RESEARCH ARTICLE

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The Methylation Patterns of the *IGF2* and *IGF2R* Genes in Bovine Spermatozoa are not Affected by Flow-Cytometric Sex Sorting

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SUMMARY

The objectives of this study were to investigate the effect of sexing by flow cytometry on the methylation patterns of the *IGF2* and *IGF2R* genes. Frozen-thawed, unsorted, and sex-sorted sperm samples from four Nellore bulls were used. Each ejaculate was separated into three fractions: non-sexed (NS), sexed for X-sperm (SX), and sexed for Y-sperm (SY). Sperm were isolated from the extender, cryoprotectant, and other cell types by centrifugation on a 40:70% Percoll gradient, and sperm pellets were used for genomic DNA isolation. DNA was used for analyses of the methylation patterns by bisulfite sequencing. Methylation status of the *IGF2* and *IGF2R* genes were evaluated by sequencing 195 and 147 individual clones, respectively. No global differences in DNA methylation were found between NS, SX, and SY groups for the *IGF2* ($P = 0.09$) or *IGF2R* genes ($P = 0.38$). Very specific methylation patterns were observed in the 25th and 26th CpG sites in the *IGF2R* gene. representing higher methylation in NS than in the SX and SY groups compared with the other CpG sites. Further, individual variation in methylation patterns was found among bulls. In conclusion, the sex-sorting procedure by flow cytometry did not affect the overall DNA methylation patterns of the *IGF2* and *IGF2R* genes, although individual variation in their methylation patterns among bulls was observed.

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INTRODUCTION

Although sexed sperm can greatly impact on breeding programs, high cost and reduced pregnancy rates are limiting factors for wider application of this procedure in cattle breeding. Indeed, using sperm sexed by flow cytometry tends to result in low pregnancy rates in artificial insemination and embryo transfer programs (Seidel et al., 1999; Sartori et al., 2004; Bodmer et al., 2005; Andersson et al., 2006; Peippo et al., 2009; Underwood et al., 2010ab), and low rates of in vitro embryo production (Morton et al.,

Abbreviations: DMR, differentially methylated region; ICR, imprinting control regions; IGF2[R], insulin-like growth factor 2 [receptor]; NS, non-sexed sperm; SX, sexed for X-sperm; SY, sexed for Y-sperm.

2007; Bermejo-Alvarez et al., 2008, 2010). These results suggest that fluorescence-activated cell sorting using flow cytometry may damage sperm, which could be due to the exposure to the laser, high speeds inside the collecting tube, exposure of the droplets to electric charges, and/or incubation at room temperature while being processed. Besides affecting physical and structural characteristics of the sperm (Blondin et al., 2009; Carvalho et al., 2010), these factors are potential hazards to the DNA, which could affect subsequent embryo development.

Sperm DNA damage may result from DNA fragmentation, abnormal chromatin packaging, and epigenetic defects (Tavalaee et al., 2009). Yet, assessment of DNA fragmentation has been the only method used for evaluating the effect of sexing on chromatin integrity (Boe-Hansen et al., 2005; Blondin et al., 2009; Carvalho et al., 2010; Gosalvez et al., 2011), and no study has assessed possible epigenetic changes that could occur as well.

In mammals, correct DNA methylation reprogramming and maintenance of genomic imprinting during gametogenesis are essential for embryo development, normal preimplantation (Newell-Price et al., 2000), and maintenance of pregnancy (Lucifero et al., 2004). DNA methylation is one of the most stable epigenetic modifications, and is the most studied, major epigenetic modification governing gene expression (Bird, 2002; Jaenisch and Bird, 2003). It occurs in regions of the genome that are generally rich in CpG dinucleotides (Bird, 2002; Jaenisch and Bird, 2003), which are located on promoter regions in differentially methylated regions (DMR) or in imprinting control regions (ICR). DNA methylation is the most well-characterized example of epigenetic contribution of the sperm nucleus to the developing embryo, and paternal methylation defects can severely compromise embryo development (Carrel and Hammoud, 2010). This modification is thought to have dramatic impacts on embryo survival.

