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2 Non-motile tetraploid spermatozoa of *Misgurnus* loach hybrids

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41 **Abstract**

42 Here, we showed poor motility, low concentration, low viability, abnormal morphology, larger  
43 volume of mitochondrial mass per cell and higher ATP content of spermatozoa with tetraploid  
44 DNA content, taken from diploid loach hybrid between *Misgurnus anguillicaudatus* female and *M.*  
45 *mizolepis* male. Hybrid males produced larger head spermatozoa with no flagellum (36.4%), one  
46 flagellum (46.7%) or two flagella (16.9%). These flagella were shorter than those of normal wild-  
47 type *M. anguillicaudatus* and often gave abnormalities in microtubule structure. Abnormally  
48 shorter flagellum is difficult to propel tetraploid spermatozoa with increased head size in normal  
49 progressive motility, although they had higher energy shown by larger volume of mitochondrial  
50 mass as well as higher ATP content. These tetraploid spermatozoa are likely produced by the arrest  
51 of regular meiotic division after replication of chromosomes, followed by abnormal  
52 spermiogenesis.

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54 **Keywords:** Meiosis; Microtubule; Polyploidy; Spermatogenesis; Spermiogenesis

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72 **Introduction**

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74 Hybrid female fishes between genetically closed species are often fertile and produce fertile eggs  
75 which can be developed after fertilization with sperm of parental species [1-5]. On the other hand,  
76 hybridizations between remotely related species often give rise to sterile progeny even if they can  
77 survive until the adult stages, but hybrids from several combinations of different species produce  
78 fertile eggs and then generate viable progeny by the occurrence of alternative atypical  
79 reproduction such as unreduced oogenesis, clonal gynogenesis or semi-clonal hybridogenesis [6-9].  
80 Hybrid male fishes are often sterile even in the combination between relatively closed species  
81 such as Japanese char *Salvelinus leucomaenis* x brook trout *S. fontinalis* [2] and other examples  
82 [5], but with a few exceptions: fertile unreduced diploid spermatozoa were reported in the Iberian  
83 minnow with a natural hybrid origin [10], common carp × crucian carp hybrids [11-13], and  
84 sex-reversed clonal loach which is considered to have a hybrid origin [14, 15].

85 In Japan, mud loach *Misgurnus mizolepis*, is a well-known exotic cobitid species (Teleostei:  
86 Cobitidae) which has been observed and recorded in several areas [16]. *M. mizolepis* can be  
87 morphologically distinguishable from *M. anguillicaudatus* distributed in Japan [17], and is  
88 considered as a synonym of *Paramisgurnus dabryanus* [16, 18]. *M. anguillicaudatus* has  $2n=50$   
89 chromosomes categorized into 10 metacentric (m), 4 submetacentric (sm) and 36 acrocentric (a)  
90 [19], while *M. mizolepis* has  $2n=48$  with a karyotype of 12 m + 4 sm + 32 a [20]. Since both  
91 species have the same arm number (NF) of 64, interspecific karyotype difference can be well  
92 explained by Robertsonian translocation, i.e., centric fusion or fission [20]. To assess genetic  
93 influence of exotic species to indigenous species, reproductive performance has been examined in

94 interspecific hybrids between *M. anguillicaudatus* and *M. mizolepis*. Park et al. [21] reported  
95 fertility of hybrid males based on the presence of spermatozoa in histological analyse. Fujimoto et  
96 al. [22] artificially induced *M. anguillicaudatus* female x *M. mizolepis* male hybrids, and then  
97 observed that some hybrid males had testis including haploid, diploid and tetraploid cell  
98 populations, while the other had mainly tetraploid cells. They also reported that only haploid  
99 spermatozoa were fertile among spermatozoa of hybrid males and generated next generation of the  
100 progeny by back-crossing [22]. In the case of haploid spermatozoa, spermatogenesis should  
101 successfully proceed due to regular meiotic division between balanced chromosomes in hybrid  
102 because no or little differences in chromosomal dosages between *M. anguillicaudatus* and *M.*  
103 *mizolepis*. On the other hand, tetraploid spermatozoa of the hybrid males had no fertility, but they  
104 might have been matured and spermiated without the completion of meiosis [22].

105 Little is known about physiological and morphological characteristics of tetraploid  
106 spermatozoa in hybrid males. Here, we investigated motility-related parameters such as total  
107 motility, progressive motility, duration of motility, concentration, and viability in spermatozoa  
108 taken from hybrid males. Next, we observed ultrastructure by electron microscopy and measured  
109 head length, head width, flagellum length and number of mitochondria of tetraploid spermatozoa.  
110 Then, volume of mitochondrial mass per cell and ATP content were estimated.

111

## 112 **Materials and methods**

113

114 Ethics and fish used

115 This study was performed in accord with the Guide for the Care and Use of Laboratory Animals in

116 Hokkaido University. The fishes were kept in the aquarium of the Environment Control  
117 Experiment Building, Faculty and Graduate School of Fisheries Sciences, Hokkaido University.  
118 Three adult normal wild-type diploid *M. anguillicaudatus* males (range of standard length (SL):  
119 75-80 mm) and three adult interspecific diploid hybrid males (range of SL: 78-83 mm) were used  
120 for this study. Normal wild-type diploid *M. anguillicaudatus* loaches were obtained from  
121 Kitamura, Iwamizawa City, Hokkaido. Interspecific diploid hybrid males were produced by  
122 fertilizing eggs of *M. anguillicaudatus* with sperm of *M. mizolepis* [22]. Pure mud loach *M.*  
123 *mizolepis* males were not available in the present study.

124

#### 125 Sperm collection

126 Sperm collection was performed according to Fujimoto et al [22]. The ploidy status of sperm  
127 sample from each individual was assessed by flow cytometry as described in [22-25]. For  
128 evaluation, the collected samples were immediately placed in 1.5 ml microtubes containing 1 ml  
129 immobilizing solution (IS) (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM CaCl<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>;  
130 Kurokura et al.[26]), followed by vortexing. Subsequently, the diluted sperm was stored at 4°C  
131 before analyses. For electron microscopy, the collected samples were immediately mixed with 2.5%  
132 glutaraldehyde in a 0.1 M phosphate buffer (pH7.2).

133

#### 134 Evaluation of motility, concentration and viability of sperm

135 Motility was assessed using our previous procedures for loach [23, 24, 27]. Total motility (%),  
136 progressive motility (%) and its duration (s) were obtained from video sequences analyzed with a  
137 video recorder (Sharp VHS VC-HF920) from subjective visualization of sperm movement based

138 on Iwamatsu et al. [28]. The proportion of total motility, progressive motility, and duration of  
139 motility were measured in triplicate for each sample evaluated. The spermatozoa diluted in IS  
140 were fixed by the fixative solution (1% formalin, 5% NaHCO<sub>3</sub>) and cell numbers were counted  
141 three times using Thoma's counting chamber in each sample after sedimentation of spermatozoa  
142 for 5 min. Then average value of concentration was also calculated. The viability of spermatozoa  
143 was assessed using DUAL-staining (SYBR-14 and propidium iodide) procedure using the  
144 LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc. Eugene, OR, USA.), following  
145 instructions from the manufacturer. The evaluation was carried out under a fluorescence  
146 microscopy (Nikon ECLIPSE E800, Tokyo, Japan). One hundred spermatozoa from each sample  
147 were counted to determine the percentage of PI-negative (live) and positive (damaged or dead)  
148 spermatozoa.

