Specific transgenerational imprinting effects of the endocrine disruptor methoxychlor on male gametes

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Abstract

Endocrine-disrupting chemicals (EDCs), among which methoxychlor (MXC), have been reported to affect the male reproductive system. This study evaluates the possible deleterious effects of MXC on imprinted genes. After administration of the chemical in adult male mice or in pregnant mice we analyzed by pyrosequencing possible methylation defects in two paternally imprinted (H19 and Meg3 (Gtl2)) and three maternally imprinted (Mest (Peg1), Snrpn, and Peg3) genes in the sperm and in the tail, liver, and skeletal muscle DNAs of the adult male mice and of the male offspring. MXC treatment of adult mice decreased the percentages of methylated CpGs of Meg3 and increased those of Mest, Snrpn, and Peg3 in the sperm DNA. MXC treatment of pregnant mice decreased the mean sperm concentrations by 30% and altered the methylation pattern of all the imprinted genes tested in the F1 offspring. In the latter case, MXC effects were transgenerational but disappeared gradually from F1 to F3. MXC did not affect imprinting in the somatic cells, suggesting that it exerts its damaging effects via the process of reprogramming that is unique to gamete development. A systematic analysis at the CpG level showed a heterogeneity in the CpG sensitivity to MXC. This observation suggests that not only DNA methylation but also other epigenetic modifications can explain the transgenerational effects of MXC. The reported effects of EDCs on human male spermatogenesis might be mediated by complex imprinting alterations analogous to those described in this study.
expression without modifying the underlying DNA sequence. The perinatal exposure to MXC has, indeed, been shown to cause an hypermethylation of the ESR2 promoter and of ten other genes in the ovary (Zama & Uzumcu 2009).

A functionally important player in epigenetic gene regulation is imprinting. In an imprinted gene, the expression of the two alleles depends on their parental origin. It occurs at specific sites within or surrounding the gene, called differentially methylated domains (DMDs). Within a DMD, one parental allele is methylated on all or the majority of its CpG dinucleotides, and the opposite parental allele is methylated on none or a small percentage of its CpG dinucleotides. One of the well-characterized consequences of DNA methylation is silencing of the corresponding allele (Li et al. 1993, Kaneda et al. 2004). An epigenetic reprogramming initiated by the erasure of preexisting DNA methylation marks takes place during the embryonic development in primordial germ cells. Imprint reestablishment follows in both female and male germ lines during gametogenesis that occurs both during embryonic development and in the gonads after puberty (Lee et al. 2002). Reprogramming differs from one imprinted gene to the other in terms of timing and extent of methylation. For instance, in the mouse paternally methylated H19, Meg3, and Rasgrf1 genes, DMD methylation is erased although not totally in Meg3, and H19 at e12.5 and DMD remethylation occurs at various rates between e12.5 and the mature sperm age (Li et al. 2004).

The effects of EDCs on imprinting have already been studied. Vinclozolin (VCZ), a fungicide possessing an anti-androgenic activity affected spermatogenesis inducing decreases in sperm number and motility and an increase in apoptosis in the seminiferous tubules germ cells (Shimamura et al. 2002, Anway et al. 2005, 2006, 2008, Buckley et al. 2006, Elzeinova et al. 2008). MXC, administered perinatally or during gestation, was also found to affect spermatogenesis in the adult or in the offspring respectively (Chapin et al. 1997, Anway et al. 2005). An intriguing observation of these studies is that the effects on male genital tract and spermatogenesis were found to be transgenerational, extending from F1 to F4 for VCZ (Anway et al. 2005, 2006, 2008) and from F1 to F2 for MXC (Anway et al. 2005). The effects of VCZ on reproduction were reported to be correlated with alterations in the germ line DNA methylation pattern (Anway et al. 2005). VCZ, administered to pregnant mice, during a period of time encompassing embryo sex differentiation, affected both spermatogenesis and the sperm methylation pattern of paternally and maternally methylated genes in the offspring. The effects of VCZ on the imprinted genes were transgenerational but disappeared gradually from F1 to F3 (Stouder & Paoloni-Giacobino 2010). However, a systematic study of the effects of MXC on imprinted genes has never been performed.