Two important imprinted genes are insulin-like growth factor 2 (*IGF2*) and insulin-like growth factor-2 receptor (*IGF2R*), which are, respectively, located on chromosome 29 and 9 in the bovine genome. Both genes are related to embryo development and placentation (Curchoe et al., 2005; Perecin et al., 2009). The *IGF2* gene is predominantly expressed by the paternal allele and silenced on the maternal allele (Feil et al., 1994). This indicates the presence of an intragenic DMR, with a high level of DNA methylation in the sperm and a low level in the oocyte (Gebert et al., 2006). The *IGF2R* gene is generally imprinted on the paternally inherited allele and expressed from the maternal allele, and it is controlled by a differentially methylated ICR (Zwart et al., 2001).

There are a variety of studies reporting that artificial reproductive technologies (ARTs), including somatic cell nuclear transfer (SCNT), in vitro fertilization, intracellular sperm injection, cryopreservation, and in vitro embryo culture, are associated with changes in DNA methylation of the *IGF2* and *IGF2R* imprinted genes (Curchoe et al., 2005; Long and Cai, 2007; Gebert et al., 2009; Poplinski et al., 2010). These changes can result in abnormal development because the acquisition and maintenance of genomic imprinting are critical for early embryogenesis and maintenance of pregnancy (Lucifero et al., 2004). The increase of the imprinting abnormalities in ARTs could arise from in vitro manipulation of gametes and embryos or other external factors, such as hormonal administration. Because sex-sorted sperm are subjected to several hazardous conditions during the sorting process, we hypothesized that these factors may induce alterations in DNA methylation patterns. Therefore, this study investigated the effect of sexing by flow cytometry on the methylation patterns of the *IGF2* and *IGF2R* genes.

RESULTS

Two imprinted genes that are normally paternally methylated (*IGF2*) or unmethylated (*IGF2R*) were examined using the quantitative bisulfite sequencing method. The methylation patterns of the *IGF2* DMR and *IGF2R* ICR2 for all treatments are shown in Figure 1. One hundred percent of the analyzed sequences for the *IGF2* or *IGF2R* genes in all treatments were hypermethylated ($\geq$50% of CpG sites on a given methylated strand) or hypomethylated ($<$50% of CpG sites on a given methylated strand), respectively. For the *IGF2* gene, 195 clones were evaluated, and in each clone, we analyzed a total of 28 CpG sites. No differences in DNA methylation were found between the non-sexed (NS) ($96.4 \pm 0.41\%$; $n = 67$ clones), sexed for X (SX) ($95.4 \pm 0.46\%$; $n = 67$ clones), and sexed for Y (SY) ($96.9 \pm 0.38\%$; $n = 61$ clones) sperm groups ($P = 0.09$).

For the *IGF2R* gene, 147 clones were evaluated, and for each clone a total of 26 CpG sites were analyzed. Similar to the *IGF2* gene, no differences in overall DNA methylation were found between the NS ($7.24 \pm 0.58\%$; $n = 44$ clones), SX ($8.0 \pm 0.64\%$; $n = 58$ clones), and SY ($7.96 \pm 0.46\%$; $n = 48$ clones) groups ($P = 0.38$). Very specific methylation patterns were, however, observed in the 25th and 26th CpG sites (Fig. 1), which were highly methylated in the NS (86.4% and 84.1%), SX (86.2% and 81.0%), and SY (89.6% and 62.5%) groups compared with the other CpG sites. Furthermore, the percentage of methylation on the 26th CpG site was lower ($P < 0.05$) in the SY group than in the NS and SX groups (Fig. 2).

We also compared the methylation patterns of the *IGF2* and *IGF2R* genes among treatments for each individual bull, and among bulls within each treatment (Tables 1 and 2). For the *IGF2* gene, there were no differences between bulls within the same group (Table 1). Bull 3 showed a difference in methylation patterns, with a higher percentage of methylation in the SY group than SX (Table 1 and Fig. 3). When the same analysis was performed for the *IGF2R* gene, there was no difference between sperm groups for any of the bulls (Table 2). Nevertheless, an effect by individual bull was observed on the methylation patterns of the *IGF2R* gene for the SY group, in which sperm from bulls 1 and 3 were less methylated than those from bulls 2 and 4 (Table 2 and Fig. 4).