149

#### 150 Electron microscopy

151 Ultrastructure of the spermatozoa was observed by scanning electron microscopy (SEM) and  
152 transmission electron microscopy (TEM), according to the procedures in loach sperm [23, 24].  
153 Morphological characteristics of spermatozoa were evaluated by using a SEM. Length of head  
154 size of spermatozoa was measured along head-tail axis from the anterior tip to the posterior tip of  
155 the head. Width was the largest transverse distance of the head of spermatozoon. For measurement  
156 of head size of spermatozoa without flagellum, major and minor diameters were used as length  
157 and width of head of spermatozoa. As flagellum length, the length of tail part without mid-piece  
158 was measured. Number of mitochondria per spermatozoon was counted in TEM image from 180  
159 different cells of wild-type diploid males and those of hybrid males.

160

161 Estimation of volume of mitochondrial mass per spermatozoon

162 Spermatozoa were stained with MitoTracker Green FM (MTGFM: Molecular Probes, Eugene, OR,  
163 USA) according to Zhao et al. [23, 24]. MTGFM is a mitochondrion-specific probe that becomes  
164 fluorescent in the lipid environment of mitochondria. MTGFM contains a thiol-reactive chloromethyl  
165 moiety, resulting in stable peptide and protein conjugates after this accumulation in mitochondria.  
166 MTGFM appears to preferentially accumulate in mitochondria regardless of mitochondrial membrane  
167 potential ( $\Delta\Psi_m$ ), making it an important tool for determining mitochondrial mass [29, 30]. Sperm was  
168 taken from each individual of three normal wild-type diploid males. Then, sperm samples were mixed  
169 and diluted to obtain a final concentration of  $10^6$  cells  $\text{ml}^{-1}$  before analysis. Samples from three hybrid  
170 males were also prepared in the same way. The spermatozoa were assessed by the flow cytometer  
171 (Beckman-Coulter EPICS ALTRA Flow Cytometer Cell Sorter, CA, USA) with an argon laser at 488  
172 nm and a 525 nm filter to detect fluorescence. Forward and side scatter (hereafter abbreviated FS and  
173 SS, respectively) from the cells were used for observation of the cell distribution profile. Flow-check™  
174 Fluorescent beads (10  $\mu\text{m}$ ; Beckman-Coulter, USA) were used for optimization of the analyzer. The  
175 data generated by the flow cytometer were plotted in a single dimension to produce a histogram. The  
176 regions on these plots can be sequentially separated, based on fluorescence intensity, by creating a  
177 series of subset extractions, termed "gates".

178

179 Measurement of ATP content

180 The collected sperm samples from three wild-type males and three hybrid males were diluted  
181 100-fold in IS, before measurement of ATP content. ATP content was measured using a

182 Bioluminescence Assay Kit HS II (Roche Diagnostics GmbH, Germany), following instructions  
183 from the manufacturer [23, 24]. Luminescence was read with a Luminescencer-JNR II AB-2300  
184 (ATTO, Japan). ATP content of each sample was expressed as nmol ATP/10<sup>9</sup> spermatozoa. For  
185 each sample ATP content was measured six times.

186

#### 187 Statistics

188 The data of morphological parameters and ATP content were tested for statistical significance  
189 using one-way analysis of variance (ANOVA) with the LSD *post hoc test* in SPSS ver 11.0.  
190 Statistical significance was set at 0.05.

191

## 192 Results

193

### 194 Ploidy status of sperm

195 When major cell population of sperm from normal wild-type diploid males ( $n = 3$ ) showed 1C  
196 DNA content (Fig. 1a), major cell population of sperm from interspecific hybrid males ( $n = 3$ )  
197 gave DNA content corresponding to 4C (Fig. 1b). These results showed that spermatozoa of  
198 hybrids were tetraploid, when control diploid produced haploid spermatozoa.

199

### 200 Viability, concentration, motility of spermatozoa

201 Parameters of spermatozoa from normal diploid and interspecific hybrid are shown in Table 1.

202 Haploid spermatozoa from normal diploids exhibited vigorous total motility (91.7 %), active  
203 progressive motility (87.3 %) and long motility duration (175.0 s). However, poor total motility

204 (<5 %), no progressive motility and short motility duration (96.7 s) were detected in sperm from  
205 the interspecific hybrid males. Hybrid males gave apparently lower average concentration of  
206 spermatozoa ( $32.4 \times 10^6$  cells/ml) than normal diploid ( $3090.0 \times 10^6$  cells/ml).

207

#### 208 Microscopy of spermatozoa

209 About half of spermatozoa from hybrids had one flagellum (46.7 %), but those without flagellum  
210 (36.4 %) or with two flagella (16.9 %) were also found (Fig. 2, Table 2). The average length of the  
211 flagella in hybrid (the cells without flagellum were not measured), with higher SD ( $12.47 \pm 7.05$   
212  $\mu\text{m}$ ), was obviously shorter than that of the normal spermatozoa (means  $\pm$  SD,  $23.85 \pm 2.29 \mu\text{m}$ )  
213 ( $P < 0.05$ ) (Fig. 2, Table 3). The head length of spermatozoa from hybrid ranged from 2.11  $\mu\text{m}$  to  
214 3.49  $\mu\text{m}$  (Fig. 2, 3), and the average head length of spermatozoa without flagellum ( $2.99 \pm 0.36$   
215  $\mu\text{m}$ ) were slightly larger than those with one flagellum or two flagella ( $2.75 \pm 0.21 \mu\text{m}$ ) in hybrid  
216 ( $P < 0.05$ ) (Table 3). The average head size (length / width of head, means  $\pm$  SD) of tetraploid  
217 spermatozoa from hybrid ( $2.83 \pm 0.24 / 2.80 \pm 0.24 \mu\text{m}$ ) was approximately 1.6 times larger than  
218 that of normal spermatozoa from wild diploid male loaches ( $1.80 \pm 0.08 / 1.80 \pm 0.07 \mu\text{m}$ ) ( $P <$   
219  $0.05$ ) (Table 3). The ratios of head length to head width were approximately 1.0, i.e. sphere like, in  
220 all spermatozoa observed and were not significantly different between wild-type diploid and  
221 hybrid males (Table 3). The ratios of the tetraploid spermatozoa head length to flagellum length in  
222 hybrid ( $0.225 \pm 0.213$ ) were significantly different from that of normal spermatozoa ( $0.075 \pm 0.09$ )  
223 ( $P < 0.05$ ) (Fig. 2, Table 3). The larger cell volumes of spermatozoa from hybrids than wild-type  
224 diploids were shown by forward light-scattering linear scale (FS Lin) from flow cytometry, but  
225 significant differences in inner morphological complexity were not detected by side

226 light-scattering linear scale (SS Lin) between the two (Fig. 4).

