

In Vitro Embryo Production Efficiency in Cattle and its Association with
Oocyte ATP Content, Quantity of Mitochondrial DNA, and Mitochondrial DNA
Haplogroup

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1 Abstract

2

3 Mitochondria have a broad range of functions that affect reproduction, and structural as well
4 as quantitative variation in mtDNA has been associated with gamete quality and reproductive
5 success. In order to investigate the mitochondria effect on in vitro embryo production we
6 collected oocytes by ultrasound guided follicular aspiration from donor cows known to differ
7 in the developmental capacity, measured by the blastocyst formation rate, of their oocytes.
8 To evaluate the potential effects of mtDNA and mitochondrial function on oocyte quality, the
9 donor cows' mtDNA control region was sequenced and after pair-wise comparisons of
10 polymorphisms animals were grouped into two major haplogroups. The number of mtDNA
11 molecules per oocyte was quantified by real time PCR, and the ATP content was measured in
12 each oocyte to identify variations between haplogroups.

13 Overall ATP-stocks in oocytes of the two haplogroups differed significantly ($p < 0.05$; mean
14 \pm sem) both at the germinal vesicle and metaphase II stages (2.8 ± 0.06 pmol vs. 2.6 ± 0.07
15 pmol and 2.9 ± 0.1 pmol vs. 2.3 ± 0.06 pmol, respectively). The proportion of development to
16 blastocyst was significantly different between haplogroups (22.3 ± 2.1 % vs. 36.7 ± 2.9 %
17 [5]). The number of mtDNA molecules per oocyte was highly variable ($377\ 327 \pm 14\ 104$
18 ranging from 2×10^3 to 1.2×10^6) but not significantly different between the two haplogroups;
19 significant differences were observed between animals without any apparent relationship to
20 blastocyst production. These data suggest that mitochondria and mtDNA haplogroup affect
21 the developmental capacity of bovine oocytes in vitro.

22

22 Introduction:

23 In bovine in vitro embryo production (IVP) programs, the aptitude of the oocyte to support
24 embryo development (i.e. oocyte quality) is routinely assessed by oocytes' morphological
25 appearance [1-4]. However, both macroscopic appearance and the maternal origin affect
26 oocyte quality for IVP. We have previously demonstrated that when oocytes recovered in
27 vivo by ovum pick-up (OPU) are submitted to the same IVP conditions, the blastocyst
28 production rate varies according to the oocyte donor and is independent of the source of
29 semen used [5]. This group of females, with known oocyte phenotypes for in vitro blastocyst
30 production provides a powerful means to further study factors affecting oocyte quality.

31

32 Since mitochondria are maternally inherited organelles and they are the main energy producer
33 in the oocyte, it is possible to hypothesize their role in the aptitude of the oocyte to support in
34 vitro embryo development. These organelles use the oxidative phosphorylation pathway to
35 supply adenosine triphosphate (ATP) for all energy-requiring cellular activities. In oocytes
36 and during the early stages of embryonic development, mitochondria are undifferentiated and
37 generate ATP at relatively low levels when compared to the morula or blastocyst stages [6, 7].
38 The energy substrates (glucose, pyruvate, lactate) as well as oxygen tension and pH in the
39 culture medium affect in vitro embryonic development through glycolysis and/or oxidative
40 phosphorylation in mitochondria [8], revealing a clear relationship between metabolism and
41 developmental competence. Adequate ATP reserves in oocytes and embryos are critical for
42 normal nucleic acid and protein synthesis and they have been suggested to be an indicator of
43 the developmental potential of mouse [9, 10] and bovine embryos [11]. Furthermore,
44 variations in ATP concentrations appear to be associated with embryo developmental
45 competence in humans [6].

46 Mitochondria have the particularity of possessing their own genome. In mammals,
47 mitochondria have retained a restricted set of independent genes in a circular genome of 16.6
48 kilobases encoding 13 proteins that are transcribed and translated in the mitochondrion. The
49 remaining genes, approximately 80, involved in the respiratory chain are located within the
50 nucleus and are governed by Mendelian rules of inheritance. Mitochondria and their DNA
51 (mtDNA) are semi-autonomous and replicate, divide, and fuse independently from somatic
52 nuclear division [12]. Each oocyte contains a huge quantity of mitochondria in their
53 cytoplasm [13, 14] each one containing one copy of the mtDNA. Recently, blastocyst
54 formation has been linked to the number of mitochondria in the oocyte [15], where the
55 average mitochondrial DNA copy number was significantly lower in cohorts of oocytes
56 suffering from fertilization failure than cohorts with a normal fertilization. However, the
57 influence of the quantity of mitochondria in the oocyte on blastocyst production is unknown.
58 Because mtDNA is exposed to free radicals generated by the electron transport chain and
59 since its genome replicates frequently with a deficient DNA proofreading mechanism, its
60 genome mutates rapidly [16, 17]. The control region (mtDNA-CR, also known as D-loop) is
61 the most rapidly evolving region of the mtDNA with a mutation rate up to 20 times that of the
62 nuclear genome [18, 19]. This results in approximately one mutation every 33 generations
63 [19]. Specific mutations in the mtDNA control region have been associated with the
64 proportion of oocytes with high developmental ability recovered by OPU as well as with the
65 rate of transferable nuclear-transfer embryos [20]. Additionally, mtDNA-CR polymorphisms
66 allow the subdivision of animals in haplogroups that have been associated with calving rate in
67 beef cattle [21].
68 Because of the uniqueness of maternal inheritance of mitochondria and their influences on
69 reproduction, the goal of the present study was to quantify the mtDNA and ATP present in
70 each oocyte recovered from animals previously selected for their IVP ability. In addition, it

71 intended to characterize the sequence of the mtDNA control region from the selected animals
72 and compare it to family lines with reported mutations affecting in vitro embryo production.

73

74 Materials and Methods:

75 Animals:

76 Animals used in the present study were previously selected according to their IVP capacity
77 [5]. They were six contemporary unrelated herd-mate Holstein cows that received the same
78 nutritional and health management from birth and throughout the experiment. Experimental
79 animals will be referred to in the text as cow 1 to 6.