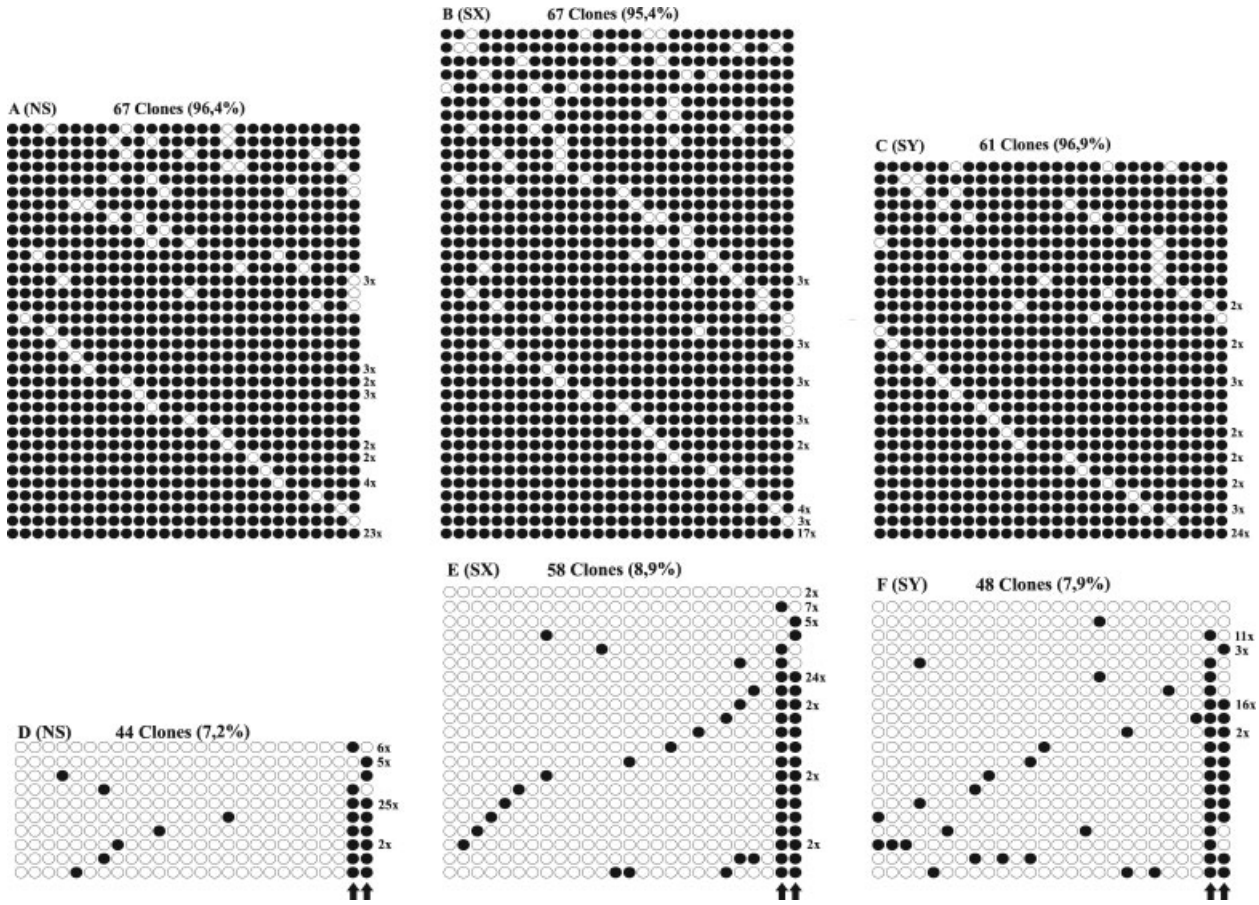

Figure 1. Methylation patterns in the DMR of the last exon of the *IGF2* gene (**A**–**C**) and the second imprinting control region (ICR2) of the *IGF2R* gene (**D**–**F**) in non-sexed (A and D), sexed-X (**B** and **E**) and sexed-Y sperm (C and F) from four Nellore bulls. The arrow indicates the very specific methylation patterns observed in the 25th and 26th CpG sites, which had high methylation. White and dark circles represent unmethylated and methylated CpGs, respectively; horizontal lines of circles represent one clone, and the number of clones with the same methylation patterns is indicated at the right end of the lines. The data are the average of four bulls (three replicates per bull for the *IGF2* gene, and two replicates per bull for the *IGF2R* gene).

DISCUSSION

During the sorting process, spermatozoa are exposed to several potential hazards that could be responsible for deleterious effects in the cell and reduced fertility rates. Indeed, some studies (Blondin et al., 2009; Carvalho et al., 2010) have focused on clarifying the effect of flow cytometric cell sorting on the viability of sperm cells. Several attributes of the spermatozoa, such as motility pattern, lifespan, acrosome integrity, viability of the plasma membrane, chromatin integrity, embryo development, and mRNA profile, have been evaluated (Boe-Hansen et al., 2005; Morton et al., 2007; Blondin et al., 2009; Carvalho et al., 2010); none of the studies have evaluated epigenetic changes—another candidate, given that cells susceptible to environmental changes are exposed to potential external risks.

Our results showed no differences in DNA methylation between the NS, SX, and SY groups for either gene (Fig. 1). All analyzed sequences for the *IGF2* and *IGF2R* genes in every treatment were hypermethylated and hypomethylated, respectively. These patterns of methylation for those specific regions in non-sexed (NS) bovine sperm have been described by Gebert et al. (2006), Long and Cai (2007), and Gebert et al. (2009).

Sexed sperm are subjected to a variety of adverse conditions during sorting, which can cause damage that may compromise embryo development and pregnancy rates. Previous studies in our laboratory have failed to detect an effect of sperm sexing on in vitro embryo development until D7 (Carvalho et al., 2010). We hypothesized that sex-sorting could cause sperm damage that is not observed in D7 embryos, but appears later in pregnancy; this possibility is supported by higher embryo loss between days 30 and 90 of pregnancy reported when sexed-sperm were used to inseminate cows and heifers (Bodmer et al., 2005). Embryonic loss between days 30 and 90 is also very common in cloned embryos (Hill et al., 2000;

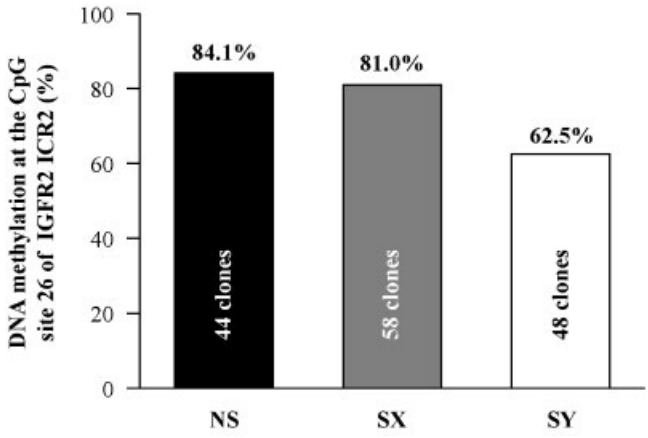

Figure 2. Percentage of methylation at the 26th CpG site in the second imprinting control region (ICR2) for the *IGF2R* gene in non-sexed (NS), sexed-X (SX), and sexed-Y (SY) bovine sperm ($P < 0.05$). Data are the average of four bulls (two replicates per bull).

Suteevun-Phermthai et al., 2009), perhaps due to abnormalities of DNA methylation in nuclear reprogramming during cloning (Kang et al., 2001; Young et al., 2001; Long and Cai, 2007). In that regard, abnormal methylation levels in sexed sperm could explain some of the problems with embryo development and implantation failures.

Based on these observations from embryo cloning, our finding that sperm sexing had no effect on methylation patterns of those regions was unexpected. We instead speculate that bovine sperm are highly resistant to DNA damage or environmental changes. It is well known that sperm DNA is extremely stable because the paternal genome is packaged and covalently modified (Hammoud et al., 2009a). This packaging in sperm DNA is supported by the high proportion of protamine proteins, with only a small portion of the DNA being associated with histone proteins (Hammoud et al., 2009a). Hammoud et al. (2009a) suggested that these remaining histones are associated with regions containing imprinted genes in bovine. Although both genes evaluated in this study are imprinted, it is not known whether these regions are packaged by histones or protamines, making these regions more or less susceptible to changes. Moreover, it is important to point out that in the present study we only assessed two regions of the genome, and we cannot assume that other regions do not have altered patterns of methylation due to sexing. Additionally, we cannot ignore that the sexing process has been improved in recent years (Sharpe and Evans, 2009), becoming more efficient and causing less damage to sperm. This is supported by recent studies that reported similar structural and/or functional quality in sorted and non-sorted bull sperm (Blondin et al., 2009; Peippo et al., 2009; Carvalho et al., 2010).

Notably, a very specific methylation patterns was observed in the 25th and 26th CpG sites of the IGF2R gene (Fig. 1). Evaluating the same region of the *IGF2R* gene in *Bos taurus* spermatozoa, Long and Cai (2007) found the same overall low methylation patterns as we did, but they did not observe hypermethylated patterns at these sites. Differences in methylation of specific CpG sites within the same region were also identified in other cell types and other genes (Kovacheva et al., 2007; Colosimo et al., 2009). We do not know the exact cause for specific CpG sites to be methylated, nor the biological implication of differential methylation patterns. One possibility that may explain the presence of high methylation in the *IGF2R* gene in our study is a specific epigenetic characteristic of *Bos indicus* compared to *Bos taurus* cattle. Furthermore, on the 26th CpG site, a difference in methylation was observed within treatments, with SY sperm having less methylation than the NS and SX sperm (Fig. 2). The reason why SY sexed sperm showed a lower resistance to demethylation of the *IGF2R* gene is not known.

We also found a difference from other reports in the number of CpG sites in the *IGF2* gene. After DNA sequencing, we identified 28 CpG sites in each clone evaluated, which was different from the 27 CpGs reported by Gebert et al. (2006) and GenBank accession no. X53553. In a study conducted in our laboratory, Fagundes et al. (2011) evaluated the methylation patterns of immature and mature bovine oocytes, and identified the same difference in the number of CpGs in relation to the study of Gebert et al. (2006). This difference may be due to the presence of a single nucleotide polymorphism. We speculate that this difference occurred because each study used a distinct genetic group of cattle (*Bos taurus* vs. *Bos indicus*). These results lead us to believe that the differences in methylation

TABLE 1. Percentage of Methylation in the Last Exon of the *IGF2* Gene in Non-Sexed (NS), Sexed X (SX), and Sexed Y (SY) Bovine Sperm From Four Different Bulls

	NS		SX		SY		
Bull	N clones	% ± SEM	N clones	% ± SEM	N clones	% ± SEM	*P*-value
1	17	97.2 ± 0.65	19	96.4 ± 0.86	19	97.5 ± 0.61	0.7
2	14	96.1 ± 1.08	15	95.2 ± 0.90	14	96.7 ± 0.59	0.5
3	20	95.7 ± 0.8[a,b]	16	93.7 ± 1.14[a]	21	97.1 ± 0.72[b]	0.03
4	16	96.6 ± 0.76	17	96.4 ± 0.6	7	94.9 ± 1.53	0.4
P-value		0.6		0.16		0.3	

Data are the average of three replicates per bull.
[a,b]Within a line means without a common superscript difference ($P < 0.05$).

TABLE 2. Percentage of Methylation in the Imprinting Control Region (ICR2) of the *IGF2R* Gene in Non-Sexed (NS), Sexed X (SX), and Sexed Y (SY) Bovine Sperm From Four Different Bulls

	NS		SX		SY		
Bull	N clones	% ± SEM	N clones	% ± SEM	N clones	% ± SEM	*P*-value
1	10	6.5 ± 2.75	16	6.7 ± 0.74	18	6.1 ± 0.74[a]	0.28
2	18	8.0 ± 0.39	15	7.4 ± 0.79	7	9.9 ± 1.85[b]	0.47
3	4	7.69 ± 0.40	17	9.1 ± 1.04	11	6.4 ± 0.58[a]	0.13
4	12	6.73 ± 0.83	10	8.8 ± 1.00	12	9.61 ± 1.30[b]	0.14
P-value		0.32		0.26		0.02	

Data are the average of two replicates per bull.
[a,b]Within a column means without a common superscript difference ($P < 0.05$).

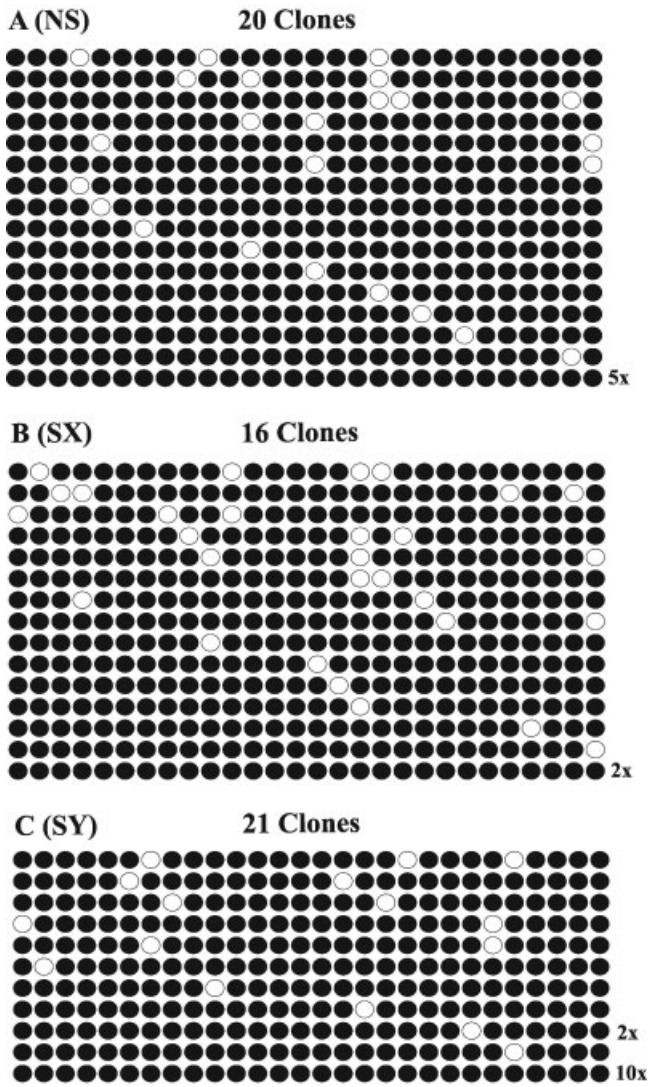

Figure 3. Methylation patterns in the last exon of the *IGF2* gene in non-sexed (**A**), sexed-X (**B**), and sexed-Y sperm (**C**) from bull 3. White and dark circles represent unmethylated and methylated CpGs, respectively; horizontal lines of circles represent one clone, and the number of clones with the same methylation patterns is indicated at the right end of the lines. Data are the average of three replicates per group.

of specific CpG sites and the difference in number of CpG sites in the studied regions may have some biological effect in the development of the embryo or even in the adult animal. This hypothesis is based on the several physiological differences reported between *Bos taurus* and *Bos indicus*, such as differences in ovarian function and in circulating hormones (Sartori and Barros, 2011). These differences could be due to epigenetic variation between the two subspecies.

When we evaluated the effect of sexing on methylation patterns of each individual bull within a treatment, only a slight difference was observed between groups. The difference in the methylation patterns found in bull 3 (Table 1; Fig. 3) or the SY group (Table 2; Fig. 4) for the *IGF2* and *IGF2R* genes, in spite of being statistically significant, was so small that it likely has no biological effect. We believe that variation found in the methylation patterns can be attributed to individual characteristics of the bulls.

In conclusion, our study showed that sex-sorting by flow cytometry did not affect the DNA methylation patterns in the intragenic DMR located in the last exon of the *IGF2* gene or the second ICR of the *IGF2R* gene. Yet, individual bull methylation variation was found. Furthermore, a very specific methylation patterns was observed in the *IGF2R* gene, probably due to an epigenetic characteristic in *Bos indicus* cattle.

MATERIALS AND METHODS

Semen Collection and Sorting

The semen used in this study was obtained from four sexually mature Nellore bulls. One ejaculate from each bull was collected by artificial vagina, and only ejaculates with >60% motility and <20% morphological abnormalities were used. Each ejaculate was divided into three fractions. One fraction was used as the non-sexed (NS) semen and the other two were subjected to flow cytometry and sorted for X (SX) and Y (SY) fractions. The proportion of semen designated for immediate freezing was diluted in a Tris-base freezing diluent with 4% egg yolk, cooled at 4°C for 90 min, and then diluted with the Bioxcell® (IMV, L'Aigle, France). Sperm were loaded into 0.5-ml straws (IMV, L'Aigle, France) and frozen in a programmable freezer TK 3000® (TK, Uberaba, MG, Brazil). At the end of