227 Although patch- or spot-like vesicles or vacuoles were seldom detected in condensed nucleus  
228 of normal spermatozoa (Fig. 5a), such structures were detected in a condensed nucleus of  
229 spermatozoa from hybrid (Fig. 5b). In spermatozoa with two flagella (Fig. 5bc), some exhibited  
230 separate cytoplasmic channels (Fig.5bd), while the other showed communal cytoplasmic channel  
231 (Fig. 5ce). Normal 9 + 2 structure was found in spermatozoa from wild-type diploid males (Fig.5f),  
232 but abnormal 9 + 1 microtubule structure was detected together with normal 9 + 2 in spermatozoa  
233 from hybrid males (Fig. 5g).

234 Mean number of mitochondria counted in spermatozoa ( $n = 180$ ) was similar between control  
235 wild-type and interspecific hybrid males, but spermatozoa with smaller numbers (4 to 6) of  
236 mitochondria appeared in hybrids (Fig. 6).

237

238 Volume of mitochondrial mass per spermatozoon

239 Total volume of mitochondrial mass per spermatozoon from interspecific hybrid was larger than  
240 that from normal diploid (Fig. 7).

241

242 ATP content of sperm

243 ATP content of sperm from interspecific hybrid males ( $257.37 \pm 8.30$  nmol/ $10^9$  spermatozoa) was  
244 higher than that from normal diploid males ( $80.06 \pm 5.16$  nmol/ $10^9$  spermatozoa) ( $P < 0.05$ ) (Fig.  
245 8). The inter-males variability was low in ATP content for all spermatozoa.

246

247 **Discussion**

248

249 In the present study, we found that interspecific *M. anguillicaudatus* female x *M. mizolepis* male  
250 hybrids predominantly produced non-motile tetraploid spermatozoa which had approximately 1.6  
251 times larger head sizes than those of normal haploid spermatozoa from wild-type diploid males. In  
252 addition, about 47% of spermatozoa had one abnormally short flagellum, but 17% had two flagella  
253 and 36% had no flagellum. These results showed that diploid hybrids generated abnormal  
254 spermatozoa (or spermatozoon-like cells without flagellum) with replicated chromosomes  
255 equivalent to 4C DNA content, which underwent the process of spermiogenesis. Above mentioned  
256 observations, i.e., production of unusual large-head spermatozoa with 4C DNA content nucleus  
257 and differentiated flagellum(a) were quite similar to the results that reported in the interspecific  
258 hybrid males between *Oryzias latipes* and *O. curvinotus* [31]. In medaka hybrids, the arrest of the  
259 meiotic cell cycle was concluded based on cytological observation, absence of the expression  
260 protamine mRNA and a cell culture of primary spermatocytes, in which production of one  
261 spermatozoon-like cell from one spermatocyte isolated from the hybrid was observed [31]. Thus,  
262 abnormal spermatozoa were also likely produced by the same meiotic arrest after the replication of  
263 chromosomes, i.e. elevation of chromosomes from 2C (diploidy) to 4C (tetraploidy), in hybrid  
264 loaches as in medaka hybrids. The meiotic arrest was presumably due to the failure of pairing  
265 between homologous chromosomes derived from different species. However, as in medaka  
266 hybrids, loach hybrids also produced unusual spermatozoa with flagellum(a) by the process of  
267 spermiogenesis.

268 Fujimoto et al. [22] reported  $13.2 \times 10^6$  cells/ml concentration, 4.1% (range 0-8%) total  
269 motility, 21.9% progressive motility (range 0-39%), and 102.9s duration of motility (range

270 63-131s) for spermatozoa of the same hybrid males. These parameters were almost same except  
271 for the 0% progressive motility in the present study. Since the previous study reported the  
272 occurrence of very small number of viable diploid progeny after back-cross of hybrid male to *M.*  
273 *anguillicaudatus* female, the difference of progressive motility should be well explained by the  
274 proportion of motile haploid spermatozoa. Moreover, the previous genetic studies using  
275 microsatellite DNA markers revealed that the above-mentioned viable diploid progeny had alleles  
276 derived from both two species and thus fertile haploid spermatozoa were formed by meiotic  
277 segregation even in the inter-specific hybrid male [22]. At present, however, it is not known why  
278 some hybrid males generate motile haploid spermatozoa by meiosis, but the other males do not  
279 due to the arrest of meiosis after the replication. What is the difference between the two cases in  
280 spermatogenesis of the *Misgurnus* hybrids? Further cytogenetic and molecular studies are required  
281 to answer this question.

282 Total volume of mitochondrial mass per spermatozoon and ATP content of sperm  
283 apparently increased in hybrids and thus it seemed to compensate probable reduced motility due to  
284 the increase of sperm head sizes by the elevation of ploidy status or the increase of genetic  
285 materials in cell nucleus. However, the spermatozoa of hybrids did not exhibit active progressive  
286 motility. Poor motility found in the present tetraploid spermatozoa may be explained by the  
287 malformations especially in flagellum which is the motor of the spermatozoon. No or double short  
288 flagella apparently inhibit to propel the movement of spermatozoa with increased head sizes. In  
289 spermatozoa with one flagellum, the length of flagellm was significantly shorter than that of  
290 wild-type diploids. Same situations were already found in abnormal hexaploid spermatozoa  
291 formed in hyper-triploid loaches [24].

292 Other factor related to the reduced motility may be abnormal microtubule structure of the  
293 axoneme. Although the axoneme of a motile flagellum has two central microtubule singlets in  
294 addition to the nine outer doublets (called a 9 + 2 axoneme), tetraploid spermatozoa often showed  
295 9 + 1 structure. Such a deviation from the typical 9 + 2 microtubule structure was reported to link  
296 to the formation of non-motile flagellum [32]. Abnormalities in flagellar number (no-flagellar or  
297 bi-flagellar) and structure (9 + 1 axonema and others) were also found in hexaploid spermatozoa  
298 of hyper-triploid loach [24]. Number of mitochondria of tetraploid spermatozoa from the hybrid  
299 was similar to that observed in hexaploid spermatozoa from hyper-triploid loach: almost same  
300 mean and SD were reported in spite of big difference in ploidy status [24]. These common  
301 morphological features are likely caused by the spermiogenesis of unusual spermatids which are  
302 formed without two successive meiotic divisions. Therefore, abnormalities of spermatozoa of  
303 hybrid males may be closely linked to the unusual proceeding of spermiogenesis without the  
304 completion of meiotic divisions.

