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Non-motile tetraploid spermatozoa of *Misgurnus* loach hybrids

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Abstract

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

Keywords: Meiosis; Microtubule; Polyploidy; Spermatogenesis; Spermiogenesis

Introduction

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

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

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

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

Materials and methods

Ethics and fish used

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

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

Sperm collection

Sperm collection was performed according to Fujimoto et al [22]. The ploidy status of sperm sample from each individual was assessed by flow cytometry as described in [22-25]. For evaluation, the collected samples were immediately placed in 1.5 ml microtubes containing 1 ml immobilizing solution (IS) (128.4 mM NaCl, 2.7 mM KCl, 1.4 mM $CaCl_2$, 2.4 mM $NaHCO_3$; Kurokura et al.[26]), followed by vortexing. Subsequently, the diluted sperm was stored at 4°C before analyses. For electron microscopy, the collected samples were immediately mixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH7.2).

Evaluation of motility, concentration and viability of sperm

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

on Iwamatsu et al. [28]. The proportion of total motility, progressive motility, and duration of motility were measured in triplicate for each sample evaluated. The spermatozoa diluted in IS were fixed by the fixative solution (1% formalin, 5% $NaHCO_3$) and cell numbers were counted three times using Thoma's counting chamber in each sample after sedimentation of spermatozoa for 5 min. Then average value of concentration was also calculated. The viability of spermatozoa was assessed using DUAL-staining (SYBR-14 and propidium iodide) procedure using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc. Eugene, OR, USA.), following instructions from the manufacturer. The evaluation was carried out under a fluorescence microscopy (Nikon ECLIPSE E800, Tokyo, Japan). One hundred spermatozoa from each sample were counted to determine the percentage of PI-negative (live) and positive (damaged or dead) spermatozoa.

Electron microscopy

Ultrastructure of the spermatozoa was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), according to the procedures in loach sperm [23, 24]. Morphological characteristics of spermatozoa were evaluated by using a SEM. Length of head size of spermatozoa was measured along head-tail axis from the anterior tip to the posterior tip of the head. Width was the largest transverse distance of the head of spermatozoon. For measurement of head size of spermatozoa without flagellum, major and minor diameters were used as length and width of head of spermatozoa. As flagellum length, the length of tail part without mid-piece was measured. Number of mitochondria per spermatozoon was counted in TEM image from 180 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 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^{TM}
174 Fluorescent beads (10 μm; 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

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

Statistics

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

Results

Ploidy status of sperm

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

Viability, concentration, motility of spermatozoa

Parameters of spermatozoa from normal diploid and interspecific hybrid are shown in Table 1. Haploid spermatozoa from normal diploids exhibited vigorous total motility (91.7 %), active 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 x 10^6 cells/ml) than normal diploid (3090.0 x 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 ± 7.05
212 μm), was obviously shorter than that of the normal spermatozoa (means ± SD, 23.85 ± 2.29 μm)
213 (P<0.05) (Fig. 2, Table 3). The head length of spermatozoa from hybrid ranged from 2.11 μm to
214 3.49 μm (Fig. 2, 3), and the average head length of spermatozoa without flagellum (2.99 ± 0.36
215 μm) were slightly larger than those with one flagellum or two flagella (2.75 ± 0.21μm) in hybrid
216 (P<0.05) (Table 3). The average head size (length / width of head, means ± SD) of tetraploid
217 spermatozoa from hybrid (2.83 ± 0.24 / 2.80 ± 0.24 μm) was approximately 1.6 times larger than
218 that of normal spermatozoa from wild diploid male loaches (1.80 ± 0.08 / 1.80 ± 0.07 μm) (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 ± 0.213) were significantly different from that of normal spermatozoa (0.075 ± 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

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

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

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

Volume of mitochondrial mass per spermatozoon

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

ATP content of sperm

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

Discussion

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

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

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

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

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

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LEGENDS

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

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

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

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

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

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

Fig. 6. Number of mitochondria (mean ± SD) counted in transmission electron microscopic sections from diploid loach (a) and hybrid (b).

