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REGULAR ARTICLE

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Fixation methods can produce misleading artifacts in sperm cell ultrastructure of diploid and tetraploid Pacific oysters, *Crassostrea gigas*

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Abstract Spermatozoa from diploid and tetraploid Pacific oysters (*Crassostrea gigas*) were examined after anisotonic fixation. Morphological anomalies, such as membrane rupture, detached tails, and the formation of tail vesicles (typically associated with damage attributable to procedures such as cryopreservation) were observed; the Mantel-Haenszel Chi-square test indicated a strong association between the anomalies and fixative osmolality ($P<0.001$). The present study also indicated that media in a range of 800 to 1,086 mOsm/kg could be assumed to be functionally isotonic to Pacific oysters, and osmolalities below or above this caused severe cell damage. For example, the maximum volume of flagella obtained after hypotonic fixation was approximately twice the volume of the flagella in isotonic fixation. Sperm cell flagellar volumes after hypertonic fixation (1,110 mOsm/kg) were 32% smaller than those in isotonic fixation, and sperm heads were 25% smaller. Although the damage associated with anisotonic fixation was evident in all parts of the sperm cells, the most vulnerable locations were the plasma membrane and flagellum motor apparatus. The formation of tail vesicles after hypotonic fixation was also examined. Because of water uptake, oyster sperm became swollen in hypotonic fixative, and bending or coiling of the axoneme within the tail vesicles led to the appearance of multiple axonemal structures in cross sections when observed by transmission electron microscopy. This phenomenon might be generally misinterpreted as the presence of double tails. This and other fixation artifacts can lead to the misinterpretation of damage caused by cryopreservation in ultrastructure studies of sperm of aquatic species, especially those in marine species.

Keywords Spermatozoa · Ultrastructure · Anisotonic · Tail vesicles · Morphological anomalies · Cryopreservation · Pacific oyster, *Crassostrea gigas* (Mollusca)

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Introduction

The Pacific oyster, *Crassostrea gigas*, is one of the most important models for ploidy (chromosome set) manipulation in bivalves. Tetraploid broodstocks (possessing four sets of chromosomes instead of the normal two sets) of the Pacific oyster have been developed by breeding tetraploid females with tetraploid males, which were originally produced through chemical interference of meiotic division (Guo and Allen 1994), and are available for commercial application for the production of reproductively sterile triploid oysters (Guo et al. 1996; Eudeline et al. 2000, 2002). Cryopreservation of sperm from tetraploid Pacific oysters has been initiated to fulfil the goal of expanding the commercial-scale application of tetraploid stocks and improving tetraploid breeding programs. Our preliminary studies of sperm cryopreservation of tetraploid oysters have shown limited success (Dong et al. 2005a) as compared with that for diploid oysters. Differential responses to cryopreservation by sperm from tetraploid and diploid oysters may arise because of differences in gonadal development or in sperm architecture of tetraploids and diploids.

Attempts to examine the ultrastructural differences between sperm from diploid and tetraploid oysters have been initiated (Dong et al. 2005b). When conventional fixation

methods (fixative osmolality of 300–400 mOsm/kg) were applied to the sperm from diploid and tetraploid oysters, morphological anomalies, such as swelling of the head and flagellar disruption, occurred in samples from both ploidies. Subsequent attempts with an adjusted osmolality of 1,110 mOsm/kg for the fixative yielded morphological anomalies, such as membrane rupture and exposed mitochondria, in samples from both ploidies. Based on these observations, we have hypothesized that these morphological changes in sperm might be attributable to osmotic shock in combination with the effects of fixation. Thus, in this study, our goal has been to evaluate the effect of the osmotic pressure of the fixative on the ultrastructural morphological changes of spermatozoa from diploid and tetraploid Pacific oysters. Our specific objectives have been: (1) to document various configurational changes occurring with fixation at various osmotic pressures; (2) to evaluate size changes of spermatozoal components after fixation at various osmotic pressures; (3) to discuss the previously unrecognized positive and negative implications of the artifacts induced by fixation methods for ultrastructure study of sperm of aquatic species; and (4) to call attention to the problems that are associated with fixation methods in morphological studies with cryopreserved sperm. We suggest that artifacts of this type have been generally misinterpreted in previous studies, especially with respect to marine species.

Materials and methods

Oysters

Tetraploid and diploid Pacific oysters were obtained in August, 2003, from Taylor Resources Quilcene Shellfish Hatchery (http://www.taylorshellfish.com) in Quilcene, Wash. (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). The ploidy level of individual oysters was verified by flow cytometry (Allen 1983).

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 (C–F HBSS) at 1,000 mOsm/kg (Dong et al. 2002). All chemicals were of reagent grade (Electron Microscopy Sciences, Fort Washington, Pa.). Osmolality was measured with a vapor pressure osmometer (model 5,500, Wescor, Logan, Utah). Three fixation methods were tested: the first batch of samples (two diploid and two tetraploid males) was fixed simultaneously with 2% glutaraldehyde and 1% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer (CB; pH 7.4; 231 mOsm/kg); the second batch of samples (one diploid and one tetraploid male) was fixed with 4% glutaraldehyde in 0.1 M CB with the addition of sucrose to bring the final osmolality to 1,110 mOsm/kg; and the third batch of samples (one tetraploid male) was fixed with 4% glutaraldehyde in distilled water (356 mOsm/kg), and 4% glutaraldehyde in 0.1 M CB with the addition of sucrose to provide final osmolalities of 623, 805, 874, 967, and 1,086 mOsm/kg. Sperm were collected on 0.2-μm polycarbonate membrane filters (Osmonics, Minnetonka, Minn.) during fixation. Rinse solutions (0.1 M CB) were also added with sucrose to provide the same osmolalities corresponding to the fixatives; an additional treatment with fixative at 356 mOsm/kg was rinsed off in 0.1 M CB without addition of sucrose (referred to as 231 mOsm/kg hereafter to differentiate it from the samples rinsed with adjusted osmolality). Samples were rinsed three times for 10 min each 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 13 SEM filters (one filter per sample) was examined by SEM (Cambridge 260 Stereoscan). Observations were made over all areas of each filter for each sample.