305

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313

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400

401 **LEGENDS**

402 Fig. 1. Flow-cytometric histograms of sperm samples from normal diploid wild type *Misgurnus*  
403 *anguillicaudatus* (a) and hybrids between *M. anguillicaudatus* female and *M. mizolepis* male (b).  
404 Fluorescent peak of haploid spermatozoa from normal wild type diploid loach indicates a DNA  
405 content of 1C (a). Fluorescent peak of tetraploid spermatozoa from hybrid loaches indicates a  
406 DNA content of 4C (b). Mean is the average relative DNA content detected automatically by the  
407 flow cytometer. Area% means the proportion of cells contained in the highest peak of histogram to  
408 the total cells analyzed. CV% is estimated by the coefficient of variation of the histogram  
409 multiplied by 100, i.e.  $CV\% = \text{Standard deviation} / \text{average} \times 100$ .

410 Fig.2. Scanning electron microscopy of spermatozoa from normal diploid (a) and hybrid (b, c, d)  
411 loach. Asterisk indicates cell without flagellum (b). Triangle indicates cell with two flagella (b).  
412 Square indicates cell with one flagellum. White arrow indicates cell with relatively small (c) or big  
413 size (d).

414 Fig. 3. Head length of spermatozoa from normal diploid (a) and hybrid (b) loaches. Columns  
415 indicate the percentages of spermatozoa with each head length. N: number of the cells measured.

416 Fig. 4. Volume and morphological complexity of spermatozoa assessed by Flow Cytometer from  
417 normal diploid (a) and hybrid (b) loach. FS Lin (forward light-scattering linear scale) indicates  
418 volume of cells; SS Lin (side light-scattering linear scale) indicates complexity of inner structure  
419 of cells.

420 Fig. 5. Transmission electron microscopic (TEM) images of spermatozoon from normal diploid (a),  
421 bi-flagellar spermatozoon with separate cytoplasmic channel from hybrid (b) and bi-flagellar  
422 spermatozoon with communal cytoplasmic channel from hybrid (c), schematic representation of  
423 bi-flagellar spermatozoon with separate cytoplasmic channels (d) and bi-flagellar spermatozoon

424 with communal cytoplasmic channel (e), and TEM images of typical 9 + 2 microtubule structure  
425 of flagellum of spermatozoon from normal diploid (f) and of abnormal hybrid (g). (d) and (e)  
426 are schematic representation of (b) and (c), respectively. F: flagellum, M: mitochondrion, N:  
427 nucleus, P: patch- or spot-like vesicles or vacuoles (a-e). White arrow indicates normal 9+2  
428 microtubule structure of the flagellum and black arrow indicates abnormal 9+1 microtubule  
429 structure of the flagellum (g).

430 Fig. 6. Number of mitochondria (mean  $\pm$  SD) counted in transmission electron microscopic  
431 sections from diploid loach (a) and hybrid (b).

432 Fig. 7. Total volume of mitochondrial mass per spermatozoon assessed by Flow Cytometer from  
433 normal diploid (a) and hybrid (b) loach. X axis represents MitoTracker Green FM intensity  
434 (logarithmic scale) and Y axis indicates cell counts (linear scale).

435 Fig. 8. ATP content of spermatozoa from loach males examined. Significant differences of ATP  
436 content were recorded between haploid spermatozoa from normal diploid males and tetraploid  
437 spermatozoa from hybrid males. Different letters on column indicate significantly different at  $P <$   
438 0.05.

Table 1. Average viability, concentration, total motility, progressive motility and duration of motility of spermatozoa from normal and hybrid loach males

Biotype	Fish No.	Viability (%)	Concentration ( $\times 10^6$ cells/ml)	Total motility (%)	Progressive motility (%)	Duration of motility (s)
Normal diploid	#1	90	2950	90	85	170
	#2	95	3200	95	91	180
	#3	90	3120	90	86	175
	Mean of #1-3	91.7	3090.0	91.7	87.3	175.0
Hybrid	#1	75	54.3	<5	0	95
	#2	75	19.2	<5	0	105
	#3	80	23.8	<5	0	90
	Mean of #1-3	76.7	32.4	<5	0.0	96.7

Table 2. Percentage of abnormal spermatozoa in the males evaluated

	Normal diploid	Hybrid
N	90	165
No-flagellar spermatozoa	0	60 (36.4%)
Mono-flagellar spermatozoa	90 (100%)	77 (46.7%)
Bi-flagellar spermatozoa	0	28 (16.9%)

N: number of the cells measured

Table 3. Morphometric characteristics of spermatozoa of normal diploid and hybrid loach males

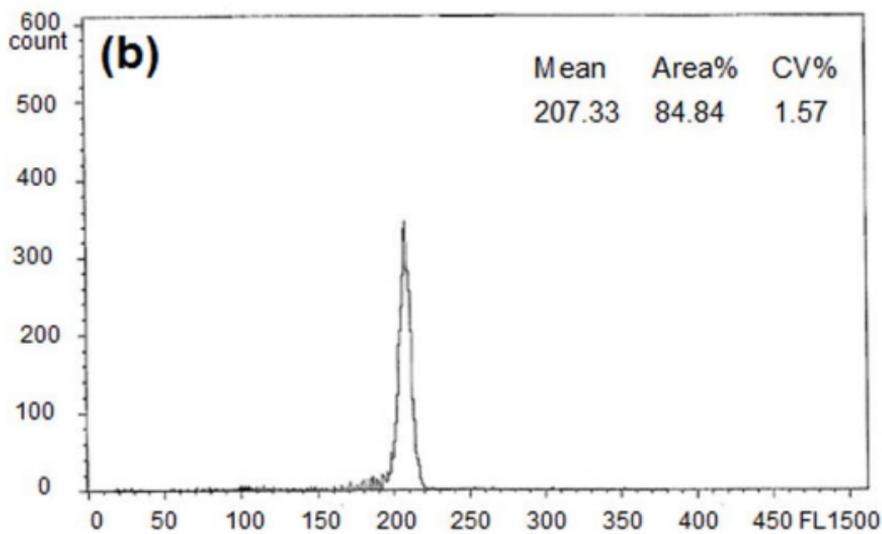
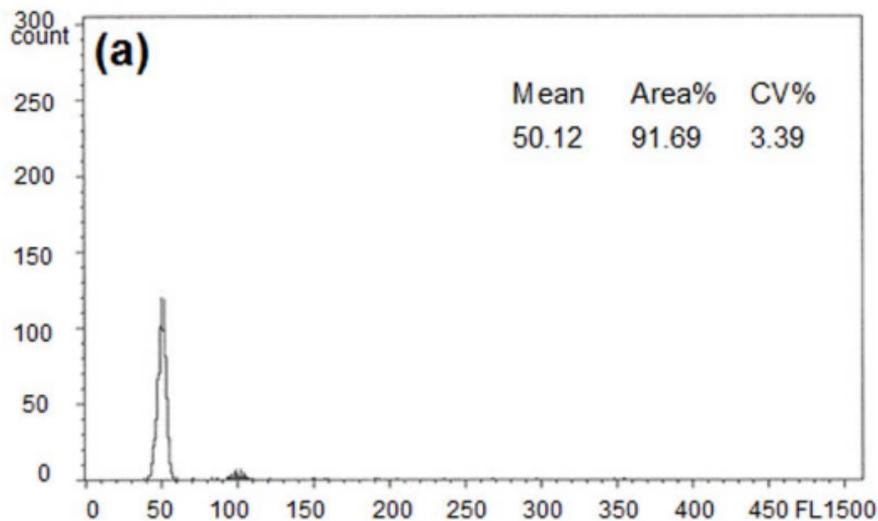
Biotype	Fish No.	Type of flagella	N	Head size of spermatozoa ( $\mu\text{m}$ )		Length of flagellum ( $\mu\text{m}$ ) mean $\pm$ SD	Head length/Head width	Head length/flagellum length
				Length of head mean $\pm$ SD	Width of head mean $\pm$ SD			
Normal diploid	#1	MonoF	30	1.80 $\pm$ 0.07 <sup>a</sup>	1.80 $\pm$ 0.07 <sup>a</sup>	23.89 $\pm$ 2.35 <sup>a</sup>	1.00 $\pm$ 0.052 <sup>a</sup>	0.075 $\pm$ 0.010 <sup>a</sup>
	#2	MonoF	30	1.81 $\pm$ 0.09 <sup>a</sup>	1.79 $\pm$ 0.06 <sup>a</sup>	23.42 $\pm$ 2.26 <sup>a</sup>	1.01 $\pm$ 0.062 <sup>a</sup>	0.077 $\pm$ 0.008 <sup>a</sup>
	#3	MonoF	30	1.80 $\pm$ 0.08 <sup>a</sup>	1.80 $\pm$ 0.07 <sup>a</sup>	24.25 $\pm$ 2.25 <sup>a</sup>	1.00 $\pm$ 0.049 <sup>a</sup>	0.074 $\pm$ 0.008 <sup>a</sup>
	Mean of #1-3		90	1.80 $\pm$ 0.08*	1.80 $\pm$ 0.07*	23.85 $\pm$ 2.29 <sup>a</sup>	1.00 $\pm$ 0.054 <sup>a</sup>	0.075 $\pm$ 0.009 <sup>a</sup>
Hybrid	#1	NoF	19	3.00 $\pm$ 0.38 <sup>b</sup>	2.97 $\pm$ 0.35 <sup>b</sup>	-----	1.01 $\pm$ 0.078 <sup>a</sup>	-----
		MonoF	24	2.76 $\pm$ 0.19 <sup>c</sup>	2.76 $\pm$ 0.20 <sup>c</sup>	11.69 $\pm$ 7.21 <sup>b</sup>	1.00 $\pm$ 0.071 <sup>a</sup>	0.240 $\pm$ 0.250 <sup>b</sup>
		BiF	9	2.74 $\pm$ 0.24 <sup>c</sup>	2.71 $\pm$ 0.22 <sup>c</sup>	13.55 $\pm$ 6.92 <sup>b</sup>	1.01 $\pm$ 0.076 <sup>a</sup>	0.209 $\pm$ 0.186 <sup>b</sup>
	#2	NoF	19	2.99 $\pm$ 0.35 <sup>b</sup>	2.96 $\pm$ 0.33 <sup>b</sup>	-----	1.01 $\pm$ 0.077 <sup>a</sup>	-----
		MonoF	26	2.74 $\pm$ 0.19 <sup>c</sup>	2.71 $\pm$ 0.19 <sup>c</sup>	12.45 $\pm$ 7.11 <sup>b</sup>	1.01 $\pm$ 0.072 <sup>a</sup>	0.224 $\pm$ 0.235 <sup>b</sup>
		BiF	10	2.74 $\pm$ 0.23 <sup>c</sup>	2.71 $\pm$ 0.21 <sup>c</sup>	11.91 $\pm$ 7.09 <sup>b</sup>	1.01 $\pm$ 0.075 <sup>a</sup>	0.232 $\pm$ 0.217 <sup>b</sup>
	#3	NoF	22	2.98 $\pm$ 0.35 <sup>b</sup>	2.98 $\pm$ 0.33 <sup>b</sup>	-----	1.00 $\pm$ 0.076 <sup>a</sup>	-----
		MonoF	27	2.77 $\pm$ 0.18 <sup>c</sup>	2.74 $\pm$ 0.20 <sup>c</sup>	13.21 $\pm$ 7.01 <sup>b</sup>	1.01 $\pm$ 0.074 <sup>a</sup>	0.211 $\pm$ 0.201 <sup>b</sup>
		BiF	9	2.76 $\pm$ 0.21 <sup>c</sup>	2.73 $\pm$ 0.19 <sup>c</sup>	12.04 $\pm$ 6.98 <sup>b</sup>	1.01 $\pm$ 0.076 <sup>a</sup>	0.238 $\pm$ 0.194 <sup>b</sup>
	Mean of NoF		60	2.99 $\pm$ 0.36 <sup>b</sup>	2.97 $\pm$ 0.34 <sup>b</sup>	-----	1.01 $\pm$ 0.077 <sup>a</sup>	-----
Mean of MonoF and BiF		105	2.75 $\pm$ 0.21 <sup>c</sup>	2.72 $\pm$ 0.20 <sup>c</sup>	12.47 $\pm$ 7.05 <sup>b</sup>	1.01 $\pm$ 0.074 <sup>a</sup>	0.225 $\pm$ 0.213 <sup>b</sup>	
Mean of #1-3		165	2.83 $\pm$ 0.24*	2.80 $\pm$ 0.24*	12.47 $\pm$ 7.05 <sup>b</sup>	1.01 $\pm$ 0.075 <sup>a</sup>	0.225 $\pm$ 0.213 <sup>b</sup>	

N: number of the cells measured

NoF: Noflagellar spermatozoa MonoF: Monoflagellar spermatozoa BiF: Biflagellar spermatozoa

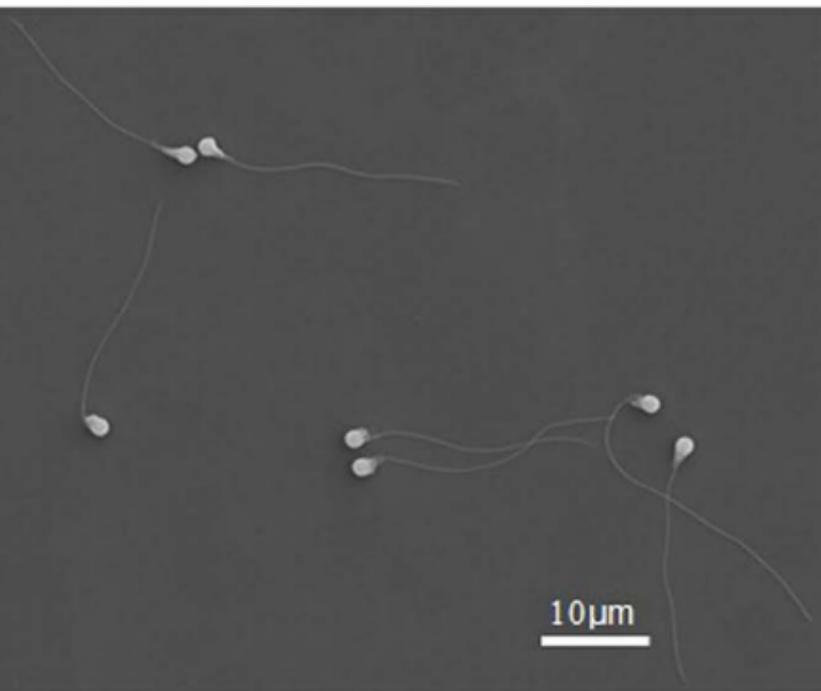
Values within a column followed by different letters or \* are significantly different at  $P < 0.05$

