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Spermatozoal ultrastructure of diploid and tetraploid Pacific oysters

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Abstract

Spermatozoal ultrastructure of tetraploid Pacific oysters, *Crassostrea gigas*, was examined by light and electron microscopy and compared to that of diploid Pacific oysters. Ultrastructure of sperm from tetraploids was compositionally similar to diploids except for overall size and the number of mitochondria. Instead of the four mitochondria always found in sperm from diploid oysters, 44% of sperm from tetraploid oysters had four mitochondria, 53% had five, and 3% had six. The linear dimensions of sperm components such as acrosome height and width, sperm head length and width, mitochondrial height, length of the main piece and end piece of flagellum, and total length of flagellum in tetraploids were approximately 1.25 times larger than corresponding measurements in diploids, which corresponds to a doubled volume in sperm of tetraploids compared to that of diploids. Tetraploid males produced half the number of sperm ($2.7 \pm 0.5 \times 10^{10}$) g^{-1} of gonad wet weight compared to diploid males. Despite the increased sperm sizes in tetraploids, the ratios of the sperm head length to head width, and sperm head length to flagellum length were the same for both ploidy levels for fixed and live samples. The implications of these findings on susceptibility of sperm from diploid and tetraploid oysters to damage from cryopreservation are discussed.

Keywords: *Crassostrea gigas*; Sperm; Ultrastructure; Tetraploid; Cryopreservation

1. Introduction

The induction of triploidy (possession of three chromosome sets instead of the normal two) in bivalves is useful for aquaculture because of the reduced gamete output (functional reproductive sterility) and improved meat quality and growth (Allen and Downing, 1991; Wang et al., 2002). The benefits of triploidy extend to traits such as enhanced culture performance, reduced risk of introduction of exotic species, and protection of intellectual property. The main advantage of farming triploid oysters in North

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America is that they do not come into spawning condition during the summer, and thus retain their marketability year round (Nell, 2002). Triploidy is usually induced by chemical treatment (Allen et al., 1989), physical stress such as heat shock (Quillet and Panelay, 1986; Yamamoto et al., 1988, 1990), or hydrostatic pressure (Chaiton and Allen, 1985; Allen and Downing, 1986; Shen et al., 1993) thereby preventing release of the second polar body in fertilized eggs. Unfortunately, none of these methods of induction are completely reliable, and the chemical methods are costly and potentially dangerous to the operator (Guo and Allen, 1994; Nell et al., 1996; Nell, 2002). Neither physical nor chemical methods can guarantee 100% triploidy, and often high mortality is associated with the treatment stress. However, crossing of gametes from tetraploids and normal diploids offers reliable, essentially 100% triploid production and high survival rates (Guo and Allen, 1994; Guo et al., 1996; Wang et al., 1999; Eudeline et al., 2000a,b).

The Pacific oyster, *Crassostrea gigas*, is one of the most successful models for ploidy manipulation in bivalves. Tetraploid broodstock (possessing four chromosome sets) of the Pacific oyster have been developed by breeding tetraploid females with tetraploid males, and are available for commercial application for triploid seed production (Guo et al., 1996; Eudeline et al., 2000a,b). Cryopreservation of sperm from these Pacific oysters has been initiated to fulfill the goal of expanding commercial-scale application of tetraploid stocks and improving tetraploid breeding programs. Our preliminary studies of sperm cryopreservation of tetraploid oysters showed limited success (Dong et al., 2005a) compared to that of diploid oysters. Differential response to cryopreservation by sperm from tetraploid and diploid oysters may due to the differences in their gonadal development or differences in the sperm architecture of tetraploids and diploids. Therefore, the goal of this study was to examine the ultrastructure of sperm in tetraploid oysters compared to that of diploid oysters by light and electron microscopy. Our specific objectives were to compare between diploid and tetraploid oysters the differences in: (1) sperm architecture; (2) the linear dimensions of sperm components; (3) the number of mitochondria, and (4) to also discuss the relationship between sperm morphology differences and the susceptibility to cryopreservation damage.