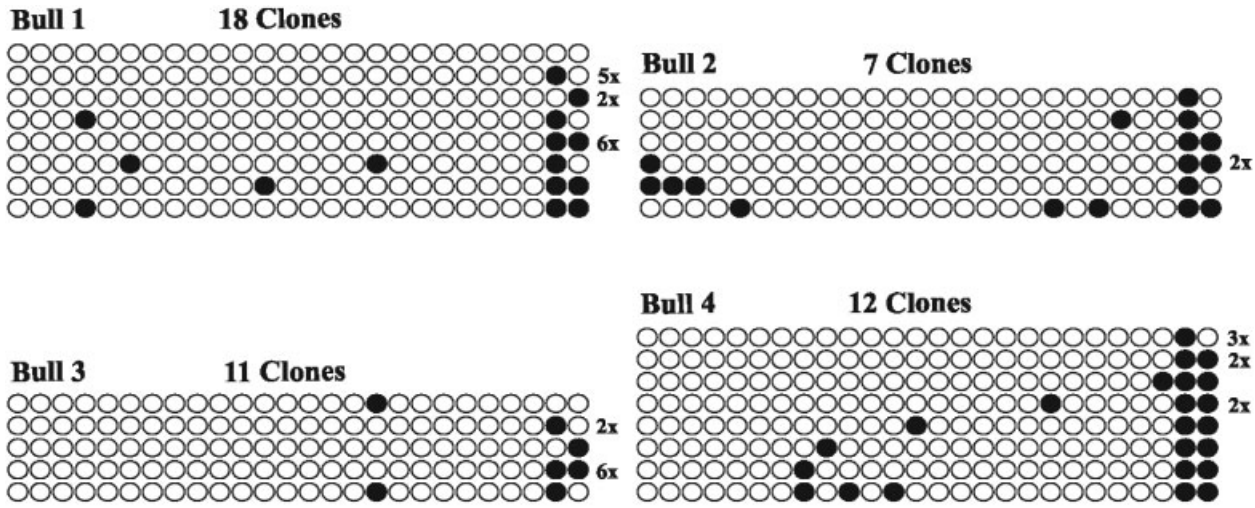

Figure 4. Methylation patterns in the second imprinting control region (ICR2) of the *IGF2R* gene in sexed-Y sperm from four Nellore bulls. White and dark circles represent unmethylated and methylated CpGs, respectively; horizontal lines of circles represent one clone, and the number of clones with the same methylation patterns is indicated at the right end of the lines. Data are the average of two replicates per bull.

the program, the straws were plunged into liquid nitrogen for storage.

The remainder of the ejaculate was diluted to 200×10^6 sperm/ml with Tris medium supplemented with 49–65 mM Hoechst 33342 (Invitrogen Molecular Probes®, Eugene, OR) and incubated for 45 min at 35°C. After staining, samples were diluted at 1:1 with Tris medium supplemented with 4% egg yolk and 0.0015% food dye (FD&C #40; Warner Jenkinson Company Inc., St. Louis, MO), and filtered through a 50-μm filter (GCAT, Fort Collins, CO) to remove any debris or agglutinated cells prior to sorting.

A high-speed cell sorter (MoFlo SX, Beckman Coulter, Fullerton, CA) was operated at 40 psi with a diode pumped solid-state pulse laser (Vanguard 350 HMD-355; Spectra Physics, Mountain View, CA) at 125 mW with bovine sheath fluid (CHATA Biosystems Inc., Fort Collins, CO). Gates were set to attain 90% purity, and sexed sperm were sorted into Tris medium. After cooling at 4°C for 90 min, the sexed sperm was centrifuged and diluted in Bioxcell® (IMV, L'Aigle, France). The semen was packaged into 0.25 ml straws and frozen as described above for non-sexed sperm.

Sperm Processing

Sperm cells were isolated from the extender, cryoprotectant, and other cell types by centrifugation on discontinuous density Percoll gradients (GE Healthcare Bio Science, Upsala, Sweden), as described by Lalancette et al. (2008). Briefly, one straw per group/bull was thawed and layered on top of a gradient composed of 2 ml 40:70% Percoll and centrifuged for 45 min at $700 \times g$ at room temperature.