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

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

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

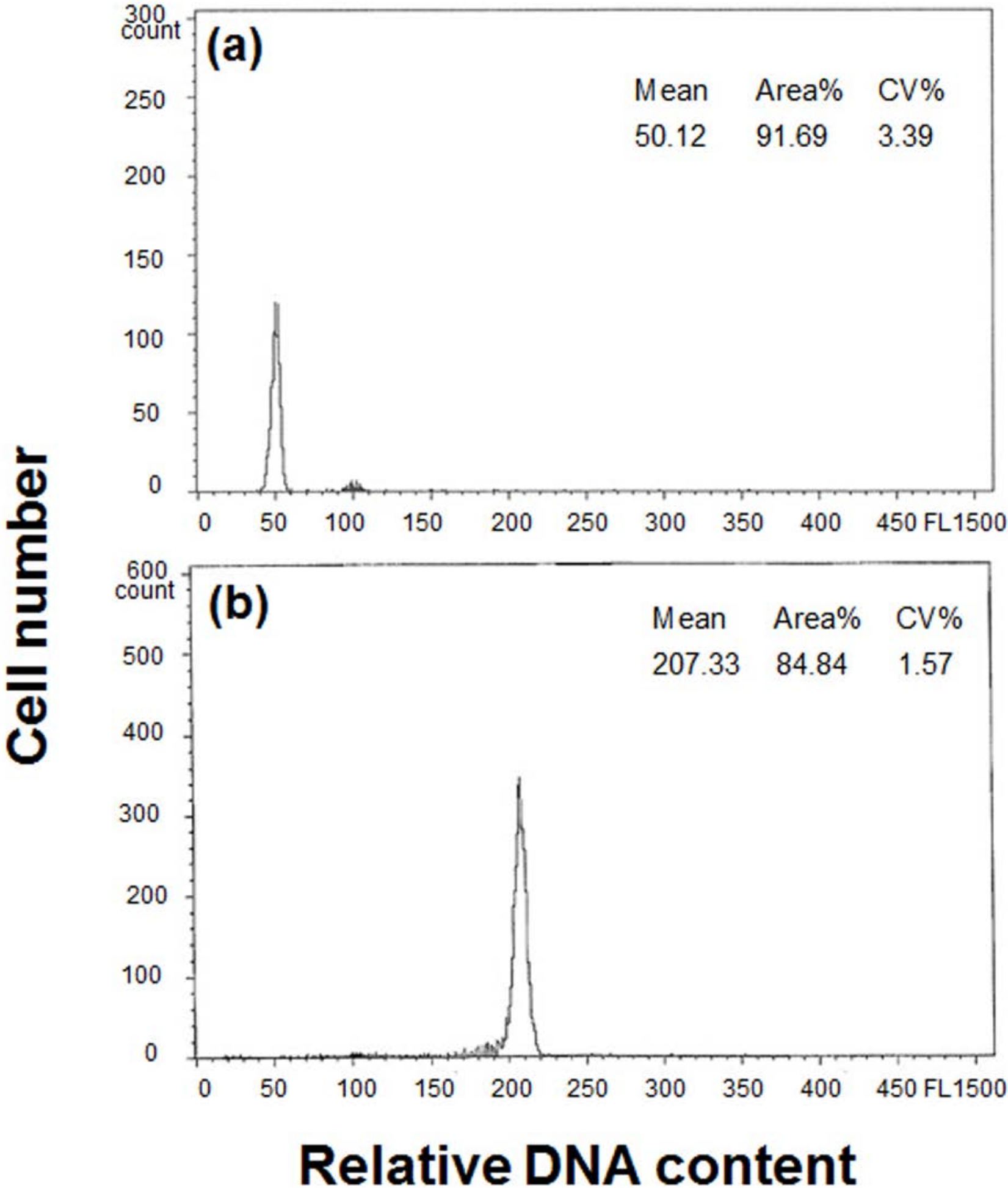
Table 3. Morphometric characteristics of spermatozoa of normal diploid and hybrid loach males

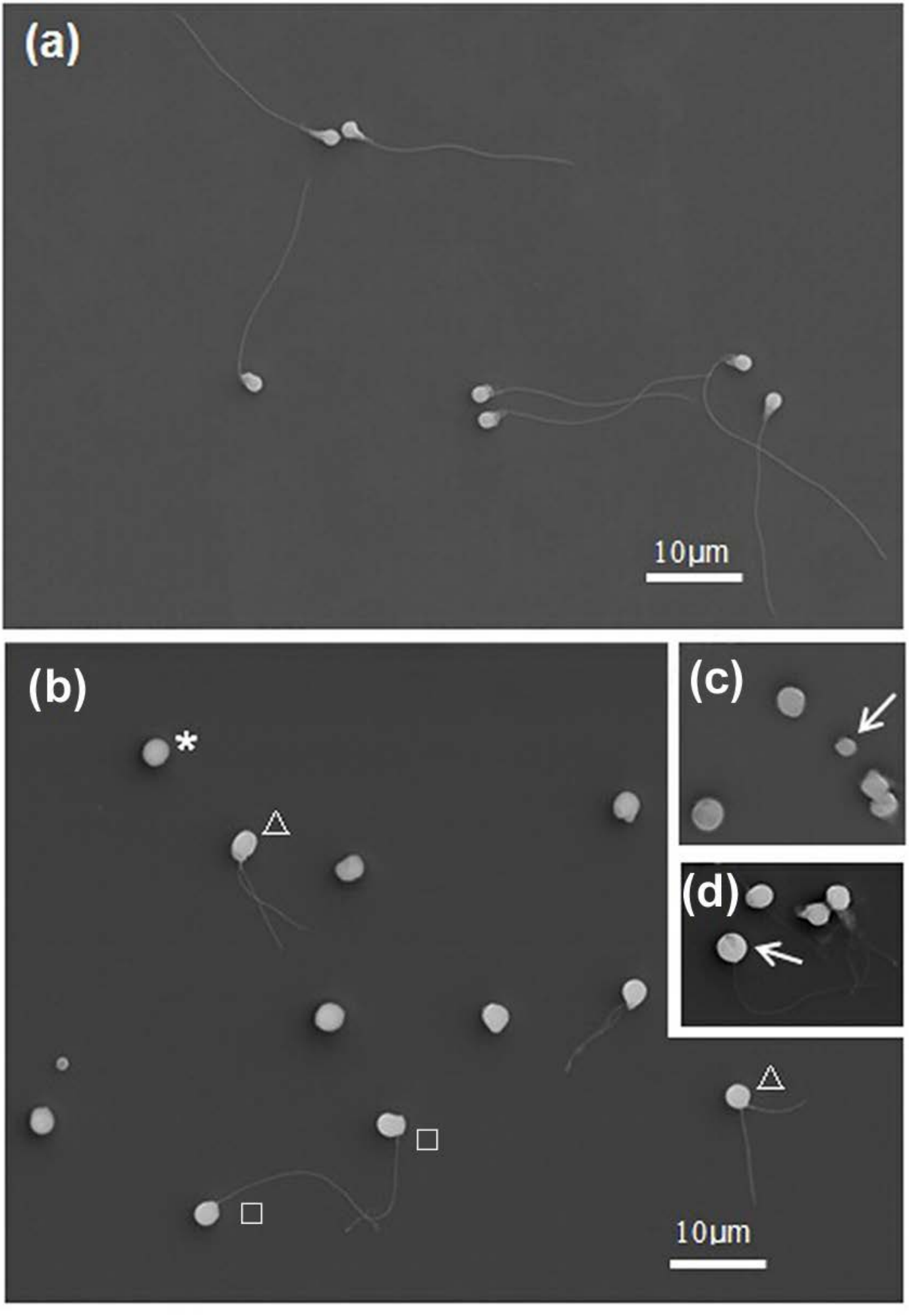
Biotype	Fish No.	Type of flagella	N	Head size of spermatozoa (μm)		Length of flagellum (μm) mean±SD	Head length/Head width	Head length/flagellum length
				Length of head mean±SD	Width of head mean±SD			
Normal diploid	#1	MonoF	30	1.80±0.07[a]	1.80±0.07[a]	23.89±2.35[a]	1.00±0.052[a]	0.075±0.010[a]
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

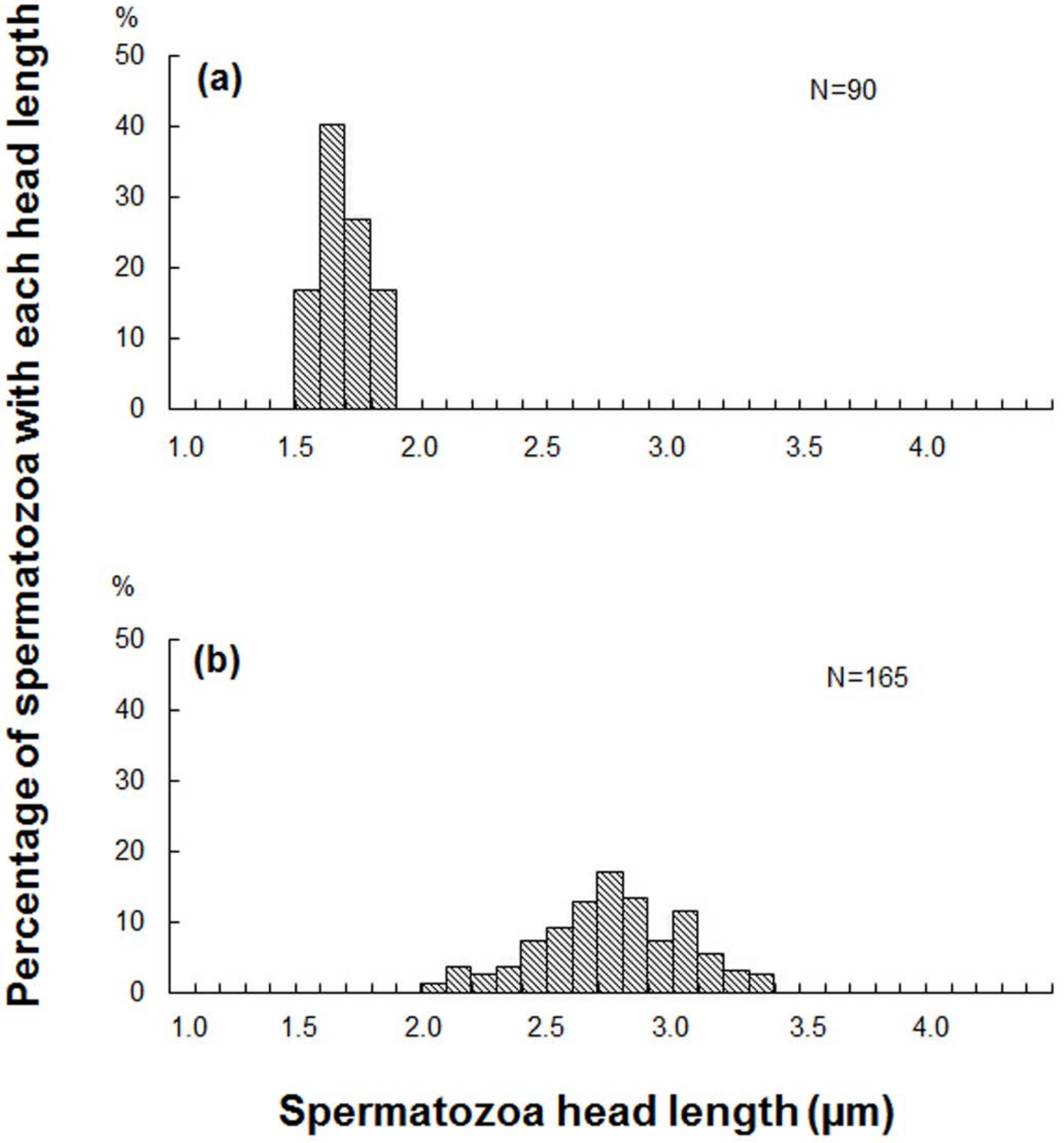
N: number of the cells measured

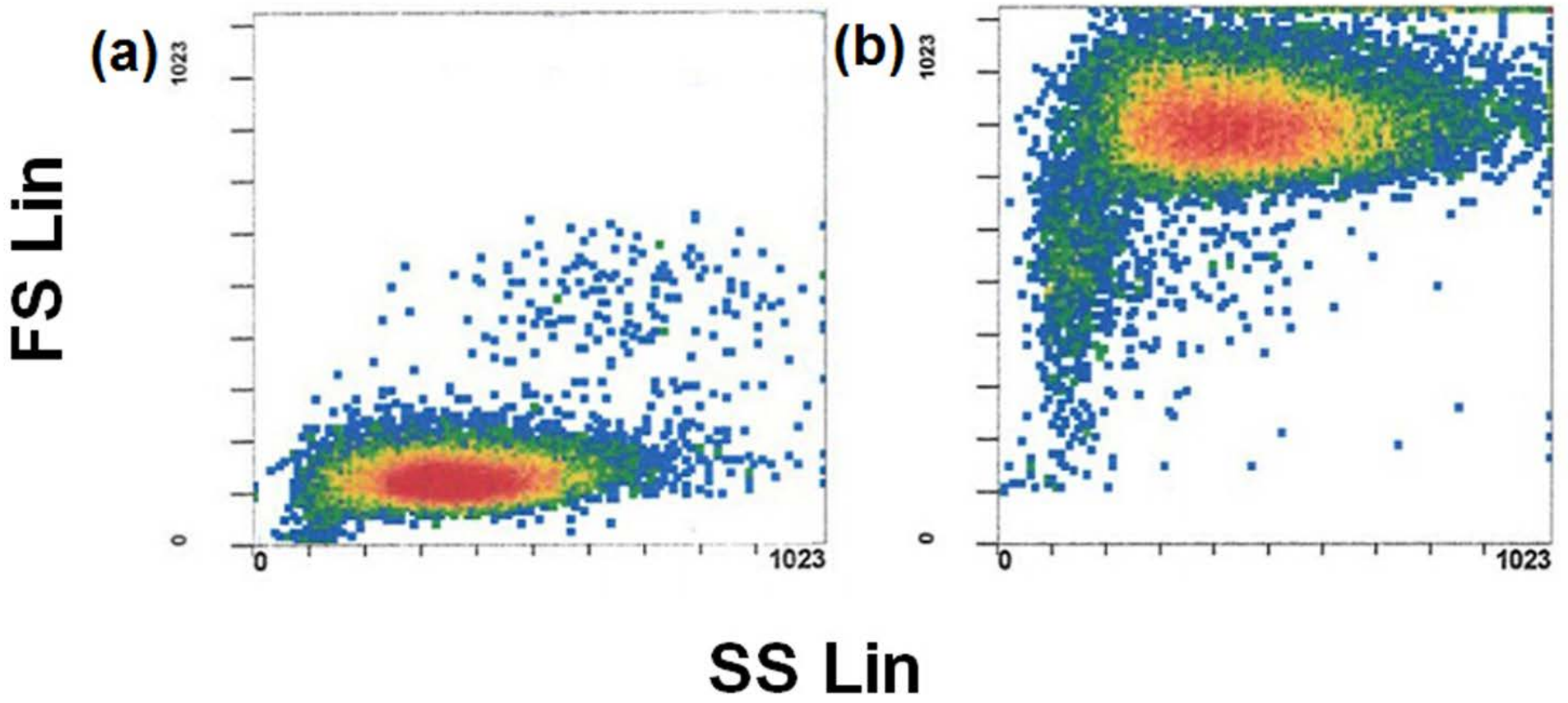
NoF: Noflagellar spermatozoa MonoF: Monoflagellar spermatozoa BiF: Biflagellar spermatozoa

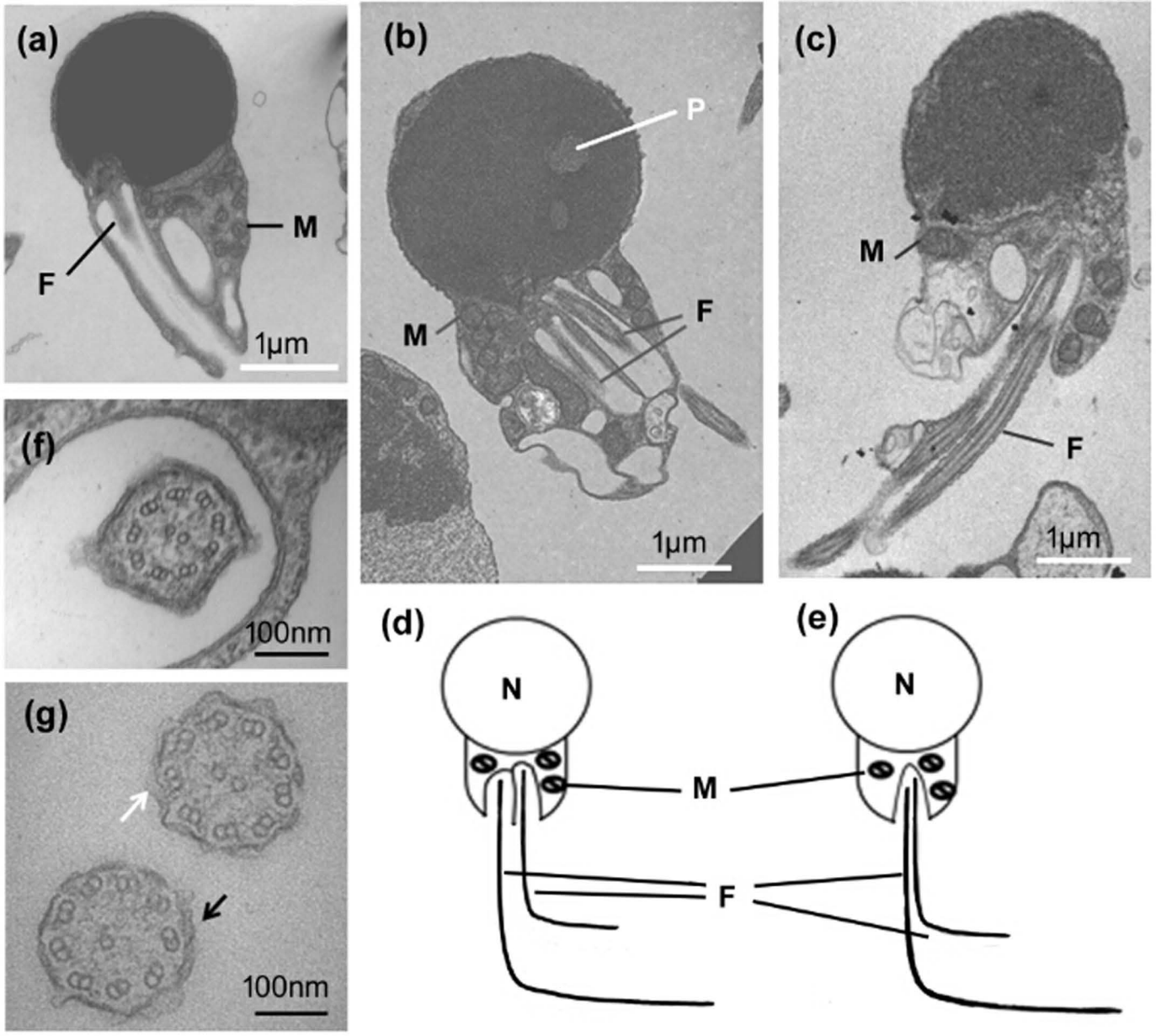
Values within a column followed by different letters or * are significantly different at $P< 0.05$

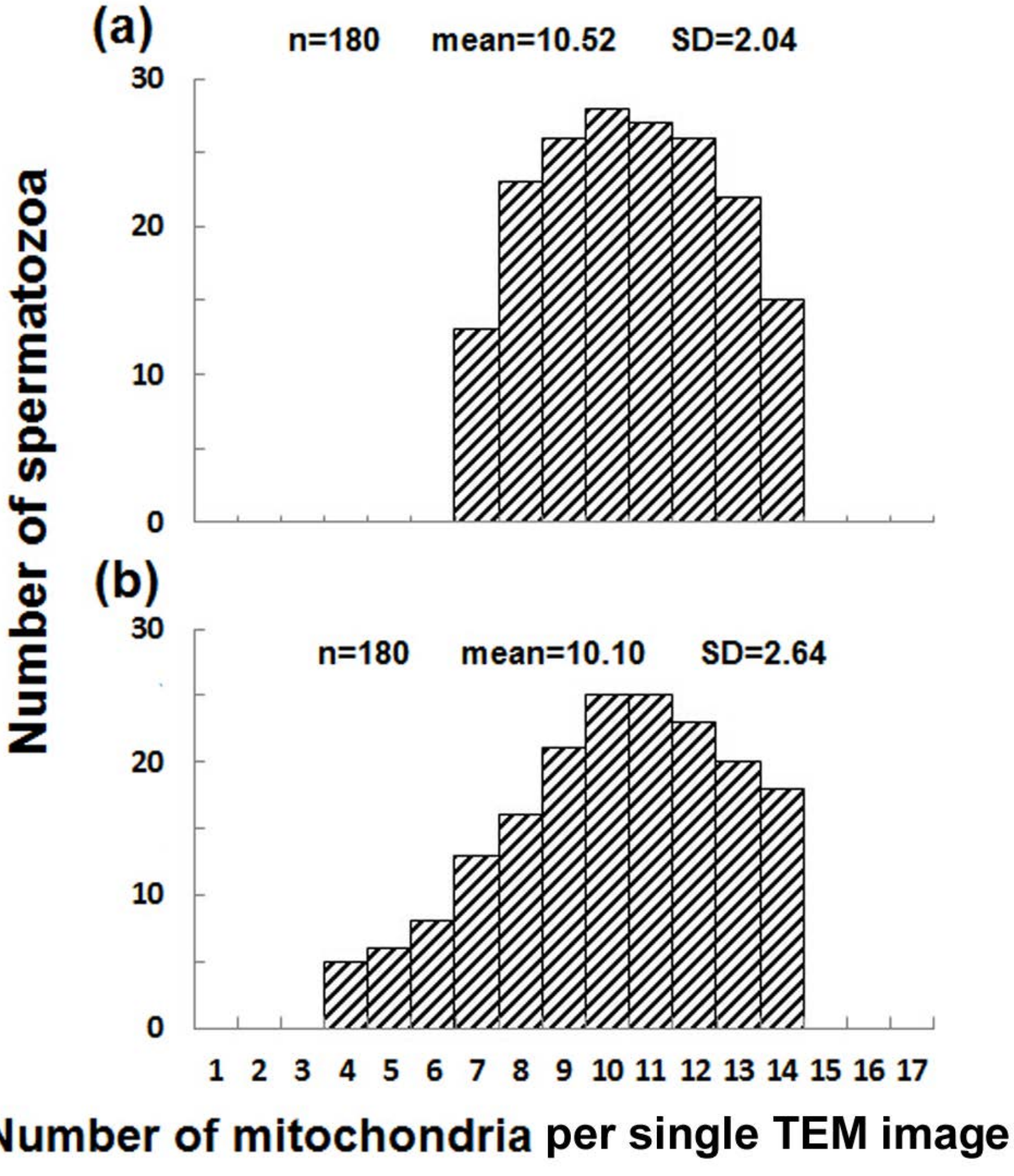

(a)
Mean Area% CV%
50.12 91.69 3.39
300
count
250
200
150
100
50
0
0 50 100 150 200 250 300 350 400 450 FL1500
(b)
Mean Area% CV%
207.33 84.84 1.57
600
count
500
400
300
200
100
0
0 50 100 150 200 250 300 350 400 450 FL1500
Cell number
Relative DNA content

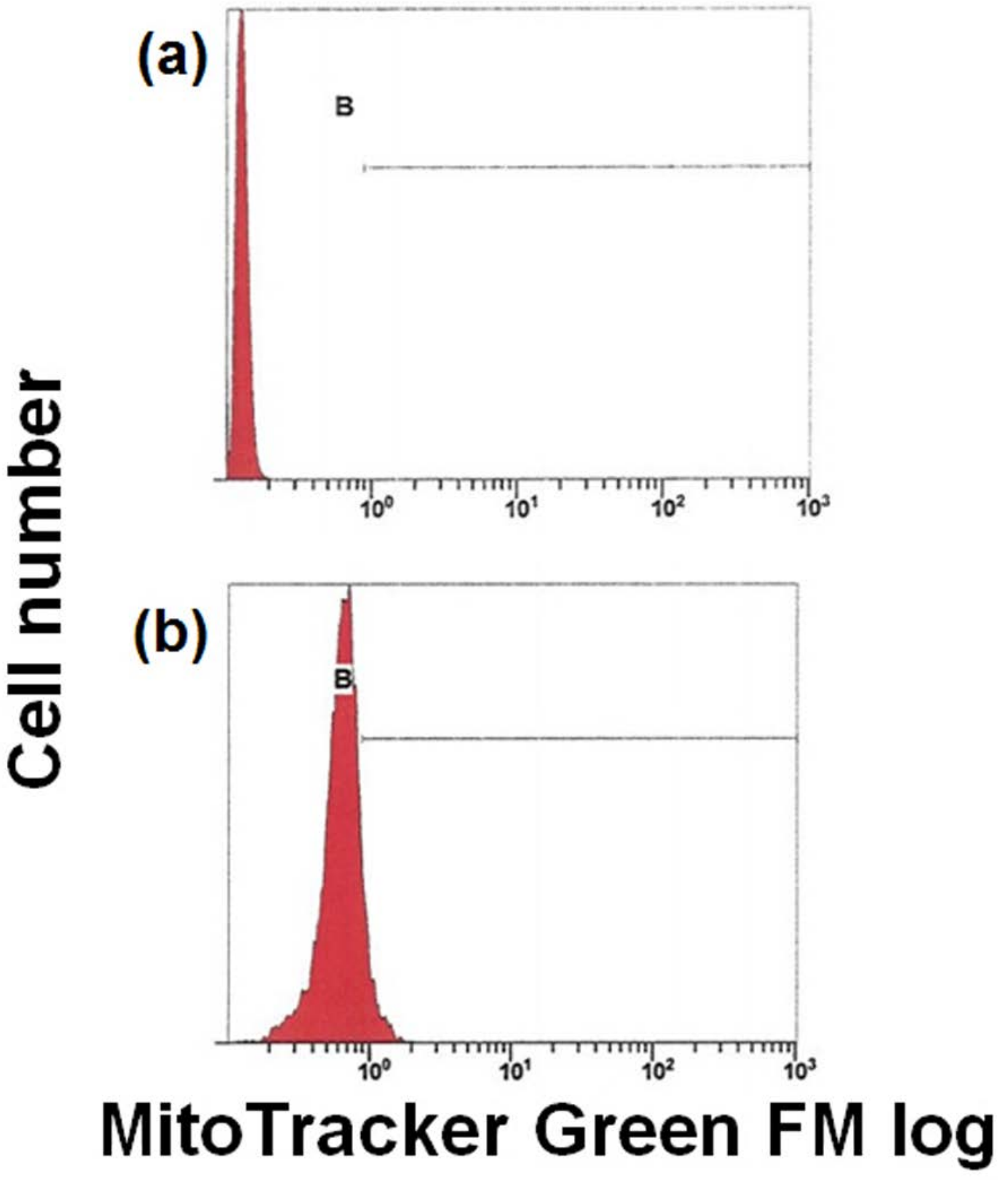

(a)
10μm
(b)
*
△
□
□
△
10μm
(c)
(d)

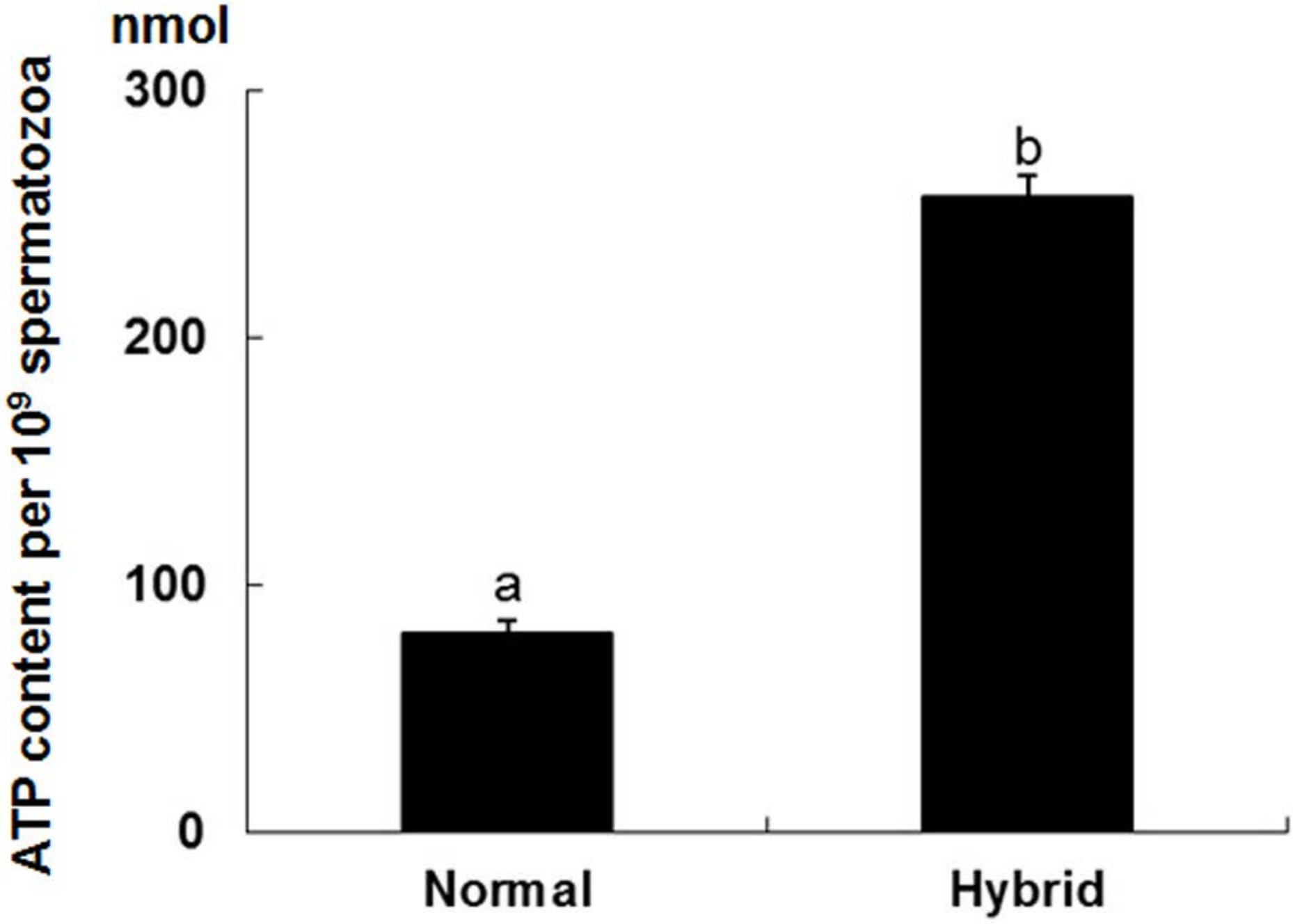

Percentage of spermatozoa with each head length
%
50
40
30
20
10
0
(a)
N=90
1.0
1.5
2.0
2.5
3.0
3.5
4.0
%
50
40
30
20
10
0
(b)
N=165
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Spermatozoa head length (μm)


(a)
(b)
FS Lin
SS Lin
1023
0
1023
0
1023
0


(a)
M
F
1μm
(b)
P
M
F
1μm
(c)
M
F
1μm
(f)
100nm
(g)
100nm
(d)
N
M
F
(e)
N


(a)
n=180 mean=10.52 SD=2.04
30
20
10
0
(b)
n=180 mean=10.10 SD=2.64
30
20
10
0
Number of spermatozoa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Number of mitochondria per single TEM image


(a)
B
(b)
B
Cell number
10^0
10^1
10^2
10^3
MitoTracker Green FM log


nmol
300
200
100
0
ATP content per 10^9 spermatozoa
a
b
Normal
Hybrid