effect (P -value: 2.87×10^{-42}) of about 5 decimal places compared with the initially most significant SNP rs109478645 (P -value: 4.07×10^{-37}).

DISCUSSION

Discussion of Whole-Genome Results

With a testing population of 2,527 Holstein bulls, we were able to detect 50 QTL. To verify which of

Table 4. Correlations between investigated traits¹

VIT/LRT _{max}	NR56-h	NR56-c	FSTC-h	CTFS	FSTC-c	DO	pCE-h	mCE-h	pSB-h	mSB-h
NR56-h	1.2%	0.83	0.76	0.23	0.49	0.38	0.45	0.56	0.49	0.49
NR56-c	0.70	1.5%	0.75	0.40	0.76	0.62	0.50	0.57	0.57	0.57
FSTC-h	0.71	0.49	1.4%	0.40	0.71	0.58	0.60	0.62	0.77	0.77
CTFS	-0.09	-0.23	0.19	3.9%	0.71	0.90	0.53	0.40	0.43	0.43
FSTC-c	0.42	0.6	0.64	0.44	1.0%	0.93	0.58	0.53	0.64	0.64
DO	0.20	0.21	0.49	0.85	0.84	2.6%	0.55	0.46	0.54	0.54
pCE-h	0.11	0.12	0.09	0.19	0.19	0.22	4.8%	0.66	0.87	0.63
mCE-h	0.06	0.07	0.08	-0.05	0.14	0.06	0.04	3.9%	0.67	0.81
pSB-h	0.02	0.04	-0.03	-0.02	0.04	0.01	0.73	0.06	2.7%	0.72
mSB-h	0.14	0.19	0.20	0.06	0.29	0.21	0.05	0.71	0.05	5.4%

¹Above the diagonal, Spearman's rank correlations between the traits at 59,179,424 bp (LRT_{max}) are reported. LRT = likelihood ratio test statistic. The diagonal contains heritabilities (Vereinigte Informationsysteme Tierhaltung, Verden, Germany; VIT), whereas the genome-wide correlations provided by VIT are shown under the diagonal. Nonreturn rate for 56 d (NR56) and days from first service to conception (FSTC) of heifer (-h) and cow (-c), days open (DO), days from calving to first insemination (CTFS), maternal (m) or paternal (p) calving ease (CE) and stillbirth (SB) are shown.

the detected QTL were already known, we searched the literature and available databases (e.g., Cattle-QTLdb, The Bovine Genome Database). To be able to compare our results with previous results that had been mapped to now obsolete genome maps, we transferred all QTL positions to UMD3.1 using ensembl and Arkdb. The UMD3.1 positions of all previous QTL that were mapped to the same region as the QTL in our

study are given in Supplemental Table S1 (<https://doi.org/10.3168/jds.2016-11506>).

In total, 6 out of the 50 QTL from this study have been published recently for the same traits (Supplemental Table S1; <https://doi.org/10.3168/jds.2016-11506>). Therefore, we were able to map 44 new QTL that, based on the literature and databases searches, have not been described for Holstein before. Our discussion

Table 5. Mixed-linear-model association (MLMA) analysis results for paternal calving ease (pCE) and stillbirth (pSB) on BTA18¹