For transmission electron microscopy (TEM), sperm of two diploids and two tetraploids from the first batch were examined. The gonads were dissected and minced into small fragments (<1 mm) while immersed in 2% glutaraldehyde in 0.1 M CB (364 mOsm/kg). Fixation took place for 1 h at room temperature (RT), during which the fixative was replaced twice. The tissues were rinsed in 0.1 M CB (3×15 min each), post-fixed in 2% OsO_4 in 0.1 M CB for 1 hr at RT, rinsed twice with distilled water, stained with 0.5% uranyl acetate in the dark for 1 h at RT, rinsed in

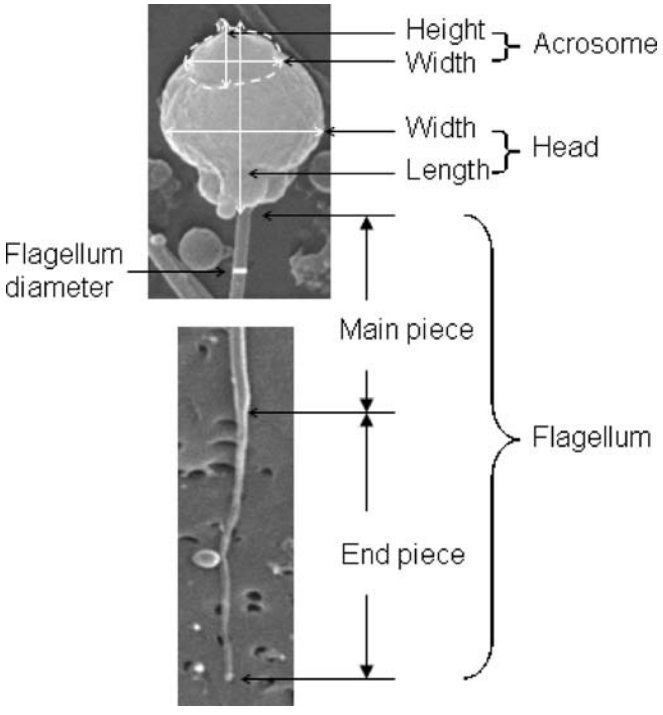

Fig. 1 Sperm components measured in samples prepared for scanning electron microscopy

distilled water, dehydrated through an ethanol series (20%, 30%, 50%, 70%, 75%, 80%, 85%, 90%, 100%, 100%, and 100%) for 10–20 min each, and infiltrated in equal volumes of LR white resin (London Resin Company, England) to 100% ethanol on a rotating shaker (Vari-Mix, Thermolyne) for 1 h at RT and then in 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. A 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 a ultramicrotome (DuPont MT 5,000, Sorvall Ultra Microtome) and stained with Reynolds' lead citrate (Reynolds 1963) for 1 min. Five grids per oyster were examined by TEM (JEOL 100CX, Japan).

Spermatozoa measurements

The height and width of the acrosome, the length and width of the sperm head, and the diameter of the main piece of the flagellum were measured (Fig. 1) by using SEM samples from the different preparation methods. Data were analyzed by one-way analysis of variance (ANOVA). The Tukey-Kramer method for unequal cell sizes was used to test for differences (α=0.05) among results for the various parameter estimates.

Quantitative analysis of morphological anomalies

For the third trial with fixatives at different osmotic pressures, ten SEM fields (~5,000× magnification) were captured randomly from each treatment for spermatozoa from one tetraploid oyster. Morphological anomalies were examined based on five criteria: membrane integrity, acrosome integrity, mitochondrial attachment with the sperm head, tail attachment with the head, and linearity of the tail. Spermatozoa were categorized as meeting or not meeting these criteria when counted for each field and were summed for the calculation of percent frequencies. Mantel-Haenszel Chi-squared test statistics for ordinal data were applied to test whether the morphological anomalies were independent of fixative osmolality. The Mantel-Haenszel Chi-square is a statistic for testing the null hypothesis of independence against a two-sided alternative hypothesis of non-zero true