Cell number

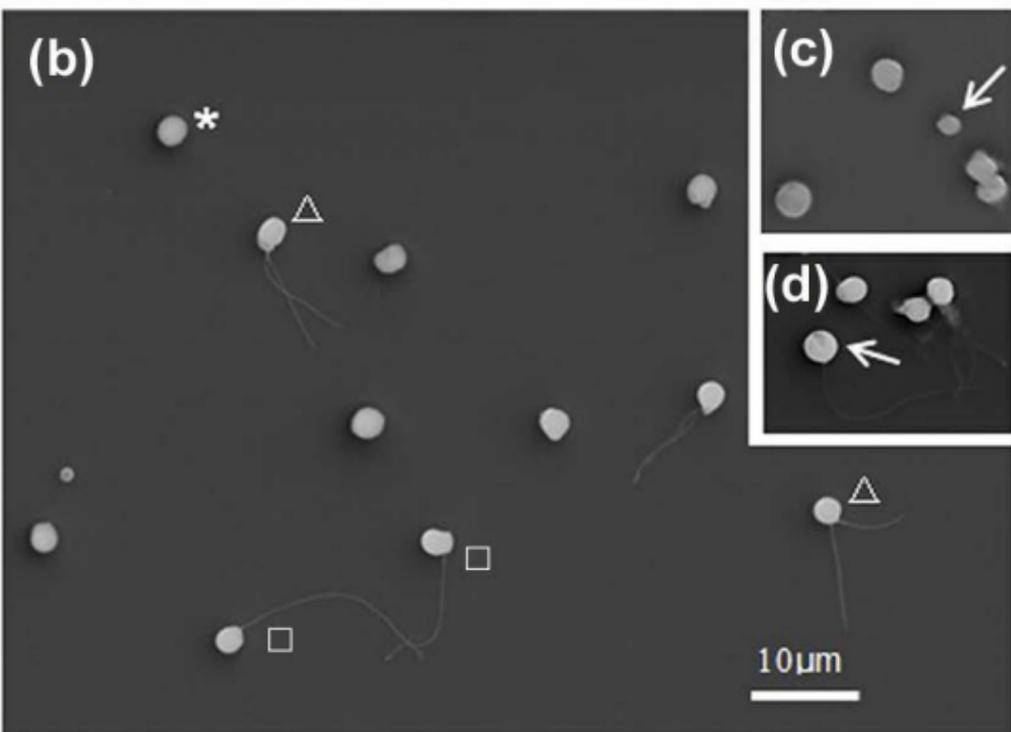


Relative DNA content

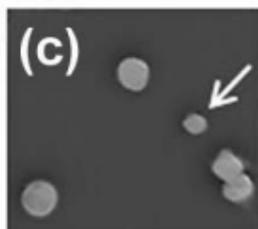
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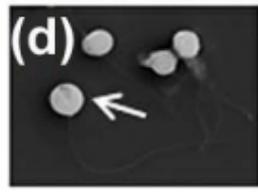
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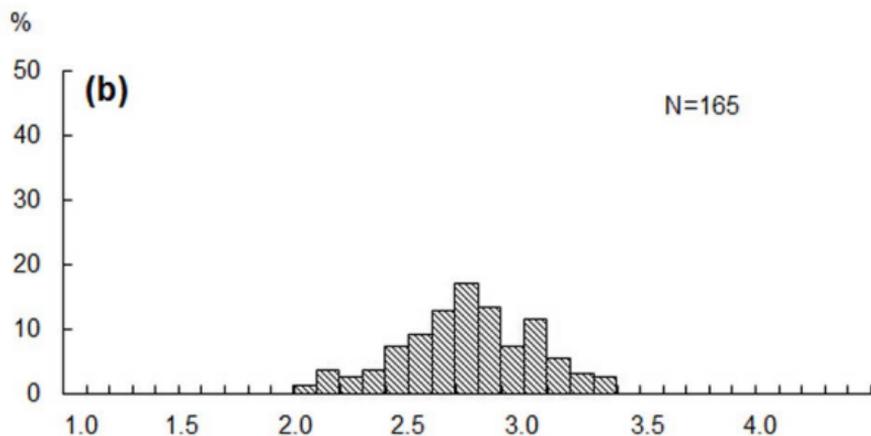
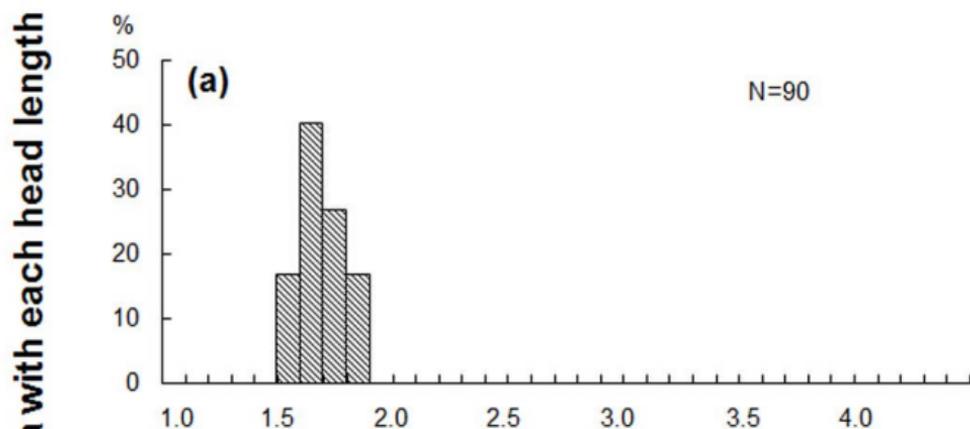


**(c)**



**(d)**

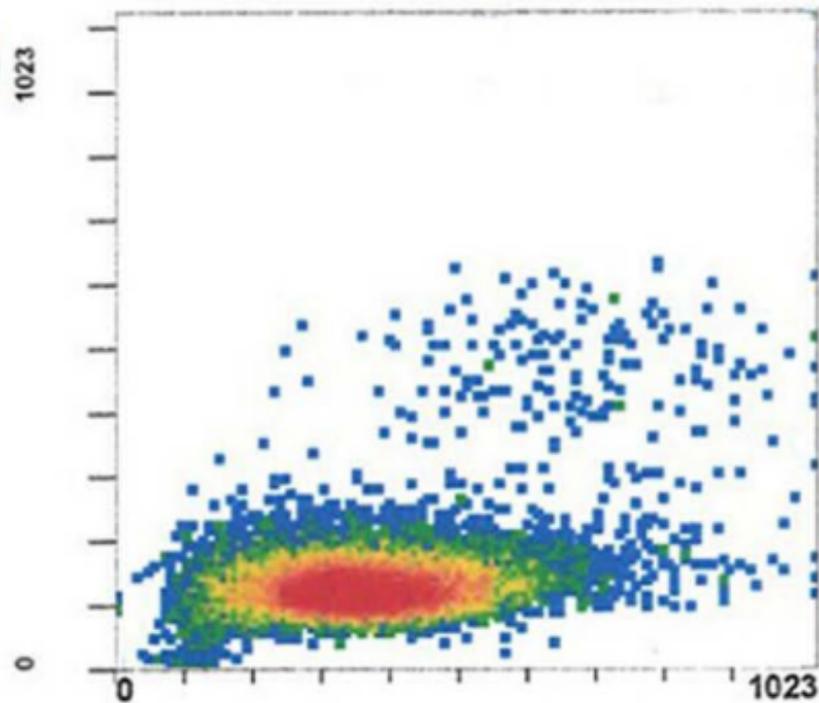




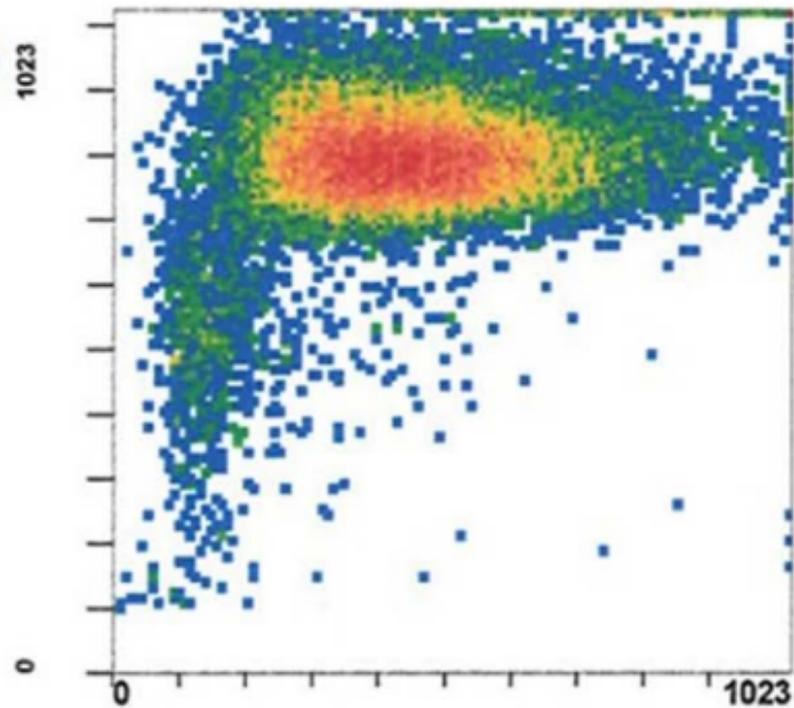
**Spermatozoa head length ( $\mu\text{m}$ )**

**FS Lin**

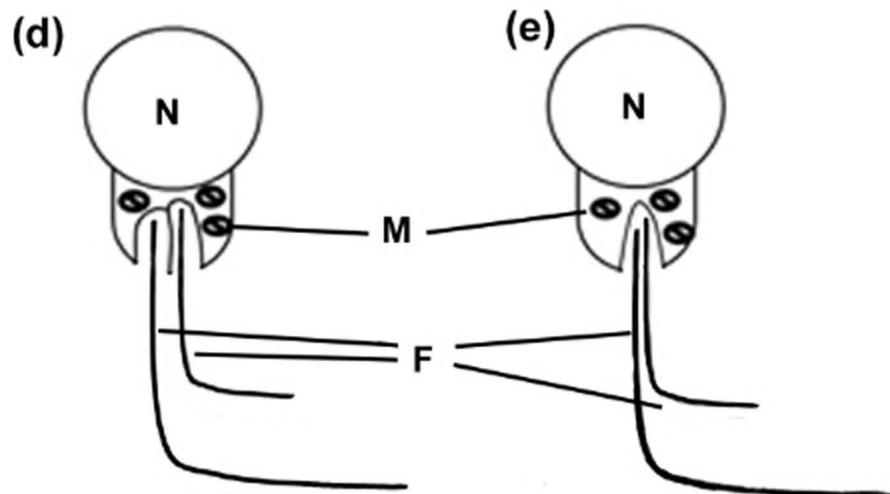
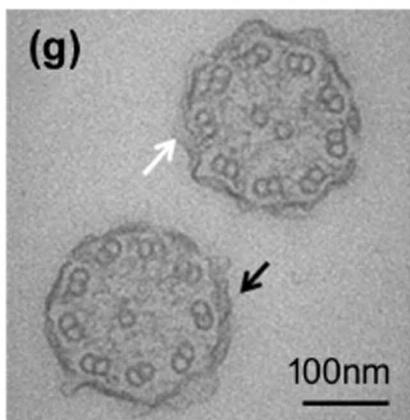
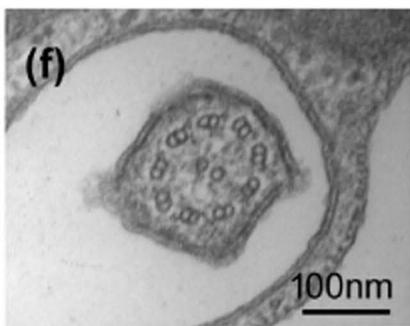
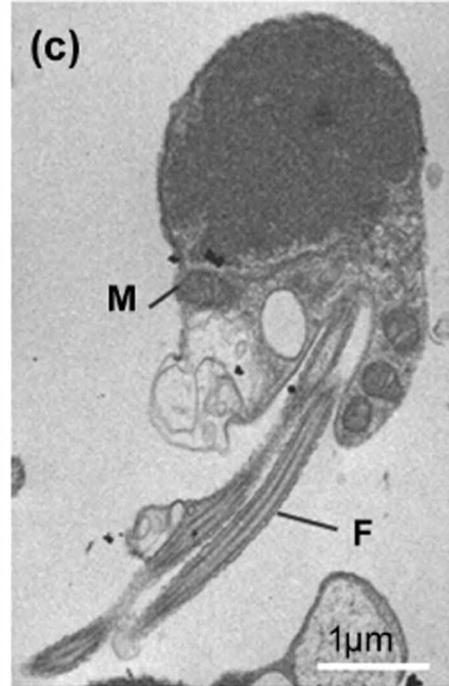
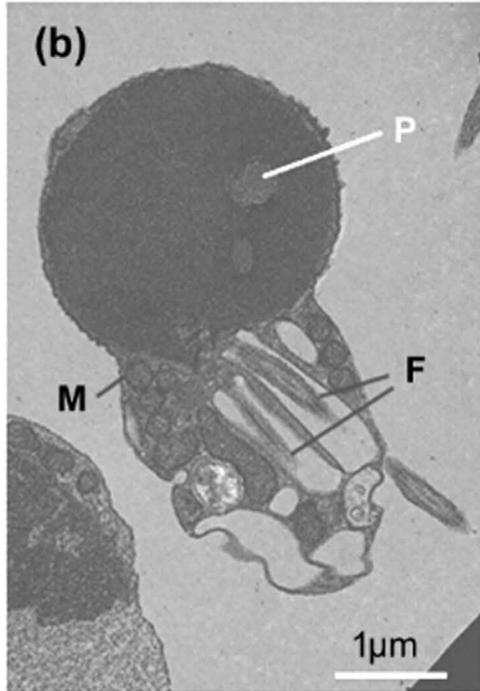
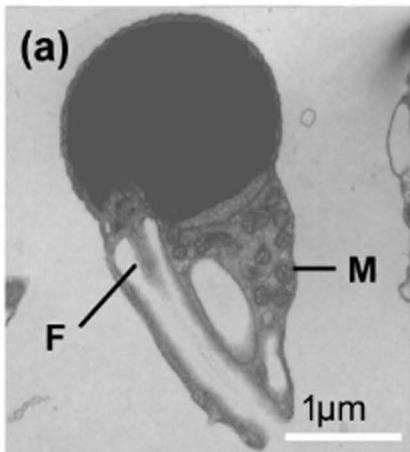
**(a)**