SNP	SNP rs no.	Position (bp)	P-value (pCE)	P-value (pSB)	QTL (if overlapping and the same trait)
ARS-BFGL-BAC-36979	rs110265528	53,571,368	1.23E-05	<u>9.59E-06</u>	
ARS-BFGL-NGS-7458	rs41897446	55,590,144	<u>7.83E-07</u>	<u>7.01E-06</u>	QTL 18.4
ARS-BFGL-NGS-93837	rs110389036	55,807,264	<u>7.92E-09</u>	<u>5.59E-07</u>	QTL 18.4
ARS-BFGL-NGS-25104	rs109341458	56,364,657	<u>2.46E-11</u>	<u>8.53E-10</u>	QTL 18.4
ARS-BFGL-NGS-104774	rs108984194	56,561,695	<u>1.14E-18</u>	<u>7.45E-15</u>	QTL 18.4
Hapmap39335-BTA-43845	rs41582494	56,642,741	<u>3.48E-06</u>	<u>2.46E-06</u>	QTL 18.4
Hapmap43854-BTA-43847	rs41636742	56,702,312	<u>7.91E-08</u>	1.35E-05	QTL 18.4
ARS-BFGL-NGS-98028	rs41636749	57,174,711	<u>9.43E-21</u>	<u>3.63E-15</u>	QTL 18.4
ARS-BFGL-NGS-25117	rs42843551	57,238,311	<u>1.63E-14</u>	<u>1.41E-10</u>	QTL 18.4
BTA-97501-no-rs	rs41665732	57,565,406	<u>1.08E-10</u>	<u>1.01E-08</u>	QTL 18.4
ARS-BFGL-NGS-109285	rs109478645	57,589,121	<u>3.92E-37</u>	<u>2.42E-30</u>	QTL 18.4
ARS-BFGL-NGS-6380	rs41890787	57,788,407	<u>6.12E-07</u>	1.30E-05	QTL 18.4
ARS-BFGL-BAC-2333	rs109882115	58,067,310	<u>1.39E-14</u>	<u>2.53E-11</u>	QTL 18.4
ARS-BFGL-NGS-66925	rs109032416	58,260,521	<u>2.84E-11</u>	<u>1.83E-11</u>	QTL 18.4
BFGL-NGS-112752	rs109173807	58,352,626	<u>1.34E-13</u>	<u>2.01E-11</u>	QTL 18.4
Hapmap45189-BTA-43948	rs41636786	58,404,041	<u>4.39E-09</u>	<u>7.94E-09</u>	QTL 18.4
Hapmap40906-BTA-121147	rs41664920	58,551,307	<u>1.42E-16</u>	<u>6.64E-16</u>	QTL 18.4
Hapmap40537-BTA-43945	rs41582522	58,666,276	<u>2.05E-08</u>	<u>1.59E-08</u>	QTL 18.4
BFGL-NGS-116069	rs42550256	59,802,319	<u>1.12E-09</u>	<u>2.27E-07</u>	QTL 18.4
BFGL-NGS-118325	rs109530681	60,103,953	<u>1.00E-15</u>	<u>6.83E-12</u>	QTL 18.4
ARS-BFGL-NGS-39594	rs110889414	60,135,318	<u>2.22E-15</u>	<u>5.81E-12</u>	
ARS-BFGL-NGS-17877	rs109998463	60,377,508	<u>3.58E-07</u>	<u>1.12E-07</u>	
ARS-BFGL-NGS-107318	rs110912084	61,024,520	<u>2.11E-06</u>	8.31E-05	
BFGL-NGS-113023	rs109374253	61,156,737	<u>6.09E-10</u>	<u>2.31E-07</u>	
ARS-BFGL-NGS-49873	rs109118771	61,267,887	<u>4.51E-08</u>	1.47E-04	
ARS-BFGL-NGS-1786	rs41900248	62,571,431	<u>2.44E-06</u>	<u>7.61E-07</u>	
ARS-BFGL-NGS-103214	rs110508656	63,096,692	<u>5.87E-14</u>	<u>4.52E-15</u>	QTL 18.7
ARS-BFGL-NGS-17826	rs41902064	64,286,141	<u>5.59E-06</u>	6.60E-04	
Sum of significant ($P < 10^{-5}$) SNP			27	23	20 SNP overlapping with QTL

¹All significant SNP ($P < 10^{-5}$) of MLMA#1 are underlined. In cases where the SNP is located within the CI of a QTL, the overlapping QTL is listed in the right column.

Figure 2. Comparison of the results for paternal calving ease in mixed linear models 1 and 2 and in the mixed-linear-model association analysis (MLMA) #1. Model 1 (solid line) covers all SNP markers used. In model 2 (dotted line with circles), rs109478645, which is the genome-wide most significant SNP in MLMA#1, is excluded. Due to the very similar likelihood ratio test statistic (LRT) values of model 1 and model 2, both curves overlap for the bigger part of the region. MLMA#1 [dash-dotted line with triangles, here unit = $-\log(P)$] shows the location of the most significant SNP. For positional orientation, QTL 18.1 to 18.7 are indicated below the x-axis. As some of the QTL were found for other traits besides paternal calving ease, this figure does not show a peak at each QTL.

will focus on certain outstanding QTL: some known and also some newly identified QTL for which possible candidate genes were examined.