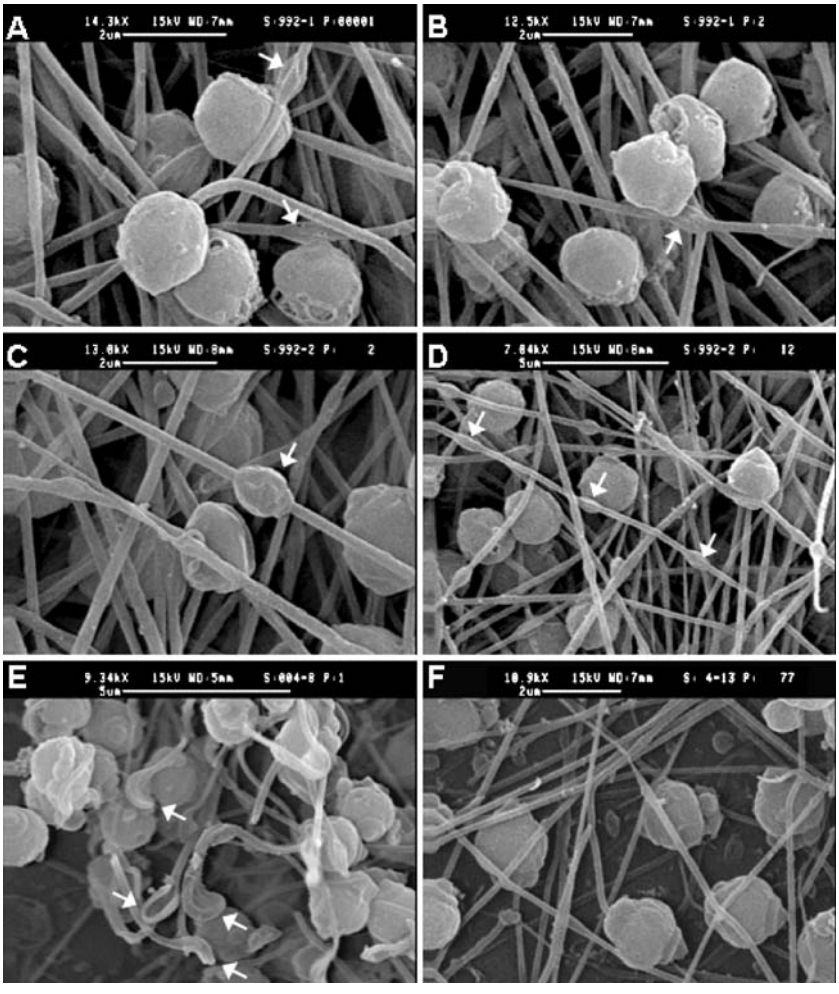

Fig. 2 Scanning electron micrographs of spermatozoa from diploid (**a**, **b**) and tetraploid (**c**–**f**) Pacific oysters (*arrows* tail vesicles formed after hypotonic fixation). **a**–**d** Samples were simultaneously fixed with 2% glutaraldehyde and 1% osmium tetroxide (~754 mOsm/kg, hypotonic). **e** Samples were fixed in 4% glutaraldehyde without addition of sucrose (356 mOsm/kg, hypotonic). **f** Samples were fixed in 4% glutaraldehyde with the addition of sucrose to bring the final osmolality of 967 mOsm/kg (isotonic)

correlation (Agresti 1996). Because there were cell counts of zero, data were transformed with the addition of a constant value of 0.5 prior to the chi-squared test. Arbitrary scores (1, 1.1, 4, 5.5, 6, 7, 8) reflecting distances between osmolality categories (231, 356, 623, 805, 874, 967, and 1,086 mOsm/kg) were chosen to perform the test after a sensitivity analysis (Agresti 1996). The Mantel-Haenszel Chi-square test was repeated with selected osmolality categories to evaluate at which point the association occurred, if present. Odds ratios (θ) were also calculated between the fixative at 874 mOsm/kg and the remaining categories.

Results

Morphological anomalies occurred with different fixation methods

With hypotonic fixative solution (<800 mOsm/kg), spermatozoa from diploid and tetraploid Pacific oysters exhibited morphological anomalies (Fig. 2a–e) compared with samples fixed with isotonic solution (~1,000 mOsm/kg, Fig. 2f). By SEM, the swelling of the sperm head and flagellum were found in samples simultaneously fixed with glutaraldehyde and OsO_4. No natural indentations, such as those found in well-preserved spermatozoa under isotonic conditions, were seen between the acrosome and nucleus, or between the mitochondria and nucleus (Fig. 2f). The plasma membranes were found to rupture, with most damage occurring around the midpiece (Fig. 2a,b). Micrographs made using this preparation method also showed single ellipsoidal-spherical swellings on the flagella (Fig. 2) and, in some cases, multiple ellipsoidal-spherical swellings on the same flagellum (Fig. 2d). Scanning electron micrographs of samples fixed with glutaraldehyde alone (hypotonic conditions) exhibited plasma membranes that were severely ruptured, and most sperm heads were found without plasma membranes, although the acrosome and mitochondria often remained intact (Fig. 2e). A striking phenomenon for these samples was that over 90% of the flagella were found to be bent into hair-pin-like forms (Fig. 2e).

The ellipsoidal-spherical swellings and hair-pin-like forms were termed "tail vesicles". Cross sections of these tail vesicles showed multiple axonemes within the same flagellum membrane in TEM micrographs (Fig. 3a–d). A single loop or a single bend of the axoneme (Fig. 3e) within the tail vesicles produced a double axoneme appearance in TEM cross section, whereas coiling inside the tail vesicles produced a triple axoneme appearance (Fig. 3f). More complicated coiling, twisting, or simple rupture of the axoneme within the tail vesicles yielded other variant appearances in TEM cross sections (Fig. 3b–d). In addition to anomalies found within the sperm head and flagellum, mitochondria were also found to rupture on hypotonic fixation (Fig. 3b). All of these morphological anomalies apparent in SEM and TEM micrographs were found for samples from both diploid and tetraploid oysters.