2. Materials and methods

2.1. Oysters

Tetraploid and diploid Pacific oysters were obtained in August, 2003, from Taylor Resources Quilcene Shellfish Hatchery (www.taylorshellfish.com) in Quilcene, WA (47°49′133″ N, 122°49′523″ W) and were shipped chilled at 5–10 °C by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Ploidy level of individual oysters was verified by flow cytometry (Allen, 1983).

2.2. Sample preparation

For scanning electron microscopy (SEM), sperm were collected by dry stripping of the gonad (Allen and Bushek, 1992) and suspended in calcium-free Hanks' balanced salt solution at 1000 mOsmol/kg (Dong et al., 2002). Two fixation methods were used for two batches of sperm samples: the first batch (two diploid and two tetraploid males) were fixed with 2% glutaraldehyde and 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer (CB, pH 7.4); the second batch of samples (one diploid and one tetraploid male) were fixed with 4% glutaraldehyde in 0.1 M CB with the addition of sucrose to bring the final osmolality to 1110 mOsmol/kg. Sperm were collected on 0.2-μm polycarbonate membrane filters (Osmonics, Inc. Minnetonka, MN) during fixation, rinsed with 0.1 M CB, and dehydrated by exposure to an ethanol series (20%, 30%, 50%, 70%, 75%, 80%, 85%, 90%, 100%, 100%, and 100%) for 10–20 min each. Samples were critical point dried and sputter coated with gold (60%) and palladium (40%) at a thickness of less than 100 Å. A total of six SEM filters (one filter per oyster) were examined using scanning electron microscopy (Cambridge 260 Stereoscan). Observations were made for all areas of the filter for each sample.

For transmission electron microscopy (TEM), the gonads were dissected and minced into small fragments (<1 mm) while immersed in 2% glutaraldehyde

in 0.1 M CB (first batch) or 4% glutaraldehyde at 1110 mOsmol/kg (second batch). Fixation occurred over 1 h at room temperature (RT), during which the fixative was replaced twice. The tissues were rinsed in 0.1 M CB (194 mOsmol/kg) three times for 15 min each, and post-fixed in 2% OsO_4 in 0.1 M CB for 1 h at RT. Tissues were rinsed twice with distilled water, stained with 0.5% uranyl acetate in the dark for 1 h at room temperature, rinsed in distilled water and dehydrated through an ethanol series (20%, 30%, 50%, 70%, 75%, 80%, 85%, 90%, 100%, 100%, and 100%) for 10–20 min each. Samples were infiltrated in equal volumes of LR white resin (London Resin Company Ltd., England) to 100% ethanol on a rotating shaker (Vari-Mix, Thermolyne) for 1 h at RT, and 100% LR white resin for 1 h. Flat embedment in LR white resin was carried out by using two aluminum 43-mm (diameter) weighing dishes (VMR Scientific, West Chester, PA). The sample pieces and 7 ml of resin were distributed in one dish and the other dish was nested into it as an air-excluding cover, and a tiny hole (~1 mm in diameter) was punched along one edge to allow air bubbles to escape while the two dishes were pressed together. The resin was polymerized at 60 °C overnight and the sample blocks were shaped by sawing. Thin sections (80 nm) were cut with an ultramicrotome (DuPont MT 5000, Sorvall® Ultra Microtome), stained with Reynolds' lead citrate (Bozzola and Russell, 1992) for 1 min, and five grids per oyster were examined using TEM (JEOL 100CX). All chemicals were of reagent grade (Electron Microscopy Sciences, Fort Washington, PA).

2.3. Sperm measurements

With samples after fixation (prior to dehydration) from the first batch, the length and width of sperm heads, and the length of the main piece and end piece of flagella were measured at ×800-magnification with light microscopy (Fig. 1). Digital images were captured through a diagnostic instrument CCD camera (SPOT RT Slider, SpectraCore, Inc. Webster, NY). Unfixed sperm samples from 10 tetraploid and 3 diploid males were measured for the height and width of the acrosome, the length and width of the sperm head, the height of the mitochondria, and the total length of flagellum at 1000-× magnification by light microscopy (Fig. 1). The number of mitochondria per sperm was counted from two samples by use of TEM (from two oysters of each ploidy in the first batch) showing transverse sections of the midpiece, and one sample by use of SEM with exposed mitochondria (from two oysters in the second batch).