A few mouse and human DMDs have been well characterized. They are, in particular, the DMDs of the maternally expressed paternally methylated H19 (Tremlay et al. 1997) and Meg3 (Li et al. 2004) genes and of the paternally expressed maternally methylated small nuclear ribonucleoprotein polypeptide N (Snrpn, Shemer et al. 1997), Mest (Kaneko-Ishino et al. 1995), Peg3 (Kuroiwa et al. 1996), and the potassium channel 1 (Kcnq1; Smilich et al. 1999) genes.

The aim of this study is to evaluate systematically the possible deleterious effects of MXC on imprinted genes. MXC was administered in adult male mice or in pregnant mice at the time of embryo sex differentiation. We investigated possible methylation defects in the DMDs of two paternally imprinted (H19 and Meg3) and three maternally imprinted (Mest, Snrpn, and Peg3) genes in the sperm as well as in the tail, liver, and skeletal muscle DNAs of the adult mice and male offspring over three generations.

Results

MXC or vehicle only was administered to 8-week-old male mice during 8 consecutive days and a sperm count was performed. As shown in Fig. 1, MXC did not alter the mouse sperm count. The methylation status of the paternally methylated H19 and Meg3 genes and maternally methylated Mest, Snrpn, and Peg3 genes was studied in the same animals by DNA bisulfite treatment. For each imprinted gene, a number of CpGs varying between 5 and 23 were analyzed. The DNA bisulfite treatment technique enables to measure the amount of methylated as compared to total (methylated and nonmethylated) CpGs. In the sperm of control mice, the number of methylated CpGs was close to the theoretical imprinted gene values of 100 and 0% of the total CpGs in paternally or maternally methylated genes respectively. MXC treatment induced significant changes in the methylation pattern of our five target genes in sperm, with the exception of H19. The number of methylated CpGs of Meg3 was decreased to a value of 96% and those of Mest, Snrpn, and Peg3 were increased

![Figure 1](https://via.placeholder.com/150)  
Figure 1 Motile sperm concentration, in millions of spermatozoa/ml of sperm, of vehicle only (control adult) or methoxychlor (MXC)-administered male mice (MXC adult) or of vehicle only (control) or MXC-administered females’ F1 and F2 offspring (MXC F1 or F2). The results are the means ± S.E.M. of six to nine mice. ***P<0.005.
lower in the F2 than in the F1 offspring for *Mest, Snrpn,* and *Peg3* \((P < 0.001, P < 0.005, \text{and } P < 0.005 \text{ respectively })\). Only *H19* was as much affected in the F2 as in the F1 offspring. In the F3 offspring, the effects of MXC could not be detected anymore in our five target genes (Fig. 4).

In the tail, liver, and skeletal muscle of control offspring the amount of methylated CpGs was close to the theoretical value of 50% of the total CpGs in all the imprinted genes tested and no decrease in this value could be detected in MXC-administered female F1–F3 offspring as compared to controls (Fig. 5A–C). Some variations in the amount of methylated CpGs between the groups can be seen in Fig. 5B and C. It consists of significantly higher numbers of methylated CpGs in liver *Meg3* F2, in muscle *Mest* F1, and in both tissue *Snrpn* F2 and F3. It is noteworthy that the amount of methylated CpGs in the *Snrpn* gene of somatic cells was systematically slightly lower than the theoretical value of 50% in most of the groups tested (Figs 3A and B and 5A–C). This incomplete imprinting status was to some extent corrected in some groups of mice.

In conclusion, MXC administration both in adult mice and during gestation altered the methylation patterns of imprinted genes in the male germline cells but not in the somatic cells.

The effects of MXC administration on the methylation status of each of the individual CpGs tested in our five imprinted genes are shown in Table 1.
This study is the first to address the possible effects of MXC administration on canonical imprinted genes. We investigated the possibility of both direct and transgenerational effects of MXC on maternally imprinted and paternally imprinted genes in mouse sperm and somatic cells.

The pyrosequencing technique was chosen and used throughout this study since it was previously shown to yield results similar to those obtained by the subcloning-sequencing technique (Stouder et al. 2009).

In adult mice, MXC administration during the 8 days did not affect the sperm count. MXC administration in adult male rats during the 28 days was found to induce a decrease in the number of sperm cells in the caudal epididymis (Okazaki et al. 2001). In our study, the short time that elapsed between the end of the MXC treatment and the sperm collection would not allow the spermatogonial cells to become sperm. This might explain why no effect of MXC on sperm counts was observed. Moreover, the discrepancy between our results and those of Okazaki et al. (2001) might be explained by the higher doses of MXC used in the latter study. Our results, however, show for the first time the direct effects of MXC on the methylation pattern of four out of the five paternally imprinted and maternally imprinted genes tested in the sperm. It is noteworthy that MXC affected specifically the imprinted genes of the germline leaving those of the somatic cells (liver and skeletal muscle) untouched.