80

81 Ovum Pick-Up:

82 Experimental animals had their oestrous cycles' synchronized (CRESTAR® method, Intervet,
83 Angers, France) before oocyte recovery. Oocyte collection was performed twice a week (two
84 sessions per week), starting 5 days after implant removal without any other additional
85 hormonal treatment to stimulate follicular growth. Oocyte recovery was realized in 3 different
86 series of 6, 10 and 6 weeks over a period of 12 months for a total of 43 OPU sessions.

87 Follicular aspiration was performed as described by Pieterse et al. (1988) [22]. In summary,
88 cows were properly restrained and a low epidural anaesthesia administered (5 ml of 2%
89 lidocaine; Xylovet®, CEVA sante animale, Libourne, France) 10 minutes before OPU.

90 Ovarian follicles were identified and aspirated using an ultrasound scanner (Starvet 3, Pie
91 Medical, Pouilly, France) equipped with a 7.5 MHz sectorial array transducer mounted on a
92 probe, specially designed for OPU and placed in the cranial vagina. Follicular contents were
93 collected into sterile conical tubes (one per animal) containing 10 ml of heparinized PBS (40
94 IU/ml) and were maintained at 37°C during the entire procedure.

95

96 Classification of COCs:

97 Cumulus oocyte complexes (COC) were identified after rinsing and filtering the recovered
98 follicular fluid using a 100- μ m mesh with warm (37°C) PBS. Once identified, COC were
99 immediately placed into warm M 199 (Sigma, St.Quentin-Fallavier, France) with 10% fetal
100 calf serum (FCS) (Life Technologies, Cergy, France). Cumulus oocyte complexes were
101 classified according to their morphology into the following categorical grades: i) COC grade
102 1 corresponding to intact immature COC with 3 or more layers of dense cumulus cells and
103 homogeneously granulated cytoplasm; ii) COC grade 2 have fewer layers of compact cumulus
104 investment or are partially denuded oocytes with a healthy cytoplasm; and iii) COC grade 3
105 are oocytes that are completely devoid of cumulus cells but with normal cytoplasm. A fourth
106 category, COC grade 4, included oocytes that did not fit into the first three categories,
107 degenerated oocytes, oocytes with irregular ooplasm (presenting dark areas) as well as COC
108 with abnormal or expanded cumulus investments. COC of this grade were discarded,
109 therefore the results represent the analysis of oocytes quality one to three. Oocytes from
110 slaughterhouse ovaries were used as controls for the ATP and mtDNA quantification studies.
111 Ovaries were collected immediately after slaughter and transported to the laboratory in a
112 temperature-controlled container. The content of the antral follicles between 2 and 8 mm was
113 aspirated and recovered in a conical 50 ml tube containing 10 ml of M 199 medium at 39°C.
114 Oocytes were selected and rinsed before utilisation. COC-1 to -3 (using the same
115 classification as described above) were used as controls.

116

117 In vitro maturation:

118 In vitro maturation conditions are described elsewhere [23]. Briefly, the COC grades 1, 2 and
119 3 from each animal were matured in vitro for 22-24 hours in 50 μ l microdrops of M199
120 supplemented with 10% FCS, 10 μ g/ml FSH, 10 μ g/ml LH and 1 μ g/ml estradiol 17 β (Sigma,

121 St.Quentin-Fallavier, France), over a layer of Vero cells (Rhône-Mérieux, Lyon, France) to
122 improve maturation of denuded oocytes [24]. One microdrop was assigned to each cow and
123 all oocytes collected from that animal in one OPU session were matured as a group. Oocytes
124 from all six animals were processed at the same time and matured under controlled
125 atmospheric conditions (5% CO₂ in air).

126

127 Oocyte storage:

128 All oocytes were frozen, either before or after maturation, and stored until analysis. Each
129 oocyte had its cumulus cells removed mechanically by gentle pipetting either before or after
130 maturation. After maturation, oocytes at the metaphase II (M II) stage (expanded cumulus and
131 first polar body) were treated with hyaluronidase (1 mg/ml in PBS; Sigma, St.Quentin-
132 Fallavier, France) for 3-4 minutes before cumulus extirpation. After being rinsed 3 times in
133 filtered (0.2 µm) PBS (oocytes for mtDNA quantification) or ATP buffer (oocytes used for
134 ATP measurement; 99.0 mM NaCl, 3.1 mM KCl, 0.35 mM NaH₂PO₄, 21.6 mM Na-lactate,
135 10.0 mM Hepes, 2.0 mM CaCl₂, 1.1 mM MgCl₂, 25.0 mM NaHCO₃, 1.0 mM Na-pyruvate,
136 0.1 mg/ml of gentamicin), oocytes were loaded into 500-µl tubes for freezing. Oocytes used
137 for ATP measurements were stored in 50 µl ATP buffer and frozen in liquid nitrogen while
138 those used for mtDNA quantification were stored in 100 µl PBS at -21°C.

139

140 ATP quantification:

141 ATP concentrations were measured in denuded oocytes at the germinal vesicle (GV) or M II
142 stage, i.e. before or after in vitro maturation. The measurement was performed using a
143 commercial assay kit based on the luciferin-luciferase reaction (Bioluminescent Somatic Cell
144 Assay Kit, Fl-ASC; Sigma, St.Quentin-Fallavier, France) following the technique described
145 by Rieger (1997) [25] and the manufacturer's recommendations. Briefly, oocytes were

146 thawed and kept on ice. One hundred μl of ice-cold somatic cell reagent (FL-SAR reagent)
147 was then added to the oocyte solution and incubated for 5 minutes on ice. Next, 100 μl of
148 diluted ice-cold assay mix (FL-AAM reagent; 1:25 with ATP assay mix dilution buffer, FL-
149 AAB reagent) was added and the tubes were kept for an additional five minutes at room
150 temperature in the dark. The solution was then transferred into appropriate plastic tubes fitted
151 for the high sensitivity (0.01 pmol) luminometer (Bioluminat Junior; Berthold, Wildbad,
152 Germany) and luminescence was measured. To obtain simultaneous measurements of
153 samples, a set of tubes with oocytes was measured once (M1) and again (M2) in reverse
154 order. The geometrical mean of the measures was calculated for final use. A seven point
155 standard curve (0-6 pmol/tube) was determined and at every 20 oocytes a negative control
156 was run. The ATP content was calculated using the formula derived from the linear regression
157 of the standard curve.