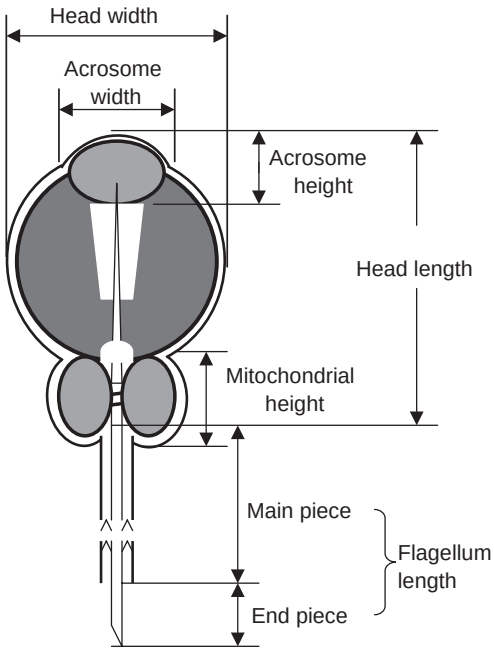

Fig. 1. Identification of sperm components used for measurement of unfixed sperm samples with light microscopy. Diagram adapted from Galtsoff and Philpott (1960).

2.4. Nomenclature and image processing

The detailed structures of spermatozoon from invertebrates and vertebrates have been described by terms used with different meanings by different authors. In the present study, the terms used were based on Franzén (1956), and the flagellum length includes the main piece and end piece (Fig. 2). For fixed samples, prior to measurement, images were processed with different enhancements to locate the tip of the end piece (Fig. 2). The "invert" command reversed a positive black-and-white image into a negative. The "equalize" command redistributed the brightness values of the pixels within an image to more evenly represent the entire range of brightness

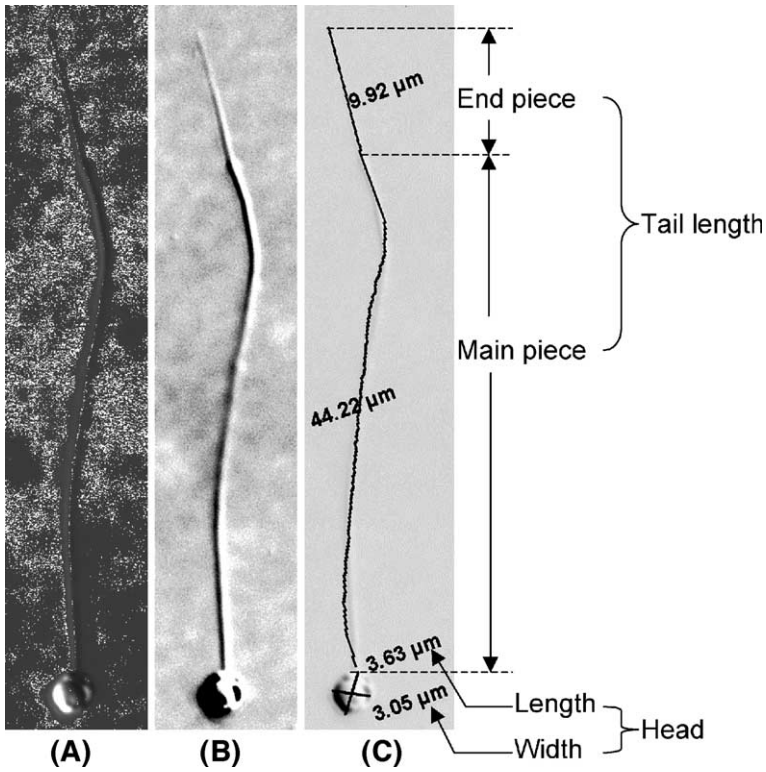

Fig. 2. Examples of image processing and measurements made for spermatozoal components with samples after fixation (prior to dehydration) at ×800-magnification with light microscopy. (A) Image after enhancement of brightness and contrast to reveal the sperm morphology; (B) image after "invert", "equalize", and "brightness/contrast" adjustments to show details of the end piece; (C): image showing the measurement made with cursor on computer screen.

levels. The flagellum components were clearly distinguishable after these enhancements (Fig. 2B). However, this processing did not enhance analysis of images produced with unfixed samples in this study.