Examples of Known QTL

On BTA5, 3 QTL for twinning that overlapped with QTL 5.2 for pCE and pSB were reported in the literature (Cruickshank et al., 2004: 53,296,546–62,138,446 bp; Kim et al., 2009: 62,125,530–71,561,898 bp; Meuwissen et al., 2002: 62,125,683–62,138,446 bp). As has been known for several years, twin pregnancies come with an increased risk of calving difficulties (Rutledge, 1975) and stillbirth (Hosseini-Zadeh et al., 2008). The QTL 5.3 for pCE and pSB also overlapped with 2 QTL mapped for twinning (Kim et al., 2009: 62,125,530–71,561,898 bp; Lien et al., 2000: 66,532,877–71,561,898 bp) and lay next to a third QTL for twinning (Kim et al., 2009:

69,000,000 bp). However, because the data set that is used for estimating the EBV by VIT routinely excludes twin births, the relation between twinning and pCE and pSB can be excluded as a direct cause of our mapping results. However, QTL 5.2 also overlapped with QTL for body height and birth weight that were both mapped at the same position (Schrooten et al., 2000: 66,532,877–97,454,531). The risk factors for dystocia were summarized in a review by Mee (2008) who also pointed out that an excessive birth weight leads to an increased risk. The strong correlation (0.9) between birth weight and calving ease has been known for over 30 yr (Philipsson et al., 1979). It is consequently not surprising that QTL for both traits can be mapped to an identical chromosomal region.

On BTA7, 2 QTL for paternal calving ease and stillbirth (Kühn et al., 2003: 30,308–8,193,566 bp) were found to overlap with QTL 7.1 for pCE and pSB.

Figure 3. Distribution of the alleles Q1 and q1 in diplotype effect groups (displayed on the x-axis). The dotted black line with triangles shows the sum of the haplotype Q1 alleles in each group of diplotype effects. The dash-dotted gray line with diamonds summarizes all other haplotypes (q1) and the solid black line with circles indicates the total frequency of all haplotypes (Q1 + q1) spread over the classes of diplotype effects.

The literature reported a QTL on BTA19 for paternal calf size, pCE, mCE, and calving index (Sahana et al., 2011: 25,837,047–41,372,598 bp) that overlapped

with QTL 19.1 for pCE. Calf size and calving ease have also been subject of recent research (Cole et al., 2009, 2014b). The general relation between calving ease, still-

Figure 4. Model 3 for paternal calving ease on BTA18 (solid line), which includes haplotype Q1 as fixed effect, compared with model 1 (dotted line). LRT = likelihood ratio test statistic.

Figure 5. Model 4 (solid line), which includes the most significant mixed-linear-model association analysis (MLMA) SNP rs109478645 (57,589,121 bp) as fixed effect, compared with model 3 (dotted with circles), which includes haplotype Q1 as a fixed effect, for paternal calving ease. LRT = likelihood ratio test statistic.

Figure 6. Model 4 (solid line), which includes rs109478645 (57,589,121 bp, the most significant SNP in the mixed-linear-model association analysis) as fixed effect, compared with model 3 (dotted with circles), which includes haplotype Q1 as a fixed effect, for paternal stillbirth. LRT = likelihood ratio test statistic.

BTA18

Figure 7. Basic model 1 (dotted with circles) compared with model 5 (solid line), which excludes rs43072554 (58,696,066 bp), for paternal calving ease. LRT = likelihood ratio test statistic.

birth, and calf size has already been mentioned above and according to Menissier and Foulley (1979), the risk of dystocia increases above a range of 42 to 45 kg.

On BTA25, we found a matching QTL for QTL 25.1 that had also been mapped for mCE (Seidenspinner et al., 2009: 6,257,409–12,159,341 bp) in a similar data set (both data sets are highly related and share a high proportion of ancestors). Another match was found for QTL 25.2 for mCE. At this position, a QTL associated with paternal calf size, maternal calf size, and calving index was published previously (Sahana et al., 2011, 16,113,126–22,890,173 bp).

New QTL and Candidate Genes

For all newly identified QTL, LRT_{max} and the confidence interval were compared with a map of annotated genes in the *Ensembl* genome browser. Based on the reported function of the genes located in the 2-LOD support intervals, we identified possible candidate genes. We would like to discuss selected QTL for which outstanding candidate genes were found in the OMIM database (www.omim.org).

On BTA7, we were able to identify a gene that might explain QTL 7.1 for pCE and pSB. Relaxin 3 (**RLN3**; 12,768,811–12,770,589 bp) is located within the 2-LOD support interval of the QTL (8,978,524–13,478,592 bp).

It acts as “a hypothalamic signal to coordinate food intake, thyroid function and reproductive function” (McGowan et al., 2009). It can be hypothesized that *RLN3* influences calving ease by coordination of feed intake. Assuming that *RLN3* is expressed prenatally during the transition phase, feed intake could be depressed beyond a regular level. Because the unborn calf has its highest energy requirement shortly before calving, depression of the feed intake is a severe problem for the cow. Furthermore, the calf naturally reduces the feed intake of the dam by taking up space and putting pressure on the rumen and the surrounding guts. As reported by Ingvarsten et al. (1992), a continuous feed depression of 1.5% per week was observed in a group of heifers between 26 and 3 wk ante partum. Feed intake was reduced from 14 to 9 kg (depression of around 30%). These data are consistent with Grummer (1995) who reported a depression of 30 to 35%. Low feed intake during a phase of high energy requirements results in energy deficiency. In this catabolic state, ketone bodies accumulate in the blood stream and the cow reaches a state of clinically manifesting ketosis. For these animals suffering from acute ketosis, calving problems could be a logical consequence.

On BTA11, we were able to identify 2 possible candidate genes for the NR56-h QTL in the OMIM database: the LH receptor (*LHCGR*) and the FSH re-

ceptor (*FSHR*) gene. The *LHCGR* is located between 30,823,435 and 30,888,003 bp and *FSHR* between 31,110,744 and 31,305,197 bp. The 2-LOD support interval for the NR56-h QTL ranges from 29,431,174 to 31,530,898 bp (Table 3, QTL 11.1). The FSH induces follicle maturation in the ovaries. It creates a cohort of growing follicles of which one follicle develops into the primary follicle that has the potential to be fertilized by male germ cells. The LH, however, induces the conversion of the ruptured follicle into a corpus luteum, which is a part of the induction and recognition of pregnancy. If heifers have problems either with the LH receptor or with the FSH receptor, the follicle-maturation cascade will not work properly. The animals will not be able to conceive if either no primary follicle is able to mature, or if LH is unable to bind to its receptor to introduce pregnancy.

On BTA14, we were able to identify a candidate gene for a FSTC-h QTL (58,467,724–62,449,528 bp): the zinc finger protein multitype 2 (*ZFPM2*) gene. Our QTL 2-LOD support interval (Table 3, QTL 14.1) overlaps with the location of *ZFPM2* (60,994,139–61,520,208 bp). The *ZFPM2* is co-expressed with GATA-binding protein 4 (*GATA4*) and thus alternatively named friend of *GATA*. To prove the function of *GATA4* and *ZFPM2*, Svensson et al. (2000) and Crispino et al. (2001) created *GATA4*-deficient mice. Tevosian et al. (2000) and Svensson et al. (2000) created mice that were only deficient for *ZFPM2*. These *GATA4*- and *ZFPM2*-deficient mice all died due to cardiac insufficiency around mid-gestation between d 8.5 and 13 (Svensson et al., 2000; Tevosian et al., 2000; Crispino et al., 2001). It can thus be assumed that calves bearing a defect in this gene might also die during gestation. If embryonic death occurs at an early stage, pregnancy might not have been recognized yet, whereas a previous identification of pregnancy might be even doubted in some cases. Consequently, the FSTC timespan might be erroneously increased.

