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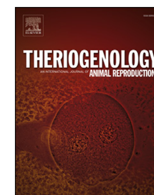

Karyomapping for simultaneous genomic evaluation and aneuploidy screening of preimplantation bovine embryos: The first live-born calves

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ABSTRACT

In cattle breeding, the development of genomic selection strategies based on single nucleotide polymorphism (SNP) interrogation has led to improved rates of genetic gain. Additionally, the application of genomic selection to *in-vitro* produced (IVP) embryos is expected to bring further benefits thanks to the ability to test a greater number of individuals before establishing a pregnancy and to ensure only carriers of desirable traits are born. However, aneuploidy, a leading cause of developmental arrest, is known to be common in IVP embryos. Karyomapping is a comprehensive screening test based on SNP typing that can be used for simultaneous genomic selection and aneuploidy detection, offering the potential to maximize pregnancy rates. Moreover, Karyomapping can be used to characterize the frequency and parental origin of aneuploidy in bovine IVP embryos, which have remained underexplored to date. Here, we report the use of Karyomapping to characterize the frequency and parental origin of aneuploidy in IVP bovine embryos in order to establish an estimate of total aneuploidy rates in each parental germline. We report an estimate of genome wide recombination rate in cattle and demonstrate, for the first time, a proof of principle for the application of Karyomapping to cattle breeding, with the birth of five calves after screening. This combined genomic selection and aneuploidy screening approach was highly reliable, with calves showing 98% concordance with their respective embryo biopsies for SNP typing and 100% concordance with their respective biopsies for aneuploidy screening. This approach has the potential to simultaneously improve pregnancy rates following embryo transfer and the rate of genetic gain in cattle breeding, and is applicable to basic research to investigate meiosis and aneuploidy.

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1. Introduction

The United Nations Department of Economic and Social Affairs (UN DESA) states that by 2030, the world population is expected to reach 8.5 billion and by 2050 it is thought that this will have increased to 9.7 billion [1]. In light of this, the Food and Agricultural Organisation predict an 85% increase in demand for animal protein and, as such, the cattle breeding industry is faced with the challenge of increasing beef and dairy production whilst minimizing environmental impact. In order to achieve this, herd productivity must be improved through selective breeding.

Traditionally, breeding programs aimed at improving herd productivity have focused on the selection of sires and dams based on estimated breeding values (EBVs) generated by scoring phenotypic records of the individual, its relatives, and on progeny phenotyping [2]. While this approach has shown success, the lengthy generation interval required to gather progeny information has encouraged the development of alternative approaches. Later work proposed a selection approach based on the assessment of

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quantitative trait loci (QTLs), sections of DNA associated with variations in phenotypes of commercial interest such as milk production [3]. More recently however, the availability of a complete genome sequence for *Bos taurus* [4] has enabled the discovery of thousands of single nucleotide polymorphisms (SNPs) associated with QTLs which can be used as high-density markers for the production of genomic estimated breeding values (GEBVs) and for the selection of livestock based on genomic data, a process termed genomic selection [5]. The accuracy of GEBVs has been shown to be as high as 85% [5], and the application of genomic selection can rapidly increase the rate of genetic gain for a given breed [6]. Indeed, a recent report from Garcia-Ruiz et al. [7] highlighted that genomic selection in Holstein cattle in the US over a period of 8 years substantially decreased generational intervals, while rapidly increasing fertility, lifespan, and udder health.

In addition to the benefits offered by genomic selection, continued advancements in reproductive technologies such as ovum pickup (OPU), *in vitro* production (IVP) and embryo transfer (ET) offer the ability to maximize the number of offspring from genetically superior animals. These technologies hold promise to reduce generational intervals further, increase selection intensity and improve the dissemination of valuable genetics. Moreover, the potential to combine IVP and genomic selection of embryos could lead to the realization of further improved rates of genetic gain, through selective transfer of embryos that are proven carriers of desirable traits [8,9].

Studies in several farm animal species have, however, suggested that the frequency of aneuploidy (extra or missing chromosomes) is increased in IVP embryos as compared to *in vivo* derived embryos [10–14], and it is well established that aneuploidy is a leading cause of IVF failure [15] and developmental arrest in humans [16,17]. As such, preimplantation genetic screening (PGS) is now commonplace in human IVF despite its use still causing some controversy [18].

Karyomapping is a method based on SNP typing in which the genotypes of an individual, the mother, father and a reference (often a sibling) are compared to describe the parental origin and haploblock inheritance of each chromosome [19]. It was designed simultaneously to detect single gene disorders and chromosomal numerical aberrations as wells as to trace the parental origin of aneuploidy and the position of recombination events [19–22]. To date however Karyomapping has not been used outside the human species, despite its potential benefits for establishing GEBVs and screening aneuploidy simultaneously. A related approach has been described by Destouni et al. [23] who applied a different haplotyping algorithm known as haplarithmisis in order to describe genome instability in chimeric and mixoploid cleavage stage bovine embryos, following validation of this methodology in human blastomeres [24]. However, this report did not describe trends in the overall incidence and origin of aneuploidy in blastocyst stage embryos.

Several reports have noted that the majority of human IVF embryos are affected by at least some degree of chromosomal abnormality [25–27] and that aneuploidy is significantly more common in oocytes than sperm cells [15]. However, similar information for domestic animals such as cattle is lacking in the current literature. The combination of IVP, genomic selection and Karyomapping in cattle thus has the potential to identify embryos with high expected breeding values, while simultaneously selecting for chromosomally normal embryos in order to maximize pregnancy rates. Moreover, the application of Karyomapping in cattle could provide insight into meiosis in a model species that, being a large monoparous mammal, should be more clinically relevant than, for example, the mouse.

Here, we use IVP blastocyst stage embryos to demonstrate the application of Karyomapping in investigating the type, level and parental origin of aneuploidy in bovine IVP embryos. We also use it to estimate the recombination frequency in parental germlines. Moreover, we report the birth of the first genomically evaluated calves born following transfer of embryos identified as chromosomally normal through Karyomapping technology.