158

159 MtDNA sequencing of the control region:

160 DNA was extracted from white blood cells and mtDNA control region sequenced after PCR
161 amplification. Amplification was performed in 30 cycles as follows: one single denaturation
162 step 94°C for 90 seconds followed by 35 cycles at 94°C (30 sec), 60°C (90 sec) and 72°C (90
163 sec). Reactions were performed using 0.5 units of TAQ-polymerase (QBIAGEN, France), 250
164 μM dNTP (Invitrogen, Cergy-Pontoise, France) 1.5 mM MgCl_2 , 12 pM of each primer
165 (named mtDA and mtDB, Table 1) [26] and 2 % formamide [27]. Sequence data were
166 obtained after purification of PCR products (GENECLEAN turbo kit, Bio 101, CA, USA)
167 using the PCR primers mtDA and mtDB and four additional primers (named mtDAP, mtDBP,
168 mtC, mtCP; Table 1). Primers were designed to overlap each other creating sequencing
169 triplicate of the amplified CR-sequence. A consensus sequence was obtained from the
170 overlapping sequences using the Pretty function of the Accelrys software (Web based

171 sequence analysis, version 2.1). Results were compared to the sequences of the control
172 regions from bovine families with documented embryo production [20] in the GenBank
173 (accession numbers AF386912 and AF386913) using the Pileup⁽⁺⁾ from the same software
174 package. The sequence obtained was used to create a dendrogram based on the differences
175 found in the mtDNA control region, creating haplogroups.

176

177 MtDNA real-time PCR quantification:

178 DNA was extracted from each oocyte with the High Pure PCR Template Preparation Kit
179 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's
180 recommendations. Quantification of mtDNA was then performed using a real-time PCR
181 method. Briefly, a Roche LightCycler was used to determine the mtDNA copy number using
182 LightCycler-Faststart DNA master SYBR Green 1 kit (Roche, Mannheim, Germany). Twenty
183 μ l PCR reaction mixtures were prepared as following: 1X buffer containing 4 mM MgCl₂, 0.2
184 mM dNTPs, 0.5 μ M of both primers (DC3 and RC1; Table 1), SYBR green I dye, 0.25 U hot
185 start Taq DNA polymerase and 10 μ l of the extracted DNA or 10 μ l of standard with a known
186 copy number. The external standard used for mtDNA quantification was the corresponding
187 189-bp PCR product cloned into PCR 2.1-TOPO[®] vector (Invitrogen, Life Technologies,
188 Groningen, Netherlands). The PCR reactions were performed with an initial denaturation at
189 95°C for 7 min and 40 cycles at 95°C for 1 s, 56°C for 5 s, and 72°C for 13 s. The SYBR
190 green fluorescence was read at the end of each extension step (72°C). A melting curve (loss of
191 fluorescence at a given temperature between 66°C and 94°C) was analysed in order to check
192 the specificity of the PCR product. For each run, a standard curve was generated using five
193 10-fold serial-dilutions (10-100,000 copies) of the external standard. This curve allowed the
194 determination of the starting copy number of mtDNA in each sample.

195

196 Statistical analysis:

197 The mean number of oocytes recovered was compared between haplogroups and between
198 animals using ANOVA. A retrospective analysis of in vitro blastocyst production at day 8
199 after in vitro fertilisation [5] was performed to evaluate the haplogroup effect on blastocyst
200 rate. ATP contents as well as the quantities of mtDNA were compared using ANOVA
201 followed by a Bonferroni post-hoc test using SPSS software (SPSS, Chicago, IL, USA). Due
202 to the large intra-animal variations found in the mtDNA copy numbers, the coefficient of
203 variation (CV; SD/mean) was calculated to allow a better estimation of inter-animal variation.
204 Results are reported as mean \pm sem (or SD when indicated) with a significance level of 0.05.

205

206 Results:

207 Characterization of the mtDNA Control Region:

208 The CR sequencing of all 6 cows revealed 21 mutations sites with 13 of them differing from
209 those published by Bruggerhoff et al. (2002) [20] (Figure 1) conferring 6 different genotypes.
210 The only insertion was in a cytosine run at the 3' end of the CR region prone to slippage. The
211 other mutation sites are transitions including one at position 191 in the stem site preceding the
212 D-loop. At position 350 a C/T transition was identified in all 6 animals. Pairwise comparison
213 of the genotypes of the animals selected for this study with those of families with different
214 aptitude for embryo production by multiple ovulation embryo transfer (MOET) and nuclear
215 transfer cloning [20] generated a dendrogram (Figure 2). This dendrogram divides the animals
216 in two major haplogroups and this information was used in the statistical analyses.
217 Haplogroup H-146 is composed of cows 1, 4 and 6 and family line AF386912, while
218 haplogroup H-235 included family line AF386913 and cows 2, 3 and 5.

219 mtDNA control regions sequences for each cow was submitted to GenBank and can be found
220 by the accession numbers AY495575, AY495576, AY495577, AY495578, AY495579, and
221 AY495580.

222

223 Oocyte recovery:

224 Nine hundred and fifty-one oocytes were recovered from 2 488 follicles (mean of 9.6 ± 0.2
225 follicles/animal) with cow 2 producing significantly more oocytes (5.3 ± 0.4 oocytes/session)
226 than the other animals (3.3 ± 0.4 , 2.8 ± 0.3 , 3.8 ± 0.3 , 3.2 ± 0.3 , and 3.7 ± 0.3 oocytes/session
227 for cows 1, 3, 4, 5, and 6 respectively). Haplogroup did not have any effect on oocyte
228 recovery with 3.6 ± 0.2 and 3.8 ± 0.2 oocytes/session for H-146 and H-235 respectively.

229

230 Oocyte ATP reserve:

231 Mean ATP concentration was 2.5 ± 0.05 pmol/oocyte at the GV stage (CV = 32.0%; n = 256)
232 and 2.4 ± 0.05 pmol/oocyte at the M II stage (CV = 33.3%; n = 245). Although no difference
233 in overall ATP was observed at the two stages of maturation, comparisons of ATP (before and
234 after maturation) between haplogroups and between animals shows significant variations.
235 Changes in ATP stored between GV and M II shows that cow 6 and control group increased
236 their ATP stores ($p < 0.05$) at M II while a decrease was observed for cows 1, 2, 3 and 5
237 ($p < 0.05$ for cows 5 and 3); ATP stored in oocytes from cow 4 remained virtually unchanged
238 (Table 2). Haplogroup 146 and H-235 differed in ATP contents at both GV and M II stages
239 (Table 3).