In pregnant mice, MXC administration during a gestational period encompassing the erasure of methylation marks and the beginning of the methylation resetting within imprinted genes decreased the sperm concentration and altered the methylation patterns of the two paternally imprinted (H19 and Meg3) and three maternally imprinted (Mest, Snrpn, and Peg3) genes in the sperm of F1 offspring. Again, MXC affected specifically the imprinted genes of the germline leaving those of the somatic cells (tail, liver, and skeletal muscle) untouched. The effects of MXC on spermatogenesis were found to be transgenerational, extending from F1 to F2 (Anway et al. 2005). They were transferred through the male germ line to males of the subsequent generation. The effects of VCZ on reproduction were reported to be correlated with alterations in the germ line DNA methylation pattern (Anway et al. 2005). VCZ administered to pregnant mice decreased the sperm concentration and affected the methylation status of imprinted genes in the sperm of F1–F3 offspring (Stouder & Paoloni-Giacobino 2010). It was also found to alter the methylation pattern of Peg3 in somatic cells of F2 and F3 offspring (Stouder & Paoloni-Giacobino 2010). Altogether, these data and those of this study show a consistent deleterious effect of EDCs on male gametogenesis and imprinting in the sperm and inconsistent effects of EDCs on somatic cells. By analogy, we can postulate that the reported effects of EDCs on human male spermatogenesis and fertility rates (Roelveld & Bretveld 2008) might involve imprinting alterations in the sperm.

**Discussion**

This study is the first to address the possible effects of MXC administration on canonical imprinted genes. MXC administered to adult male mice significantly affected, in the sperm, the methylation pattern of all the CpGs in the maternally methylated genes Mest, Snrpn, and Peg3. However, it did not affect 2 out of 6 CpGs of the maternally methylated gene Peg3 and 3 out of 6 and 5 out of 15 CpGs in the paternally methylated genes H19 and Meg3, respectively.

MXC administered to pregnant female mice significantly affected, in the sperm of the F1 offspring, the methylation pattern of all the CpGs in the maternally methylated genes Mest, Snrpn, and Peg3. It did not affect, however, 1 out of 6 and 4 out of 15 CpGs in the paternally methylated genes H19 and Meg3, respectively.

If the methylation patterns of the CpGs in F1 and F2 are compared, it can be seen, as expected, that when a significant effect of MXC is seen in F2, it always preexists in F1. It can also be seen, as expected, that the significance of MXC effects are either similar in F1 and F2 or more marked in the former. These expected observations confirm the reliability of the CpG methylation analysis. In the maternally methylated genes of the F2 offspring, MXC affected significantly the methylation pattern of all the CpGs of Mest but in Snrpn and Peg3 one out of four and four out of six CpGs were rescued respectively. In the paternally methylated genes H19 and Meg3, 2 out of 6 and 11 out of 15 CpGs were rescued respectively. The possible effects of MXC administration on the methylation status of each of the individual CpGs in our five imprinted genes were not analyzed and were not shown in F3.
Since MXC affects only the sperm cells, we can consider that it exerts its damaging effects via the process of reprogramming that is unique to gamete development.

It is generally admitted that, due to the mechanisms of erasure and reprogramming, abnormal epigenetic states are not transmitted to the next generation. However, it has been shown previously that VCZ administration or superovulation in pregnant female mice induces epigenetic mark defects that can survive transgenerational reprogramming (Anway et al. 2005, Stouder et al. 2009, Stouder & Paoloni-Giacobino 2010). It has been shown in this study that the imprinting defects induced by MXC are also transgenerational. The resistance to reprogramming in MXC-exposed fetus is partly reversed in the reprogramming period of the first subsequent gestation (F2) and does not resist the reprogramming period of the second subsequent gestation (F3). The reason for the partial and temporary failure of the reprogramming system is far from being elucidated.