2.5. Data analysis

Data were analyzed using either the Student's *t*-test (when there were two means) or by one-way analysis of variance (ANOVA, when there were more than two means). The Tukey–Kramer method for unequal cell sizes was used to test for differences ($\alpha = 0.05$) among results for the various parameter estimates. Chi-squared test statistics were applied to test whether the number of mitochondria was independent of sample preparation methods for SEM and TEM, and ploidy levels. Due to the presence of cell counts of zero (e.g., for sperm from diploid oysters, cell counts for mitochondrial numbers of 5 and 6 were zero), data were transformed by the addition of a constant value of 0.5 prior to the chi-square test (Agresti, 1996).

3. Results

3.1. Sperm architecture

General ultrastructural architecture of sperm produced by tetraploids (Figs. 3A and 4A, B) was similar to that of sperm produced by diploids (Figs. 3C and 4E,F) except for differences in size. Sperm from diploids and tetraploids possessed a cap-shaped acrosome filled with fine granular substances with the axial body extended into the nucleus. The nucleus

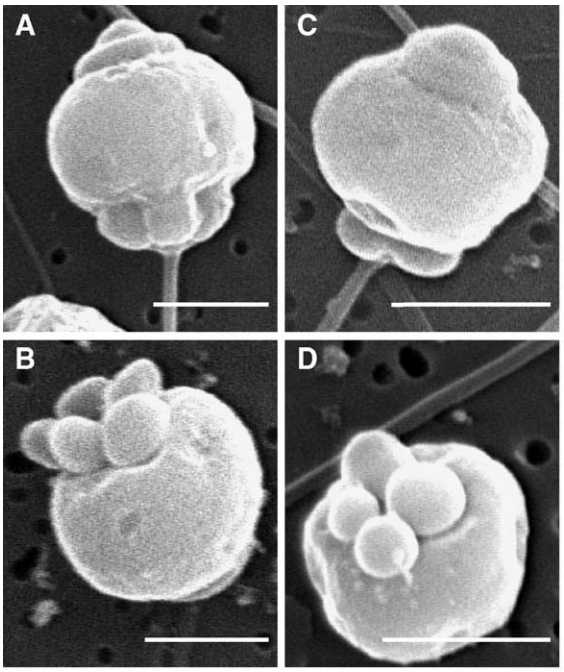

Fig. 3. Scanning electron micrographs of sperm from tetraploid (A, B) and diploid (C, D) Pacific oysters, *Crassostrea gigas*. (A) Sperm head from tetraploid. (B) Five mitochondria exposed after membrane disruption under hypertonic condition in tetraploids. (C) Sperm head from diploid. (D) Four mitochondria exposed after membrane disruption under hypertonic condition in diploids. Bar equals 1 μm.

was more electron dense than the acrosome in sperm from both ploidies, and the typical 9+2 microtubule structure of the flagellum observed in diploids (Bozzo et al., 1993) was found in tetraploids.

3.2. Linear dimensions of sperm components

The linear dimensions of sperm components were significantly larger in sperm from tetraploids than in those from diploids (Table 1). The height and width of the acrosome of sperm from tetraploids were significantly ($P<0.001$) larger than the height and width of acrosomes in diploids. However, the ratio of acrosome height to width in sperm from both ploidies was not significantly different ($P=0.606$). Similarly, the lengths and widths of the sperm head in tetraploids were significantly larger than those in diploids ($P<0.001$). Except for the head width in diploids, significant differences were also found in lengths and widths between fixed and live samples (Table 1). The ratios of sperm head length to width from fixed and live samples of both ploidies were not significantly different ($P>0.150$) except for sperm from live samples in diploids ($P<0.001$).