240 Mean ATP in oocytes recovered by OPU at GV (2.7 ± 0.05 pmol/oocyte, CV = 25.2%, n =
241 204) was significantly higher than ATP from slaughterhouse oocytes (1.5 ± 0.07 pmol/oocyte,
242 CV = 32.6%, n = 52). This difference ($p < 0.05$) persisted at the M II stage (2.5 ± 0.06
243 pmol/oocyte, CV = 31.7%, n = 159 and 2.0 ± 0.06 pmol/oocyte, CV = 27.0%, n = 86 for OPU

244 and slaughterhouse ovaries respectively). OPU oocytes showed a significant decrease in ATP
245 stocks after maturation (2.7 ± 0.05 to 2.5 ± 0.06 pmol/oocyte) while control oocytes had a
246 significant increase (1.5 ± 0.07 to 2.0 ± 0.06 pmol/oocyte) in ATP stocked in the cytoplasm
247 (Table 2).

248 ATP concentrations were not statistically different between different morphological quality
249 COC at the GV stage (COC-1: 2.6 ± 0.07 pmol/oocyte, n = 119; COC-2: 2.4 ± 0.08
250 pmol/oocyte, n = 99; COC-3: 2.4 ± 0.1 pmol/oocyte, n = 38) or at the M II stage (COC-1: 2.4
251 ± 0.08 pmol/oocyte, n = 76; COC-2: 2.4 ± 0.07 pmol/oocyte, n = 128; COC-3: 2.2 ± 0.1
252 pmol/oocyte, n = 41).

253 Overall ATP stocks in the two haplogroups (all cows in each haplogroup combined) differed
254 significantly both at the GV stage (2.8 ± 0.06 pmol/oocyte, n = 101; and 2.6 ± 0.07
255 pmol/oocyte, n = 103, for H-146 and H-235 respectively) and at the M II stage (2.9 ± 0.1
256 pmol/oocyte, n = 76; and 2.3 ± 0.06 pmol/oocyte, n = 83, for H-146 and H-235 respectively;
257 Table 3).

258

259 Quantification of mtDNA:

260 On average, each of the oocytes analysed carried $377\,327 \pm 256\,607$ copies of mtDNA (Mean
261 \pm SD; Table 4). Although the two haplogroups did not differ significantly in mtDNA (Table
262 3), there was a significant variation among animals within haplogroup H-235 (Table 4). No
263 effect of oocyte quality was observed in mtDNA copy numbers. COC-1 and 2 had $379\,117 \pm$
264 $14\,980$ copies/oocyte (n = 282) and were not different from COC-3 ($367\,022 \pm 40\,938$
265 copies/oocyte n = 49). Nevertheless, mtDNA show a non-significant trend towards a lower
266 quantity of mtDNA as oocyte quality decreases. However, a very large intra-animal variation
267 in mtDNA copy number was observed (range from 2 000 to 1 200 000 copies/oocyte) which
268 was shown by the large CV values (Table 4). Quantities of mtDNA did not differ between

269 oocytes recovered by OPU ($382\,938 \pm 15\,341$ copies/oocyte) or from slaughterhouse ovaries
270 ($337\,639 \pm 34\,304$ copies/oocyte; $p > 0.05$).

271

272 Discussion:

273 The objective of this study was to examine whether oocyte ATP and mtDNA contents vary
274 between cows and if these parameters could explain the oocyte donor effect observed on
275 blastocyst production [5]. Furthermore it was also intended to observe whether
276 polymorphisms in the mtDNA-CR of these selected animals matched those that have
277 previously been linked to in vitro fertility [20].

278

279 Mitochondrial DNA sequencing analysis has been widely used in evolutionary studies as a
280 molecular clock to identify common female ancestor. Cattle lineages have been characterized
281 based on mtDNA-CR mutations [28, 29] and many studies have suggested that maternal
282 lineage affects production and reproduction traits of cattle [30-33]. Schutz et al. (1994) found
283 that polymorphisms in the CR was associated with the number of days open in dairy cattle
284 [34] while more recently, mtDNA polymorphisms have been associated with calving rate in
285 beef cattle [21]. Sequencing of the mtDNA-CR in this study revealed that the six animals that
286 were selected for having oocytes with different abilities for IVP, had six different mtDNA-CR
287 genotypes. This enabled, after pairwise alignments, the separation of these animals into two
288 haplogroups and analysis of blastocyst production showed that animals in H-146 had
289 significant lower blastocyst rate at day 8 than H-235 (22.3 ± 2.1 % and 36.7 ± 2.9 %
290 respectively). Further statistical analysis showed that animals in H-235 had a higher
291 blastocyst rate at days 6 and 7 ($p < 0.05$, no difference detected at day 8) than animals in H-
292 146, indicating a difference in kinetics of blastocyst formation. This suggests the existence of
293 difference in embryo quality between the two haplogroups, as early blastocyst formation is a

294 sign of embryo quality [35]. However, we cannot confirm this observation because blastocyst
295 quality was not evaluated.

296

297 These two haplogroups were then compared (based on polymorphisms in the mtDNA control
298 region) to the two family lines AF386912 and AF386913 described in Germany as having
299 different in vitro fertility [20]. In contrast to the findings reported by Bruggerhoff et al. [20],
300 we observed no difference in the number of oocytes collected or variation in oocyte
301 morphological quality between haplogroups. The different experimental design employed by
302 Bruggerhoff et al. (e.g. paternally related individuals in two haplotype groups, each group
303 derived from a single founder female and thus with completely identical mtDNA type), in
304 addition to OPU frequency, equipment, and the oocyte grading system used, may account for
305 this difference in results. Mitochondrial DNA phenotypes are strongly dependent on the
306 nuclear genetic background [36-38], and the different breeds employed by Bruggerhoff et al.
307 (Simmental) and in the present study (Holstein) could explain some of the observed
308 differences. However, comparison of embryo production by MOET [20] shows that family
309 line AF386912 produced roughly three times less embryos than AF386913; those results are
310 complimentary to ours. It is nevertheless striking that an mtDNA haplotype that was
311 previously associated with superior cytoplasm for embryo production in somatic cell nuclear
312 transfer (SCNT), was assigned to haplogroup H-146 of the present study, as this haplogroup
313 yielded a significantly lower blastocyst rate in IVP. Conversely, an mtDNA haplotype
314 previously associated with inferior cytoplasm in SCNT, was assigned to our haplogroup H-
315 235, which yielded a significantly higher blastocyst rate in IVP. This points to fundamental
316 differences in cytoplasmic factors important for SCNT and IVP embryo production. The
317 ability to produce embryos in vitro from oocytes with defined mtDNA polymorphisms may in
318 the future serve as a tool for the selection of oocyte donor animals superior for IVP. We

319 acknowledge that a small number of animals were used in this experiment and that a more
320 comprehensive study is needed. It will be performed as more animals with different
321 phenotypes for IVP are identified.