The systematic analysis performed in this study at the CpG level in our target imprinted genes should allow a better understanding of the mechanism of the deleterious effects of MXC. We will discuss, first, the sensitivity to MXC and, second, the resistance to reprogramming of the individual CpGs. An important finding of this study is a heterogeneity of the CpG sensitivity to MXC or resistance to reprogramming. Differences in the response of the various CpGs of a DMD to exogeneous factors have never been described, except in an unpublished study of our group showing that only three out of six CpGs of H19 gene were affected by alcohol exposure (C.Stouder, E.Somm & A.Paolini-Giacobino, unpublished observations).

First, in F1 offspring sperm, though all CpGs were affected by MXC in the maternally methylated DMDs, a few CpGs resisted MXC in the paternally methylated DMDs. This observation suggests that the methylation process in the paternally methylated DMDs is less sensitive to MXC than the inhibition of remethylation in the maternally methylated genes. A few CpGs, CpG 5 of H19 and Meg3, were not affected by MXC in both adult and embryo. In most of the cases, however, the CpGs resisting MXC in the adult or embryo were different. The CpG specificity of the MXC effects suggests that the DNA methyltransferases (DNMTs; Jia et al. 2007) might be involved. If this is the case, the DNMTs involved in the adult and/or embryo DNA methylation should be different.

Second, in F2 offspring sperm, if the paternally methylated genes are considered, it can be seen that most of the CpGs of the H19 (4 out of 5) and a minority of the CpGs of Meg3 (4 out of 15) resisted reprogramming. If the maternally methylated genes are considered, all the CpGs of Mest, most of the CpGs of Snrpn (three out of four), and a minority of the CpGs of Peg3 (two out of six) resisted reprogramming. Therefore, the dichotomy observed between paternally methylated and maternally methylated genes in the sensitivity to MXC is not observed when the resistance to reprogramming is considered, suggesting a different mechanism for the
Table 1 Effects of methoxychlor (MXC) on the methylation patterns of the different CpGs of sperm imprinted genes. MXC was administered to adult male (direct) or to pregnant female (in utero) mice and the methylation pattern of a series of CpGs (numbered 1–15) in five imprinted genes was measured in the adult or male offspring sperm respectively.

<table>
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<th>#2</th>
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<th>#4</th>
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<td>P&lt;0.005</td>
<td>P&lt;0.001</td>
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NS, not significant.
latter. Li et al. (2004) have shown that a relatively high level of methylation was retained in Meg3 and H19 of e12.5 germ cells when the methylation of other imprinted genes was totally removed. It could, therefore, be proposed that a resistance to reprogramming occurs when the erasure step of reprogramming is incomplete. Our results, obtained on a different genetic background, showing that most of the CpGs of the H19 but a minority of the CpGs of Meg3 resisted reprogramming are not in line with this hypothesis. It has been postulated that, in male and female germ cells, epigenetic marks other than DNA methylation, perhaps those based on histone modifications, are not removed during reprogramming, allowing parental alleles to remember their origin (Lucifero et al. 2004, Morgan et al. 2005, Arnaud 2010). If MXC affects histone modifications, it might create an epigenetic mark that, by resisting reprogramming, would explain why its effects for some CpGs are transgenerational and for some or most of the CpGs still observed in F2. The Meg3 DMD of the silent paternal allele has been reported to be hypoacetylated on histones 3 and 4 (H3 and H4), while the Dlk1 DMD of the active maternal allele was highly acetylated on both histones, indicating that both histone acetylation and DNA methylation are involved in maintaining the imprinting of Meg3–Dlk1 genes (Carr et al. 2007). It would be interesting to study the possible effects of MXC on H3 and H4.

The discrepancy observed between the effects of MXC on the methylation status of sperm imprinted genes, which persisted in the F2 offspring, and the effect of MXC on the sperm count, which had already disappeared in the F2 offspring, does not necessarily mean that the decrease in the sperm count observed in the F1 offspring is not related to the changes in the methylation status imprinted genes of the sperm. The same discrepancy has been reported after VCZ administration (Stouder & Paoloni-Giacobino 2010). It suggests that a certain threshold of imprinting imbalance has to be reached to translate into a detectable decrease in spermatogenesis.

This study shows that MXC administration to pregnant female mice induces alterations in the methylation pattern of five canonical paternally and maternally imprinted genes in the sperm of the offspring, which are transgenerational but disappear in F3. This study does not give clues on the possibility that the observed methylation changes modify the imprinting status, i.e. the selection of one parental allele for expression. It would definitely be of great interest to answer this question. These effects on the methylation pattern of the five imprinted genes tested were paralleled by a decrease in the sperm concentration observed only in F1. We report a heterogeneity of the responses to MXC of the various CpGs tested, suggesting that, in addition to DNA methylation, MXC might affect specific DNMTs or histone marks.

Materials and Methods

Mice

Normal FVB/N mice were purchased from Charles River (Arbresle, France). Two-month-old female mice were naturally mated with male mice of the same age. Next morning, females with a copulation plug were separated into two groups. The dose of a developmental exposure to MXC, with which no adverse effect was observed, has been shown to vary between 20 mg/kg per day (Okazaki et al. 2001) and <20 μg/kg per day (Armenti et al. 2008) in rats. It has been reported to be about 40 mg/kg per day in mice (Swartz & Eroschenko 1998). In this study, we wanted to investigate the possible effects of MXC on imprinted genes, if possible, in the absence of effects on the sexual development of the offspring. Considering the above literature, we chose a relatively low dose of 10 mg/kg per day. MXC (Sigma–Aldrich) was suspended in sesame oil and administered intraperitoneally, in doses of 10 mg/kg per day, to a first group (n = 6–7) of 8-week-old adult male mice during 8 consecutive days. The corresponding control group (n = 6–7) consisted of 8-week-old adult male mice injected for the same period with the sesame oil only. The mice were killed by cervical dislocation the day after the last injection. In a second group (n = 9–10) pregnant mice received MXC in doses of 10 mg/kg per day from days 10 to 18 of pregnancy, i.e. during a period encompassing the erasure of imprinted gene methylation marks (when primordial germ cells enter the gonads) and the beginning of the imprinted gene methylation resetting in prospermatogonia (Trasler 2009). The control mothers (n = 9–10) were injected with sesame oil only, from days 10 to 18 of pregnancy and their F1 offspring were used at the age of 2 months as controls. To study the possible transgenerational effects of MXC treatment, F1 and F2 outcross were performed in this group. For F1 outcross, 9–10 F1 males were crossed with 9–10 FVB/N females in order to obtain F2 male offspring and for F2 outcross 9–10 F2 males were crossed with 9–10 FVB/N females to obtain F3 male offspring. F1–F3 offspring were used at the age of 2 months. Only one offspring from each mother was analyzed. Animal protocols used in these studies were approved by the Commission d’Ethique de l’Expérimentation Animale of the University of Geneva Medical School and by the Geneva Veterinarian Office.

Sperm collection

The mouse vas deferens and epididymis were dissected out, placed into a Petri dish, scored with a razor blade in a droplet of PBS to allow sperm to diffuse into the medium. The latter was then transferred to a microcentrifuge tube and the fragments were allowed to sediment during 30 min at 37 °C. The supernatant was carefully transferred to another tube, and this procedure was repeated three times. The final supernatant, containing the motile sperm fraction, was carefully removed and centrifuged at 2800 g for 3 min to pellet the sperm.

Sperm count

In some experiments, an aliquot of the supernatant’s motile sperm fraction was used for sperm counting, using
Table 2 Characteristics of the analyzed fragments of the five genes.

**H19**  
GB # U19619, nt 1481–17306 CpGs, 254 bp amplicon  
Oligonucleotide forward: 5'-GGGGGTTATAAATGTTATTAGGGGGAAG-3'; oligonucleotide reverse: 5'-biotin-7AAAACCCCTAACCCTCATAAAAAACCTAAACTTAAATCA-3'  
Amplified sequence:  
GGGGGTTATAAATGTTATTAGGGGGAAGTTATTTTTATATTGTTTATGCCTGGATTTTTAAATTAATAAGGTCGGTTTATTTTTTGTAAAGAATTTTTTGTGTGTAAAGATTA- 
GGGTTGTTCGTACGGCGGTAGTGAAGTTTCGTATATCGTAGTTTTAAAACGGATTGTAATTGATTGAGTTTTTTTTTTTTATTATTATTTATATTTTATAGTTATGGGTTTTATGAGGTTAGGGGTT  