The height of mitochondria (Fig. 1) was significantly larger ($P<0.001$) for sperm from tetraploids than from diploids (Table 1). For the flagellum, the length of main piece and end piece (Fig. 2) were significantly larger ($P<0.001$) for sperm from tetraploids than from diploids. Significant differences were observed for the total tail length in fixed and live samples from both ploidies ($P<0.001$). The ratio of the length of the flagellum main piece to the end piece was similar in sperm from tetraploids and diploids though they were significantly different ($P=0.020$). The ratio of acrosome height to head length was significantly larger ($P=0.010$) in spermatozoa from tetraploids, while the ratio of acrosome width to head width was significantly larger ($P<0.001$) in spermatozoa from diploids. However, ratios of sperm head length to total flagellum length in fixed and live samples from both ploidies were all close to 0.06 and were not significantly different ($P>0.276$) except for the fixed samples from tetraploids ($P<0.001$).

3.3. Number of mitochondria

The number of mitochondria per sperm produced by tetraploids ranged from four to six (Table 2; Figs. 3B and 4C,D) with a modal number of five, while the number of mitochondria in diploids was always four (Table 2; Figs. 3D and 4G). Chi-squared test statistics for the counts of mitochondria number in sperm from diploids ($\chi^2=0.246$; $P=0.884$) and tetraploids ($\chi^2=1.087$; $P=0.581$) indicated that the number of mitochondria was independent of the SEM and TEM preparation methods, which means the frequency distribution of the number of mitochondria observed from SEM samples was not different from the two TEM samples for each ploidy. Therefore, the depressions sometimes observed on the SEM micrographs (Fig. 3B,D) suggested other effects, perhaps from hypertonic fixative treatment, rather than missing mitochondria. Subsequent combination of the data into a single table indicated that the number of mitochondrial