322 In this study, we explored the relationship between in vitro embryo developmental
323 competence not only with specific mtDNA mutations but also with mtDNA oocyte content.
324 During oocyte growth, as the cytoplasm increases in volume, so does the number of
325 mitochondria [39, 40] with a mature oocyte containing on average 1.6×10^5 and 3.1×10^5
326 mtDNA molecules in mouse and human oocyte respectively [13]. However, the absolute
327 quantity of mtDNA copies in a human oocyte is quite variable. Some authors published mean
328 values of 1.9×10^5 copies/oocyte [15] while others 8.0×10^5 copies/oocyte [14]. The present
329 results showed a mean 3.7×10^5 mtDNA per bovine oocyte and a very large intra-animal
330 variation. Other authors have also reported a large variation between oocytes from the same
331 individual [13, 15]. The mean quantity of mtDNA copies per oocyte reported here in the cow
332 is not very different from the 2.6×10^5 reported by Michaels et al. (1982) [41] but if we
333 consider the mean quantity of mtDNA in oocytes from each cow individually, the difference
334 varied between 1.2 to 2.1 fold.

335 The quantity of mitochondria in the oocyte affects its ability to produce ATP [42], to escape
336 atresia [43] and to support embryo development [15]. However, our data did not provide any
337 evidence that the quantity of mitochondria influence embryo production. This is confirmed by
338 the observation that despite a significant difference in blastocyst formation rate, the two
339 haplogroups had equivalent numbers of mtDNA per oocyte. Our data shows that oocyte
340 morphological quality is a poor indicator of the quantity of mitochondria in the cytoplasm. A
341 trend towards a lower quantity of mtDNA in oocytes of poorer quality was observed but it
342 was not statistically significant. Additionally, the best and the worst blastocyst producer had
343 virtually the same mean number of mtDNA copies, and the animal with the highest numbers

344 of mitochondria (cow 3) only had an average embryo production rate. The similar quantity of
345 mtDNA between oocytes from OPU and slaughterhouse indicate that the time elapsed
346 between slaughter and oocyte collection does not affect the quantity of mtDNA. Nevertheless,
347 a very large variation in the number of mtDNA per oocyte was evidenced between animals;
348 this large variation demands further investigation to permit us to correlate it with the also
349 large intra-animal variation usually observed for in vitro blastocyst production. To observe a
350 definitive effect of the quantity of mitochondria on IVP, taking into consideration the large
351 inter-oocyte intra-animal variation, one should perform mtDNA quantification and IVF on the
352 same oocyte. This is practically impossible using the techniques described above. The real
353 significance of the number mitochondria in the oocyte, on bovine in vitro embryo production,
354 remains to be proven.

355

356 Mitochondrial activity is responsible for ATP production and energy accumulation during
357 oogenesis, which is a crucial factor for successful development [6, 44, 45]. Oocyte ATP
358 contents have been suggested to be an indicator for the developmental potential of human,
359 mouse and bovine oocytes [6, 11, 46, 47]. Although oocyte mitochondria are small, round to
360 oval in shape, microscopically dense, and contain a few underdeveloped cristae, which are
361 inherent characteristics of cells with low metabolic rate [7, 48], they can produce enough ATP
362 to sustain embryo development. ATP equilibrium (production vs. consumption) was studied
363 by comparing ATP stocks at GV and M II stages. Our results show that the ATP stored in the
364 oocyte was not influenced by oocyte quality (either at GV or M II) in opposition to the results
365 observed by Stojkovic et al. [11] where a significant difference between COC-1 and COC -3
366 was found. The reason for this difference could be related to the differences in the grading
367 systems used. At M II the results of the two studies are in agreement and both studies
368 observed an increase in ATP stocks in oocytes from slaughterhouse. The ATP reserves in

369 slaughterhouse oocytes at the GV stage were significantly smaller than oocytes from OPU.
370 This is probably due to the period of nutrient deprivation to which they were submitted
371 between slaughter and arrival in the laboratory. However, ATP reserves were restored to OPU
372 levels after in vitro maturation. This significant increase after in vitro maturation indicates
373 that mitochondria are functional and support embryo development at a rate of 45.7 %
374 (blastocyst rate with oocytes collected at the same time as those used for ATP measurement in
375 our IVF laboratory, data not shown). Nevertheless, it is known that mouse oocytes can sustain
376 maturation with very low net ATP reserves [6]. The ATP levels observed in the present
377 experiment on slaughterhouse oocytes were similar to those reported by others, either before
378 or after in vitro maturation [6, 11]. An interesting phenomenon was observed for ATP levels
379 at the two maturation stages studied in the two haplogroups. Haplogroup H-235, with a
380 significantly higher blastocyst rate, had significantly higher stores of ATP at the GV stage
381 than at the M II stage indicating that ATP utilization is beyond the oocyte capacity to
382 replenish its reserves while in haplogroup H-146 these values were not statistically different.
383 The two haplogroups were also different in their ATP reserves at M II stage with more ATP
384 in the haplogroup with higher embryo production. We also showed that ATP reserves
385 behaved differently between cows; ATP contents increased, decreased or remained constant
386 between the GV and M II stages. This demonstrates that the oocyte donor influenced the ATP
387 contents of oocytes at GV and M II. Cow 5 (52.4% blastocyst rate) and cow 6 (20.3%
388 blastocyst rate) had the same levels of ATP at GV but there was a marked difference at M II.
389 This raises questions about mitochondrial function. It is unclear if the observed increase in
390 ATP for cow 6 is related to production of ATP that surpasses consumption or due to
391 decreased utilization of ATP by a metabolically compromised oocyte. The fact that
392 embryonic metabolism remains constant in normally developing oocytes until the 12-16 cell
393 stage [48] suggests that on the one hand oocytes from this animal are not consuming enough

394 ATP, resulting in positive net ATP production; on the other hand, oocytes from cow 6 had
395 significantly more mtDNA than the average oocyte (33% more mtDNA than cow 5). Cow 4,
396 with a blastocyst rate of only 12.4% had the same level of ATP before and after maturation.
397 This could be due to a perfect equilibrium between production and consumption of ATP or
398 due to a metabolically and developmentally incompetent oocyte. Future studies using
399 differential staining of mitochondria can help in the interpretation of this finding.