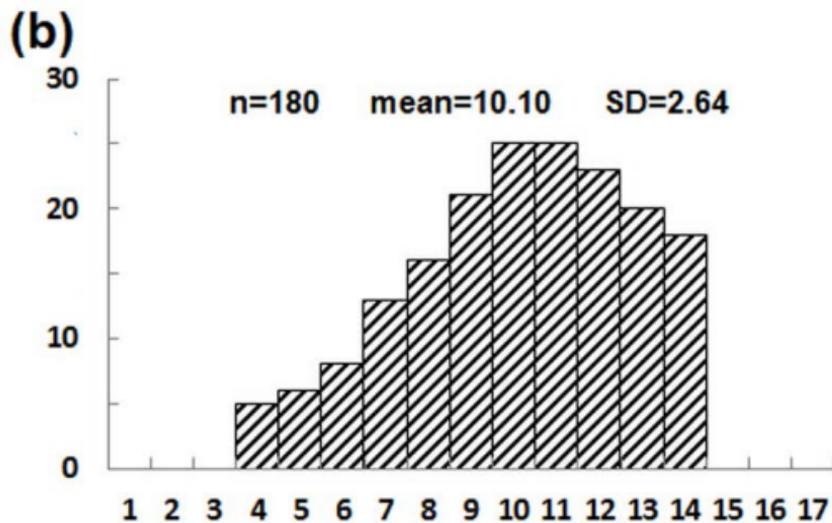
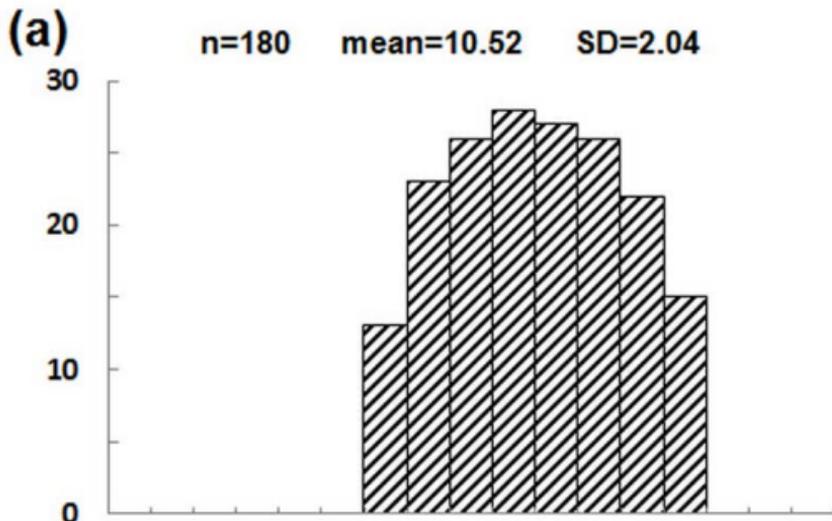
**(b)**



**SS Lin**

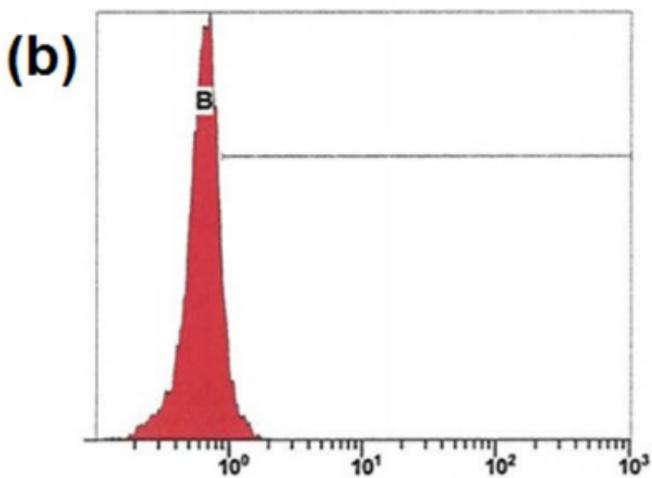
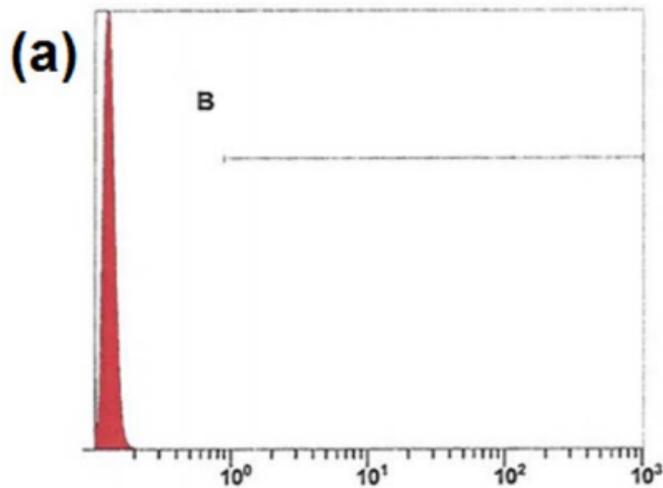


**Number of spermatozoa**



**Number of mitochondria per single TEM image**

Cell number



MitoTracker Green FM log