2. Materials and methods

Embryos were produced *in vitro* following one of two protocols, depending on whether oocytes were collected from donors included in active commercial breeding programs or from ovaries that were surplus to requirement subsequent to animal slaughter for the meat industry. In the latter case, embryos were assessed for research purposes only and were not transferred to recipient animals. In each case, oocyte collection, IVF and embryo culture was carried out as described in the following sections. A total of 33 embryos from commercial donors and total of 28 embryos from abattoir-derived sources were assessed for meiotic recombination frequency and chromosome copy number abnormalities. In all cases, pedigree analysis confirmed that sires and dams did not share a common relative within at least the previous 2 generations.

2.1. Production of embryos from abattoir derived sources

The protocol used followed the indications of Nivet et al. [28], with some modifications. Briefly, abattoir sourced bovine ovaries were transported to the laboratory in warm (36–38 °C) PBS in a thermos flask within 3 h of culling. Follicles ranging between 3 and 8 mm were manually aspirated using a 5 ml syringe equipped with a 19G needle. Cumulus-oocyte-complexes (COCs) showing homogenous ooplasm and at least 2 compact layers of cumulus cells, were selected under 20x observation and removed to HEPES modified tissue culture medium 199 (TCM-199) supplemented with 10% FBS, 0.2 mM pyruvate and antibiotics (Pen/Strep, Gibco, Grand Island, NY). The COCs were then washed twice and cultured in groups of 20–30 in 90 μl drops of TCM-199 medium supplemented with 10% FBS, 10 IU/ml pregnant mare serum gonadotrophin (PMSG) and 5 IU/ml human chorionic gonadotrophin (hCG) (PG600, Intervet, Milton Keynes, UK), 0.2 mM pyruvate, and antibiotics. The culture was carried out at 38.5 °C and under 6.5% CO2 in air for 18–22 h.

COCs showing a homogenous ooplasm and an expanded cumulus were selected for fertilisation and removed to glucose-free TALP supplemented with antibiotics, 10 μg/ml heparin and 1:25 penicillamine, hypotaurine and epinephrine (PHE) solution prepared as described by Miller et al. [29]. Frozen/thawed bull spermatozoa (Semex, Monkton, UK) were selected using the commercial discontinuous density gradient system BoviPure™ (Nidacon, Mölndal, Sweden) according to manufacturer's instructions. The motility of the sample was confirmed by x200 observation under modulation contrast microscopy and only samples with high (>70%) progressive motility were further employed. An appropriate volume of the sperm preparation was added directly to the TALP drop containing the matured oocytes to give a final concentration of $10^{\hat{}5}$ motile sperm cells/ml. Finally, the gametes were co-cultured at 38.5 °C under 6.5% CO_2 in air overnight.

After IVF, the putative zygotes were removed to HEPES modified, glucose-free TALP medium and mechanically denuded with a 125 μm wide tip (EZ-Tip, RI, Falmouth, UK). Zygotes were then washed two times and cultured in SOFaaci [30] supplemented with 5% FBS, 5 mg/ml BSA and antibiotics. Finally, zygotes were cultured in groups of 20–30 in 90 μl drops of SOFaaci at 38.5 °C and under 6.5% CO_2 and 5% O_2 for 7 or 8 days subsequent to fertilisation.

2.2. Production of embryos from commercial donors

Oocytes were collected following standard ovum pick-up (OPU) procedures. Briefly, dominant follicles were aspirated prior to administration of FSH hormone. Following superstimulation and a brief coasting period as previously described [28], follicles were assessed using a Prosound 2 with WTA OPU probe handle transvaginal ultrasound device (Hitachi Aloka Medical, Tokyo, Japan). Cumulus-Oocyte-Complexes (COCs) were aspirated by transvaginal puncture under epidural using an 18G needle, 50 mmHg pressure and a 16 mls/min flow rate using a Cook Aspiration Unit (Cook Medical, Bloomington, In). COCs were washed in Boviteq's proprietary Oocyte Wash medium supplemented with 3 mg/ml fatty acid free BSA and antibiotics. Washed COCs were then matured overnight at 38.5 °C in Boviteq's proprietary Oocyte Maturation medium supplemented with 10% FBS, 4 mg/ml BSA, pyruvate, antibiotics, FSH, LH and estradiol.

Matured oocytes were washed in Oocyte Wash medium (Boviteq) and placed in Boviteq's proprietary Fertilisation medium supplemented with 6 mg/ml fatty acid free BSA, pyruvate, and antibiotics. Semen was prepared using the BoviPure discontinuous density gradient system, checked for morphology and motility and adjusted to give a final concentration of 1×10^6 sperm/ml. Finally, PHE and heparin were added to the fertilisation medium and zygotes were incubated overnight at 38.5 °C, 5.6% CO_2 under oil.

Presumptive zygotes were washed in Oocyte Wash medium and transferred to Boviteq's proprietary stripping medium supplemented with 3 mg/ml fatty acid free BSA, antibiotics and pyruvate. Cumulus cells were removed mechanically using a 122–124 μM denuding pipette (Vitrolife) until all cells were removed before washing the zygotes once more and transferring them to Boviteq's Proprietary Dev1 media supplemented with 4 mg/ml fatty acid free BSA, antibiotics and pyruvate. Zygotes were incubated for up to 48 h at 38.5 °C, 6.9% CO_2 and 5% O_2 under oil. After this time, 16-cell stage embryos were transferred to Boviteq's proprietary Dev2 media supplemented with 4 mg/ml fatty acid free BSA, antibiotics and pyruvate and incubated up to 48 h at 38.5 °C, 6.9% CO_2 and 5% O_2 under oil. Morulae were then incubated in Boviteq's proprietary Dev3 media supplemented with 4 mg/ml fatty acid free BSA, pyruvate and antibiotics at 38.5 °C, 6.9% CO_2 and 5% O_2 under oil overnight.