400

401 In the present study, we showed that genetic variation in the mtDNA-CR successfully
402 generated haplogroups with different phenotypes for in vitro embryo production. Haplogroups
403 also differed in their ATP levels at GV and M II. Oocyte ATP reserves and oocyte energy
404 metabolism were distinctly different between animals; the significance of this variation is still
405 unclear but should be clarified as more animals, selected for blastocyst production, become
406 available. A maternal family line study could confirm the repeatability of the results reported
407 here. It is clear that oocytes that grew in follicles large enough to be aspirated by OPU at a 3-4
408 day interval have enough mitochondria to sustain development. Variations in in vitro embryo
409 production, if related to the oocytes' mitochondria population, would mainly be due to factors
410 other than inadequate oocyte mitochondrial load. Nevertheless, a large intra-animal variation
411 in the quantity of mtDNA was observed.

412

413

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415

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References

1. Boni R, Cuomo A, Tosti E. Developmental potential in bovine oocytes is related to cumulus-oocyte complex grade, calcium current activity, and calcium stores. *Biol Reprod* 2002; 66: 836-842.
2. Gandolfi F, Luciano AM, Modina S, Ponzini A, Pocar P, Armstrong DG, Lauria A. The in vitro developmental competence of bovine oocytes can be related to the morphology of the ovary. *Theriogenology* 1997; 48: 1153-1160.
3. Van Soom A, Tanghe S, De Pauw I, Maes D, de Kruif A. Function of the cumulus oophorus before and during mammalian fertilization. *Reprod Domest Anim* 2002; 37: 144-151.
4. Hashimoto S, Saeki K, Nagao Y, Minami N, Yamada M, Utsumi K. Effects of cumulus cell density during in vitro maturation of the developmental competence of bovine oocytes. *Theriogenology* 1998; 49: 1451-1463.
5. Tamassia M, Heyman Y, Lavergne Y, Richard C, Gelin V, Renard JP, Chastant-Maillard S. Evidence of oocyte donor cow effect over oocyte production and embryo development in vitro. *Reproduction* 2003; 126: 629-637.
6. Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod* 1995; 10: 415-424.
7. Sathananthan AH, Trounson AO. Mitochondrial morphology during preimplantational human embryogenesis. *Hum Reprod* 2000; 15 Suppl 2: 148-159.
8. Barnett DK, Bavister BD. Inhibitory effect of glucose and phosphate on the second cleavage division of hamster embryos: is it linked to metabolism? *Hum Reprod* 1996; 11: 177-183.

9. Quinn P, Wales RG. The relationships between the ATP content of preimplantation mouse embryos and their development in vitro during culture. *J Reprod Fertil* 1973; 35: 301-309.
10. Calarco PG. Polarization of mitochondria in the unfertilized mouse oocyte. *Dev Genet* 1995; 16: 36-43.
11. Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Goncalves PB, Wolf E. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod* 2001; 64: 904-909.
12. Bereiter-Hahn J, Voth M. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. *Microsc Res Tech* 1994; 27: 198-219.
13. Steuerwald N, Barritt JA, Adler R, Malter H, Schimmel T, Cohen J, Brenner CA. Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. *Zygote* 2000; 8: 209-215.
14. Barritt JA, Kokot M, Cohen J, Steuerwald N, Brenner CA. Quantification of human ooplasmic mitochondria. *Reprod Biomed Online* 2002; 4: 243-247.
15. Reynier P, May-Panloup P, Chretien MF, Morgan CJ, Jean M, Savagner F, Barriere P, Malthiery Y. Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol Hum Reprod* 2001; 7: 425-429.
16. Ankel-Simons F, Cummins JM. Misconceptions about mitochondria and mammalian fertilization: implications for theories on human evolution. *Proc Natl Acad Sci USA* 1996; 93: 13859-13863.

17. Clayton DA. Nuclear gadgets in mitochondrial DNA replication and transcription. *Trends Biochem Sci* 1991; 16: 107-111.
18. Brown WM, George M, Jr., Wilson AC. Rapid evolution of animal mitochondrial DNA. *Proc Natl Acad Sci USA* 1979; 76: 1967-1971.
19. Parsons TJ, Muniec DS, Sullivan K, Woodyatt N, Alliston-Greiner R, Wilson MR, Berry DL, Holland KA, Weedn VW, Gill P, Holland MM. A high observed substitution rate in the human mitochondrial DNA control region. *Nat Genet* 1997; 15: 363-368.
20. Bruggerhoff K, Zakhartchenko V, Wenigerkind H, Reichenbach HD, Prella K, Schernthaner W, Alberio R, Kuchenhoff H, Stojkovic M, Brem G, Hiendleder S, Wolf E. Bovine somatic cell nuclear transfer using recipient oocytes recovered by ovum pick-up: effect of maternal lineage of oocyte donors. *Biol Reprod* 2002; 66: 367-373.
21. Sutarno, Cummins JM, Greeff J, Lymbery AJ. Mitochondrial DNA polymorphisms and fertility in beef cattle. *Theriogenology* 2002; 57: 1603-1610.
22. Pieterse MC, Kappen KA, Kruip TA, Taverne MA. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. *Theriogenology* 1988; 30: 751-762.
23. Menck MC, Guyader-Joly C, Peynot N, Le Bourhis D, Lobo RB, Renard JP, Heyman Y. Beneficial effects of Vero cells for developing IVF bovine eggs in two different coculture systems. *Reprod Nutr Dev* 1997; 37: 141-150.
24. Grocholova B, Petr J, Marek J, Tepla O. Beneficial influence of Vero cells on in vitro maturation and fertilization of bovine oocytes. *Theriogenology* 1995; 44: 199-207.
25. Rieger D. Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin-luciferase reaction. *Anal Biochem* 1997; 246: 67-70.