DNA Isolation

Genomic DNA was isolated from the pellet obtained after passage through a Percoll gradient using the *Salting out* procedure. Briefly, 300 μl of a lysis solution (50 mM Tris, pH 7.8, 5 mM EDTA, pH 8.0, 100 mM NaCl, and 2% SDS) with proteinase K (0.5 mg/ml) and 0.3% β-mercaptoethanol was added to the sperm pellet. The lysis proceeded overnight at 55°C. Genomic DNA was separated using a 6 M NaCl solution and precipitated using isopropanol. The DNA pellet was washed with 1 ml 70% ethanol followed by air-drying. The DNA was resuspended in about 50 μl sterile water. Finally, the DNA was stored at −80°C until needed for sodium bisulfite treatment.

Sodium Bisulfite Treatment

Genomic DNA (80–200 ng) treatment with sodium bisulfite was performed using the EZ DNA methylation kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol, except that the conversion temperature was changed to 55°C. Samples were diluted in 10 μl of distilled water and maintained at −80°C until needed for PCR amplification.

PCR Amplification, Cloning, and Bisulfite Sequencing

Nested PCR using bisulfite-treated DNA was performed to amplify a DMR located into the last exon of the *IGF2* gene and an ICR located in intron 2 of the *IGF2R* gene. The primers used for *IGF2* were designed by Gebert et al. (2006), and PCR was performed using 1× Taq buffer (Invitrogen, São Paulo, Brazil), 2.0 mM $MgCl_2$, 0.4 mM

dNTP, 10 pmoles of each primer, 1 U Platinum Taq Polymerase (Invitrogen), 3 μl template DNA for the first round, and 0.5 μl template DNA for the second round, in a total volume of 20 μl. Both rounds of nested PCR were carried out with an initial denaturing step of 94°C for 3 min, and then 45 cycles at 94°C for 40 sec, 45°C (first round) or 40°C (second round) for 1 min, and 72°C for 1 min, with a final extension at 72°C for 15 sec. For the *IGF2R* gene, the outer primers were designed according to Long and Cai (2007), and we designed the inner primers: forward 5′AAATGAAAGYAGTGAATTA3′ and reverse 5′CCAACCRAACCRCTAACCCT3′. PCR was performed using 1× Taq buffer (Invitrogen), 2.0 mM $MgCl_2$, 0.4 mM dNTP, 10 pmoles of primer, 1 U Platinum Taq Polymerase (Invitrogen), 3 μl template DNA for the first round, and 0.5 μl template DNA for the second round in a total volume of 20 μl. Both rounds of nested PCR were carried out with an initial denaturing step of 94°C for 3 min and then 15 cycles at 94°C for 40 sec, 55°C (first round) or 50°C (second round) for 1 min, and 72°C for 1 min. Next, 20 more cycles were performed at 94°C for 40 sec, 50°C (first round) or 45°C (second round) for 1 min and 72°C for 1 min, with a final extension at 72°C for 15 sec. The PCR products were recovered from an agarose gel and purified using the GenClean III kit (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. Then, the purified products were cloned into the pGEMT-Easy vector (Promega) and transformed into *Escherichia coli* cells (XL-1 Blue).

Individual clones were sequenced using a dideoxy fluorescence terminator system (ABI 3130xl). Only sequences originated from clones with ≥95% cytosine conversion were used. For each bull three (*IGF2*) and two (*IGF2R*) independent series of DNA extraction, amplification, cloning, and sequencing were carried out. Methylation status was determined by the BiQ Analyzer software (Bock et al., 2005) using the sequences in GeneBank (accession no. NM_174087.3 and no. DQ835615 for the *IGF2* and *IGF2R* genes, respectively). Percentages of methylation were calculated by counting the number of methylated CpGs out of the total number of CpG sites in each clone.

Statistical Analysis

The methylation patterns of the different treatments (NS, SX and SY) and different bulls (1, 2, 3, and 4) were compared using the Kruskal–Wallis test in the Prophet Program, version 5.0 (BBN Systems and Technologies, 1996). The methylation patterns for the 25th and 26th CpG sites of *IGF2R* were analyzed using the Chi-square test. Data are presented as mean ± SEM. A *P*-value of <0.05 was considered significant.

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