Association between Single Nucleotide Polymorphism in RelA with Somatic Cell Count and Longevity Supports Importance of NF-κB Signalling in Cattle Health

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Abstract

Mastitis reduces milk production and causes culling. The NF-κB transcription factor RelA plays a central regulatory role in innate immunity. This study used a candidate gene approach to investigate associations between the synonymous C/G SNP rs48035703 in RELA with somatic cell count (SCC) and survival time. Blood samples were collected from 337 Holstein-Friesian heifers on 19 farms and genotyped by PCR-restriction fragment length polymorphism. Animals were monitored from 6 months until 2340 d of age. Pedigree, milk production and disease records were obtained. Genotype frequencies were CC 0.63, CG 0.30 and GG 0.06. The C allele had a favourable additive effect on survival: average longevities from birth were CC, 1872 d; CG, 1745 d and GG 1596 d (P < 0.003). Log transformed first lactation somatic cell count (SCC) data showed a significant association with this SNP using an allele substitution model (mean residuals ± SD: GG 0.30 ± 1.263; CG 0.22 ± 0.994, CC −0.04 ± 0.803, P < 0.05). More CC cows than expected were classified as intermediate and fewer as mastitic (30.4% v 45.9%) with respect to SCC class when categorised as 0 (unaffected), 1 (intermediate) and 2 (mastitic), whereas for CG heterozygotes fewer were intermediate and more were mastitic (12.1% v 60.3%) (p = 0.05). RELA rs48035703 CC genotype cows were therefore less likely to experience a high SCC and survived longer. These results support a role for RelA in combating mammary gland infection and warrant further studies in additional populations.

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1. Introduction

Mastitis is an inflammatory condition of the udder, most commonly due to bacterial infection. It causes major economic loss worldwide through reduced milk production, infertility and early culling and can be fatal in severe cases [1] [2] [3]. Environmental pathogens such as *Escherichia coli* and *Streptococcus uberis* can invade the gland, multiply, induce a rapid host response and are then generally eliminated [4] [5]. The inflammation is associated with a distinct acute phase response, which is accompanied by a reduction in milk yield, altered milk composition and damage to mammary tissue. Alternatively, contagious organisms, in particular *Staphylococcus aureus*, can survive in the udder and establish a chronic sub-clinical infection which manifests as an increase of somatic cells in the milk [6].

The different species of bacteria therefore elicit differential responses by the host. Gram negative bacteria, in particular *E. coli*, activate the TLR4 transduction pathway via lipopolysaccharide (LPS), causing inflammatory and apoptotic responses [6]. The severity of the ensuing disease is influenced mainly by the ability of the cow to control bacterial growth in the first few hours after infection [7], although the rapidity with which leukocytes are recruited into the affected quarter may also be important [2] [6]. The cow is less able to mount an effective immune response in the early postpartum period [8]. In contrast, *S. aureus* infection elicits a slower response and some key cytokines including TNFα and IL1 are barely up-regulated [9]. All toll-like receptor (TLR) signal transduction pathways eventually activate members of the NF-κB family of transcription factors [10]. Early phase NF-κB responses involve MyD88 (myeloid differentiation primary-response protein 88) dependent pathways, whereas later responses utilize MyD88-independent pathways. *S. aureus* probably acts through MyD88-independent NF-κB activation. NF-κB is known to be a central orchestrator of immune defence and many pro-inflammatory genes (e.g. *IL1B, IL6, IL8, TNFA*) have NF-κB attachment sites in their promoter.

The NF-κB family comprises five structurally homologous transcription factors p50 and p52 (which are activated by proteolysis of the larger precursors p105/NF-κB1 and p100/NF-κB2), RelA (also known as p65), RelB and c-rel. They all carry a Rel homology domain which contains a nuclear localization signal and they bind to κB enhancers as homo- or hetero-dimers [11]. The NF-κB members are usually present in an inactive form in the cytoplasm, complexed with members of a family of inhibitory IκB proteins. The IκB protein is degraded during pathway activation allowing the NF-κB complex to enter the nucleus to activate gene transcription [12] ([Figure 1](#)). The precursor proteins NF-κB1 and NF-κB2 also function as IκB-like molecules, which inhibit the nuclear translocation of associated NF-κB members [11].
The RELA gene drives pro-inflammatory responses by promoting the expression of inflammatory cytokines. It also maintains autocrine IFN-β signalling in uninfected cells, facilitates inflammatory and adaptive immune responses following infection, and promotes infected-cell survival during this process [13]. RelA is known to play a key role in mastitis. An elegant study by Notebaert et al. [14] utilized real-time imaging of NF-κB activation in response to mammary gland.
infection in transgenic mice. This experiment showed that RELA mRNA expression peaked at 6 - 8 h post infection with *E. coli*, returning to basal by 24 h. This expression was nearly all located in mammary epithelial cells as there was little contribution from neutrophils at this early stage. The evidence suggested induction of RelA homodimers rather than classic p65/p50 heterodimers. Maximal systemic NF-κB activation in liver also occurred at 6 - 8 h, together with increased circulating concentrations of serum amyloid A and TNFα. In cattle, Chen *et al.* [15] measured expression of NF-κB pathway genes in blood and a selection of tissues taken from Chinese Holstein cows and found higher RELA expression in animals with mastitis caused by *S. aureus*. Sun *et al.* [16] tested the ability of LPS binding protein to block LPS-induced apoptosis of bovine mammary epithelial cells. In this system TLR4 and RelA protein were both down-regulated and NF-κB transcription activity was weakened.

In the cow, the gene for RELA is located at 44.5 Mb on BTA29. It is highly polymorphic with 166 missense SNP, four nonsense SNP and one frameshift mutation reported ([https://www.ncbi.nlm.nih.gov/snp](https://www.ncbi.nlm.nih.gov/snp)). The frequency with which the majority of these SNP are present in cattle populations has not, however, been determined. Despite the central role for RelA in the immune response to mastitis, the majority of quantitative trait loci (QTL) and genome wide association studies (GWAS) have not identified a marker in this region associated with SCC, udder development or udder health ([17] [18] [http://www.animalgenome.org/](http://www.animalgenome.org/)). Meredith *et al.* [19] did, however, find that a SNP rs110252785 at 44.6 Mb, was significantly associated with milk protein percent. More recently, Durán Aguilar *et al.* [20] undertook a GWAS for somatic cell score (SCS) in Holsteins using copy number variation (CNV) as marker. This approach yielded 18 candidate genes which included RELA. The CNVR_1640_P located on BTA29 extended from 44.41 to 44.50 Mb and was associated with eight genes including RELA and *MAP3K11*. The latter is part of the MAPK family, which are also key signalling molecules in innate immunity, controlling both pro- and anti-inflammatory processes including cytokine production in an orderly manner [21].

We undertook this study as part of a candidate gene approach focusing on genes which are of known importance in innate immunity. Their potential genetic associations with milk production, disease and survival times were investigated in a starting population of Holstein-Friesian cows in which extensive phenotypic data were obtained from birth until death or culling. Information on the animals and farms has been described previously [22] [23].

2. Material and Methods

2.1. Animals and Farms

All procedures involving animals were carried out under the Home Office Animals (Scientific Procedures) Act 1986 and approved by the Royal Veterinary College’s (RVC) Ethics and Welfare Committee. Briefly, samples and data from Holstein-Friesian heifers born between August 2003 and October 2004 on 18 commercial UK dairy farms and one primarily research farm were used. Each
farm had a four-month enrolment period during which all consecutively live-born dairy heifers were recruited. The mean cohort size was 24 animals per farm, range 15 to 30. These farms provided a variety of management practices representative of those commonly encountered on dairy farms in the UK. Whole blood samples used in this study for SNP identification (10 ml into heparinized vacutainers, BD Vacutainer System, Devon, UK) were collected from 337 Holstein heifers at 6 months of age and stored at −20°C. For each cow, pedigree information for the preceding three generations was collected from the Holstein UK website (www.ukcows.com), creating a total pedigree containing 2251 animals of which there were 93 sires and 181 maternal grandsires.

Once these animals had calved data were obtained through the herd management software on milk production traits (yield and SCC from monthly records provided by commercial milk recording services), fertility, health, and longevity. The number of records used in each analysis varied depending on the availability of information for each trait and the longevity of the individual cow. Animals were monitored until 2340 d (6.4 yr) or until the time of death or culling if that occurred earlier. Reasons for culling were obtained where possible and classified into the following categories: infertility, abortion, mastitis/high SCC, legs/feet/lameness, other illnesses (e.g. abscess, displaced abomasum), or no reason for culling given. Some animals were placed in more than one category, for example having a combination of poor fertility and high SCC [23].

2.2. SNP Selection and Genotyping

All reagents were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) unless otherwise stated. The method used for SNP identification has been described previously [24]. DNA from blood samples was extracted using a Flexi-Gene DNA Kit (Qiagen, North Manchester, UK) following the protocol supplied and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, USA). A DNA pooling sequence strategy was initially used to identify SNP in the bovine RELA gene by direct sequencing of PCR amplification products. Five primers were designed to sequence the exons and their flanking regions using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) based on the published gene sequence of RELA from the Ensembl database (ENSBTAG00000013895), with NCBI Genbank accession number NM_001080242.2. DNA samples (1 μl each of 50 ng·μL⁻¹ DNA) from each of 10 British Holstein cows were used to construct the DNA pool. PCR was performed using a Qiagen Multiplex PCR kit as described previously [25]. Sequence files were assessed using the Chromas Pro software package (release 2.33; Technelysium Pty Ltd, South Brisbane, QLD 4101, Australia).

Based on the results generated in the above pooling sequence analysis, one synonymous SNP rs48035703 from RELA intron 5 was subsequently genotyped on all individual cow samples using PCR-restriction fragment length polymorphism (PCR-RFLP). Primers were designed as follows using Primer Premier 5.0:
Forward, 5’-TGTCAGCCAAAGTAAGGAGTG-3’; Reverse, 5’-TCTCCTTTGCTCCTGCTTT-3’. The appropriate enzyme was selected using the WatCut online tool (http://watcut.uwaterloo.ca/template.php?act=snp_new). PCR was then performed using a Qiagen multiplex PCR kit as described previously [25]. Thereafter, 10 μl PCR products were digested with 2 U restriction enzyme BsrBI (New England Biolabs Inc, Hitchin, Herts, UK) in a 20 μl reaction volume at 37 °C. The digested products were detected by electrophoresis with 1.5% agarose gel and alleles were indicated by bands of different sizes (Figure 2).

### 2.3. Data Analysis

Milk production traits were analyzed for each animal by measuring the total milk produced, 305 d milk yield and peak daily milk yield. Data on SCC from monthly milk analysis records were averaged over each lactation. Milk phenotype data for lactation 1 only and “all lactations” were calculated as residuals (L1) or cow effect (LAll) after fitting a mixed model to all herd data [25]. A trait to represent mastitis status was derived from the SCC data after Yoshida et al. [26]. This classified animals as unaffected (class 0, never had a SCC > 200,000 cells∙mL\(^{-1}\)), affected (class 2 mastitic, cows with a SCC > 300,000 cells∙mL\(^{-1}\) in two consecutive tests or in four non-consecutive tests, or with a SCC > 500,000 cells∙mL\(^{-1}\) in any one test during any lactation period, regardless of parity) or intermediate between these two extremes (class 1).

The genotype frequency of the polymorphism was examined for deviations from Hardy-Weinberg equilibrium within the population using the χ\(^2\) test. Association analyses were carried out for all traits except SCC, separately in each lactation using the mixed effect model in Equation (1).

\[
Y_{ijl} = \mu + H_i + SNP_j + a_k + e_{ijkl}
\]

with: \(Y\) = trait analyzed; \(\mu\) = overall mean; \(H_i\) = fixed effect of the \(i\)th herd \((i = 1 – 20)\); \(SNP_j\) = fixed effect of the \(j\)th genotype of the SNP \((j = 1 – 3)\), \(a_k\) = the random animal term fitted using a pedigree file and \(e_{ijkl}\) = random residual error.

Significant differences between the genotypes within the SNP were tested using t-statistics and standard errors of differences were calculated for each pair of means.

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**Figure 2.** The PCR-RFLP analysis of the G/C polymorphism in intron 5 of the bovine RELA gene. Electrophoresis was performed using 1.5% agarose gels. Arrows on left hand side show the positions of the undigested 964 bp PCR product and the two PCR products digested with BsrBI restriction endonuclease. Examples of the heterozygous genotype GC, homozygous genotype GG and homozygous genotype CC are shown.
tested. SCC was analyzed using a trinomial generalized linear model with a logit link function. All analyses were carried out in ASReml (http://www.vsni.co.uk/). SCC as a tertiary trait was analyzed by Fisher’s Exact Test in a 3 × 3 table of SCC class by genotype. Mean SCC for heifers and all lactations was also analyzed by an allele substitution model using the command-assoc in PLINK [27] with a quantitative trait.

Differences between genotypes in the number of animals that survived were measured by Kaplan-Meier survival analysis using the Statistical Package for the Social Sciences 17.0 (SPSS Inc., Chicago, IL, USA). Censored animals were those cows that survived > 2340 d (6.4 years). The proportions of cows censored were compared using the Cox proportional hazards regression model. The fixed effects of the herd and sire of the animal were initially included in the analyses but were found to have a non-significant effect on survival (P > 0.20) so were removed from the final model.

3. Results

The initial search in our population of British Holstein-Friesian cows identified a synonymous C/G SNP rs48035703 in RELA intron 5 on BTA29 at position 44,502,474 bp. This SNP was taken forward to genotyping 315 6-month old heifers using PCR-RFLP. Of these, there were 200 CC homozygotes, 95 CG heterozygotes and 20 GG homozygotes with respective genotype frequencies of 0.63, 0.30 and 0.06. These were distributed with a $\chi^2 = 0.18$ against the Hardy-Weinberg Equilibrium. This SNP in RELA was associated with survival time from birth to death or culling (Figure 3).

The C allele was favourable with an additive effect. The average survival times were: CC, 1872 d; CG, 1745 d and GG 1596 d. On average, the GG homozygotes therefore had lives that were about 4.9 and 9 months shorter than those of animals carrying one or two C alleles, respectively ($P < 0.003$). The survival curves showed that the genotypes did not start to diverge until after about 900 d of age (2.5 yr), by which time most cows had started their first lactation. For the 20 GG genotype animals, three were culled as heifers, three in lactation (L) 1, six in L2 and four in each of L3 and L4. Of these, 11/17 (65%) had at least one recorded episode of clinical mastitis. The reasons for culling were available for 13 animals with the rs48035703 GG genotype. The predominant reason given was mastitis ($n = 6$), followed by infertility ($n = 4$), with one each due to respiratory disease, abscess and injury.

The basic statistics of the milk traits analysed in this study (total milk produced, 305 d milk yield and peak daily milk yield in L1 and L2) are summarised in Table 1. None of these traits was significantly associated with the RELA SNP rs48035703.

The overall mean ± SD SCC data (log residuals) were 0.052 ± 0.894 in L1 and −0.00056 ± 0.250 when all lactations were included (log cow effect). These data were analysed separately using three different models. The first lactation only
Figure 3. Kaplan-Meier analysis showing the proportion of animals surviving from birth through to 2340 d of age. A population of 315 Holstein Friesian cows were genotyped for the RELA SNP rs48035703. Survival time was significantly lower for animals with the GG genotype (P < 0.003).

Table 1. Basic statistics of the milk traits (in kg) analysed in this study.

<table>
<thead>
<tr>
<th>Traits(^a)</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total milk yield(_{L1})</td>
<td>307</td>
<td>9,303</td>
<td>3,957</td>
</tr>
<tr>
<td>305d milk yield(_{L1})</td>
<td>200</td>
<td>8,723</td>
<td>1,542</td>
</tr>
<tr>
<td>Peak milk yield(_{L1})</td>
<td>253</td>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>Total milk yield(_{L2})</td>
<td>237</td>
<td>10,109</td>
<td>3144</td>
</tr>
<tr>
<td>305d milk yield(_{L2})</td>
<td>160</td>
<td>9,983</td>
<td>1,675</td>
</tr>
<tr>
<td>Peak milk yield(_{L2})</td>
<td>206</td>
<td>42</td>
<td>7</td>
</tr>
</tbody>
</table>

\(^a\): L1, lactation 1; L2, lactation 2.

Data showed a significant association with SCC using an allele substitution model (Table 2). The mean log residuals ± SD by genotype were: GG 0.30 ± 1.263 (n = 13); CG 0.994 ± 0.994 (n = 59) and CC −0.039 ± 0.803 (n = 124; P < 0.05). Significance was lost when data from all lactations was included in the analysis (P = 0.33).

A Fisher’s Exact Test categorical model was used to determine the cumulative probability of genotype affecting SCC categorised as Class 0 (unaffected), 1 (intermediate) and 2 (mastitic) (Table 3). This analysis showed a trend for more CC genotype cows than expected to be classified as intermediate (45/148, 30.4%) and fewer as mastitic (68/148, 45.9%), whereas for the heterozygote CG cows fewer were intermediate (7/58, 12.1%) and more were mastitic (35/58, 60.3%) (P = 0.05). Data were only available for 13 GG genotypes, so the frequency estimates are not reliable, but of these 15.4% were classed as intermediate and 53.8% were mastitic. The third model, using Equation (1), resulted in a non-significant
Table 2. Summary results from fitting an allele substitution model and a genotypic model to the analysis of SCC data with respect to SNP rs48035703 in RELA intron 5 on BTA29.

<table>
<thead>
<tr>
<th>Trait</th>
<th>n</th>
<th>Allele substitution effect</th>
<th>SE</th>
<th>T</th>
<th>P: Allele substitution</th>
<th>P: Genotypic</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCa</td>
<td>191</td>
<td>0.224</td>
<td>0.10</td>
<td>2.14</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>SCCL</td>
<td>232</td>
<td>0.026</td>
<td>0.03</td>
<td>0.97</td>
<td>0.33</td>
<td>0.24</td>
</tr>
</tbody>
</table>

a: SCCH included data from the first lactation only whereas for SCCL all lactations were included.

Table 3. Summary results from fitting a Fisher’s Exact Test categorical model to determine the cumulative probability of genotype for SNP rs48035703 in RELA intron 5 on BTA29 affecting SCC categorised as unaffected, intermediate or mastitic.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Counts</th>
<th>Unaffected</th>
<th>Intermediate</th>
<th>Mastitic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>35a (23.6%)</td>
<td>45b (30.4%)</td>
<td>68a (45.9%)</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Expected</td>
<td>37.2</td>
<td>36.5</td>
<td>74.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>16a (27.6%)</td>
<td>7b (12.1%)</td>
<td>35a (60.3%)</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>Expected</td>
<td>14.6</td>
<td>14.3</td>
<td>29.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>4a (30.8%)</td>
<td>2a (15.4%)</td>
<td>7a (53.8%)</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Expected</td>
<td>3.3</td>
<td>3.2</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>54</td>
<td>110</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a & b: Proportions within rows differ from expected, P < 0.05.

effect of genotype on categorical SCC (P = 0.09). Together these data indicated that cows with the CC genotype at RELA rs48035703 were less likely than the heterozygotes to experience a high SCC of >200,000 cells·mL⁻¹, classified as mastitis.

4. Discussion

These results show that the RELA SNP rs48035703 was significantly associated with both survival time and SCC in a population of British Holstein Friesian cows. This region on BTA29 has not previously been associated with either productive life, SCC or SCS in traditional GWAS studies (http://www.animalgenome.org/).

There are a number of possible reasons for this discrepancy. SCC is a complex multi-locus trait and involves a large number of variants with differing actions which together only explained 5.4% of the total genetic variance in a population of German Holstein cattle [18]. We, however, found convincing evidence that cows carrying the G allele of rs48035703 had a shorter survival time in the herd. In our data the significant association with SCC was detected when only the first lactation data were considered. If cows with the less favourable genotype have a shorter survival time, then fewer of these animals will have SCC data available in later lactations, which may impact on the results. The other trait analysed was survival time from birth. Others have previously reported significant associations of the trait “Length of productive life” (which starts when cows calve for the first time) with SNP in BTA29 [28] [29]. These were rs110734670, located at
48.4 Mbp, rs109761676 at 37.6 Mbp and rs109447102 at 37.7 Mbp. Although these SNP are at some distance away from RELA, they do flank the region of interest.

Durán Aguilar et al. [20] discovered recently that there is a CNV region on BTA29 with a length of 86,267 which stretches from 44.4 to 44.5 Mbp. CNVs are less frequent than SNP, but involve larger genomic regions that may affect gene structure and regulation [30]. As CNVs are heritable it is possible that they may be associated with animal health and production traits under recent selection [31]. When deletions occur there is only one, or occasionally no, copies of the gene present in the genome, which can result in dosage imbalance [32]. The particular CNVR in BTA29 reported by Durán Aguilar et al. [20] was associated with eight genes SSSCA1, FAM89B, EHBP1L1, KCNK7, MAP3K11, PCNXL3, SIPA1 and RELA. This study compared Mexican Holstein cows with estimated breeding values for somatic cell score (SCS) which were ± 2 SD from the average SCS score, and included 140 high and 140 low phenotype samples. Of these 14 cows (5.8%) had this particular CNV loss. If the same CNV is also present in other Holstein populations, then it would clearly affect SNP genotyping results.

We focused on RELA, as this is a plausible candidate gene to influence an innate immune response involved in mastitis. The SNP analysed was located in intron 5, so is unlikely to be the causative mutation, although synonymous mutations have been shown to alter the functions of translated protein through a variety of mechanisms such as influencing splice sites, miRNA and exonic transcription factor binding sites, affecting mRNA stability and altering translational speed [33]. It could, however, have been in linkage with another functional SNP in either RELA, which is known to be highly polymorphic (https://www.ncbi.nlm.nih.gov/snp), or in adjacent genes. Of those mentioned above, MAP3K11 (also known as MLK3) is of most interest. This gene encodes a member of the serine/threonine kinase family which activates the NF-κB signalling pathway by directly phosphorylating and so activating inhibitory IkB proteins. It can also activate both MAPK8/JNK kinase and AMPK [34]. Other genes adjacent to RELA from 44.5 to 44.6 Mb are LOC101904027, KAT5, RNASEH2C, AP5B1, OVOL1 and LOC101904071. Of these KAT5 encodes a histone acetylase that has a role in DNA repair, apoptosis and signal transduction but none of these genes have established roles in immunity.

Bacteria such as E. coli can cause a variety of other important cattle diseases apart from mastitis. In this study two cows with the GG genotype were culled due to respiratory disease and an abscess respectively, and another died with no reason given. Several animals had reported metritis or endometritis and four were culled for infertility. The numbers are too low to draw conclusions, but further work is warranted to assess the linkage between this SNP and immune responses to other infections. In humans, a SNP rs1049728 in the vicinity of RELA was significantly associated with the soluble ICAM-1 concentration in the circulation [35]. ICAM-1 is a member of the immunoglobulin superfamily of
adhesion receptors: the soluble form is thought to arise from proteolytic cleavage
of the extra-cellular domains and is a recognized genetic marker for inflamma-
tion [36].

5. Conclusion
In conclusion, we have reported a significant association between an SNP in
RELA, longevity and SCC in a population of Holstein-Friesian dairy cows. As
NF-κB activation is known to play a central role in the mammary response to
bacterial infection, this link seems plausible. The SNP investigated was, however,
a synonymous SNP located in RELA intron 5. Further work is therefore required
to confirm the gene and establish the mutation which influence the traits re-
ported.

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Conflicts of Interest
The authors declare no conflicts of interest regarding the publication of this pa-
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