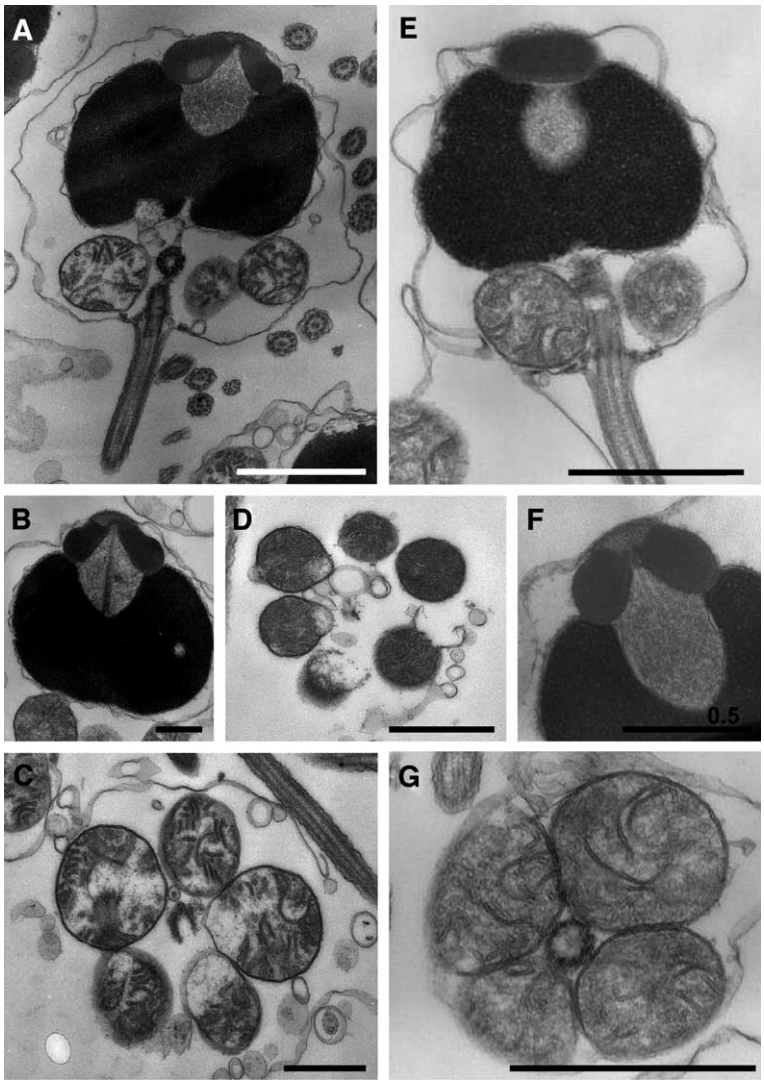

Fig. 4. Transmission electron micrographs of sperm from tetraploid (A–D) and diploid (E–G) Pacific oysters, *Crassostrea gigas*. (A) Longitudinal section through sperm from tetraploid. (B) Longitudinal section through acrosome showing axial body and axial rod from tetraploid. (C), (D) Transverse section through midpiece showing five (C) and six (D) mitochondria from tetraploid. (E) Longitudinal section through sperm from diploid. (F) Longitudinal section through acrosome showing axial body and axial rod from diploid. (G) Transverse section through midpiece showing four mitochondria from diploid. Bar equals 1 μm unless specified.

was strongly related to ploidy levels ($\chi^2 = 242.950$; $P < 0.001$).

4. Discussion

Generally, the architecture of sperm produced by tetraploid Pacific oysters was larger but similar to sperm from diploids with respect to the presence of a broad, cup-shaped acrosome, subacrosomal material including an axial rod, a relatively spherical nucleus, and a typical 9+2 microtubule structure. These characteristics were also consistent with previous ultrastructural studies within the family Ostreidae of *C. virginica* (Galtsoff and Philpott, 1960), *C. angulata* (Sousa and Oliveria, 1994), *C. gigas* (Bozzo et

Table 1
Size (μm) and ratios of spermatozoal components (mean ± SD) of diploid and tetraploid Pacific oysters *Crassostrea gigas*

Component	Parameter	Diploid		Tetraploid	
		Fixed ($n=40$)	Live ($n=30$)	Fixed ($n=40$)	Live ($n=90$)
Acrosome	Height	–	0.69 ± 0.08^{a}	–	0.76 ± 0.10^{b}
	Width	–	1.22 ± 0.10^{a}	–	1.38 ± 0.10^{b}
	Height/Width	–	0.56 ± 0.07^{a}	–	0.55 ± 0.09^{a}
Head	Length	2.61 ± 0.12^{a}	2.40 ± 0.14^{b}	3.42 ± 0.17^{c}	3.19 ± 0.22^{d}
	Width	2.32 ± 0.07^{a}	2.26 ± 0.16^{a}	3.04 ± 0.19^{b}	2.79 ± 0.21^{c}
	Length/Width	1.12 ± 0.06^{a}	1.06 ± 0.07^{b}	1.13 ± 0.06^{a}	1.14 ± 0.08^{a}
Mitochondria	Height		0.72 ± 0.08^{a}		0.82 ± 0.08^{b}
Flagellum	Main piece	33.2 ± 1.1^{a}	–	43.4 ± 1.6^{b}	–
	End piece	7.9 ± 0.7^{a}	–	9.7 ± 2.0^{b}	–
	Total length	41.1 ± 1.3^{a}	37.3 ± 1.9^{b}	53.1 ± 2.9^{c}	50.4 ± 2.0^{d}
	Main/End piece	4.23 ± 0.46^{a}		4.70 ± 1.18^{b}	
Acrosome height/Head length		–	0.29 ± 0.04^{a}	–	0.31 ± 0.05^{b}
Acrosome width/Head width		–	0.54 ± 0.06^{a}	–	0.50 ± 0.04^{b}
Head length/Flagellum length		0.064 ± 0.003^{a}	0.064 ± 0.005^{a}	0.057 ± 0.005^{b}	0.063 ± 0.005^{a}

Numbers within parentheses indicate the sampling size (n).
Numbers in rows sharing the same superscript were not significantly different at $P=0.05$.

al., 1993), and *Saccostrea commercialis* (Healy and Lester, 1991).

The primary differences in sperm ultrastructure between tetraploids and diploids were size related. Although by definition the chromosome set number is doubled in the sperm from tetraploids compared to that of diploids, the linear dimensions of the sperm components ranged from 1.10 to 1.35 (approximately 1.25 on average) times of that of sperm from diploids. Similar findings were observed for sperm from diploid and tetraploid rainbow trout *Oncorhynchus mykiss*, in which the average width of sperm from tetraploids was 1.30 (and length was 1.20) times that of sperm from diploids (Chourrout et al., 1986). These values corresponded to a doubled sperm volume with the assumption of a spherical configuration of the sperm head, and a theoretical value of $\sqrt[3]{2}$(~1.26 times) for the increase of linear dimensions (e.g. radius) given a double-fold increase of volume. Earlier studies have indicated that tetraploid males produced $2.7 \pm 0.5 \times 10^{10}$ sperm g^{-1} of gonad wet weight, which was half the number produced by diploid males (Dong et al., 2005b). The fewer number of sperm per gram of gonad wet weight in tetraploids is also explainable by the doubled sperm volume, which is similar to the findings with egg production from tetraploid and diploid females in *C. gigas* (Guo and Allen, 1997).

Table 2
Frequency distribution (percent) of the number of mitochondria in sperm observed in diploid and tetraploid Pacific oysters (one male for SEM, and two males for TEM for each ploidy with 100–102 sperm counted for each male)

Ploidy[a]	Preparation[b]	Number of mitochondria		
		4	5	6
Diploid	SEM ($n=100$)	100	0	0
	TEM ($n=200$)	100	0	0
	Average	100 ± 0	0 ± 0	0 ± 0
Tetraploid	SEM ($n=102$)	47	51	2
	TEM ($n=202$)	42	54	4
	Average	44 ± 3	53 ± 2	3 ± 1

[a] Chi-squared test for ploidy level: $\chi^2 = 242.950$; $P<0.001$.
[b] Chi-squared test for preparation method in diploids ($\chi^2 = 0.246$; $P=0.884$) and tetraploids ($\chi^2 = 1.087$; $P=0.581$).

The length and width of the sperm heads from diploids in the present study were in good agreement with those reported for diploid Pacific oysters in Japan (Komaru et al., 1994), however, a difference was observed for the length of the flagellum: 34.2 ± 1.2 μm (Komaru et al., 1994) compared to 41.1 ± 1.3 μm in the present study. It is possible that measurement in the previous study only included the main piece of the flagellum (excluding the poorly visible end piece) because the flagellum length in the previous study was close to the length of the main piece (33.2 ± 1.1 μm) in the present study. It is also possible that the flagellum was defined only as the main piece (unlike our definition) as controversies on nomenclature existed in early studies (Franzén, 1956), unfortunately,

no nomenclature was provided for clarification in the previous study. The end piece of the flagellum was difficult to visualize under light microscopy without image processing, especially with unfixed samples. However, there is also a possible geographical or genetic difference between the populations studied as the observed acrosome height was also smaller (0.51 ± 0.03 μm) in the previous study, compared to that of the present study (0.69 ± 0.08 μm). Another study from Spain, however, reported a smaller head size (2 μm diameter) and longer flagellum length (~48 μm) for mature spermatozoon from *C. gigas* (Bozzo et al., 1993) compared to either the previous or present study. Despite the larger values observed with sperm from tetraploid Pacific oysters compared with the diploids in the present study, the ratios of the different sperm components were not different such as for the ratios of head length to width, and head length to flagellum length.

In addition to the difference found for the linear dimensions of sperm components, the major difference observed was in the number of mitochondria. The first report of more than the conventional four mitochondria in oyster sperm was documented in *C. virginica* "on rare occasions" (Eckelbarger and Davis, 1996). However, in the present study, more than half of the sperm produced by tetraploids had 5 or 6 mitochondria instead of the constant 4 mitochondria observed for sperm from diploid Pacific oysters and other oysters (Galtsoff and Philpott, 1960; Healy and Lester, 1991; Sousa and Oliveria, 1994). Ultrastructural study of sperm from diploid and tetraploid Mediterranean mussels (*Mytilus galloprovincialis* Lamarck) revealed similar findings for the number of mitochondria, in which 5–7 mitochondria were observed in tetraploids compared to only 5 in diploids (Komaru et al., 1995). The increased number of mitochondria in the sperm of the tetraploid mussels was suggested to offset the size increase of the sperm head with resultant motility similar to sperm from diploids because the majority (75%) of sperm has six or seven mitochondria. Previous study of sperm from triploid Pacific oysters, however, did not show an increase in the number of mitochondria, and it was suggested that motility of sperm produced by triploid Pacific oysters could be reduced because the head was significantly larger (compared to sperm from diploids) and the number of mitochondria was the same (Komaru et al., 1994). If the number of mitochondria does play a role in sperm motility, the increased number of mitochondria in only roughly half (56%) of the sperm from tetraploid Pacific oysters in the present study may explain their consistently relatively lower motility ($45 \pm 18\%$) compared to the sperm from diploids ($57 \pm 27\%$) (Dong et al., 2005a, and our other unpublished observations). Increases in the number of mitochondria may be regulated by increased nuclear size, but why the increased nuclear size of triploid Pacific oysters was not associated with an increased number of mitochondria such as in tetraploids merits further study.

In this study, although the height of mitochondria was estimated by use of light microscopy, mitochondria were sometimes observed of different sizes in the SEM micrographs (e.g. Fig. 3D) although measurement of the sizes was not attempted here for SEM samples. Future efforts may be required to compare the sizes (e.g. diameter or volume) of individual mitochondria prepared with SEM among sperm with 4, 5 or 6 mitochondria within tetraploid oysters, and between tetraploid and diploid oysters especially if a comparison of total volume or metabolic capacity could be made. It would also be informative to evaluate differences among sperm having four mitochondria and those with five or six in tetraploid oysters, for example, in motility speed and duration, and their susceptibility to cryopreservation damage. Flow cytometric assays have been used to estimate the mitochondrial function of sperm samples prior to freezing and after thawing (e.g. Graham et al., 1990; Sutovsky et al., 1996; Segovia et al., 2000). If mitochondrial size was uniform and their differences in numbers would reflect differences in fluorescence intensity, it might be possible to sort sperm with five or six mitochondria from those with four prior to freezing. Therefore subsequent freezing and thawing could evaluate differential susceptibility to cryopreservation. An alternative approach would be to examine mitochondrial number distribution in sperm that survive cryopreservation compared to the distributions prior to freezing.

With respect to cryobiological theory, the larger linear dimensions of the sperm components found in tetraploid Pacific oysters yielded a smaller ratio of surface area to volume compared with sperm from diploids, which has been associated with differences in

membrane permeability parameters between these two types of sperm cells when studied with differential scanning calorimetry (He et al., 2004), a physical method of estimating water movement based on thermal properties. Although this same study suggested similar optimal cooling rates for sperm from diploid (~44 °C/min) and tetraploid (~43 °C/min) oysters, our previous empirical trials have shown that sperm from tetraploids are more susceptible to cryopreservation at these cooling rates than are those from diploids (Dong et al., 2005a). It is possible that variables other than surface area-to-volume ratio may play important roles in sperm cryopreservation, for example, membrane fluidity. A recent study of human sperm suggested that sperm adaptability to the stresses induced by freezing and thawing could be dependent on initial membrane fluidity (Giraud et al., 2000). It is also possible that sperm from tetraploids are more sensitive to osmotic shock than those from diploids. This would be especially important if plasma membrane thickness or composition was not different between the ploidies, although cell size was, yielding effectively a compromised membrane in tetraploids. Based on these and other questions, diploid and tetraploid oysters provide a useful model for study of a variety of factors, and future research can seek improved experimental control of variables to examine the different susceptibility to cryopreservation between sperm from diploid and tetraploid Pacific oysters.

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