**Meg3**  
GB # NT_166318, nt 21719523–21719838  
23 CpGs, 316 bp amplicon  
Oligonucleotide forward: 5'-GGGGGTTATAAATGTTATTAGGGGGAAG-3'; oligonucleotide reverse: 5'-biotin-7AAAACCCCTAACCCTCATAAAAAACCTAAACTTAAATCA-3'  
Amplified sequence:  
GGGGGTTATAAATGTTATTAGGGGGAAGTTATTTTTATATTGTTTATGCCTGGATTTTTAAATTAATAAGGTCGGTTTATTTTTTGTAAAGAATTTTTTGTGTGTAAAGATTA- 
GGGTTGTTCGTACGGCGGTAGTGAAGTTTCGTATATCGTAGTTTTAAAACGGATTGTAATTGATTGAGTTTTTTTTTTTTATTATTATTTATTTTATAGTTATGGGTTTTATGAGGTTAGGGGTT  

**Mest**  
GB # AF017994, nt 1300–1479  
5 CpGs, 180 bp amplicon  
Oligonucleotide forward: 5'-GGGGGTTATAAATGTTATTAGGGGGAAG-3'; oligonucleotide reverse: 5'-CCCTCTCTCTAACTAATTAAACCTA-3'  
Amplified sequence:  
GGGGGTTATAAATGTTATTAGGGGGAAGTTATTTTTATATTGTTTATGCCTGGATTTTTAAATTAATAAGGTCGGTTTATTTTTTGTAAAGAATTTTTTGTGTGTAAAGATTA- 
GGGTTGTTCGTACGGCGGTAGTGAAGTTTCGTATATCGTAGTTTTAAAACGGATTGTAATTGATTGAGTTTTTTTTTTTTATTATTATTTATTTTATAGTTATGGGTTTTATGAGGTTAGGGGTT  

**Snrpn**  
GB # AF130843, nt 4432–4634  
5 CpGs, 202 bp amplicon  
Oligonucleotide forward: 5'-GGGGGTTATAAATGTTATTAGGGGGAAG-3'; oligonucleotide reverse: 5'-CCCTCTCTCTAACTAATTAAACCTA-3'  
Amplified sequence:  
GGGGGTTATAAATGTTATTAGGGGGAAGTTATTTTTATATTGTTTATGCCTGGATTTTTAAATTAATAAGGTCGGTTTATTTTTTGTAAAGAATTTTTTGTGTGTAAAGATTA- 
GGGTTGTTCGTACGGCGGTAGTGAAGTTTCGTATATCGTAGTTTTAAAACGGATTGTAATTGATTGAGTTTTTTTTTTTTATTATTATTTATTTTATAGTTATGGGTTTTATGAGGTTAGGGGTT  

**Peg3**  
GB # AF105262, nt 3094–3197  
6 CpGs, 103 bp amplicon  
Oligonucleotide forward: 5'-GGGGGTTATAAATGTTATTAGGGGGAAG-3'; oligonucleotide reverse: 5'-CCCTCTCTCTAACTAATTAAACCTA-3'  
Amplified sequence:  
GGGGGTTATAAATGTTATTAGGGGGAAGTTATTTTTATATTGTTTATGCCTGGATTTTTAAATTAATAAGGTCGGTTTATTTTTTGTAAAGAATTTTTTGTGTGTAAAGATTA- 
GGGTTGTTCGTACGGCGGTAGTGAAGTTTCGTATATCGTAGTTTTAAAACGGATTGTAATTGATTGAGTTTTTTTTTTTTATTATTATTTATTTTATAGTTATGGGTTTTATGAGGTTAGGGGTT
a hemocytometer. The corresponding sperm concentration was calculated using the following formula: cells/ml = the average count per square x dilution factor x 10^4 (counts 10 squares).

**Tissue collection**

Fragments of 5×5 mm size of tail, liver, and tibialis anterior muscle were collected, cut into small pieces in a Petri dish, and transferred to DNA-extraction buffer.

**DNA isolation**

Motile sperm fraction DNA was extracted using the QIAampDNA microkit (Qiagen). Total genomic DNA was extracted from tail, liver, and tibialis anterior muscle tissues by incubation for 4 h at 56 °C in DNA-extraction buffer (50 mM Tris–HCl, 0.1 mM EDTA, pH 7.5). In all, 2

bisulfite treatment and PCR amplification followed directly by pyrosequencing, with PCR amplification followed by amplicon subcloning and sequencing. The results were found to be similar.

**Statistical analysis**

Significances were evaluated using the unpaired Student's t-test and set at P<0.05.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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