26. Hiendleder S, Schmutz SM, Erhardt G, Green RD, Plante Y. Transmitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Mol Reprod Dev* 1999; 54: 24-31.
27. Sarkar G, Kapelner S, Sommer SS. Formamide can dramatically improve the specificity of PCR. *Nucleic Acids Res* 1990; 18: 7465.
28. Wu J, Smith RK, Freeman AE, Beitz DC, McDaniel BT, Lindberg GL. Sequence heteroplasmy of D-loop and rRNA coding regions in mitochondrial DNA from Holstein cows of independent maternal lineages. *Biochem Genet* 2000; 38: 323-335.
29. Hauswirth WW, Laipis PJ. Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. *Proc Natl Acad Sci USA* 1982; 79: 4686-4690.
30. Boettcher PJ, Gibson JP. Estimation of variance of maternal lineage effects among Canadian Holsteins. *J Dairy Sci* 1997; 80: 2167-2176.
31. Gibson JP, Freeman AE, Boettcher PJ. Cytoplasmic and mitochondrial inheritance of economic traits in cattle. *Livest Prod Sci* 1997; 47: 115-124.
32. Abdel-Azim G, Freeman AE. Superiority of QTL-assisted selection in dairy cattle breeding schemes. *J Dairy Sci* 2002; 85: 1869-1880.
33. Faust MA, McDaniel BT, Robison OW. Genetics of reproduction in primiparous Holsteins. *J Dairy Sci* 1989; 72: 194-201.
34. Schutz MM, Freeman AE, Lindberg GL, Koehler CM, Beitz DC. The effect of mitochondrial DNA on milk production and health of dairy cattle. *Livest Prod Sci* 1994; 37: 283-295.
35. Van Soom A, Ysebaert MT, de Kruif A. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol Reprod Dev* 1997; 47: 47-56.

36. Zeviani M, Amati P, Savoia A. Mitochondrial myopathies. *Curr Opin Rheumatol* 1994; 6: 559-567.
37. Guan MX, Fischel-Ghodsian N, Attardi G. Nuclear background determines biochemical phenotype in the deafness-associated mitochondrial 12S rRNA mutation. *Hum Mol Genet* 2001; 10: 573-580.
38. Battersby BJ, Loredó-Osti JC, Shoubridge EA. Nuclear genetic control of mitochondrial DNA segregation. *Nat Genet* 2003; 33: 183-186
39. Jansen RP, de Boer K. The bottleneck: mitochondrial imperatives in oogenesis and ovarian follicular fate. *Mol Cell Endocrinol* 1998; 145: 81-88.
40. Smith LC, Alcivar AA. Cytoplasmic inheritance and its effects on development and performance. *J Reprod Fertil Suppl* 1993; 48: 31-43.
41. Michaels GS, Hauswirth WW, Laipis PJ. Mitochondrial DNA copy number in bovine oocytes and somatic cells. *Dev Biol* 1982; 94: 246-251.
42. Van Blerkom J, Sinclair J, Davis P. Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy. *Hum Reprod* 1998; 13: 2857-2868.
43. Perez GI, Trbovich AM, Gosden RG, Tilly JL. Mitochondria and the death of oocytes. *Nature* 2000; 403: 500-501.
44. Thompson JG, McNaughton C, Gasparrini B, McGowan LT, Tervit HR. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. *J Reprod Fertil* 2000; 118: 47-55.
45. Van Blerkom J. Intrafollicular influences on human oocyte developmental competence: perifollicular vascularity, oocyte metabolism and mitochondrial function. *Hum Reprod* 2000; 15 Suppl 2: 173-188.

46. Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 1984; 72: 9-13.
47. Slotte H, Gustafson O, Nylund L, Pousette A. ATP and ADP in human pre-embryos. *Hum Reprod* 1990; 5: 319-322.
48. Motta PM, Nottola SA, Makabe S, Heyn R. Mitochondrial morphology in human fetal and adult female germ cells. *Hum Reprod* 2000; 15 Suppl 2: 129-147.
49. Khurana NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod* 2000; 62: 847-856.

Figure Legends:

Figure 1: Complete mtDNA CR sequence. Previously described mutations (PDM) and different mutation sites (NMS) are punctually identified. The stem-loop structure, the control of the L-strand transcription and the D-loop strand termination site (term), as well as the sequence element (TAS-A) associated with the bovine D-loop strand terminations are marked. The conserved sequence block (CSB) 1 and 2+3, the promoters for the H- and L-strand transcription (HSP and LSP) and the origin of the H-strand replication (O_H) are also identified. MtDNA sequences for each cow has been submitted to the GenBank.

Figure 2: Multiple alignment dendrogram based on mutations in the mtDNA control region; oocyte donor cows are compared to family lines with known mtDNA control region mutations previously associated with oocyte production by OPU and in vitro embryo production by somatic cell nuclear transfer cloning

Table 1: Primers used for amplification and sequencing of the mtDNA control region

Primer	Orientation	Sequence	Fragment size
mtDA	Upstream*	5' - CTCACCATCAACCCCAAAGCT - 3'	435 bp
mtDAP	Downstream	5' - CTTAATTACCATGCCGCGTGAA - 3'	
mtDB	Downstream*	5' - TCATCTAGGCATTTTCAGTG - 3'	388 bp
mtDBP	Upstream	5' - GCTCACACATAACTGTGCTGTC - 3'	
mtC	Upstream	5' - CACGAGCTTAATTACCATGCC - 3'	270 bp
mtCP	Downstream	5' - GGGGGATGCTTGGACTCA - 3'	
DC3	Upstream	5' - AAATAATATAAGCTTCTGACTCC - 3'	190 bp
RC1	Downstream	5' - TCCTAAAATTGAGGAAACTCC - 3'	

*primers described by Hiendleder et al. (1999)

Table 2: ATP content and blastocyst production; animals are ranked by blastocyst rate and haplogroup