2.3. Embryo biopsy, cryopreservation and transfer

Blastocysts were biopsied 7–8 days post-IVF by one of two operators using either a blade-assisted or a laser-assisted method. All biopsy procedures (blade and laser assisted) were carried out on an Integra TI micromanipulator platform (Research Intruments, Falmouth, UK), equipped with a heated stage (set to 38.5 °C). On day 7 or 8 after fertilisation, blastocysts were individually transferred into 60 μl droplets of pre-warmed Vigro Flushing Media (Eggtech, Chilmark, UK) using a 290 μm EZ-TIP (Research Instruments). Embryos were immobilised in this droplet by gentle aspiration on the side in which the inner cell mass was located using a 15 μm flat Holding Pipette with a 35° bend (Research Instruments). For blade assisted biopsy, a microblade (Feather, Osaka, Japan) was employed to deliver a cut by moving the blade laterally against the holding pipette, creating an uneven split. For laser assisted biopsy, a Saturn fixed point laser (Research Instruments) was pulsed at 400 mW for 0.25 ms through its 40x objective in order to drill a hole into the zona pellucida at a site directly opposite the inner cell mass. Once a hole of sufficient size was achieved, trophectoderm cells were aspirated using a 30 μm, 35° angle Biopsy Pipette (Research Instruments). The biopsy was retrieved by mechanical force exerted during aspiration with the aid of one or two laser pulses when necessary in order to disrupt cell junctions between trophectoderm cells. In all cases the biopsy resulted in the recovery of approximately 8–12 cells, which were washed through three droplets of DNA free PBS (Qiagen RepliG Single Cell Kit) under observation and finally transferred into a DNA free PCR tube containing 1 μl of PBS using a 125 μm EZ-Tip (Research Instruments). Presence of the biopsied cells within the tube was confirmed by observation under 40x total magnification.

After biopsy, blastocysts from commercial donors were either transferred fresh or cryopreserved using Boviteq's proprietary Freezing media supplemented with 0.5 mg/ml fatty acid free BSA, 2% FBS, pyruvate and antibiotics. Embryos were rinsed in Boviteq's Rinse media, transferred to Boviteq's Holding media supplemented with 0.5 mg/ml fatty acid free BSA, 2% FBS, pyruvate and antibiotics and then incubated in Freezing media for 20 min. During this time, embryos were loaded into straws following previously published procedures [31] and placed in a BioCool Freezer stabilized at −6.5 °C for 1 min. Ice formation was seeded using tweezers and monitored over 10 min. After this time, embryos were cooled at a rate of 0.3 °C/minute to −33 °C, held for 5 min and plunged into liquid nitrogen. Thawing was achieved by exposure to ambient temperature for 4 s followed by warming at 20 °C for 10 s. Transfer into synchronized recipients was performed with the aid of a Cassou ET gun.

2.4. Whole genome amplification

Embryo biopsies were subject to DNA extraction and whole genome amplification in order to generate enough material for analysis using the RepliG single cell kit (Qiagen, Manchester, UK) as per manufacturer's instruction. Briefly, cell lysis was achieved by incubating biopsies at 65 °C for 10 min in buffer DLB supplemented with 100 mM DTT. Following addition of Stop Solution and master mix containing DNA polymerase, samples were incubated at 30 °C for 8 h. Finally, samples were incubated at 6 °C for 3 min to inactivate the DNA polymerase. Samples displaying a DNA concentration of 100 μg/ml or above as determined on a Qubit 2.0 Fluorometer dsDNA BR assay (ThermoFisher, Waltham, MA) were considered successfully amplified and were stored at −20 °C until submission for genotyping.

2.5. Dam, sire and calf DNA extraction

For sires, DNA was extracted from sperm pellets leftover from IVF procedures. Briefly, the sperm pellets were washed in 1 ml buffer I (150 mM NaCl, 10 mM EDTA, pH 8.0), and centrifuged at 6000 g for 10 min before discarding the supernatant. The sperm pellets were then suspended in 500 μl buffer II (500 mM NaCl, 100 mM TRIS, 10 mM EDTA, 1% SDS, 100 mM DTT, pH 8.0) and incubated at 65 °C for 90 min. After this time, 150 μl of ammonium acetate 7.5 M was added to the mixture to precipitate proteins and the sample was spun at 15,000 g for 10 min. The supernatant was transferred to a clean tube, mixed with 900 μl of isopropanol and spun at 20,000 g for 10 min. The resulting DNA pellet was washed in 500 μl ethanol then spun as before. Finally, all supernatant was discarded and the DNA was re-hydrated overnight at 4 °C in 20 μl of TE buffer (1 mM EDTA, 10 mM TRIS, pH 8.0).

For dams and calves, DNA was extracted from blood samples collected by standard technique into lithium heparin vacutainers using the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) following manufacturer's instruction. Alternatively, maternal DNA was extracted from abattoir-derived ovarian tissue in the same way.

2.6. SNP interrogation and Karyomapping

For all samples, SNP interrogation and genotyping was performed by Neogen Europe (Auchincruive, UK) using a GGP 50K SNP array (Illumina, Cambridge, UK). Raw data from embryo biopsies was submitted to the Agriculture and Horticulture Development Board (ADHB) for genomic evaluation.

Samples showing a call rate of at least 80% and for which a sibling (including a sibling embryo) was available were considered suitable for Karyomapping. Raw SNP data from embryo biopsies and parental DNA samples were processed in-house using the Excel macro BoVision (version 3) based on the original Karyomapping program (developed and adapted by Alan Handyside, Illumina, Cambridge, UK). For the diagnosis of chromosomal abnormalities, three independent operators reviewed each case. The number of crossover events and aneuploidy events were determined on a per chromosome basis for all the assessed samples. A crossover event was recorded when at least three consecutive informative SNPs were observed. Intuitively, because the Y chromosome is only present in a single copy in the paternal genome, it cannot be used for haploblock-based investigations like Karyomapping and therefore was excluded from our analysis. Similarly, because sires only have one copy of the X chromosome, the paternal X chromosome will be passed more or less unchanged from father to female offspring, once again preventing haploblock analysis.

3. Results

Overall, 111 embryo biopsies (n = 80 from commercial donors and n = 31 from abattoir derived material) were produced of which 93 were successfully amplified by WGA, 89 were successfully SNP typed and 61 (33 from commercial donors and 28 from abattoir sources) fulfilled all the requirements for Karyomapping (high enough call rate, availability of a sibling). The embryo biopsies were obtained from 15 IVP rounds and a total of 9 different sires and 11 different dams. At the time of writing, we have a pregnancy rate of 41.4% (12 pregnancies out of 29 transfers and currently five liveborns following ET of Karyomapped embryos).

3.1. Frequency and type of chromosomal abnormalities in the trophectoderm of bovine blastocysts

Following Karyomapping analysis of trophectoderm biopsies, 68.8% of the embryos (n = 42/61) appeared euploid while the rest displayed one or more abnormalities including monosomy, trisomy, uniparental disomy, triploidy and parthenogenetic activation. A trend appeared to be present with embryos displaying either up to three abnormal chromosomes (n = 10/61) or a large set of abnormalities (n = 9/61) with no in-between clusters.

Overall, trisomies were detected with a frequency of 2.12% (n = 70/3304 chromosomes) while monosomies with a frequency of just 0.30% (n = 10/3304 chromosomes) resulting in a statistically significant difference between these two groups (chi-square, $\chi^2_1 = 45.5$, $P = 1.5 \times 10^{-11}$). However, no chromosome appeared to be more commonly affected by aneuploidy (Fisher's exact test, df = 58, $P > 0.05$). These results are displayed in Fig. 1. Finally, triploidy and uniparental disomy were only detected once (n = 61) while parthenogenesis occurred in four embryos. Example Karyomaps are shown in Fig. 2.

3.2. Parental origin of aneuploidy

When the parental origin of the aneuploidy was considered, monosomies were detected with a frequency of 0.24% (4/1653) and 0.36% (6/1653) for chromosomes of paternal and maternal origin,

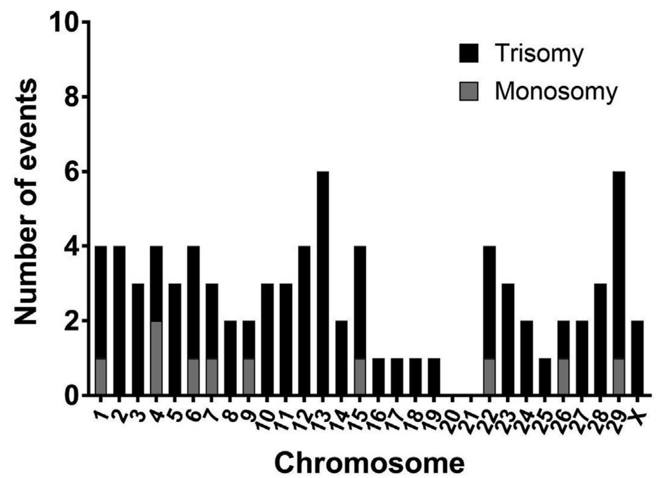

Fig. 1. Number of monosomies and trisomies detected per chromosome by Karyomapping of cattle embryo biopsies. The results were collected from n = 56 embryos and a total of 3304 chromosomes. No information on the paternal X chromosome was available.

respectively, and no statistically significant differences were found between these two groups (chi square, $\chi^2_1 = 0.40$, $P = 0.52$). On the other hand, trisomies appeared over represented in the maternally derived chromosome population where they were observed with a frequency of 2.60% (n = 43/1653) as opposed to a frequency of 1.51% (n = 25/1653) for the paternally derived chromosome population, resulting in a statistically significant difference (chi-square, $\chi^2_1 = 4.7$, $P = 0.03$). These findings are summarized in Fig. 3. Due to the nature of the sex chromosomes and of Karyomapping, however, abnormalities of the X chromosome could not be compared between sexes. Parthenogenetically activated embryos and triploid embryos were also excluded from comparison.

3.3. Frequency of crossover in the maternal and paternal germline

On average, 87.7 ± 1.4 (SEM) crossover events were detected per euploid embryo, of which 44.6 ± 0.9 were of paternal and 43.1 ± 1.0 of maternal germline origin. While there was no difference in the total number of crossover events between the two germ lines (*t*-test, $t_{96} = 1.06$, $P = 0.29$), significant differences became apparent at a chromosomal level. A complete breakdown of this analysis is presented in Fig. 4.

Assuming that, on average, one crossover event occurs in a chromosome of size 1 Morgan (M) as discussed by Weng et al. [32], based on the number of crossover events detected in this set of tests (as reported in Table 1), the genetic length of the bovine genome was estimated to be 4453 cM (sex averaged length across 29 autosomes plus female chromosome X). By dividing the genetic length by the physical genome length measured in mega base pairs (Mb), the average genome-wide recombination distance per Mb was calculated as 1.67 cM/Mb. Furthermore, linear regression analysis found that a significant correlation exists between chromosome size and average recombination distance, suggesting smaller chromosomes have shorter distances between crossover events (linear regression, $R^2 = 0.45$ $F = 22.02$, $P = 6.96 \times 10^{-5}$). Interestingly, the maternal chromosome X appeared as an outlier, showing a much greater space between adjacent events than other chromosomes of similar size. This is detailed in Fig. 5. Moreover, when only euploid embryos were taken into account, Karyomapping analysis detected a higher genetic length in abattoir derived embryos (4695 cM, n = 19) as opposed to commercially derived embryos (4159 cM, n = 23) (*t*-test, $t_{40} = 3.21$, $P = 0.0026$).

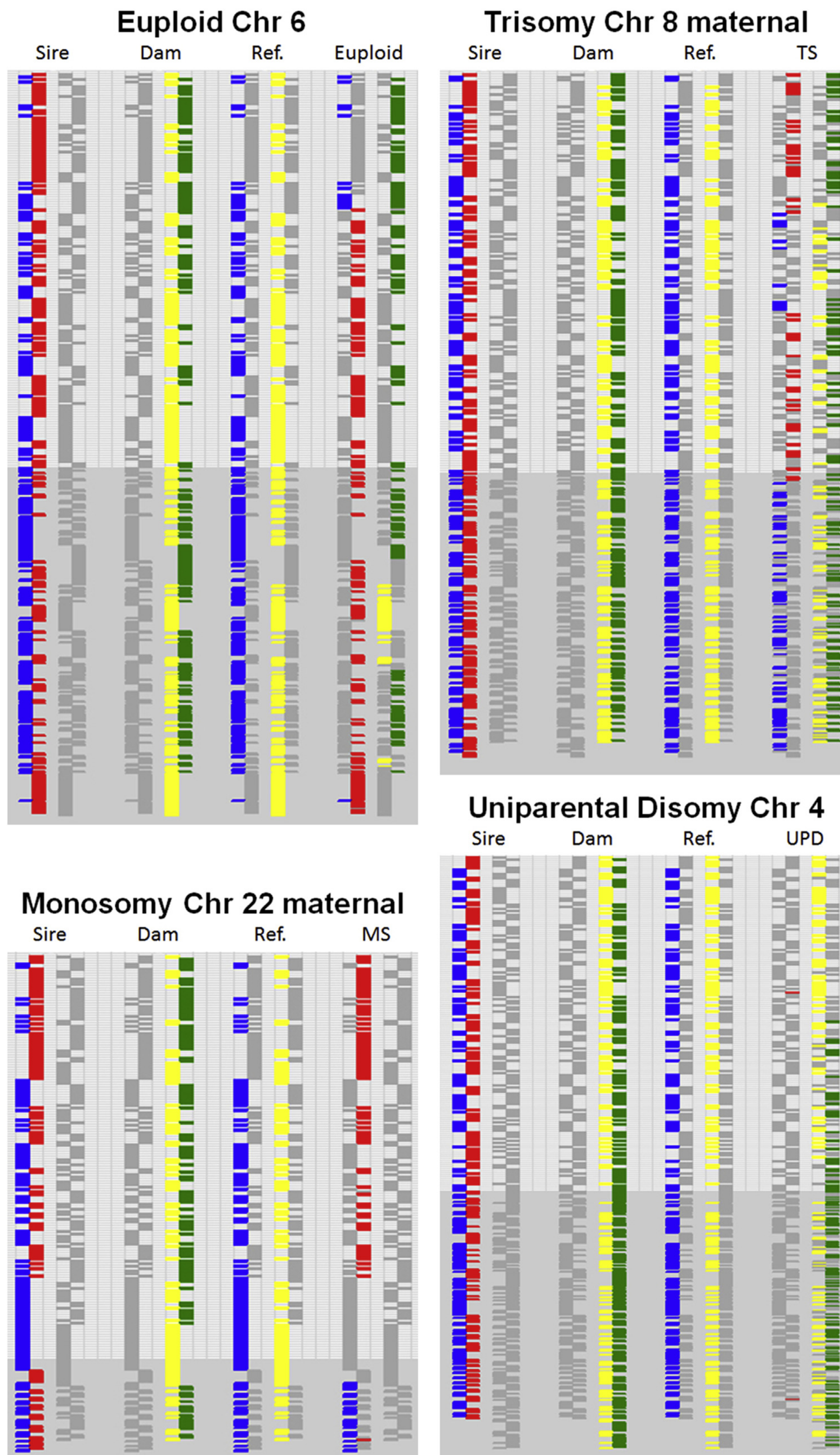

Fig. 2. Example Karyomaps. A series of Karyomaps are presented from both normal and abnormal cases. Each case is presented together with information from both its parents and a sibling embryo. Gray bands imply absence of information. Euploid chromosomes are characterized by few, large alternating blocks of blue/red (for paternal) or green/yellow (for maternal) bands, representing alternating haplotypes. Monosomies appear as complete or almost complete lack of information for a full chromosome, while trisomies appear as frequent and short blocks of alternating haplotypes. Uniparental disomies appear as a monosomy for one chromosome and a trisomy for its homologue, and, finally, parthenogenetic activation events appear similar to a monosomy of paternal origin at all loci. Chr: Chromosome; Ref.: Reference sibling; MS: Monosomy; TS: Trisomy; UPD: Uniparental Disomy. These Karyomaps were produced by BoVision (version 3).

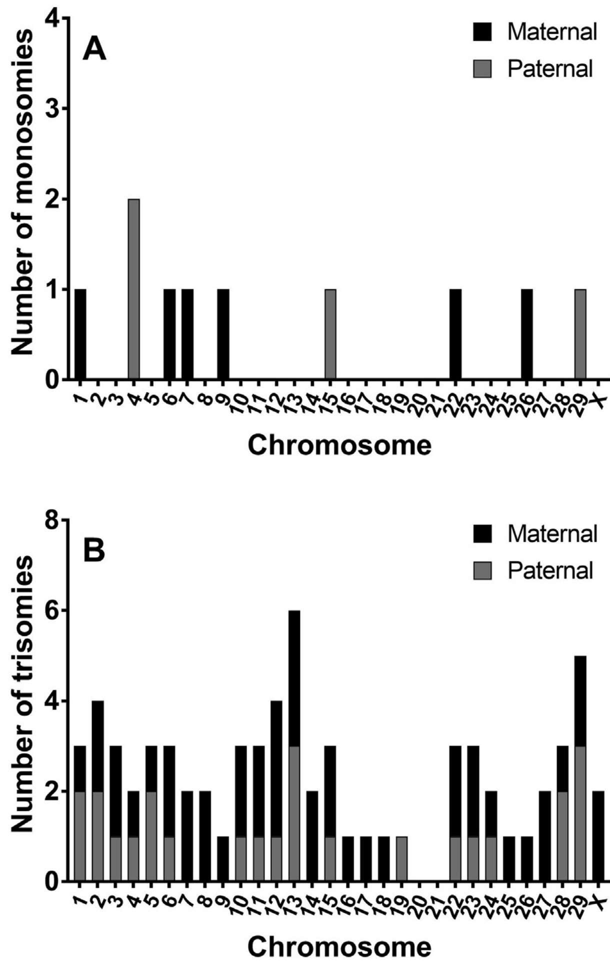

Fig. 3. A) Number of monosomies per chromosome by parent of origin. No obvious pattern was detected. B) Number of trisomies per chromosome by parent of origin. Overall, trisomies appeared more common in the maternal chromosome population (chi-square, $P < 0.05$). Data from $n = 56$ embryo biopsies and $n = 3304$ chromosomes. No information available for the paternal X chromosome.

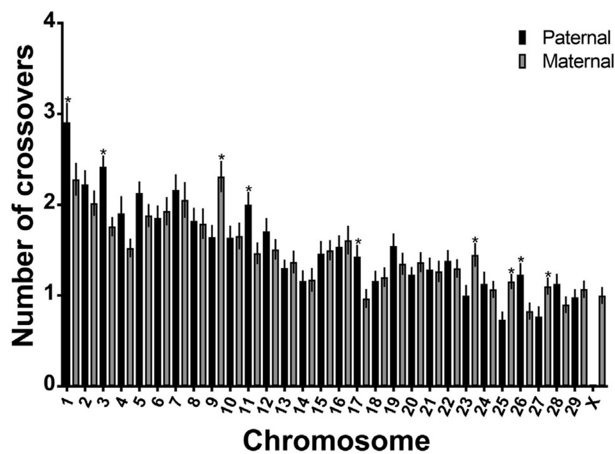

Fig. 4. Average number of crossovers per chromosome and parent of origin. The analysis was completed on a total of 56 embryos. For each chromosome pair, a star (*) indicates that a significant difference was found between the number of events per parent (paired student t-test, $P < 0.05$). The paternal X chromosome could not be analyzed by Karyomapping. Data given as mean ± S.E.M.

3.4. Concordance between embryo biopsy and live born calves

To verify whether the SNP typing and Karyomaps produced from embryo biopsies were a true representation of the embryo's genotype, the results obtained from the biopsied embryos that later resulted in live births (see Fig. 6) were compared against the SNP typing and Karyomaps obtained from the respective live born calves.

All five embryo biopsies appeared euploid at Karyomapping, a result that matched the live born calves. Moreover, when the SNP results were compared, it was found that the biopsy and the live born were concordant for $98.2 \pm 1.4\%$ of SNP loci. ADO events (where one sample was homozygous and the other heterozygous) represented $1.8 \pm 1.4\%$ of loci and complete mismatches (one sample homozygous for allele A, the other sample homozygous for allele B) affected between 0% and 0.02% of loci. SNP loci that produced no call in either the biopsy, the live born or both were excluded from the analysis above.

4. Discussion

Here, we present the first successful application of Karyomapping to the cattle breeding industry, which has led to the birth of five healthy calves. We have combined the use of SNP data generated from the biopsy and whole genome amplification of DNA extracted from trophectoderm cells from preimplantation embryos for both genomic selection and PGS. Furthermore, we have employed this technology to investigate the frequency of meiotic recombination and the frequency of meiotic errors in both dam and sire lines. Combined, these efforts provide new insight to our existing knowledge of bovine genetics and have the potential to significantly improve the rate of genetic gain in beef and dairy herds. Genotyping of embryo biopsies by SNP genotyping has been reported occasionally in the literature [9] and has the potential to improve cattle breeding by introducing new genetics to the breeding herd more rapidly by shortening generation times. By combining chromosomal screening, Karyomapping also has the potential to improve overall live birth rates. Suboptimal fertility in cattle is a huge issue from a commercial and industry point of view. If aneuploidy is the cause of much of this failure of implantation or early embryonic death then this approach would be massively informative, and would lead to us developing PGS-based techniques to improve conception rates. Our reported pregnancy rate in this study (32.8%) is, by necessity, low, as much of the work was developmental and only latterly did we act on the Karyomapping result when performing ET. Future controlled studies will be able to assess the efficacy of this approach with screened and non-screened cohorts.

4.1. Characterisation of chromosomal abnormalities in bovine preimplantation embryos by Karyomapping

Karyomapping revealed that a total of 42/61 (68.9%) embryos were euploid, 4/61 (6.6%) were parthenogenetically activated and 15/61 (24.6%) embryos had at least one chromosome aberration. Interestingly, uniparental disomy was identified in one of these cases, demonstrating the applicability of Karyomapping for the discovery of this condition in bovine embryos, the diagnosis of which normally escapes detection with other methodologies such as array comparative genomic hybridization for aneuploidy [22].

Overall, the incidence of aneuploidy was lower than that of some reports in the literature [14,33] and higher than that of others [34,35]. Remarkably, a significantly higher number of trisomies

Table 1
Average number of crossovers and calculated recombination distance by chromosome.

Chromosome	Size (Mb)	Average crossover count	Recombination distance (cM/Mb)
1	158.34	2.58	1.63
2	137.06	2.18	1.59
3	121.43	2.08	1.71
4	120.83	1.70	1.41
5	121.19	2.00	1.65
6	119.46	1.89	1.58
7	112.64	2.11	1.87
8	113.39	1.81	1.59
9	105.71	1.98	1.87
10	104.31	1.65	1.58
11	107.31	1.72	1.61
12	91.16	1.23	1.34
13	84.24	1.34	1.59
14	84.65	1.17	1.38
15	85.30	1.48	1.74
16	81.72	1.57	1.92
17	75.16	1.19	1.58
18	66.00	1.18	1.79
19	64.06	1.44	2.25
20	72.04	1.30	1.81
21	71.60	1.27	1.78
22	61.44	1.34	2.18
23	52.53	1.23	2.34
24	62.71	1.10	1.75
25	42.90	0.95	2.21
26	51.68	1.03	1.98
27	45.41	0.94	2.07
28	46.31	1.01	2.18
29	51.51	1.05	2.04
X (maternal)	148.82	1.00	0.67
TOTAL	2660.91	44.53	–
Average	–	–	1.67

than monosomies were detected. Karyomapping is able to detect meiotic but not post-zygotic trisomies, whilst being able to detect both meiotic and post-zygotic monosomies [22]. This might suggest that some of the trisomies recorded were in fact false positives. However, to mitigate this risk, three independent operators were required to agree on the diagnosis before a trisomy was called. Moreover, our study includes an evaluation of blastocyst stage embryos, whereas others have assessed cleavage stage embryos at either the 2-cell or 8-cell stage. It is possible that trisomies are better tolerated than monosomies by preimplantation cattle embryos, allowing them to reach the blastocyst stage more often, a hypothesis supported by evidence gathered from studies in humans [36,37]. Therefore, the population examined here could have been partially depleted of monosomies, and to the best of our knowledge, this is the first report to describe this phenomenon in cattle. The observations discussed above would imply that the numerical aberration rates presented here are not informative of the original number of monosomy and trisomy events occurring in bovine germlines. However, total blastocyst aneuploidy rates appear very similar to the total gamete aneuploidy rates in humans [38], suggesting that the trisomy rates recorded here could be a good estimate of trisomy rates in the parental germline. It is also important to note that this study has utilised a more sensitive technique than those that identified lower incidences of aneuploidy at the cleavage stage, and therefore it is possible that the results of these other studies are under-represented.

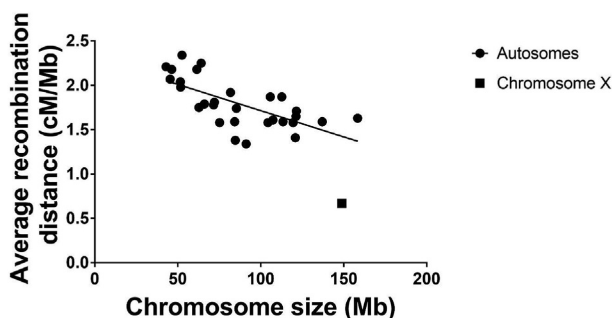

Fig. 5. A moderate but significant association was found between chromosome size and average recombination distance, suggesting that smaller chromosomes allow for less space between events. Chromosome X appeared as an outlier due to its reduced recombination frequency.

Fig. 6. Birth of the first Karyomapped calf. Crossfell Cinder Candy is the daughter of the sire Cinderdoor (Semex) and the dam Crossfell Uno Cookie (Paragon Veterinary Group) and was born in Cumbria following the transfer of a Karyomapped embryo.

In this data set, no sex-related difference was discovered in the prevalence of monosomies; however, trisomies were 1.7 times more common in the maternal chromosome population, somewhat less than the 9:1 ratios observed in humans [15] but similar to that previously reported for cattle [39]. Moreover, our data sheds new light on the incidence of meiotic errors for individual chromosomes in the bovine karyotype; a topic barely covered in the current literature. Although some information is described in oocytes, studies have largely used dated technologies and therefore more up-to-date investigations are warranted [39–41]. In bulls, very little information is available regarding the total aneuploidy levels in sperm, with only two studies suggesting a low incidence of aneuploidy in bull sperm [42,43]. Karyomapping, thanks to its ability to assign the parental origin of aneuploidy, can be used to report on all bull sperm chromosomes to fill the current gap in the literature. A total aneuploidy rate of 1.75% was estimated for bull sperm in this study, albeit this figure was established on a small population of sires. In order to investigate this further in the bovine model, future efforts should focus on investigating the incidence of aneuploidy in bull spermatozoa and cow/heifer oocytes. This will form the basis of our future studies.

Additionally, in this data set four embryo biopsies showed evidence of parthenogenetic activation. Mammalian oocytes are known to have the potential to become activated and progress to cleavage divisions and even form a blastocyst without the need for a male gamete contribution [44]; however, the spontaneous activation of an oocyte is a rare occurrence [45]. It is also interesting to note that three of these parthenotes originated from the same IVF cycle, which used semen from the same sire. A spike in the calcium concentration in an oocyte is sufficient to induce parthenogenetic activation [46]. Therefore, it is possible that some oocytes become activated after a fertilisation-induced calcium spark, but problems with sperm decondensation, perhaps due to a bull-specific factor, prevented syngamy and resulted in a parthenogenetically activated oocyte instead, an event that has been described before in bovine embryos fertilized by intracytoplasmic sperm injection (ICSI) [47].

Finally, it is important to note that our results (and those of others) could be affected by the presence of mosaicism, which is defined as the presence of two or more cell lineages of different chromosome complement and is well-reported in humans [48–50] and in cattle [23,33]. In addition, it has been established for some time that the trophectoderm is often affected by some degree of aneuploidy, which is not necessarily mirrored by the inner cell mass [48]. At present the extent to which mosaicism confers detrimental effects (or not) remains unknown and is heavily debated, however it should be pointed out that it is not possible to screen the entire embryo and therefore analysis must be carried out on only a portion of the embryo. In the current study, an evaluation of mosaicism from TE biopsies was not undertaken; however, methodologies such as aCGH and NGS could be applied to study the nature and impact of mosaicism on development [51].

4.2. Recombination events

The number of crossovers per meiotic event is known to differ significantly between the two sexes in several mammalian species [52–54]. In cattle, Ma et al. [55] reported an increased recombination frequency in bulls; however, the results presented here failed to detect a clear difference in the recombination rate of bulls and cows in agreement with several other studies [56–58].

Using Karyomapping, we estimated that the genetic length of the 29 autosomes plus the maternal X chromosome in the bovine genome is 4453 cM. This largely exceeds previously published estimates of 3097 cM [32] and 2435 cM [55]. Similarly, the recombination frequency estimated in our study using Karyomapping (1.67 cM/Mb) was much greater than the recombination frequency previously estimated by other groups, which fell in the range of 0.8–1.12 cM/Mb [32,55,58]. This could be explained by a higher number of crossover events detected by Karyomapping, a different sample population, or by false positives; however, to reduce false positives crossover events were only counted if supported by at least three consecutive informative SNPs.

Interestingly, no crossovers could be detected on a number of euploid chromosomes despite the fact that a minimum of one chiasma per chromosome is required during meiosis [59]. Karyomapping can only detect crossover events that generate different haplotypes between the sample and reference and therefore juxtaposing crossovers (events occurring in the exact or similar location in both sample and reference) will remain undetectable. In addition, crossovers tend to accumulate close to so-called "recombination hot-spots" [60] so that sample and reference might regularly display juxtaposing crossovers. Moreover, Karyomapping relies on the presence of heterozygous markers to track haploblocks; therefore, the presence of shared homozygosity regions between parents could have masked some events. Therefore, these considerations suggest that the genetic length calculated here by Karyomapping could be an underestimation. This hypothesis is also backed up by the finding that abattoir derived embryos displayed an apparently higher recombination rate, possibly thanks to a reduced genetic relationship between sire and dam, allowing Karyomapping to obtain more accurate crossover estimates in this more heterogeneous population.

Additionally, the findings presented suggest that smaller chromosomes display a higher number of recombination events per Mb, in agreement with previous studies in cattle [32,61]. Our data shows a more pronounced effect than those previously described, however it is possible that this can be explained by differences in sample population, SNP array used and breeds assessed. Interestingly, the X chromosome displayed a smaller number of crossover events than expected for its size. This is in agreement with previous studies detecting this phenomenon in humans [20], rat, and mouse [62].

4.3. Accuracy of Karyomapping in preimplantation bovine embryos

To the best of our knowledge, this is the first study to assess the accuracy of SNP calls from whole genome amplified biopsies from blastocyst stage bovine embryos in comparison to the respective live-born animals. We report that over 98% of SNP calls were concordant in embryo biopsies with their respective live-born calves, indicating the ability to accurately determine the genetic merit of the embryo. These results are in line with the accuracy obtained by Fujii et *al.* [63], who compared SNP genotype calls in whole genome amplified biopsied sections from two elongating bovine embryos compared to the remaining conceptus. In addition, our results exceeded the accuracy achieved by Lauri et al. [64] who assessed ADO and allele drop-in (ADI) in a single known heterozygous and single known homozygous loci respectively in biopsies from cloned blastocyst stage bovine embryos, or by comparing the genotypes of blastocyst biopsies to expected outcomes based on parent SNP calls. In this respect, our own results offer a more robust assessment of the accuracy of SNP genotyping from whole genome amplified biopsies from bovine preimplantation embryos than results currently reported in the literature.

4.4. Live born calves

We show for the first time that results from commercial SNP arrays for the purpose of genomic selection may be simultaneously applied using Karyomapping technology in order to screen for

chromosome copy number in bovine preimplantation embryos. Use of Karyomapping led to the birth of five healthy calves, with 100% concordance found between results from the embryo biopsy and the respective live-born animal. Since healthy live calves were born, it is unsurprising that in these cases both the embryo and the live-born animals produced euploid results for all autosomes and the X chromosome. In this study, we did not assess the accuracy of aneuploid calls as aneuploid embryos were either not transferred or resulted in no pregnancy. Again, this would be expected given the evidence available from studies in humans, which indicates that aneuploidy is a leading cause of IVF failure. Nonetheless, other reports indicate a high degree of accuracy in detecting aneuploidy using SNP analysis from whole genome amplified human DNA [65,66].

5. Conclusions and future perspectives

The work described demonstrates the value of Karyomapping for both PGS and genomic selection, and for undertaking fundamental biological studies with bovine embryos. The birth of the first five Karyomapped calves opens the way to a method of breeding that combines IVP with simultaneous genetic screening for chromosomal errors (PGS) and genomic selection for livestock improvement. Given that aneuploidy is recognized as a leading cause of pregnancy failure, at least in humans, Karyomapping offers the prospect of selecting more karyotypically normal and, therefore, 'developmentally competent' embryos for transfer; thereby improving pregnancy rates and enhancing overall breeding efficiency (i.e. rate of genetic gain) in cattle. This, however, remains to be confirmed and details pertaining to the nature and extent of aneuploidy in different embryonic-cell lineages, and mosaicism more generally, will need to be established for this species. Larger scale studies that relate pregnancy outcomes following transfer to chromosomal anomalies in embryo biopsies are therefore required. Ultimately, this could lead to the development of a low-cost, high-throughput platform for chromosome screening that focuses on the most important and/or frequent errors as they relate to pregnancy outcomes. Finally, knowledge gained from such studies can be readily translated to other domestic animal species (e.g. pigs and sheep) as high-density SNP chips are already commercially available. While each species will no doubt present its own challenges, research efforts to improve IVP success are ongoing and it is likely that Karyomapping will find a use in these species as well.

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Declarations of interest

The authors have no conflict of interest to declare.

Contributors

KJT, GS, GD and CS acquired the data reported in this study. DHB, AHH, KDS and DKG conceived the study. The analysis of the data was performed by GS, KJT, AHH and DKG. GS, KJT, DHB, KDS and DKG drafted the manuscript which was critically reviewed and approved by all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2018.11.014.

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