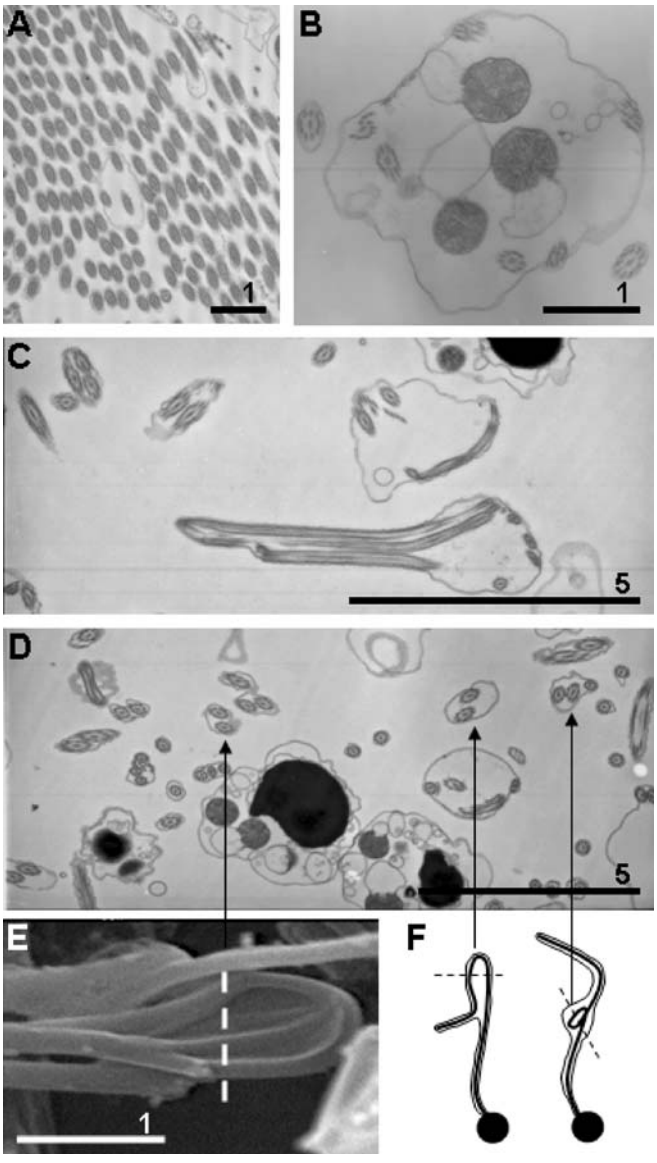

Fig. 3 Transmission and scanning electron micrographs of the tail vesicles formed in tetraploid Pacific oyster spermatozoa exposed to hypotonic fixation (*arrows* respective cross sections). **a** Transverse section of flagella showing single axoneme and triple axonemes. **b** Transverse section of the tail vesicle formed around midpiece showing different sections of the coiled motor apparatus, the axoneme. **c**, **d** Transverse section of tail vesicles frequently showing two or three sections of coiled axonemes. **e** Tail vesicle observed by SEM showing the loops formed by the axoneme within the plasma membrane of the flagellum part. **f** Representations showing bending or coiling of the axoneme within the tail vesicles resulting in double and triple axoneme cross sections in the transmission electron micrographs. *Bars* are given in micrometers

Sperm samples fixed in a hypertonic solution (1,110 mOsm/kg) also displayed ultrastructural changes (Fig. 4). Diploids (Fig. 4a–d) and tetraploids (Fig. 4e–h) both showed the same morphological anomalies. Most tails were detached from the sperm heads. Plasma membranes at the head region were frequently observed to have peeled away from the cytoplasm at different angles (Fig. 4a,c–e,g) with some sperm heads showing complete loss of plasma membranes (Fig. 4b). As a consequence, the number and

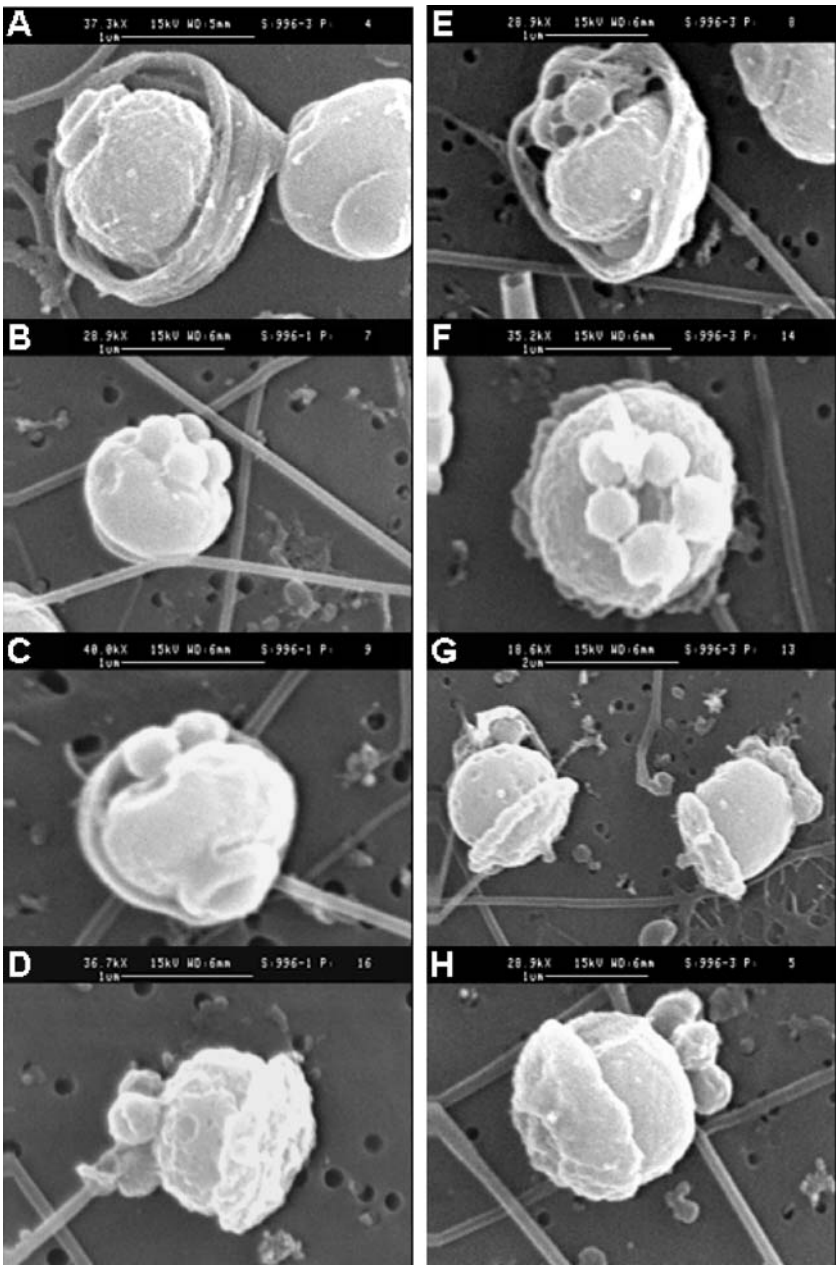

Fig. 4 Scanning electron micrographs of morphological anomalies associated with spermatozoa fixed in hypertonic solution in diploid (**a**–**d**) and tetraploid (**e**–**h**) Pacific oysters. **a**, **c**–**e**, **g**, **h** Peeling of plasma in different directions. **b**, **f** Exposed mitochondria after the loss of plasma membrane

shape of mitochondria became visible (Fig. 4b,f). Complete separation of the plasma membrane and acrosome were also found in SEM micrographs three times among the 13 samples examined.

Sperm size variation with different fixation methods

The linear dimensions of spermatozoal components were consistent with the morphological observations described

Table 1 Size of spermatozoal components (mean±SD, μm) of three diploid (*2C*) and three tetraploid (*4C*) Pacific oysters *Crossastrea gigas* (*Hypotonic* fixation at 754 mOsm/kg, *Hypertonic* fixation at 1,110 mOsm/kg, *numbers in parentheses* sperm sample size)

Ploidy	Preparation	Acrosome		Head		Flagellum
		Height	Width	Length	Width	Diameter
2C	Hypotonic	0.39±0.07[a] (5)	0.86±0.07[a] (8)	1.99±0.08[a] (27)	1.68±0.07[a] (38)	0.22±0.03[a] (236)
	Hypertonic	0.50±0.08[b] (50)	0.81±0.05[a] (52)	1.54±0.13[b] (53)	1.48±0.07[b] (59)	0.12±0.02[b] (162)
4C	Hypotonic	0.50±0.08[b] (8)	1.00±0.08[b] (8)	2.42±0.11[c] (37)	2.07±0.08[c] (37)	0.24±0.03[c] (234)
	Hypertonic	0.59±0.10[b] (47)	1.05±0.07[b] (50)	2.02±0.20[a] (46)	1.79±0.08[d] (72)	0.14±0.02[d] (186)

[a–d]Numbers in columns sharing the same superscript were not significantly different at P=0.05

Table 2 Size of spermatozoal components (mean±SD, μm) of one tetraploid Pacific oyster, *C. gigas* (numbers within parentheses indicate sperm sample size)

Fixative osmolality (mOsm/kg)	Acrosome		Head		Flagellum
	Height	Width	Length	Width	Diameter
231[f]	0.57±0.08[a] (25)	1.00±0.08[e] (27)	2.34±0.16[ab] (26)	1.85±0.12[bc] (27)	0.20±0.03[a] (94)
356	0.55±0.07[a] (28)	1.01±0.08[de] (28)	2.29±0.10[bc] (28)	1.81±0.09[bcd] (28)	0.19±0.03[a] (79)
623	0.56±0.08[a] (29)	1.04±0.05[cde] (29)	2.24±0.09[c] (29)	1.75±0.06[d] (29)	0.20±0.03[a] (112)
805	0.56±0.06[a] (27)	1.06±0.06[bcd] (27)	2.30±0.08[abc] (27)	1.81±0.07[cd] (27)	0.18±0.03[b] (78)
874	0.60±0.10[a] (26)	1.12±0.06[a] (27)	2.38±0.09[a] (26)	1.91±0.07[a] (27)	0.17±0.02[b] (119)
967	0.59±0.08[a] (49)	1.09±0.05[ab] (50)	2.27±0.08[bc] (46)	1.87±0.06[abc] (49)	0.16±0.02[c] (220)
1,086	0.57±0.08[a] (27)	1.07±0.08[abc] (27)	2.28±0.12[bc] (28)	1.87±0.07[ab] (28)	0.16±0.02[c] (131)

[a–e]Numbers in columns sharing the same superscript were not significantly different at $P=0.05$

[f]Instead of 0.1 M CB with addition of sucrose to produce the same osmolality as the fixative for the rinse solution in other treatments, the rinse solution used in this treatment was 0.1 M CB (231 mOsm/kg) after dilution with distilled water

above, with the largest values observed for swollen samples and the smallest values observed for shrunken samples. For samples from the first two batches (Table 1), the linear dimensions of spermatozoal components, except for the acrosome, were significantly larger ($P<0.001$) in samples after hypotonic fixation than in those after hypertonic fixation and were also larger in tetraploids than in diploids. Acrosome heights were smaller after hypotonic fixation than after hypertonic fixation, although they were not significantly different for the spermatozoa of tetraploids ($P=0.170$). The acrosome width, however, did not show a significant difference between the hypertonic and hypotonic treatments for spermatozoa from diploids ($P=0.792$) and tetraploids ($P=0.669$), although tetraploid values were significantly larger than the diploid values ($P<0.001$).

For samples from the third batch with tetraploid oyster sperm fixed at different osmolalities (Table 2), the linear dimensions of the acrosome and sperm head were highest at 874 mOsm/kg, and samples with osmolalities either below or above 874 mOsm/kg showed decreased values. Samples fixed at 356 mOsm/kg but rinsed at different osmolalities (231 versus 356 mOsm/kg) did not show a significant difference ($P>0.050$) in any of the parameters measured. Except for the flagellum diameter, no significant differences ($P>0.050$) in other measurements were found between spermatozoa fixed at 623 and 805 mOsm/kg, but with increased osmolality, the diameter of the flagellum main piece decreased consistently, from 0.20±0.03 μm to 0.16±0.02 μm (Table 2). No significant differences ($P>0.050$) were found for measurements between spermatozoa fixed at 967 and 1,086 mOsm/kg; these had the same measurements as those fixed at 874 mOsm/kg except for a significantly larger ($P<0.050$) head length and flagellum diameter found at 874 mOsm/kg.

Quantitative analysis of morphological anomalies

The percent frequency of intact plasma membranes, intact acrosomes, attached mitochondria, attached tails, and straight tails showed the highest values at fixative osmolalities between 874 and 1,086 mOsm/kg (Table 3). Plasma membrane and flagellum attachment and straightness were more sensitive to variations in osmotic pressure than were acrosome intactness and mitochondrial attachment. Because the least cellular damage was observed when using a fixative osmolality of 874 mOsm/kg, data

Table 3 Percent frequency of spermatozoa with intact plasma membrane, intact acrosome, mitochondria attached to sperm head, tail attached with sperm head, and straight tail of samples fixed under various osmotic pressures in tetraploid Pacific oyster, *Crossastrea gigas*, and odds ratio (θ) between treatment at 874 mOsm/kg and others

Treatment (mOsm/kg)	Intact membrane		Intact acrosome		Mitochondria attached to head		Tail attached with sperm head		Straight tail	
	Percent	θ	Percent	θ	Percent	θ	Percent	θ	Percent	θ
231[a]	7	0.012	94	0.317	82	0.033	8	0.021	9	<0.001
356	10	0.009	96	0.207	83	0.033	12	0.014	4	<0.001
623	24	0.036	97	0.358	93	0.091	36	0.083	94	0.032
805	69	0.251	99	0.883	98	0.405	49	0.142	99	0.286
874[b]	90	–	99	–	99	–	87	–	100	–
967	81	0.471	100	4.464	98	0.407	66	0.286	100	0.699
1,086	67	0.232	99	0.893	100	2.635	64	0.268	100	0.872

[a]Instead of 0.1 M CB with addition of sucrose to produce the same osmolality as the fixative for the rinse solution in other treatments, the rinse solution used in this treatment was 0.1 M CB (231 mOsm/kg) after dilution with distilled water

[b]Data from this treatment were used as the reference standard for calculation of the odds ratios for the other treatments

Table 4 *P*-values for the Mantel-Haenszel Chi-squared test statistics testing the null hypothesis of independence against the two-sided alternative hypothesis of non-zero true correlation

Treatment (mOsm/kg)	Membrane integrity	Acrosome integrity	Mitochondrial attachment	Tail attachment	Tail straightness
Across all treatments	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
231, 356, 623, 805	<0.001[a]	0.060	<0.001[a]	<0.001[a]	<0.001[a]
874, 967, 1,086	<0.001[a]	0.914	0.690	<0.001[a]	0.943

[a]Morphological anomalies of spermatozoa were associated with fixative osmolality, and the non-zero true correlations between them were significant

from this treatment were used as a reference standard (presumptive isotonic condition) for statistical comparisons. The odds ratio (θ) between this reference and the other treatments were calculated to evaluate the effect of anisotonic fixatives on sperm morphology. For example, for the parameter of intact membranes, a value of θ=0.009 (between the treatments at 356 and 874 mOsm/kg) indicated that the estimated odds of intact membranes at 356 mOsm/kg was 0.009 times the odds estimated for 874 mOsm/kg, suggesting a profound impact (a value of 1.00 would indicate no difference) of hypotonic treatment (356 mOsm/kg) on sperm membrane integrity when compared with isotonic treatment (874 mOsm/kg). Smaller odds ratios for treatments below 874 mOsm/kg than for those above 874 mOsm/kg suggested a larger effect from hypotonic fixation than from fixation at osmolalities of 967 and 1,086 mOsm/kg.

Morphological anomalies of the plasma membrane, acrosome, mitochondria, and flagellum were significantly associated with fixative osmolality across the tested range (Table 4). However, among hypotonic fixatives (≤805 mOsm/kg), acrosome integrity was independent of fixative osmolality (*P*=0.060), whereas membrane integrity, mitochondrial attachment, tail attachment, and tail straightness were significantly associated with fixative osmolality (Table 4). For fixative osmolalities of 874, 967, and 1,086 mOsm/kg, only plasma membrane integrity and tail attachment were associated with fixative osmolality.

Discussion

The formation of a bent or convoluted appearance of flagella in semen diluted with water was reported for bull, ram, stallion, and dog as early as 1856 by Kölliker. However, this phenomenon was long neglected, and mammalian spermatozoa were often considered not to respond to osmotic gradients (for a review, see Drevius 1973). The bending and coiling of the flagellum of bull spermatozoa in hypotonic media was studied extensively (e.g., Drevius 1963, 1971, 1972a,b,c,d; Lindahl and Drevius 1964; Drevius and Eriksson 1966), and when viewed by phase-contrast microscopy, the ellipsoidal-spherical shapes and hair-pin-like forms on the flagellum were termed "tail vesicles" (Drevius and Eriksson 1966). Initially, the whole flagellum, instead of the axoneme inside the tail vesicles, was thought to curve, bend, and coil into spirals (Drevius 1963; Lindahl and Drevius 1964). Later, when two to three or even six to eight axoneme cross sections were found within the same membrane, the hypothesis of the coiling of the motor apparatus (axoneme) inside the tail vesicles was proposed and tested (Drevius and Eriksson 1966). However, most of this work was performed with light microscopy and TEM, and no SEM micrographs were available from these studies. In the present investigation, micrographs obtained by both SEM and TEM clearly support the coiled axoneme hypothesis.

The coiling and twisting of the axoneme within the cell membrane under hypotonic conditions also offers one explanation for the suppressed motility of sperm from tetraploid Pacific oysters in C–F HBSS at low osmolalities (<500 mOmsol/kg; Dong et al. 2002), and this may also be true for diploid oysters. Hypotonicity-induced flagellar coiling has been shown to vary in duration and effect: (1) coiling and loss of function can be permanent possibly because of a "leaky" membrane; (2) recovery of normal flagellar shape and function (uncoiling) can occur if exposure to hypotonic medium is of short duration; (3) normal flagellar shape can be recovered (despiralization) without restoration of function after prolonged hypotonicity (Drevius and Eriksson 1966). Despite replacement of isotonic media, sperm of the eastern oyster (*C. virginica*) permanently lose motility after exposure to hypotonic media (Paniagua-Chavez et al. 1998), perhaps because of despiralization. However, similar studies in mammalian spermatozoa showing regained motility indicate an uncoiling process after moderate or mild hypotonic treatment (Drevius and Eriksson 1966). Therefore, if species respond to the same hypotonic gradients differently, then different osmotic sensitivities may exist among species.

A differential response of spermatozoa to hypotonic gradients has also been seen in the present study, e.g., the formation of tail vesicles, in which the swelling in samples simultaneously fixed with both glutaraldehyde and OsO_4 (ellipsoidal-spherical shapes) was not as pronounced as those fixed with 4% glutaraldehyde alone (hair-pin-like forms). One possible reason is that these two fixatives combined have a higher osmolality (754 mOsm/kg) and thus are more isotonic with spermatozoa compared with 4% glutaraldehyde alone (356 mOsm/kg). The other possible reason is that the rapid fixative action of OsO_4 renders the sperm membrane porous and therefore osmotically inactive and consequently prevents the progression of tail vesicles from the ellipsoidal-spherical shape to the hair-pin-like form. If the latter is true, we suspect that the process of volume expansion for oyster sperm in hypotonic glutaraldehyde and CB solution is rapid (in seconds), which indeed has

been shown to be the case with bull spermatozoa suspended in distilled water; the time for bull epididymal spermatozoa to undergo abrupt hypotonic spermolysis is approximately 2 s (Drevius 1971, 1972c).

The shrinkage of spermatozoa in hypertonic media was also associated with ultrastructural changes of the cell membrane and flagellum. The damage to the sperm head (e.g., membrane rupture, peeling or loss of membranes, acrosome loss) and tail (e.g., flagellar detachment from the head) associated with hypertonic fixation (1,110 mOsm/kg) is in agreement with the observation of the decreased motility of sperm from tetraploid Pacific oysters in C–F HBSS at high osmolalities (>1,100 mOmsol/kg; Dong et al. 2002), and this may be true for diploid oysters. The present study also indicates that osmolalities in a range of 800 to 1,086 mOsm/kg can be assumed to be isotonic to oyster sperm, and media with osmolalities below or above this range could cause damage. This is reasonable given that Pacific oysters are euryhaline estuarine organisms.

We have noted that the linear dimensions of the acrosome and sperm head in hypotonic solutions, which should have caused swelling, are smaller than those in isotonic solutions (Table 2), a finding contradictory to that expected. This apparent contradiction is probably attributable to the combined effects of initial swelling and the subsequent loss of the plasma membrane resulting from continuous swelling with prolonged suspension in hypotonic solutions (Table 3). However, no membrane loss has been observed for the flagellum, and flagellar diameters increased (Table 2), suggesting a different degree of elasticity of the plasma membrane between the head and tail regions. The maximum volume of flagella in sperm from tetraploids (calculated as a simple cylinder with the length of 53 μm; He et al. 2004) obtained with hypotonic fixation (diameter: 0.24 μm) in the present study was approximately twice the volume of the cells in isotonic fixation (diameter: 0.17 μm). The magnitude of this swelling is similar to that reported for bull spermatozoa examined microscopically following suspension in hypotonic solutions (Drevius and Eriksson 1966) and for bull spermatozoa aged in seminal plasma (Foote and Bredderman 1969). A lesser degree of volume change has been observed for the head region, which when exposed to hypotonic fixation (364 mOsm/kg) expands to approximately 1.19 times the volume of that in isotonic fixation (calculated as the volume of ellipsoidal-sphere, $V = \frac{4}{3}\pi ab^2$, where a is the major semi-axis and b is the minor semi-axis). Hypertonic fixation (1,110 mOsm/kg) resulted in flagella that are ~32% smaller in volume than those in isotonic fixation and in heads that are 25% smaller. These results also support the hypothesis that the plasma membrane of the tail region has more elasticity than that of the head region.

Although the damage associated with anisotonic fixation is clear, the most vulnerable places were the plasma membrane and flagellum motor apparatus. The acrosome and mitochondria remain generally intact and in close association with the nucleus. Considering cryopreservation as a form of osmotic shock (Mazur 2004), it seems reasonable to suggest that similar responses to osmotic gradients can occur when sperm are subjected to cryopreservation. This also implies that membrane integrity assays (e.g., Graham et al. 1990; Garner et al. 1994) and motility estimates routinely used to evaluate sperm quality after thawing can be combined to serve as valid tests for general osmotic damage associated with cryopreservation or any accompanying osmotic changes.

The phenomena associated with the response of sperm cells to anisotonic solutions have been used to characterize sperm sensitivity to osmotic pressure in mice (Songsasen and Leibo 1997), boars (Quintero-Moreno et al. 2005), and marine fish (Suquet et al. 2000). The resistance of sperm to osmotic shock is considered to be correlated with cryopreservation tolerance (Maisse 1996). In relation to the difficulty of cryopreservation of sperm from tetraploid Pacific oysters compared with those from diploids (Dong 2005), comparisons of sperm responses to different osmotic pressures and between fresh and thawed sperm samples in a quantitative manner may offer a clearer understanding of their membrane properties (such as permeability or stretching capacity). In addition, the variation in the degree of swelling of spermatozoa in a given hypotonic medium may also reflect variations of the elasticity of the cell membrane and the axoneme among the spermatozoa in a given population (Drevius and Eriksson 1966). The same techniques could also help to characterize sperm cells from different males (Bredderman and Foote 1969) and offer explanations for male-to-male variations arising in different studies (e.g., Rana 1995; Leibo and Bradley 1999; Dong 2005). Another advantage of anisotonic fixation as a research tool is that it enables the easy observation of the number and arrangement of mitochondria in SEM micrographs; this would be impossible for well-preserved samples under isotonic conditions (Dong et al. 2005b).