Discussion of Major Results on BTA18

Using the haplotype identified for QTL 18.4 as a fixed effect (model 3), we observed that besides QTL 18.4 itself, QTL 18.2, 18.3, 18.6, and 18.7 were also affected by QTL18.4 alleles (Q1 and q1). They might thus represent so-called carry-over effects (Meuwissen and Goddard, 2004; Olsen et al., 2004). Therefore, only QTL 18.1 (CTFS) and 18.5 (DO and FSTC-c) remain independent of the strong QTL 18.4, which is primarily associated with pCE and pSB. These results were confirmed by the estimated Spearman's correlations. The QTL associated with pCE and pSB, which completely disappeared in model 3, showed a high Spearman's correlation of 0.87 between diplotype effects of both

traits at that locus. The QTL effects for mCE, which were corrected by QTL alleles of QTL 18.4, showed medium correlations with pCE ($r_{[mCE,pCE]} = 0.66$) and pSB ($r_{[mCE,pSB]} = 0.67$) at that locus (Table 4). On the other hand, QTL effects for DO and FSTC, which both remained significant in model 3, showed relatively low correlations ($r_{[DO,pCE]} = 0.47$, $r_{[DO,pSB]} = 0.38$; $r_{[FSTC-c,pCE]} = 0.55$; $r_{[FSTC-c,pSB]} = 0.52$). Therefore, only the following 3 QTL are discussed in further detail: 18.1, 18.4, and 18.5.

Previous mapping studies with lower resolution showed that QTL 18.1 (CTFS) and 18.5 (DO and FSTC-c) both intersected with QTL for paternal calving ease of heifers (43,245,906–62,106,261 bp) and for paternal stillbirth (43,245,906–62,106,261 bp; Seidenspinner et al., 2009). Another QTL (44,853,955–55,860,871 bp) for maternal stillbirth (Thomassen et al., 2008) also overlapped with QTL 18.1. Additionally, QTL 18.5 overlapped with 4 QTL from a study by Sahana et al. (2011) for paternal stillbirth in first and later lactations, birth index, paternal calving ease for first and later lactations, and paternal calf size for first and later lactations, which had also been mapped to an interval between the same markers (rs110389036, rs110889414; 55,807,264–60,135,318 bp).

Calving ease is known to influence DO (Thompson et al., 1983; Mangurkar et al., 1984), which is a combination of CTFS and FSTC-c. This is at least partly due to the fact that the bacterial load of the uterus rises with manipulation in the course of calving assistance (Tenhagen et al., 2007; Wagener et al., 2014). The more manipulation takes place, the more the uterus is contaminated with pathogenic bacteria and the more the health status of the cow will be affected, which in turn prolongs the average FSTC timespan. As stated by Tenhagen et al. (2007), cows with severe dystocia conceive later (resulting in an elongated FSTC) and are also more likely to be open by 200 DIM. Due to the close link between these traits, stillbirth also has similar effects. When cows that suffered from stillbirth come into heat the next time, there is an elevated probability that service will not induce conception (Tenhagen et al., 2007), which in turn leads to an elongated DO timespan. Therefore, QTL for maternal stillbirth and CTFS are at least partly connected. However, the comparison of model 1 and model 3 results proves that QTL 18.1 and 18.5 are independent from QTL 18.4 and thus provide another important piece for resolving the BTA18 fertility puzzle in the near future.

In addition to previously described QTL for stillbirth and calving ease in the region of QTL 18.4, the literature search also revealed important correlations between stillbirth and calving ease. In the German and American Holstein population, Cole et al. (2014b)

estimated BTA18-specific genetic correlations between pCE and pSB of 0.63 and 0.56, respectively (VIT predicted $r = 0.73$ genome-wide). We estimated an even higher positive Spearman's correlation (0.87) of diplotype effects for both paternal traits (pCE and pSB) at the putative QTL 18.4. The fact that the genetic correlations estimated in this study at 59,179,424 bp on BTA18 outperformed even those that were estimated by Cole et al. (2014b) for the whole chromosome is not surprising. The fact that the locus-specific correlation exceeds the genome-wide correlations indicates that on a genome-wide level, more regions have an influence on these traits and the majority of them affect both traits conjointly.

For the same region, Cole et al. (2009) reported a positive correlation ($r_{[pCE-mCE]} = 0.81$) between paternal and maternal effects for CE and SB. The positive correlation suggests a QTL with pleiotropic and aligned effects (pointing to the same direction) on both the paternal and maternal traits of CE and SB. Our results agreed with Cole et al. (2009) because we also detected a peak for mCE in the same sliding window where the major peaks for pCE and pSB were found. Moreover, we also estimated strong positive Spearman's correlations of 0.66 between mCE and pCE diplotype effects at the LRT peak (59,179,424 bp, $LRT_{max} = 160.92$) for pCE.

Calving ease and stillbirth are genetically associated because larger calves are at increased risk to be born with more difficulties (Hansen et al., 2004). This is supported by the fact that compared with calves of normal weight, a higher percentage of big calves die during or shortly after calving as a result of the long-lasting and exhausting delivery (Cooke et al., 2009; Roffeis and Krehl, 2011). Genome-wide genetic correlations between maternal and paternal calving traits of zero or even negative values, on the other hand, could be explained by the widely accepted assumption that calves with a high birth weight, which is generally associated with a greater risk of dystocia, grow in larger heifers that usually experience fewer difficulties during delivery (Waurich, 2013; for stillbirth see p. 11, Table 1, and for calving ease see p. 13, Table 2). Quite contrary to the genome-wide expectation, the corresponding locus Spearman's correlation between pSB and mSB is 0.72. The discrepancy between the negative genome-wide and the positive locus-specific correlations of paternal with maternal calving traits suggests that this QTL deviates from mainstream effects. The theory that haplotype Q1 might at least partly be responsible for maternal calving traits in this region is thus confirmed. The assumption of a partial effect on maternal traits is further supported by the fact that model 3 reduced the LRT value at QTL 18.2 by about two-thirds. A

more detailed investigation of haplotype Q1 should enable the identification of the causal variant(s) for pCE and pSB and thus help investigate the residual variance more precisely, for example, by improving mapping results for QTL that segregate independently of 18.4 (QTL 18.1 for CTFS and QTL 18.5 associated with FSTC-c and DO). Uncovering these haplotypes and their causal variants will therefore need additional research. The same applies to improved QTL mapping in other genomic regions and for related traits (e.g., Holmberg and Andersson-Eklund, 2006).

Discussion of the Most Outstanding Candidate Region

On a genome-wide level, the most outstanding candidate region is the 2-LOD support interval of QTL 18.4 (between 55,282,968 and 60,119,636 bp). Referring to the same region, Cole et al. (2009) are convinced "that there is a QTL on BTA18 that has significant effects on body size, dystocia, longevity, and economic merit. The QTL results in high calf birth weights." They also agree with Meijering (1984) and Mee (2008) that high birth weights, which are associated with a greater risk of dystocia, have a negative effect on fertility and the animals' lifetime economic merit. In 2009, Cole et al. (2009) showed that rs109478645 (BTA18: 57,589,121 bp) was the most significant SNP for the following traits: body depth, maternal CE, maternal SB, rump width, paternal CE, paternal SB, stature, and strength.

Cole et al. (2011) identified rs43072554 (BTA18: 58,696,066 bp) as the most significant SNP for pSB, the second most significant SNP for mCE, and the fourth most significant SNP for pCE. This SNP was identical with SNP 20 of the most significant 40-SNP window and was also confirmed as the most significant SNP by our mapping results. However, Cole et al. did not replicate this association in their later studies. In 2014, a GWAS that focused only on birth weight identified rs109882115 (BTA18: 58,067,310 bp) as the most significant SNP. However, the previously detected SNP rs109478645 was confirmed as the third most significant SNP in the generalized linear model and the most significant in the mixed linear model. Furthermore, it was found to be in high linkage disequilibrium with rs109882115. Consequently, Cole et al. (2014b) again declared rs109882115 to be the SNP with the highest association with the effects.

Moreover, Maltecca et al. (2011) found rs109478645 to be the most significant SNP for direct gestation length in the American Holstein population. This SNP is located in an intron of *SIGLEC-5* (BTA18: 57,938,695–57,953,490 bp). *SIGLEC-5* binds to the mouse leptin

receptor. High expression levels of *SIGLEC-5* could result in a deficiency of leptin and thus affect gestation length, which in turn leads to larger fetuses (Cole et al., 2009). Similar connections could also be made for higher birth weights in cattle fetuses as suggested by Cole et al. (2014b). Because big calves are more likely to cause problems during birth, the connections and mutual influences between calving ease, stillbirth, and birth weight are obvious (Mee, 2008; Roffeis and Krehl, 2011; Sahana et al., 2011). A long list of published studies (see Waurich, 2013; for stillbirth see p. 11, Table 1, and for calving ease see p. 13, Table 2) explains the nonexistent to negative genetic correlations between maternal and paternal calving ease and stillbirth. However, QTL 18.4 with $r_{[\text{PSB-mSB}]} = 0.72$, $r_{[\text{pCE-mCE}]} = 0.66$, and $r_{[\text{pCE-mSB}]} = 0.63$ is fairly different from genome-wide mainstream correlations between these maternal and paternal traits (Table 4). Therefore, other reasons for stillbirth or dystocia besides high birth weight should also be considered. Studies by Burfening et al. (1981), McClintock et al. (2003), Jamrozik et al. (2005), and Van Pelt et al. (2009) described moderate to strong positive genome-wide correlations between maternal calving ease and birth weight. They also indicated the existence of genetic variants with a maternally induced positive effect on prenatal growth at a magnitude that, in turn, is problematic for the cow itself when calving (Waurich, 2013, p. 16).

Cole et al. (2014b) replicated the association of rs109478645 with stillbirth from 2009 and also mapped 4 additional SNP with a large influence on this trait: rs109490807 (at 55,309,510 bp near *LIG1*), rs41665732 (at 57,565,406 bp near *CD33*), rs109882115 (58,067,310 bp near *VOM1R1*) and BTB-01438985 (60,258,991 bp, located in a large cluster of genes). Additionally, the authors' sequence data suggested that rs109478645 might be misplaced on UMD3.1 (Cole et al., 2014a). Within the range of our haplotype (58,157,343–60,978,019 bp), the genes *ZNF432*, *ZNF614*, *ZNF350*, *PPP2R1A*, *VN1R4*, and *ZNF877* were identified as positional candidate genes. None of these genes have been linked to calving problems or similar traits so far. *PPP2R1A* (Shih et al., 2011) and *ZNF350*, which is also called *ZBRK1* (Lin et al., 2013), are known to be associated with breast-ovarian cancer, ovarian clear cell carcinoma, and uterine carcinomas and are thus at least associated with reproductive tissues.

In summary, these results indicate that there has to be a region with major influence on calving in Holstein dairy cows on BTA18. However, despite considerable efforts, a causal variant within the location favored by Cole et al. (2009, 2014b; 57,589,121 bp) has not been identified yet. Consequently, the QTL position should

be reassessed. Our haplotype-based design, suggested a slightly different QTL position and rather confirmed the results of Cole et al. from 2011 than those of 2009 and 2014 (Cole et al., 2009, 2011, 2014a).

BTA18: MLMA Compared with cLDLA

To improve the discussion on the correct QTL position and to compare cLDLA results with a commonly used association analysis, we carried out a genome-wide MLMA analysis within the same data set. Similar to Cole et al. (2009), the strongest association in MLMA#1 was found between pCE and rs109478645. Moreover, SNP rs109478645 was verified to be one of the most significant SNP in the GWAS studies for calving traits of, for example, Sahana et al. (2011), Höglund et al. (2012), and Purfield et al. (2014), and for direct gestation length, which certainly has a strong influence on calving ease, in Maltecca et al. (2011). Similar results close to this position were also obtained by older studies using microsatellite markers [e.g., Kühn et al. (2003), Thomasen et al. (2008), and Seidenspinner et al. (2009)].

All in all, the cLDLA (model 1) results were identical to the main results of MLMA#1. Because we were also able to replicate QTL that had been mapped with other GWAS methods, we are convinced that the cLDLA is a reliable method for QTL mapping. To allow for better comparison of the cLDLA and MLMA mapping results and their soundness, we conducted additional cLDLA (models 2–5) and an additional MLMA (MLMA#2) analyses. Model 2 formally demonstrated that the exclusion of the most significant SNP (rs109478645) does not have an effect on haplotype-based mapping results. Model 3 included haplotype Q1 as a cofactor and clearly pointed out the presence of large and partly distant carry-over effects of this strong QTL. It was thus demonstrated that appropriate cLDLA modeling is capable of reducing the number of false positives that are caused by distant carry-over effects. In general, the presence of high LD between an unknown underlying quantitative trait nucleotide (QTN), which is embedded in an old (common) haplotype, and some relatively distant SNP could result in a discrepancy between the cLDLA and MLMA results. Moreover, because a single haplotype (Q1) represents QTL allele 1 and the remaining 363 haplotypes represent QTL allele 2 in model 3, it can be suggested that QTL allele 1 is the derived allele (Q1) and QTL allele 2 is ancestral (q1). Furthermore, model 4 met our expectations and verified that the alleles of the most significant SNP in MLMA#1 (rs109478645) are in LD with the QTN as the modeled fixed effects were able to correct the effect seen for pCE (and other traits) along the entire region.