	Maturation status	N	Mean ATP content pmol/oocyte	MtDNA haplogroup	Mean blast. rate (%) §
Cow 5 ^{bc}	GV	30	3.0 ± 0.1*	H-235	52.4 ^k ± 6.2
	M II	23	2.4 ± 0.1		
Cow 2 ^c	GV	40	2.7 ± 0.09	H-235	32.4 ^{lm} ± 3.6
	M II	33	2.5 ± 0.05		
Cow 3 ^{de}	GV	33	2.1 ± 0.08*	H-235	25.5 ^{lmn} ± 4.9
	M II	27	1.8 ± 0.05		
Cow 1 ^b	GV	42	3.1 ± 0.09	H-146	35.2 ^{kl} ± 4.6
	M II	22	2.8 ± 0.08		
Cow 6 ^a	GV	31	3.1 ± 0.06	H-146	20.3 ^{mn} ± 3.1
	M II	24	3.8 ± 0.1*		
Cow 4 ^d	GV	28	2.1 ± 0.9	H-146	11.7 ⁿ ± 2.6
	M II	30	2.1 ± 0.06		
Control ^e	GV	52	1.5 ± 0.07	NA	NA
	M II	86	2.0 ± 0.06*		

Values with * indicate that ATP contents for the same animal differ at the 0.05 level before and after maturation

Cows with the same superscript are not statistically different at the 0.05 level for total ATP (GV +M II)

Mean blastocyst rate with the same superscript are not statistically different at the 0.05 level

§ Reference Tamassia et al. (2003)

Table 3: mtDNA and ATP contents of oocytes for each haplogroup; values given are mean \pm sem

Haplogroup	MtDNA copy/oocyte (N)	Developmental stage (N)	ATP pmol/oocyte	Blastocyst % (N) §
H-235	391 582 \pm 21 029 (141)	GV (103)	2.6 ^{a*} \pm 0.07	36.7 ^d \pm 34.0 (135)
		M II (83)	2.3 ^{b**} \pm 0.06	
H-146	374 758 \pm 22 310 (149)	GV (101)	2.8 [*] \pm 0.06	22.3 ^c \pm 2.2 (164)
		M II (76)	2.9 ^{**} \pm 0.1	
Total/Mean	382 938 \pm 15 341 (290)	(363)	2.6 \pm 0.7	28.8 \pm 31.4 (299)

Values in the same column with the different superscript differ at the 0.05 level

Comparison between haplogroups at GV and M II; values with * and ** are statistically different at the 0.05 level

§ Reference Tamassia et al. (2003)

Table 4: mtDNA content and blastocyst production; animals are ranked by blastocyst rate and haplogroup

	N	MtDNA copy/oocyte Mean \pm SD	CV (%)	mtDNA Haplogroup	Mean Blast. Rate (%) §
Cow 5	41	311 607 ^y \pm 33 809	69.5	H-235	52.4 ^k \pm 6.2
Cow 2	64	361 087 ^y \pm 29 853	66.1	H-235	32.4 ^{lm} \pm 3.6
Cow 3	36	536 878 ^z \pm 41 591	46.5	H-235	25.5 ^{lmn} \pm 4.9
Cow 1	48	391 171 ^{yz} \pm 43 058	76.3	H-146	35.2 ^{kl} \pm 4.6
Cow 6	44	416 320 ^{yz} \pm 46 922	74.8	H-146	20.3 ^{mn} \pm 3.1
Cow 4	57	328 853 ^y \pm 27 508	63.2	H-146	11.7 ⁿ \pm 2.6
Control	41	337 639 ^y \pm 34 034	65.0	NA	NA
Total/Mean	331	377 327 \pm 14 104	68.0	NA	NA

Values in the same column with the same superscripts are not statistically different at the 0.05 level

Cows with the same superscript are not statistically different at the 0.05 level for mtDNA

§ Reference Tamassia et al. (2003)

Coefficient of variation (CV); Not applicable (NA)

Figures:

Figure 1:

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1                               50
mtDNA CR AACACTATTA ATATAGTTCC ATAAATACAA AGAGCCTTAT CAGTATTAAA

51                               100
mtDNA CR TTTATCAAAA ATCCCAATAA CTCAACACAG AATTTGCACC CTAACCAAAT

101                              150
mtDNA CR ATTACAAACA CCACTAGCTA ACATAACACG CCCATACACA GACCACAGAA
PDM                                     G

151                              200
mtDNA CR TGAATTACCT ACGCAAGGGG TAATGTACAT AACATTAATG TAATAAAGAC
NMS                                     C

201 loop stem term 250
mtDNA CR ATAATATGTA TATAGTACAT TAAATTATAT GCCCATGCA TATAAGCAAG

251                              300
mtDNA CR TACATGACCT CTATAGCAGT ACATAATGCA TATAATTATT GACTGTACAT
PDM                                     G A
NMS C A C T

301 TAS-A 350
mtDNA CR AGTACATTAT GTCAAATTCA TTCTTGATAG TATATCTATT ATATATTCCC
NMS C T

351                              400
mtDNA CR TACCATTAGA TCACGAGCTT AATTACCATG CCGCGTGAAA CCAGCAACCC

401                              450
mtDNA CR GCTAGGCAGG GATCCCTCTT CTCGCTCCGG GCCCATAAAC CGTGGGGGTC
NMS T

451                              500
mtDNA CR GCTATCCAAT GAATTTTACC AGGCATCTGG TTCTTTCTTC AGGGCCATCT

501                              550
mtDNA CR CATCTAAAAC GGTCCATTCT TTCCTCTTAA ATAAGACATC TCGATGGACT
PDM C
NMS T

551                              600
mtDNA CR AATGGCTAAT CAGCCCATGC TCACACATAA CTGTGCTGTC ATACATTTGG
NMS A

601                              650
mtDNA CR TATTTTTTTA TTTTGGGGGA TGCTTGACT CAGCTATGGC CGTCAAAGGC

651                              700
mtDNA CR CCTGACCCGG AGCATCTATT GTAGCTGGAC TTAAGTGCAT CTTGAGCACC
NMS C

701 < OH CSB 1 750
mtDNA CR AGCATAATGA TAAGCGTGGA CATTACAGTC AATGGTTACA GGACATAAAT
PDM A G C

751 CSB 2+3 < LSP 800
mtDNA CR TATATTATAT AT.CCCCCCT TCATAAAAAT TTCCCCCTTA AATATCTACC
PDM C

801                              850
mtDNA CR ACCACTTTTA ACAGACTTTT CCCTAGATAC TTATTTAAAT TTTTCACGCT

851                              900
mtDNA CR TTCAATACTC AATTTAGCAC TCCAAACAAA GTCAATATAT AAACGCAGGC
NMS G

901 913
mtDNA CR CCCCCCCCC CCC
PDM ---
NMS C~~

```

Figure 2:

