A splice donor variant in *CCDC189* is associated with asthenospermia in Nordic Red dairy cattle

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Bovine SNP50 Bead chip data are available as Supporting File 1. Raw sequencing data of two asthenospermic bulls are available at the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) under the primary accession number PRJEB29487.

Short running title: Recessive asthenospermia in cattle

Key words: male infertility, sterility, immotile sperm, cattle, CCDC189

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Abstract

Cattle populations are highly amenable to the genetic mapping of male reproductive traits because longitudinal data on ejaculate quality and dense microarray-derived genotypes are available for many artificial insemination bulls. Two young Nordic Red bulls delivered sperm with low progressive motility (i.e., asthenospermia) during a semen collection period of more than four months. The bulls were related through a common ancestor on both their paternal and maternal ancestry. Thus, a recessive mode of inheritance of asthenospermia was suspected. Both bulls were genotyped at 54,001 SNPs using the Illumina BovineSNP50 Bead chip. A scan for autozygosity revealed that they were identical by decent for a 2.98 Mb segment located on bovine chromosome 25. This haplotype was not found in the homozygous state in 8,557 fertile bulls although five homozygous haplotype carriers were expected (P=0.018). Whole genome-sequencing uncovered that both asthenospermic bulls were homozygous for a mutation that disrupts a canonical 5' splice donor site of *CCDC189* encoding the coiled-coil domain containing protein 189. Transcription analysis showed that the derived allele activates a cryptic splice site resulting in a frameshift and premature termination of translation. The mutated CCDC189 protein is truncated by more than 40%, thus lacking the flagellar C1a complex subunit C1a-32 that is supposed to modulate the physiological movement of the sperm flagella. The mutant allele occurs at a frequency of 2.5% in Nordic Red cattle. Our study in cattle uncovered that CCDC189 is required for physiological movement of sperm flagella thus enabling active progression of spermatozoa and fertilization.

Background

Impaired male fertility is a well-recognized and highly heterogeneous condition in many species including humans, mice and cattle (*e.g.*, [1],[2],[3],[4],[5]). Males with reduced reproductive ability may produce ejaculates with anomalous characteristics such as low sperm count, impaired progressive motility or morphological aberrations of the spermatozoa [6]. However, standard semen parameters are only poor predictors of male reproductive ability because many fertility disorders are caused by aberrations of the spermatozoa that are not immediately apparent or regularly monitored (e.g., [7],[8],[4]).

The normal movement of the sperm flagellum, i.e., activated and hyperactivated motility, facilitates active progression of spermatozoa through the oviduct. Morphological

aberrations of the sperm tail such as stump tail, short tail, dysplasia of the fibrous sheath or disorganized flagellar microtubules may compromise sperm motility and fertilization success [9]. Genetic variants causing morphological abnormalities of the sperm tail have been identified in several species including humans [10],[11], pigs [12], cattle [13], and mice [14] where they impede motility. The fertilization success of ejaculates containing a high proportion of spermatozoa with low progressive motility (i.e., asthenospermia) is often reduced, both *in vivo* and *in vitro* [15],[16], albeit fertilization anecdotally being reported as possible *in vivo* [17].

Due to the low heritability of male reproductive performance, detecting genetic variants that underpin impaired male fertility requires large cohorts with phenotypic records that, moreover, have been genotyped [18]. Comprehensive data on male fertility have become available for many cattle populations due to the wide-spread use of artificial insemination (AI) [19]. The availability of both longitudinal data on semen quality and dense microarray-derived genotypes makes cattle populations highly amenable to the mapping of genetic determinants that underpin male reproductive performance [4],[13],[20],[21].

Prospective bulls for AI are selected at an early age and reared at AI centers under controlled conditions. When the bulls reach an age of approximately one year, their semen is collected once or twice per week. Immediately after semen collection, macroscopic, microscopic and computer-assisted analyses are carried out to assess whether the ejaculates are suitable for AI [22]. Up to 20% of all collected ejaculates are rejected because they do not fulfill the requirements for AI [23]. The most common pathological findings in ejaculates of insufficient quality include absence or low number of spermatozoa, reduced sperm motility, impaired sperm viability or aberrant morphology of heads and/or tails in many spermatozoa [24]. Usually, it takes a few weeks until young bulls produce semen that is considered suitable for AI, owing to individual differences in epididymal sperm maturation [22]. Young bulls that permanently produce ejaculates that do not fulfill the requirements for AI are sporadically noticed at semen collection centers. It is plausible that some of these bulls carry deleterious genetic variants that are responsible for the low semen quality they deliver (e.g., [4],[13]).

Here we present the phenotypic and genetic analysis of a recessively inherited form of sterility in Nordic Red bulls, which primarily manifests itself through asthenospermia. The availability of dense genotype data of two affected bulls enabled us to localize the disorder within a 2.98 Mb interval on bovine chromosome 25. Whole-genome sequencing of

138 affected bulls revealed that a mutation disrupting a canonical splice donor site of the
139 *CCDC189* gene is the most likely cause of the observed condition.

140

141 Material and Methods

142 Ethics statement

143 Ejaculates of two asthenospermic bulls were collected by employees of an AI center in
144 Finland as part of their routine practice under veterinary supervision. Both bulls were
145 eventually culled because their impaired sperm motility precluded the use of the collected
146 ejaculates for artificial inseminations. The decision to cull the bulls was at the sole
147 discretion of representatives of the AI center. Testicular tissue was collected immediately
148 after slaughter. Oocytes used for *in vitro* fertilization experiments were collected from offal
149 cow ovaries at a commercial slaughterhouse. No animal experiments were performed in
150 this study, and, therefore, approval from an ethics committee was not required.

151 Animals

152 Two fifteen-months old bulls from the Nordic Red dairy cattle breed that were born in 2012
153 and 2013 were housed at an AI center in order to collect their semen for AI. Both bulls
154 were apparently healthy and their andrological status was considered as normal, i.e., they
155 showed no signs of disease, the size of their testicles was within the normal range and
156 their libido was normal. During a period of more than four months, 11 and 27 ejaculates,
157 respectively, of both bulls were collected once or twice a week by technicians at the AI
158 center. Semen from bulls of documented normal sperm quality and fertility, of the same
159 breed and age interval, was likewise collected, processed and examined. Testicular tissue
160 samples were collected immediately after slaughter, frozen in liquid nitrogen and stored at
161 -80°C.

162 Analysis of fresh semen

163 Immediately after semen collection, sperm motility, viability and morphology of fresh
164 ejaculates were subjectively assessed by technicians at the AI center. Sperm motility was
165 evaluated using a microscope equipped with phase contrast optics. Sperm concentration
166 and plasma membrane viability were assessed using a flow-cytometer [25]. None of the
167 collected ejaculates fulfilled the requirements for AI because most spermatozoa had
168 reduced or missing progressive motility. Upon our request, one ejaculate from each of the

169 asthenospermic bulls was frozen using the commercial method routinely used by the AI
170 center.

171 **Analysis of frozen-thawed semen samples**

172 Velocity (µm/sec), and percentages of spermatozoa with total and progressive motility
173 were assessed using frozen-thawed ejaculates. Semen droplets (2.4 x 10^5 spermatozoa in
174 10 µL) were placed on a pre-warmed 76 x 26 mm Menzel-Gläser microscope slide
175 (ThermoFisher Scientific, Waltham, MA, USA) covered by a pre-warmed coverslip on a
176 thermal plate (38 ºC). Slides were analyzed using an upright Zeiss Axio Scope A1 light
177 microscope equipped with a 10x phase contrast objective (Carl Zeiss, Stockholm,
178 Sweden) connected via a CMOS camera (UEye, IDS Imaging Development Systems
179 GmbH, Obersulm, Germany) to a computer running the Qualisperm™ software (Biophos
180 SA, Lausanne, Switzerland). The Qualisperm™ technology is based on fluorescence
181 correlation spectroscopy-analysis of single particles (spermatozoa) in confocal volume
182 elements. Individual spermatozoa are projected on a pixel grid of the CMOS camera and
183 the algorithm calculates the number of fluctuations in each pixel using a correlation
184 function of sperm numbers and translation classes, thus determining the speed (velocity)
185 distribution of spermatozoa, from which proportions of motility classes are calculated [26].

186 Sperm viability (membrane integrity) and early membrane destabilization changes (YO-
187 PRO-1+/PI-), degree of mitochondrial activity (Mitotracker Deep Red+) and superoxide
188 production (MitoSOX+), production of reactive oxygen species (ROS; CellROX+) and lipid
189 peroxidation (BODIPY+) were assessed using a Gallios™ flow cytometer (Beckman
190 Coulter, Bromma, Sweden) equipped with standards optics. In all investigations, we
191 assessed 25,000 events per sample with a flow rate of 500 cells per second.

192 The total ATP concentration of frozen-thawed spermatozoa was analyzed using the ATP
193 Determination Kit A22066 (Molecular Probes TM Invitrogen detection technologies)
194 according to the manufacturer's protocol. Samples of an asthenospermic and
195 normospermic bull were analyzed using a Kikkoman Lumitester C-100 luminometer
196 (Kikkoman Corporation, Nishishinbashi, Tokyo, Japan).

197 Transmission electron microscopy on spermatozoa of two asthenospermic and one
198 normospermic bull was performed using frozen-thawed semen samples. After thawing,
199 semen samples were washed twice in phosphate buffered saline (PBS), fixed with 2.5%
200 glutaraldehyde and 2% paraformaldehyde in PBS for 2 hours on ice and pelleted at 6000g.

201 Pellets were washed in PBS, post-fixed in 1% osmium tetroxide, washed in ddH_2O,
202 stained in 1% uranyl acetate, dehydrated in graded series of ethanol (25%, 50%, 75%,
203 90%, and 100%), and embedded in Epoxy resin through increasing concentrations (25%,
204 50%, 75%, and 100%) using PELCO Biowave+ tissue processor, and then cured at 60°C
205 for 3 days. Embedded blocks were sectioned using Leica FC6 microtome and a DIATOME
206 diamond knife with 45° angle into 60nm sections and mounted on Quantifoil copper grids
207 with formvar carbon films. Sections were post-stained with 2% uranyl acetate followed by
208 lead citrate. Grids were imaged using FEI Morgani 268 electron microscope operated at
209 100 kV at 20k magnification.

210 ***In vivo* and *in vitro* fertilization success**

211 Fertility of two asthenospermic bulls was tested *in vivo* at a conventional Finnish cattle
212 herd within one breeding season; ninety-eight cows in oestrus were inseminated by the
213 farmer either with semen from one of two bulls with asthenospermia (n=9) or a control bull
214 with normal semen quality (n=89). Insemination success was controlled by a trained
215 veterinarian.

216 *In vitro* fertilization (IVF) success, with embryo cleavage rate at 42 hours post insemination
217 and embryo yield at 9 days post insemination as end-points, was tested for an
218 asthenospermic and a normospermic bull, using 470 offal oocytes. Ovaries of slaughtered
219 cows were collected at a commercial slaughterhouse and transported to the laboratory in
220 saline at room temperature. In the laboratory, follicles of 2-8 mm in diameter were
221 aspirated with an 18 G needle and a 20 ml syringe. Cumulus-oocyte complexes (COC)
222 were collected from the aspirated follicular fluid and 470 good quality COC were selected
223 for maturation. Unless stated otherwise, all the chemicals were purchased from Sigma-
224 Aldrich (St. Louis, MO, USA). COC were matured for 24h in TCM199 with glutamax-I
225 (Gibco™; Invitrogen Corporation, Paisley, UK) supplemented with 0.25mM Na-pyruvate,
226 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 ng/ml FSH (Puregon, Organon, Oss,
227 Netherlands), 1 µg/ml β-estradiol (E-2257) and 10% heat inactivated fetal bovine serum
228 (FBS) (Gibco™, New Zealand) at 38.5°C in maximal humidity in 5% CO_2 in air. Following
229 maturation, 320 and 150 COC were inseminated using washed frozen–thawed semen
230 from an asthenospermic and a normospermic control bull of proven fertility in IVF,
231 respectively, at a concentration of 1 million spermatozoa per ml at 38.5°C in maximal
232 humidity in 5% CO_2 in air for 20h. During IVF spermatozoa capacitation and movement
233 were stimulated with 10 µg/ml heparin and PHE (20 µM penicillamine - 10 µM hypotaurine

234 – 1 μM epinephrine), respectively. After fertilization, presumptive zygotes were cultured in
235 G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with fatty-acid-free bovine
236 serum albumin (4 mg/ml) at 38.5°C in maximal humidity in an atmosphere of 5% O_2 and
237 5% CO_2. Cleavage rates of oocytes were recorded at 30 and 42 hours post insemination.
238 The number and quality of blastocysts were recorded at day 8 and 9 (IVF=day 0).

239 **Genotyping, quality control and haplotype inference**

240 The two asthenospermic bulls were genotyped at 54,001 SNPs using the Illumina
241 BovineSNP50 Bead chip using DNA purified from semen samples following standard DNA
242 extraction protocols. To facilitate haplotype inference, we also considered genotypes of
243 119 fertile bulls from the Nordic Red dairy cattle breed, including the sires (n=2) and 17
244 paternal half-sibs of the affected bulls. Genotypes for these 119 bulls were also
245 determined using the Illumina BovineSNP50 Bead chip comprising genotypes at 54,001
246 (version 1) or 54,609 SNPs (version 2). The chromosomal positions of the SNPs
247 corresponded to the University of Maryland reference sequence of the bovine genome
248 (UMD3.1) [27]. Following quality control, we retained 41,594 autosomal SNPs with a call-
249 rate greater than 95%. The per-individual call-rate was between 96.07% and 100% with an
250 average call-rate of 99.73%. The *Beagle* [28] software (version 3.2.1) was used to impute
251 sporadically missing genotypes and infer haplotypes. Genotype data are available in *plink*
252 binary format [29] (.bed/.bim/.fam) as **Supplementary File 1**.

253 **Detection of runs of homozygosity**

254 Runs of homozygosity (ROH) were identified in both asthenospermic bulls using the
255 *homozyg*-function that was implemented in the *plink* (version 1.9) software tool [29]. Due
256 to the relatively sparse SNP coverage (approximately 1 SNP per 60 kb), we considered
257 segments with a minimum length of 500 kb *(--homozyg-kb 500*) and at least 20 contiguous
258 homozygous SNPs (*--homozyg-snp 20*) as ROH. ROH were identified within sliding
259 windows of 40 SNPs (*--homozyg-window-snp 40*). For the identification of ROH, no
260 heterozygous SNP were allowed within ROH (*--homozyg-het 0*). The R script that was
261 used to identify and visualize ROH is available as **Supplementary File 2**.

262 **Identification of segments of autozygosity in two bulls with asthenospermia**

263 Genotypes contained in ROH in both asthenospermic bulls were manually inspected in
264 order to determine whether the bulls were identical by descent (IBD) for the same
265 haplotype (i.e., autozygous). In regions where both affected bulls were IBD, the haplotypes

266 of 119 fertile bulls (see above) were also inspected. Assuming recessive inheritance of a
267 deleterious mutation, we considered IBD haplotypes as associated with asthenospermia if
268 they were not detected in the homozygous state in 119 fertile bulls.

269 **Generation of whole genome sequencing data and sequence variant detection**

270 Genomic DNA was prepared from semen samples of the two asthenospermic bulls
271 according to standard DNA extraction protocols. Paired-end libraries were prepared using
272 the paired-end TruSeq DNA sample preparation kit (Illumina) and sequenced using a
273 HiSeq 2500 instrument (Illumina). We collected 252 and 270 million paired-end reads (125
274 base pairs) corresponding to an average (raw) genome coverage of 12- and 13-fold,
275 respectively. The raw sequencing data of both asthenospermic bulls are available at the
276 European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) under primary accession
277 number PRJEB29487. The raw sequencing reads were trimmed and filtered using *fastp*
278 (version 0.19.4) [30] and subsequently aligned to the UMD3.1 reference sequence
279 (GCA_000003055.3) of the bovine genome [27] using the *BWA mem* algorithm (version
280 0.7.13) [31]. Individual files in SAM format were converted into BAM format using
281 *SAMtools* (version 1.8) [32]. Duplicate reads were marked with the *MarkDuplicates*
282 command of the *Picard tools* software suite (https://broadinstitute.github.io/picard/).
283 Polymorphic sequence variants were identified using the *Genome Analysis Toolkit* (*GATK*,
284 version 4.0) [33]. Sequencing coverage along the genome was calculated based on the
285 aligned reads using the *Mosdepth* software [34].

286 **Identification of candidate causal variants**

287 Assuming that both bulls with asthenospermia were homozygous for the same recessively
288 inherited variant, we screened the sequence data of the affected bulls for variants that
289 were: a) located within the 2.98-Mb segment of autozygosity, b) homozygous for the non-
290 reference allele in both bulls and c) never observed in the homozygous state in a control
291 group of 237 animals from breeds other than Nordic Red dairy cattle that were obtained
292 from Schwarzenbacher *et al.* [35]. 126 variants that fulfilled these three criteria were
293 considered as candidate causal variants. The functional consequences of the candidate
294 causal variants were predicted according to the Ensembl transcripts database (release 91)
295 using the *Variant Effect Predictor* (VEP) [36] tool. Genotypes of candidate causal variants
296 were subsequently inspected in whole-genome sequencing data of 2,669 animals that
297 have been sequenced in Run6 of the 1000 Bull Genomes Project [37].

298 **Validating the CCDC189 splice donor variant in Nordic Red cattle**

299 We estimated the frequency of the asthenospermia-associated haplotype in 8,557 Nordic
300 Red cattle from the Nordic genomic selection reference population that had been
301 genotyped at more than 50,000 SNPs [38]. Using Sanger sequencing, we genotyped the
302 splice donor variant (GCA_000003055.3:Chr25:g.27138357C>T) in *CCDC189* (transcript-
303 ID: ENSBTAT00000045037) in 118 sires that were either identified as heterozygous
304 carriers of the asthenospermia-associated haplotype (N=30) or have been used frequently
305 in AI (N=88). PCR reactions for genotyping were done with the DyNAzyme II DNA
306 Polymerase (Thermo Fisher, MA, US) in a 10µl volume of 1x PCR buffer, 0.2mM dNTPs,
307 10pmol primer mix (forward 5'- CAAGGTCCTGCCCTTAAGAA -3') and reverse primer:
308 (reverse 5'- ATGCCATCACTCTGGACCTC-3') and 150pg of DNA. The cycling conditions
309 were the following: a) an initial denaturation at 95°C for 3 min, b) 29 cycles of 30 sec
310 denaturation (94°C), 30 sec hybridization (58°C), 30 sec elongation (72°C), and c) a final 3
311 min elongation (72°C). PCR products were purified and directly sequenced using the
312 BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, US). Electrophoresis
313 of sequencing reactions was performed on 3500xL Genetic Analyzers (Applied
314 Biosystems, CA, US), and sequences were visualized with Sequencher 5.4.6 (Gene
315 Codes Corporation, MI, US).

316 **Transcription analysis**

317 Total RNA from two bulls homozygous for the *CCDC189* splice donor variant
318 (GCA_000003055.3:Chr25:g.27138357C>T) and two control bulls that were homozygous
319 for the wild type allele was extracted from testis tissues using the RNeasy Mini Kit
320 (Qiagen) following manufacturer's instructions. The quality and concentration of total RNA
321 was analysed using Agilent's 2100 Bioanalyzer and the Nanodrop ND-2000
322 spectrophotometer (Thermo Scientific). An amount of 500 ng of total RNA from each
323 sample was reversely transcribed using QuantiTech Rev. Transcription kit (Qiagen). PCR
324 reactions were done with the DyNAzyme II DNA Polymerase (Thermo Fisher, MA, US) in a
325 30 µl volume of 1 x PCR buffer, 0.2 mM dNTPs, 10 pmol primer mix (forward 5'-
326 ACTGAGGAGATGAGGGAGGT-3' and reverse primer: reverse 5'-
327 TAGAGCTTGAAGTGGCGGAA-3') and 50 ng of cDNA. The cycling conditions were the
328 following: 1) an initial denaturation at 95°C for 3 min, 2) 35 cycles of 30 sec denaturation
329 (94°C), 30 sec hybridization (61.8°C), 1 min elongation (72°C) and 3) a final 3 min
330 elongation (72°C). PCR products were separated on a 4% agarose gel and the length of

331 the products was analysed using the Image Lab version 5.0 software (Bio-Rad
332 Laboratories). PCR products were purified with QIAquick Gel Extraction kit (QIAgen) and
333 sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, CA,
334 US). Electrophoresis of sequencing reactions was performed on 3500xL Genetic Analyzer
335 (Applied Biosystems, CA, US), and sequences were visualized with Sequencher 5.4.6
336 (Gene Codes Corporation, MI, US).

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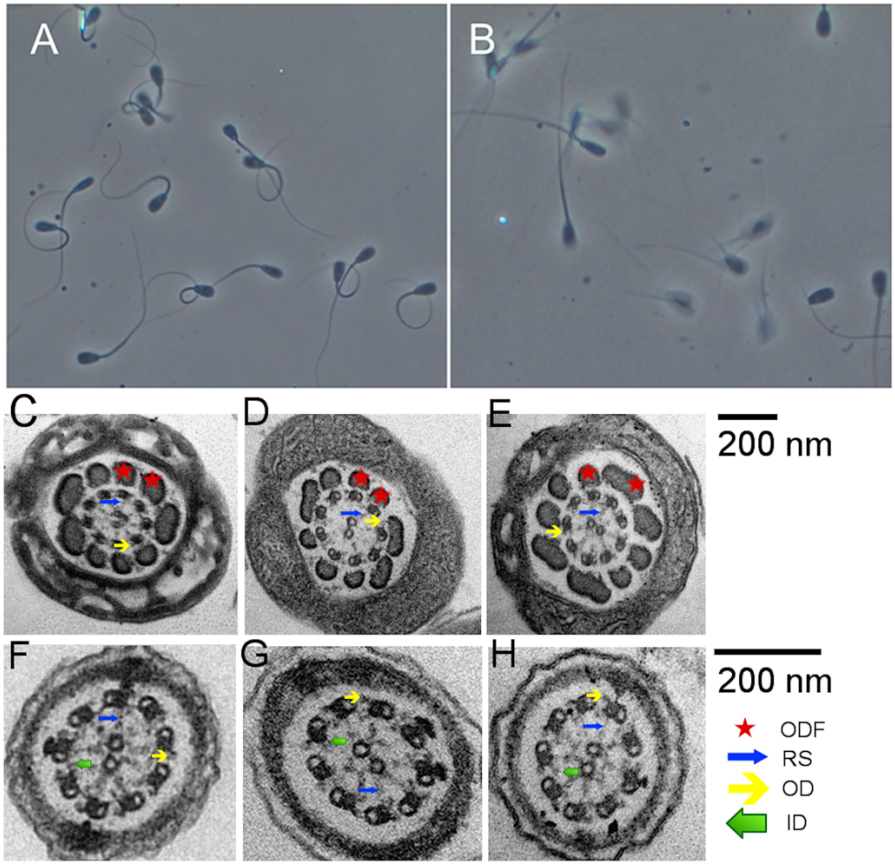
339 ## Results

340 **Aberrant mitochondrial activity in ejaculates of asthenospermic bulls**

341 Two young bulls from the Nordic Red dairy cattle breed producing ejaculates with immotile
342 spermatozoa were noticed at a semen collection center. The average ejaculate volumes
343 (3.16 ml) and sperm concentrations (1.94 Mio/µl) were within physiological ranges for bulls
344 of their age. More than 80% of the spermatozoa in the ejaculates of both bulls were
345 classified as viable and had typical morphology. However, less than 5% of the
346 spermatozoa were progressively motile, thus not fulfilling the requirements for AI, whose
347 threshold is at least 40%.

348 Frozen semen samples from both asthenospermic bulls were thawed and subjected to
349 computer-assisted motility analysis and flow cytometric studies. Post-thawing, the velocity
350 of the spermatozoa was between 10 and 14 µm/s. The velocity of spermatozoa in
351 normospermic control bulls was between 38 and 48 µm/s (sperm with progressive motility
352 typically move at a velocity faster than 25 µm/s). In two normospermic control bulls, more
353 than 50% of the spermatozoa showed progressive motility after thawing while in the
354 affected bulls, the proportion of motile spermatozoa was less than 10%. The discrepancy
355 between computer-assisted and subjective motility evaluations (10 vs 5%) results from
356 spermatozoa displaying minor movement, i.e., the sperm tails bended peculiarly which
357 was not classified as progressive motility in subjective microscopic sperm evaluation
358 (**Figure 1**, **Supplementary File 3 & 4**). The ATP content was numerically higher, though
359 not significant, in semen of asthenospermic than normospermic bulls. As expected, flow
360 cytometry analysis revealed that viable post-thawed spermatozoa examined in either bull
361 type had a tendency for destabilizing changes in the plasmalemma. However, the
362 asthenospermic bulls had more spermatozoa with high mitochondrial membrane potential

363 indicating increased mitochondrial activity. This leads to more spermatozoa producing
364 superoxide and reactive oxygen species. This in turn causes a higher proportion of
365 spermatozoa with lipid peroxidation in asthenospermic than normospermic bulls (**Table 1**).
366 Transmission electron microscopy of the sperm flagella from asthenospermic bulls showed
367 the typical arrangement of nine outer microtubule doublets surrounding the central pair
368 (9x2 + 2). No ultrastructural abnormalities of the axonemes were immediately apparent
369 (**Figure 1**).



370

371 **Figure 1 – Phenotypic manifestation of asthenospermia.** Microscopic view of a representative
372 sample of frozen-thawed spermatozoa from an asthenospermic (A) and normospermic (b) bull.
373 Bending of many spermatozoa of the asthenospermic bull and the lack of motility projected as
374 clear pictures of spermatozoa (A). Foggy (double pictured) representation of many spermatozoa in
375 the normospermic bull indicate actively moving spermatozoa (B). Both figures were photographed
376 using the same settings. Transmission electron microscopy of spermatozoa from two
377 asthenospermic (C, D, F, G) and an normospermic (E, H) bull. Representative cross-sections of
378 the midpiece (C-E) and principal piece (F-H) of the flagella revealed the typical arrangement of
379 nine outer microtubule doublets surrounding the central pair (9x2 + 2) in asthenospermic and
380 normospermic bulls. No aberrations of the axonemal ultrastructures including outer dense fiber
381 (ODF), radial spokes (RS), inner (ID) and outer dynein arms (OD) were apparent in cross-sections
382 of asthenospermic bulls.

383 **Table 1 - Results of the flow cytometric analysis of frozen thawed semen samples.**

Animal	Early membrane destabilization	Increased mitochondrial activity	Increased mitochondrial superoxide production	Increased ROS production	Lipid peroxidation degree
Asthenospermic-1	2.0	72.3	24.9	39.0	22.0
Asthenospermic-2	4.2	71.9	25.9	17.8	23.3
Normospermic-1	10.7	47.3	4.9	5.1	3.1
Normospermic-2	8.3	54.3	1.8	6.7	3.2

384 Proportions (%) of viable spermatozoa post-thawing from two asthenospermic and two
385 normospermic bulls of the Nordic Red breed depicting sperm early membrane destabilization (YO-
386 PRO-1+/PI-), degree of mitochondrial activity (Mitotracker+/PI-), and increased production of
387 mitochondrial superoxide (MitoSOX+/PI-), reactive oxygen species (ROS, CellROX+) and lipid
388 peroxidation (Bodipy-C11 + (green)) assessed using flow cytometry.

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390

391 **Bulls with asthenospermia are sterile *in vivo* and *in vitro***

392 None of the 9 cows inseminated with semen of an asthenospermic bull conceived,
393 whereas 42 of the 89 cows inseminated with normospermic semen did.

394 *In vitro* fertilization success was tested using 470 offal oocytes. Using post-thaw semen
395 from an asthenospermic bull, the embryo cleavage rate at 42 hours post-insemination was
396 only 11% (36 out of 320) compared to 70% (105 out of 150) using spermatozoa from a
397 normospermic control bull. At day nine post-insemination, the embryo yield after embryo
398 culture was only 0.9% (3 out of 320) for the asthenospermic bull compared to 23% (34 out
399 of 150) for the normospermic bull. Moreover, the three embryos obtained using semen
400 from the asthenospermic bull reached blastocyst stage only at day 9, indicating aberrantly
401 slow development kinetics.

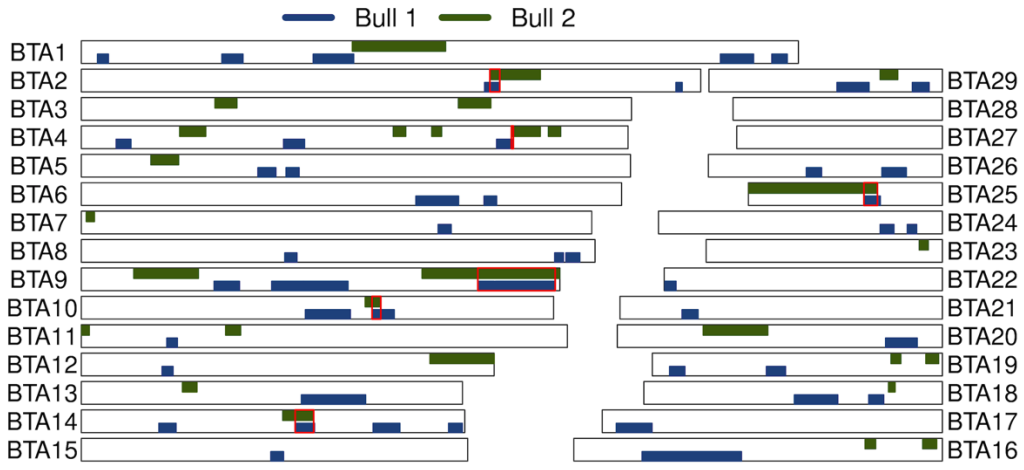
402 Because the semen analyses revealed consistent sperm deviations and deficient
403 fertilization capacity both *in vivo* and *in vitro* in both asthenospermic bulls, we suspected
404 that this type of sterility may represent a genetic condition. The analysis of pedigree
405 records revealed a common male ancestor (born in 1990) that was present either as great-
406 great-great-grandfather or great-great-grandfather in both the maternal and paternal
407 ancestry of both affected bulls (**Supplementary File 5**). The sires of the two
408 asthenospermic bulls had more than 300 and 2,000 daughters, respectively, indicating that
409 they were frequently used for AI. According to the AI centers consulted, their semen
410 quality was normal. Seven and twelve paternal half-sibs of the asthenospermic bulls were
411 used for 6,257 and 36,689 artificial inseminations, respectively. According to the AI center,

412 their semen qualities were normal and their average calving rates (number of births per
413 insemination) were 0.39 and 0.44, i.e., similar to average values in Nordic Red cattle.
414 Because a common male ancestor was in the maternal and paternal ancestry of both
415 asthenospermic bulls and fertility was normal in their first- and second-degree relatives, we
416 suspected that the disorder might follow an autosomal recessive mode of inheritance.

417 **Asthenospermia maps to a 2.98 Mb segment on bovine chromosome 25**

418 To identify the genomic region associated with asthenospermia, the two affected bulls
419 were genotyped using the BovineSNP50 Bead chip. Following quality control, genotypes
420 of 41,594 autosomal SNPs were available for genetic analyses. Assuming recessive
421 inheritance of a deleterious mutation, the genotypes of both asthenospermic bulls were
422 screened for the presence of long ROH (**Supplementary Files 1 & 2**). We identified 49
423 and 28 ROH in the two bulls that covered 258 and 210 Mb of their genomes (**Figure 2**).
424 The average length of the ROH was 6.1 Mb ranging from 1.4 to 30.4 Mb. ROH at
425 chromosomes 2, 4, 9, 10, 14 and 25 were present in both asthenospermic bulls. However,
426 the ROH at chromosomes 4, 9, 10 and 14 were homozygous for different haplotypes and
427 thus not compatible with the presumed inheritance of a recessive allele from a common
428 ancestor. Two haplotypes on chromosomes 2 (between 90,155,450 and 92,350,004 bp)
429 and 25 (between 25,490,468 and 28,470,779 bp) were identical-by-descent (IBD) in both
430 bulls with asthenospermia. The IBD haplotype located on chromosome 2 consisted of 28
431 adjacent SNPs of the BovineSNP50 Bead chip. In 100 randomly chosen fertile bulls of the
432 Nordic Red cattle population, the haplotype had a frequency of 28%. Moreover, the
433 haplotype occurred in the homozygous state in four bulls with normal fertility. Considering
434 its high frequency and occurrence in the homozygous state in fertile bulls, it is unlikely that
435 this haplotype was causal for the asthenospermia of two bulls. The IBD haplotype located
436 on chromosome 25 consisted of 45 adjacent SNPs of the BovineSNP50 Bead chip
437 (**Supplementary File 6**). It was not detected in the homozygous state in normospermic
438 bulls. Both the sires and ten (out of 17) half-sibs of the asthenospermic bulls carried the
439 haplotype in the heterozygous state. The average calving rate was 0.42 and 0.41 in half-
440 sibs that were haplotype carriers and non-carriers, respectively, indicating that fertility is
441 normal in heterozygous bulls. In 8,557 fertile sires from the Nordic Red cattle genetic
442 evaluation reference population, the haplotype was observed in the heterozygous state in
443 426 animals corresponding to a frequency of 2.5%. Homozygous haplotype carriers were
444 absent among the fertile bulls although five were expected assuming Hardy-Weinberg

445 Equilibrium proportions (P=0.018), corroborating recessive inheritance of a deleterious
446 allele.



447

448 **Figure 2 – Homozygosity mapping in two asthenospermic bulls.** Homozygosity mapping in
449 two asthenospermic bulls. Blue and green color represent autosomal runs of homozygosity (ROH)
450 that have been detected in the two asthenospermic bulls. The red frames highlight six ROH that
451 had been detected in both affected bulls. Only the 2.98-Mb segment located on chromosome 25
452 was never found in the homozygous state in normospermic bulls.
453
454

455 **A variant disrupting a splice donor site in *CCDC189* is associated with**
456 **asthenospermia**

457 In order to detect the causal mutation for the asthenospermia, we sequenced both affected
458 bulls at approximately 12-fold genome coverage. The sequencing depth in the interval
459 between 25,490,468 and 28,470,779 bp on chromosome 25, where both bulls shared an
460 extended segment of autozygosity, did not differ from the average sequencing coverage
461 (**Supplementary File 7**). Visual inspection of the aligned reads in the 2.98 Mb interval
462 revealed no evidence for a large deletion or structural variant that might segregate with the
463 haplotype. We identified 3,683 single nucleotide and short insertion and deletion
464 polymorphisms that were homozygous for the non-reference variant in both bulls in the
465 2.98 Mb IBD interval. Out of 3,683 sequence variants, 3,657 occurred in the homozygous
466 state in at least one out of 237 male control animals from breeds other than Nordic Red
467 that were not affected by the disorder. Such variants are not compatible with the presumed
468 recessive inheritance of a fully penetrant allele that causes asthenospermia, thus retaining
469 126 candidate causal variants. Variants annotation using the *Variant Effect Predictor* tool
470 revealed that 120 of 126 candidate causal variants were located in non-coding regions of
471 the genome (**Supplementary File 8**). Six variants were predicted to affect protein-coding

472 regions or splice sites including four synonymous mutations, a missense variant
473 (GCA_000003055.3:Chr25:g.26184191C>G) in *RABEP2*
474 (ENSBTAT00000008595:p.P413R, SIFT-score [39] 0.26) and a variant disrupting a
475 canonical 5' splice donor site (GCA_000003055.3:Chr25:g.27138357C>T) in *CCDC189*
476 (transcript-ID: ENSBTAT00000045037) encoding the coiled-coil domain containing protein
477 189. Of the 126 candidate causal variants, only the splice donor site mutation in *CCDC189*
478 was predicted to be deleterious to protein function.

479 The polymorphism Chr25:27138357C>T alters the first position of the fourth intron of
480 bovine *CCDC189*. The derived T-allele disrupts a canonical splice site (GT>AT; *CCDC189*
481 is transcribed from the reverse strand, **Figure 3**). The T-allele did not segregate in 237
482 healthy animals from cattle breeds other than Nordic Red that were sequenced in
483 Schwarzenbacher *et al.* [35]. In 2'669 animals that had been sequenced in the framework
484 of the 1000 Bull Genomes Project, the Chr25:27138357 T allele occurred in the
485 heterozygous state only in four animals of the Finnish Ayrshire and Swedish Red cattle
486 breeds (**Supplementary File 8**), i.e., two breeds that were used to form the Nordic Red
487 dairy cattle breed [38]. The T allele was never observed in the homozygous state in cattle
488 other than the two bulls with asthenospermia.

489 The effect of the Chr25:27138357C>T variant on *CCDC189* transcription was examined by
490 RT-PCR using RNA extracted from testicular tissues of the two asthenospermic and two
491 normospermic control bulls that were homozygous for the wild type allele. Using primers
492 located at the junction of exons 3 and 4 (forward) and in exon 6 (reverse) we obtained 358
493 and 368 bp RT-PCR products in asthenospermic (mt/mt) and normospermic (wt/wt) bulls,
494 respectively (**Figure 3D**). Sequence analysis of the RT-PCR product of mt/mt bulls
495 revealed that the Chr25:27138357C>T substitution activates a cryptic splice site in exon 4.
496 It alters the resulting CCDC189 protein sequence from amino acid residue 155 onwards
497 and creates a premature stop codon at amino acid residue 200, thus truncating 40% of the
498 332-amino acid CCDC189 protein. The truncated protein lacks domains that are supposed
499 to be essential for physiological protein function including the flagellar C1a complex
500 subunit C1a-32, which modulates ciliary and flagellar beating [40].

501 **Prevalence of the splice donor variant in Nordic Red cattle**

502 In order to confirm the association between the candidate causal splice site mutation in
503 *CCDC189* and the asthenospermia-associated haplotype, we used Sanger sequencing to
504 obtain genotypes for the polymorphism in 118 normospermic control bulls of the Nordic

Red cattle breed. The variant was in perfect linkage disequilibrium with the 2.98 Mb haplotype corroborating that it might be causative for the disorder. All bulls carrying the associated haplotype in the heterozygous state were also heterozygous for the Chr25:27138357 T allele. The T allele was not found in bulls that did not carry the asthenospermia-associated haplotype.

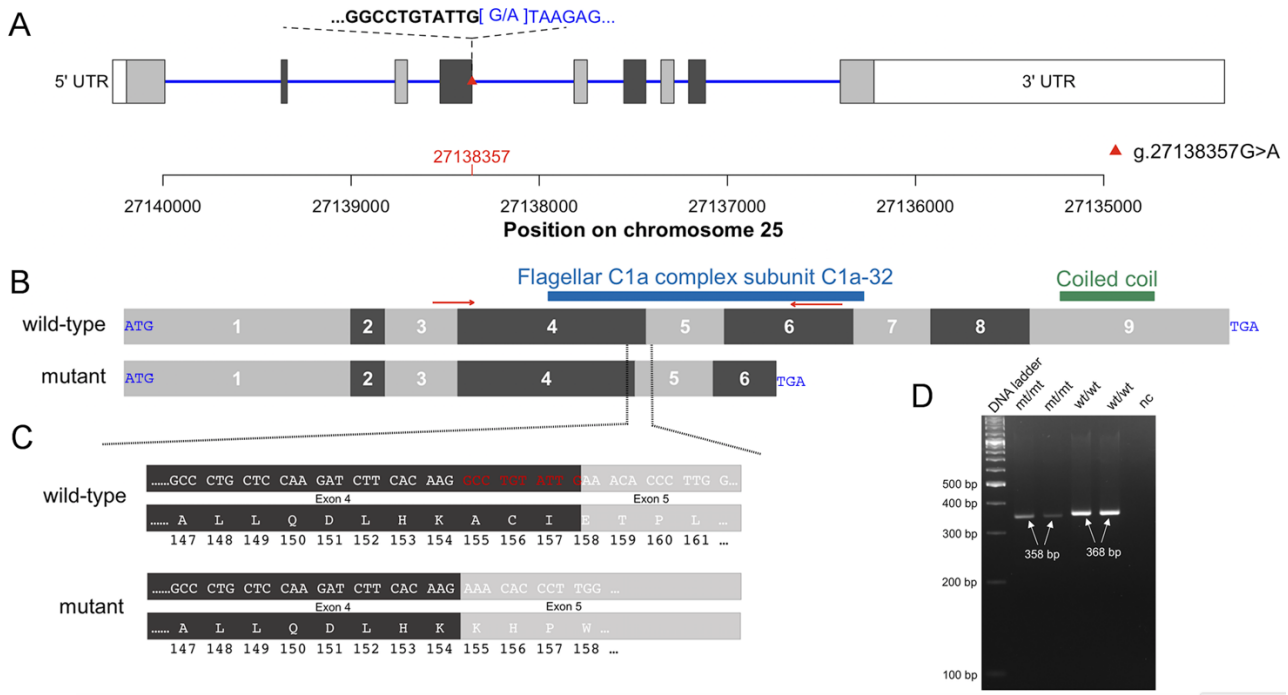


Figure 3 – Schematic overview of bovine *CCDC189*. Structure of the bovine *CCDC189* gene (transcript-ID: ENSBTAT00000045037). The bovine *CCDC189* gene encoding the coiled-coil domain containing protein 189 is transcribed from the reverse strand. It consists of nine exons (grey and dark-grey boxes) (A). A mutant allele (red triangle) at 27,138,357 basepairs disrupts a canonical 5' splice donor variant in the fourth intron. Schematic view of the cDNA sequence of *CCDC189* (B). The coiled-coil domain containing protein 189 contains the flagellar C1a complex subunit C1a-32 between amino acids 127 and 221 and a coiled coil domain between amino acids 280 and 308. Red arrows indicate the positions of the primers used for RT-PCR. The mutant allele activates a cryptic splice site in exon 4 that truncates 10 basepairs from exon 4 (red colors) and introduces a frameshift in translation that eventually creates a premature stop codon (C). The mutant protein, if retained, lacks a large fraction of the flagellar C1a complex subunit C1a-32 and the coiled coil domain. Effect of the mutant allele on CCDC189 transcription (D). The RT-PCR products from testis tissue were shorter from two mt/mt (358 bp) than wt/wt (368 bp) bulls. Lane 5 (nc) is the negative PCR control. The length of the bands was defined using the Image Lab version 5.0 software (Bio-Rad Laboratories).

Discussion

A splice donor variant in bovine *CCDC189* is the likely cause of asthenospermia in the Nordic Red cattle breed. An autosomal recessive mode of inheritance was suspected

531 because the two affected bulls were related through a common ancestor on both their
532 paternal and maternal paths, the spermatological findings were very similar in their
533 ejaculates, and their first- and second-degree relatives were fertile. Increasing rates of
534 inbreeding and the widespread use of individual sires in artificial insemination make cattle
535 populations susceptible to the propagation and phenotypic manifestation of recessive
536 alleles [41],[42]. Considering that only two bulls of the breed were affected by
537 asthenospermia, explanations other than recessive inheritance were also plausible
538 including, e.g., allelic heterogeneity [43],[44] and non-genetic causes of disease. However,
539 both bulls were housed at the AI center under controlled environmental conditions at the
540 same time as many fertile bulls and their health and andrological parameters were normal
541 indicating that non-genetic factors were less likely to cause this condition. Previous studies
542 showed that autozygosity mapping is a powerful approach to identify genomic regions
543 associated with recessive conditions even if only few affected individuals with genotype
544 information are available [13],[45]. Using autozygosity mapping in the two affected bulls,
545 we identified a 2.98 Mb segment of extended homozygosity located at chromosome 25
546 that was identical by descent in both bulls. This segment, encompassing a variant
547 disrupting a canonical 5' splice donor site in *CCDC189*, was never found in the
548 homozygous state in fertile bulls, in agreement with the presumed recessive inheritance of
549 an allele with detrimental phenotypic consequences.

550 Verifying the effect of candidate causal mutations in independent populations is critical
551 evidence to supporting variant causality [46],[35]. The splice donor variant in *CCDC189* did
552 not segregate in breeds distantly related to Nordic Red cattle indicating that it likely arose
553 after the formation of breeds thus precluding an immediate validation of the mutation in an
554 unrelated cattle population. Our study thus exemplifies difficulties inherent in validation and
555 functional investigation of deleterious sequence variants that manifest severe phenotypic
556 consequences such as impaired male fertility in livestock populations. Due to purifying
557 selection, deleterious sequence variants are rare in natural populations [46] unless they
558 either show pleiotropic effects on desired traits [43], are in linkage disequilibrium with
559 quantitative trait loci [47], or are propagated through the widespread use of heterozygous
560 mutation carriers in AI [48],[4]. The splice donor variant in *CCDC189* is rare in the Nordic
561 Red dairy cattle breed. We detected the derived allele in its homozygous state in only two
562 bulls. Assuming an allele frequency of the derived allele of 2.5% and random mating, only
563 1 out of 1,600 males is expected to be homozygous and thus sterile due to spermatozoa

564 with impaired motility. The disorder remains undetected in most homozygous animals
565 because sterility becomes evident only in breeding bulls. Apart from producing
566 asthenospermic ejaculates, both bulls homozygous for the derived T allele were healthy.
567 However, genetic variants that cause asthenospermia may also compromise the function
568 of motile cilia thus manifesting themselves in chronic detrimental diseases [11],[49],[35].
569 Further research is warranted to determine if DNA variants in *CCDC189* are associated
570 with ciliopathies particularly in animals that are kept in conventional farms under normal
571 husbandry conditions. A direct gene test will facilitate detection of and selection against
572 carriers in order to prevent the birth of homozygous males that are sterile. Due to the low
573 frequency of the deleterious allele, negative effects on genetic gain and genetic diversity
574 are likely negligible when carrier animals are excluded from breeding in the Nordic Red
575 cattle breed. However, sophisticated genome-based mating strategies are required to take
576 into account the steadily increasing number of deleterious sequence variants discovered in
577 breeding populations [50].

578 Functional and pathophysiological investigations were possible in two AI bulls only. Sperm
579 analysis revealed an identical pattern in both bulls homozygous for the deleterious allele;
580 sperm motility was impaired in both fresh ejaculates and post-thaw, e.g., the process of
581 handling the semen, even with dilution of the seminal plasma did not ameliorate the low
582 progressive motility. Slightly elevated ATP concentrations in post-thaw semen of the
583 affected bulls might result from lower sperm metabolism or accumulation of ATP due to
584 impaired progressive motility. However, elevated ATP concentrations might also derive
585 from the inactive sperm tails. Moreover, a normal proportion of viable albeit
586 asthenospermic spermatozoa resulted in high superoxide and reactive oxygen species
587 production indicating defective mitochondria [51]. This further impairs sperm motility and
588 eventually leads to lipid peroxidation and DNA damage and/or cell death, thus precluding
589 fertilization [52]. Our study showed that the affected bulls were infertile *in vivo* and *in vitro*,
590 precluding their use in AI. Fertilization might be possible *in vitro* using intracytoplasmic
591 sperm injection, though DNA damage could impair embryo development. We have not
592 explored this possibility, since the sperm phenotypic deviations were decisive to cull
593 breeding sires from AI.

594 Most likely the causal variant for asthenospermia disrupts a canonical 5' splice donor site
595 of bovine *CCDC189* encoding the coiled-coil domain containing protein 189. To the best of
596 our knowledge, this is the first time that a phenotypic consequence has been associated

597 with a variant of *CCDC189* in any organism. CCDC189 (alias Spergen-4) is a sperm tail-
598 associated protein that is mainly located in the principal piece and acrosome region of
599 mature spermatozoa indicating that it may be required for their physiological activated
600 progressive movement and acrosome reaction [53]. The velocity of spermatozoa was
601 slower and their progressive motility was severely compromised in bulls that were
602 homozygous for the Chr25:27138357 T allele indicating that flagellar movement was
603 defective. Mutations disrupting components of the dynein arms or radial spokes of
604 axonemal dyneins cause disorganized axonemes of motile cilia and flagella, thus
605 compromising their physiological movement [11],[54],[55]. In spite of an impaired motility,
606 the ultrastructural organization of the sperm flagella including the assembly of the dynein
607 arms was normal in both asthenospermic bulls, indicating that disorganized axonemes
608 were less likely to be responsible for the lack of progressive movement of the sperm. We
609 showed that the Chr25:27138357 T allele introduces a cryptic splice site in the fourth exon
610 of *CCDC189* leading to a frameshift in translation and premature translation termination.
611 The resulting CCDC189 protein, if not degraded via nonsense-mediated mRNA decay,
612 lacks a large part of the flagellar C1a complex subunit (C1a-32), that is an integral
613 constituent of the central pair of flagellar microtubules. Aberrations of the small C1a
614 microtubule projection of the central pair impair the physiological beating of flagella whose
615 axonemes are normally organized otherwise [56],[57]. It has been shown that the C1a-32
616 subunit mediates the beating of cilia and flagella by modulating the activity of both the
617 inner and outer dynein arms [40],[58]. Possibly, the resolution of the transmission electron
618 microscopy was too low to reveal potential aberrations of the small C1a-32 subunit of the
619 C1a projection [40]. Both bulls homozygous for the splice donor variant were sterile
620 because they produced asthenospermic ejaculates corroborating that CCDC189 is
621 important for physiological movement of spermatozoa and fertilization. However,
622 spermatozoa of both asthenospermic bulls bended slightly which is not classified as
623 progressive motility in automated semen analysis. The minor movement of affected
624 spermatozoa resembles uncoordinated twitching of flagella in *Chlamydomonas* mutants
625 lacking constituents of the flagellar C1a projection [57].

626

627 ## Conclusions

628 Our study in cattle revealed that a naturally occurring deleterious allele of *CCDC189*
629 encoding the coiled-coil domain containing protein 189 causes immotile sperm with

630 defective mitochondria thus resulting in male sterility both *in vivo* and *in vitro*. The mutation
631 occurs in Nordic Red dairy cattle at a low frequency. Direct gene testing facilitates the
632 efficient monitoring of the deleterious allele in the population.

633

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638

639 Availability of data

640 Genotype data of two asthenospermic bulls are available in *plink* binary format as
641 supporting information. Whole-genome sequence data of both asthenospermic bulls have
642 been deposited at the European Nucleotide Archive (ENA) of EMBL-EBI under primary
643 accession PRJEB29487.

644

645 Author contributions

646 Sample collection: HV, MA; Analysis of genotyping data: TIT HV AS GS BG HP; Analysis
647 of whole-genome sequencing data: CW RF HP; RNA analysis: TIT AS; Sperm functional
648 analyses: AVC MAR SN HS HRM MA MH HV KFi KFI TS; In vitro fertilization experiment:
649 JP MM; In vivo fertilization experiment: JT; Transmission electron microscopy: MH; Wrote
650 the paper: TIT MA HRM HP; Conceived and designed the experiments: HV MA HP; Read
651 and approved the final version of the manuscript: all authors

652

653 References

654 1. Irvine DS. Epidemiology and aetiology of male infertility. Hum Reprod. 1998;13 Suppl
655 1:33–44.

656 2. Singh P, Schimenti JC. The genetics of human infertility by functional interrogation of
657 SNPs in mice. Proc Natl Acad Sci USA. 2015;112:10431–6.

658 3. Shorter JR, Odet F, Aylor DL, Pan W, Kao C-Y, Fu C-P, et al. Male Infertility Is
659 Responsible for Nearly Half of the Extinction Observed in the Mouse Collaborative Cross.
660 Genetics. 2017;206:557–72.

661 4. Pausch H, Kölle S, Wurmser C, Schwarzenbacher H, Emmerling R, Jansen S, et al. A
662 Nonsense Mutation in TMEM95 Encoding a Nondescript Transmembrane Protein Causes
663 Idiopathic Male Subfertility in Cattle. PLOS Genetics. 2014;10:e1004044.

664 5. Charlier C, Li W, Harland C, Littlejohn M, Coppieters W, Creagh F, et al. NGS-based
665 reverse genetic screen for common embryonic lethal mutations compromising fertility in
666 livestock. Genome Res. 2016;26:1333–41.

667 6. Silber SJ. The relationship of abnormal semen parameters to male fertility. Hum
668 Reprod. 1989;4:947–53.

669 7. Gredler B, Fuerst C, Fuerst-Waltl B, Schwarzenbacher H, Sölkner J. Genetic
670 parameters for semen production traits in Austrian dual-purpose Simmental bulls. Reprod
671 Domest Anim. 2007;42:326–8.

672 8. Ren D, Navarro B, Perez G, Jackson AC, Hsu S, Shi Q, et al. A sperm ion channel
673 required for sperm motility and male fertility. Nature. 2001;413:603–9.

674 9. Lim CC, Lewis SEM, Kennedy M, Donnelly ET, Thompson W. Human sperm
675 morphology and in vitro fertilization: sperm tail defects are prognostic for fertilization
676 failure. Andrologia. 30:43–7.

677 10. Ben Khelifa M, Coutton C, Zouari R, Karaouzène T, Rendu J, Bidart M, et al. Mutations
678 in DNAH1, which Encodes an Inner Arm Heavy Chain Dynein, Lead to Male Infertility from
679 Multiple Morphological Abnormalities of the Sperm Flagella. Am J Hum Genet.
680 2014;94:95–104.

681 11. Merveille A-C, Davis EE, Becker-Heck A, Legendre M, Amirav I, Bataille G, et al.
682 CCDC39 is required for assembly of inner dynein arms and the dynein regulatory complex
683 and for normal ciliary motility in humans and dogs. Nat Genet. 2011;43:72–8.

684 12. Sironen A, Thomsen B, Andersson M, Ahola V, Vilkki J. An intronic insertion in KPL2
685 results in aberrant splicing and causes the immotile short-tail sperm defect in the pig. Proc
686 Natl Acad Sci USA. 2006;103:5006–11.

687 13. Pausch H, Venhoranta H, Wurmser C, Hakala K, Iso-Touru T, Sironen A, et al. A
688 frameshift mutation in ARMC3 is associated with a tail stump sperm defect in Swedish Red
689 (Bos taurus) cattle. BMC Genetics. 2016;17:49.

690 14. Zhang Z, Kostetskii I, Tang W, Haig-Ladewig L, Sapiro R, Wei Z, et al. Deficiency of
691 SPAG16L causes male infertility associated with impaired sperm motility. Biol Reprod.
692 2006;74:751–9.

693 15. Pusch HH. The importance of sperm motility for the fertilization of human oocytes in
694 vivo and in vitro. Andrologia. 1987;19:514–27.

695 16. Donnelly ET, Lewis SE, McNally JA, Thompson W. In vitro fertilization and pregnancy
696 rates: the influence of sperm motility and morphology on IVF outcome. Fertil Steril.
697 1998;70:305–14.

698 17. Hellander JC, Samper JC, Crabo BG. Fertility of a stallion with low sperm motility and
699 a high incidence of an unusual sperm tail defect. Vet Rec. 1991;128:449–51.

700 18. de Kretser D. Male infertility. The Lancet. 1997;349:787–90.

701 19. Thibier M, Wagner H-G. World statistics for artificial insemination in cattle. Livestock
702 Production Science. 2002;74:203–12.

703 20. Taylor JF, Schnabel RD, Sutovsky P. Review: Genomics of bull fertility. animal.
704 2018;12:s172–83.

705 21. Ferenčaković M, Sölkner J, Kapš M, Curik I. Genome-wide mapping and estimation of
706 inbreeding depression of semen quality traits in a cattle population. J Dairy Sci.
707 2017;100:4721–30.

708 22. Rodríguez-Martínez H. State of the art in farm animal sperm evaluation. Reprod Fertil
709 Dev. 2007;19:91–101.

710 23. Patrick V, L US, Catherine D, Nadine B, Tom K, Patrick B. Bovine Semen Quality
711 Control in Artificial Insemination Centers. In: Bovine Reproduction. Wiley-Blackwell; 2014.
712 p. 685–95. doi:10.1002/9781118833971.ch74.

713 24. Tanghe S, Van Soom A, Sterckx V, Maes D, de Kruif A. Assessment of different sperm
714 quality parameters to predict in vitro fertility of bulls. Reprod Domest Anim. 2002;37:127–
715 32.

716 25. Christensen P, Stenvang JP, Godfrey WL. A flow cytometric method for rapid
717 determination of sperm concentration and viability in mammalian and avian semen. J
718 Androl. 2004;25:255–64.

719 26. Tejerina F, Buranaamnuay K, Saravia F, Wallgren M, Rodriguez-Martinez H.
720 Assessment of motility of ejaculated, liquid-stored boar spermatozoa using computerized
721 instruments. Theriogenology. 2008;69:1129–38.

722 27. Zimin AV, Delcher AL, Florea L, Kelley DR, Schatz MC, Puiu D, et al. A whole-genome
723 assembly of the domestic cow, Bos taurus. Genome Biology. 2009;10:R42.

724 28. Browning BL, Browning SR. A Unified Approach to Genotype Imputation and
725 Haplotype-Phase Inference for Large Data Sets of Trios and Unrelated Individuals. Am J
726 Hum Genet. 2009;84:210–23.

727 29. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation
728 PLINK: rising to the challenge of larger and richer datasets. GigaScience. 2015;4:7.

729 30. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor.
730 Bioinformatics. 2018;34:i884–90.

731 31. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-
732 MEM. arXiv:13033997 [q-bio]. 2013. http://arxiv.org/abs/1303.3997. Accessed 25 May
733 2018.

734 32. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence
735 Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.

736 33. DePristo MA, Banks E, Poplin RE, Garimella KV, Maguire JR, Hartl C, et al. A
737 framework for variation discovery and genotyping using next-generation DNA sequencing
738 data. Nat Genet. 2011;43:491–8.

739 34. Pedersen BS, Quinlan AR. Mosdepth: quick coverage calculation for genomes and
740 exomes. Bioinformatics. 2018;34:867–8.

741 35. Schwarzenbacher H, Burgstaller J, Seefried FR, Wurmser C, Hilbe M, Jung S, et al. A
742 missense mutation in TUBD1 is associated with high juvenile mortality in Braunvieh and
743 Fleckvieh cattle. BMC Genomics. 2016;17:400.

744 36. McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, et al. The Ensembl
745 Variant Effect Predictor. Genome Biology. 2016;17:122.

746 37. Bouwman AC, Daetwyler HD, Chamberlain AJ, Ponce CH, Sargolzaei M, Schenkel
747 FS, et al. Meta-analysis of genome-wide association studies for cattle stature identifies
748 common genes that regulate body size in mammals. Nat Genetics. 2018;50:362–7.

749 38. Makgahlela ML, Mäntysaari EA, Strandén I, Koivula M, Nielsen US, Sillanpää MJ, et
750 al. Across breed multi-trait random regression genomic predictions in the Nordic Red dairy
751 cattle. J Anim Breed Genet. 130:10–9.

752 39. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants
753 on protein function using the SIFT algorithm. Nature Protocols. 2009;4:1073–81.

754 40. Goduti DJ, Smith EF. Analyses of functional domains within the PF6 protein of the
755 central apparatus reveal a role for PF6 sub-complex members in regulating flagellar beat
756 frequency. Cytoskeleton (Hoboken). 2012;69:179–94.

757 41. Charlier C, Coppieters W, Rollin F, Desmecht D, Agerholm JS, Cambisano N, et al.
758 Highly effective SNP-based association mapping and management of recessive defects in
759 livestock. Nat Genet. 2008;40:449–54.

760 42. Pausch H, Schwarzenbacher H, Burgstaller J, Flisikowski K, Wurmser C, Jansen S, et
761 al. Homozygous haplotype deficiency reveals deleterious mutations compromising
762 reproductive and rearing success in cattle. BMC Genomics. 2015;16:312.

763 43. Sartelet A, Klingbeil P, Franklin CK, Fasquelle C, Géron S, Isacke CM, et al. Allelic
764 heterogeneity of Crooked Tail Syndrome: result of balancing selection? Anim Genet.
765 2012;43:604–7.

766 44. Medugorac I, Seichter D, Graf A, Russ I, Blum H, Göpel KH, et al. Bovine Polledness –
767 An Autosomal Dominant Trait with Allelic Heterogeneity. PLoS One. 2012;7.
768 doi:10.1371/journal.pone.0039477.

769 45. Pausch H, Ammermüller S, Wurmser C, Hamann H, Tetens J, Drögemüller C, et al. A
770 nonsense mutation in the COL7A1 gene causes epidermolysis bullosa in Vorderwald
771 cattle. BMC Genetics. 2016;17:149.

772 46. MacArthur DG, Manolio TA, Dimmock DP, Rehm HL, Shendure J, Abecasis GR, et al.
773 Guidelines for investigating causality of sequence variants in human disease. Nature.
774 2014;508:469–76.

775 47. Fujii J, Otsu K, Zorzato F, de Leon S, Khanna VK, Weiler JE, et al. Identification of a
776 mutation in porcine ryanodine receptor associated with malignant hyperthermia. Science.
777 1991;253:448–51.

778 48. Sartelet A, Druet T, Michaux C, Fasquelle C, Géron S, Tamma N, et al. A Splice Site
779 Variant in the Bovine RNF11 Gene Compromises Growth and Regulation of the
780 Inflammatory Response. PLOS Genetics. 2012;8:e1002581.

781 49. Zariwala MA, Knowles MR, Omran H. Genetic defects in ciliary structure and function.
782 Annu Rev Physiol. 2007;69:423–50.

783 50. Cole JB. A simple strategy for managing many recessive disorders in a dairy cattle
784 breeding program. Genet Sel Evol. 2015;47. doi:10.1186/s12711-015-0174-9.

785 51. Rodriguez-Martinez H. Semen evaluation and handling: emerging techniques and
786 future development. In: Chenoweth P, Lorton S, editors. Animal andrology: theories and
787 applications. Wallingford: CABI; 2014. p. 509–49. doi:10.1079/9781780643168.0509.

788 52. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of
789 mitochondrial reactive oxygen species in the generation of oxidative stress in
790 spermatozoa. J Clin Endocrinol Metab. 2008;93:3199–207.

791 53. Howida A, Salaheldeen E, Iida H. Molecular Cloning of Spergen-4, Encoding a
792 Spermatogenic Cell-Specific Protein Associated with Sperm Flagella and the Acrosome
793 Region in Rat Spermatozoa. Zoological Science. 2016;33:195–203.

794 54. Alsaadi MM, Erzurumluoglu AM, Rodriguez S, Guthrie PAI, Gaunt TR, Omar HZ, et al.
795 Nonsense mutation in coiled-coil domain containing 151 gene (CCDC151) causes primary
796 ciliary dyskinesia. Hum Mutat. 2014;35:1446–8.

797 55. Bower R, Tritschler D, Mills KV, Heuser T, Nicastro D, Porter ME. DRC2/CCDC65 is a
798 central hub for assembly of the nexin-dynein regulatory complex and other regulators of
799 ciliary and flagellar motility. Mol Biol Cell. 2018;29:137–53.

800 56. Rupp G, O'Toole E, Porter ME. The Chlamydomonas PF6 locus encodes a large
801 alanine/proline-rich polypeptide that is required for assembly of a central pair projection
802 and regulates flagellar motility. Mol Biol Cell. 2001;12:739–51.

803 57. Wargo MJ, Dymek EE, Smith EF. Calmodulin and PF6 are components of a complex
804 that localizes to the C1 microtubule of the flagellar central apparatus. J Cell Sci. 2005;118
805 Pt 20:4655–65.

806 58. Nakazawa Y, Ariyoshi T, Noga A, Kamiya R, Hirono M. Space-Dependent Formation
807 of Central Pair Microtubules and Their Interactions with Radial Spokes. PLoS One. 2014;9.
808 doi:10.1371/journal.pone.0110513.

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Supplementary Information

Supplementary File 1 – Genotypes of two asthenospermic bulls

Format: .zip-archive

Genotypes at 41,594 SNPs for two asthenospermic bulls in *plink* binary format. The positions of the SNP correspond to the UMD3.1 assembly of the bovine genome.

Supplementary File 2 – R script to identify IBD segments in two asthenospermic bulls

Format: .R-file

R script to visualize runs of homozygosity in the genotype data of two asthenospermic bulls. This program uses as input the files provided as Supplementary File 1. Please note that this R script calls the fast algorithm implemented in *plink* (version 1.9) to identify runs of homozygosity.

Supplementary File 3 – Post-thaw sperm motility in an asthenospermic bull

Format: .mov

Video of frozen-thawed spermatozoa of an asthenospermic bull. Please note the minor movement of some spermatozoa that is typically not classified as progressive motility during the subjective eye-evaluation of ejaculates.

Supplementary File 4 – Post-thaw sperm motility in a normospermic control bull

Format: .mov

Video of frozen-thawed spermatozoa of an normospermic bull.

Supplementary File 5 – Pedigree of two asthenospermic bulls

Format: .png

Pedigree of two affected bulls. Ovals and boxes represent female and male ancestors, respectively. The grey box highlights the most recent common ancestor (MRCA) that was present on both the maternal and paternal ancestry of both asthenospermic bulls.

Supplementary File 6 – Asthenospermia-associated haplotype

Format: .csv

842 ID, position and asthenospermia-associated allele of 45 adjacent SNPs of the
843 BovineSNP50 Bead chip. The coding of the alleles is based on the Illumina TOP/BOT
844 format. The chromosomal position of the SNP corresponds to the UMD3.1 assembly of the
845 bovine genome.
846
847 **Supplementary File 7 – Sequencing depth along chromosome 25 in two**
848 **asthenospermic bulls**
849 *Format: .png*
850 The depth of sequencing coverage in two asthenospermic bulls was calculated at bovine
851 chromosome 25 in sliding windows of 5,000 base pairs using the aligned reads. Grey
852 background indicates the segment of extended homozygosity. The red line represents the
853 position of the candidate causal variant-
854
855 **Supplementary File 8 – Candidate causal variants compatible with recessive**
856 **inheritance of asthenospermia**
857 *Format: .txt*
858 Functional consequences of 126 candidate causal variants that were compatible with
859 recessive inheritance of asthenospermia, i.e., they were homozygous for the alternate
860 allele in two asthenospermic bulls and either heterozygous or homozygous for the
861 reference allele in 237 healthy control animals. Variants were annotated using the *Variant*
862 *Effect Predictor* tool. The quality of the sequence variant genotypes for both
863 asthenospermic bulls is shown in columns 7 and 8. The number of heterozygous control
864 animals (out of 237) is shown in column 9. The last column indicates the genotype
865 distribution of 126 candidate causal variants (homozygous for the reference allele |
866 heterozygous | homozygous for the alternate allele) in 2,669 animals of the 1000 bull
867 genomes dataset.