Nevertheless, the comparison of model 3 with model 4 suggested a higher association between the putative QTN and Q1 than between the QTN and the alleles of the most significant SNP (the LRT of model 3 is 2.16, the LRT of model 4 is 10.979, Figure 5). These results were supported by the results of MLMA#2 that showed a 5-decimal lower P -value (2.87×10^{-42}) for the modeled Q1-SNP compared with the most significant SNP rs109478645 (4.07×10^{-37}).

Model 5 pointed out that the SNP rs43072554, which was the genome-wide most significant SNP for pSB, the second significant SNP for mCE and the fourth significant SNP for pCE (Cole et al., 2011), had a much larger influence in the cDLA than the SNP rs109478645, which was the genome-wide most significant SNP in Cole et al. (2009, 2014b). As described in the results and seen in Figure 2 (model 2) and Figure 7 (model 5), exclusion of SNP rs43072554 changes the shape of the LRT statistic curve. However, although the LRT statistic is significantly depressed at $LRT_{\max}$, it still remains clearly significant with a $LRT_{\max}$ value of 99.16 (P -value of 4.59×10^{-22}).

Because the influence of this QTL region on calving traits has been known for years, a lot of research has already been conducted to identify the causal variant (Kühn et al., 2003; Cole et al., 2009, 2014b; Brand et al., 2010, for direct gestation length; Thomasen et al., 2008; Seidenspinner et al., 2009; Maltecca et al., 2011; Sahana et al., 2011; Purfield et al., 2014). It is thus quite striking that the causal variant still remains unknown. However, as the peaks for pCE in model 1 and MLMA#1 are about 1.5 Mb apart, it can be hypothesized that a possible failure in identifying the causal variant could be due to examination of the wrong chromosomal segment. Another obstacle in detecting the causal QTN could be the lack of a precise local assembly. Cole et al. (2014a) stated that the sequenced ~8-kb region downstream of rs109478645 appeared to be misassembled and that the SNP seemed to be misplaced. Consequently, some SNP in the candidate region might be misplaced on UMD 3.1. Our results suggested that the cDLA approach, which is based on reconstructed haplotypes, is less sensitive to the absence of error-free marker maps and perfectly associated SNP than the GWAS. For reasons of higher accuracy, we therefore prefer haplotype-based methods like the cDLA over single SNP-based GWAS approaches for QTL mapping. Other methods such as resampled predictive models (e.g., Biffani et al., 2015) or run of homozygosity-based approaches (e.g., Biscarini et al., 2014) also aim to detect more robust association signals than the GWAS. These methods as well as the cDLA approach should thus be considered as complementary approaches to the standard GWAS in cattle.

CONCLUSIONS

With a cDLA-based QTL mapping approach, we were able to detect 50 significant QTL. Our results partly confirmed previous findings and, moreover, identified new QTL. The most significant QTL was found for pCE on BTA18 in a QTL region that has already been mapped by other authors (Kühn et al., 2003; Thomasen et al., 2008; Cole et al., 2009, 2014b; Seidenspinner et al., 2009; Brand et al., 2010; Maltecca et al., 2011; Sahana et al., 2011; Purfield et al., 2014). However, we mapped the most probable position of this QTL to a location about 1.5 Mb away from the published position at rs109478645 (Cole et al., 2009, 2014b). In additional analyses that also included a GWAS using the MLMA method, we showed that this difference was due to the chosen method and that the cDLA results seem to be superior to those of GWAS. Furthermore, we identified the presumably harmful haplotype. Despite a lot of research that has been done on this QTL so far, the causal variant has still not been successfully identified. It can thus be hypothesized that a false chromosomal segment or some misassembled SNP (or both) in the candidate region were examined in earlier studies. Our results add important new facts to the current knowledge and will contribute to an efficient identification of the causal variant(s). Until then, they enable haplotype-based selection against the most harmful haplotype for pCE.

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