Ultrastructural studies of cryopreserved spermatozoa of aquatic species have been used to investigate possible causes of low motility and reduced fertility following cryopreservation and to understand and improve cryopreservation procedures (e.g., Billard 1983; Gwo and Arnold 1992; Lahnsteiner et al. 1992; Yao et al. 2000; Taddei et al. 2001; Li et al. 2002). To gauge the potential for misinterpretation attributable to fixation artifacts in this existing body of scientific literature, we have performed a literature search through http://www.csa.com (using the keywords “ultrastructure” and “cryopreservation”), through http://www.scholar.google.com (with the search words of “ultrastructure”, “cryopreservation”, “sperm”, “aquatic”), and through the reference citations of the papers obtained. The search addressing publications since 1960 has revealed 16 published reports for ultrastructural studies of cryopreserved sperm in aquatic species during the time span 1983–2004 (Table 5). These 16 reports compose, or are at least representative of, the majority of publications in this research area. Among these, 94% of the studies are limited to qualitative descriptions or a semi-quantitative approach that provides numbers but no statistical analysis, rather than a quantitative approach such as that in the study of the

Table 5 Literature review of fixation methods and analysis approach of ultrastructural studies in cryopreserved sperm from aquatic species (*PF* paraformaldehyde, *Glu* glutaraldehyde, *PB* phosphate buffer, *ASW* artificial sea water, *NR* not reported, *CB* sodium cacodylate buffer, *DI* distilled water, *SW* sea water, *Ql* qualitative, *Qt-1* quantitative in that numbers were provided but no statistical analysis, *Qt-2* quantitative in that numbers and statistical analysis were provided, *SW* swelled, *D* dehydration, *PM* rupture/loss of plasma membrane, *MD* middle piece destroyed, *CC* clumped chromatin, *DF* damaged flagellum that was bent, twisted, broken, or detached from head, *AA* axonemal alternation showing displacement or multiple axonemal appearance, *AR* damaged/activated acrosome)

Species	Habitat	Initial fixation (%)			Fixative buffer	Osmolality (mOsm/kg)	Morphological changes (analysis)
		PF	Glu	OsO_4			
Rainbow trout (*Oncorhynchus mykiss*)[1]	Freshwater	0	4	0	PB	NR	PM, MD (Ql)
Rainbow trout[2]	Freshwater	3	0.5	0	0.1 M CB+ 0.3 M Sucrose	NR	SW; PM, CC; AA (Ql)
Rainbow trout[3]	Freshwater	6.6	3.3	1.32	DI	NR	SW, PM, MD (Qt-1)
Grayling (*Thymallus thymallus*)[4]	Freshwater	6.6	3.3	1.32	DI	NR	SW, PM, MD (Qt-l)
Common carp (*Cyprinus carpio*); black carp (*Mylopharyngodon piceus*); blunt snout bream (*Megalobrama amblycephala*)[5]	Freshwater	0	4	0	N/A	NR	SW, PM, MD, DF (Ql)
Common carp, grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix)*[6]	Freshwater	0	3	0	N/A	NR	SW, PM, DF (Ql)
Polychaete (*Arenicola marina*, *Nereis virens*)[7]	Intertidal	0	2.5	0	0.2 M PB+ 0.14 M NaCl	NR	SW, PM, DF, AR (Ql)
Pacific oyster (*Crassostrea gigas*)[8]	Estuarine	0	3	0	2/3 ASW	NR	PM, MD, AR (Qt-1)
Pacific oyster[9]	Estuarine	0	5	0	N/A	NR	SW, PM, MD, DF, AR (Ql)
Atlantic croaker (*Micropogonias undulates*)[10]	Estuarine	0	2	0	0.12 M PB	NR	SW, PM, MD, DF, AA, (Ql)
Striped bass (*Morone saxatilis*)[11]	Anadromous	6.6	3.3	1.32	DI	NR	SW, PM, MD (Ql)
Chinese shrimp (*Penaeus chinensis*)[12]	Marine	0	2.5	0	ASW	NR	PM, AR (Ql)
Ocean pout (*Macrozoarces americanus*)[13]	Marine	Karnovsky's solution (Karnovsky 1965)				2,010	SW, D (Ql)
Sharpsnout seabream (*Diplodus puntazzo*)[14]	Marine	4	5	0	0.1 M CB	NR	SW, PM, DF, AA (Qt-2)
Flounder (*Paralichthys olivaceus*)[15]	Marine	0	2.5	0	SW	NR	SW, PM, MD, DF (Ql)
Sea perch (*Lateolabrax japonicus*)[16]	Marine	0	3	0	SW	NR	PM, MD (Ql)

References: [1]Billard 1983; [2]Conget et al. 1996; [3]Lahnsteiner et al. 1996; [4]Lahnsteiner et al. 1992; [5]Zhang et al. 1991; [6]Zhao et al. 1992; [7]Bury and Olive 1993; [8]Kurokura et al. 1990; [9]Li et al. 2002; [10]Gwo and Arnold 1992; [11]He and Woods 2004; [12]Ke and Cai 1996; [13]Yao et al. 2000; [14]Taddei et al. 2001; [15]Zhang et al. 2003; [16]Ji et al. 2004

sharpsnout seabream (*Diplodus puntazzo*; Taddei et al. 2001) and the present study (Table 5). Another major shortcoming of the current aquatic species literature is the lack of reports of fixative osmolality (Table 5). As illustrated above, anisotonic fixation induces artifacts in sperm ultrastructure, which can compromise the establishment of treatment effects and can lead to misinterpretations.

For example, in the study of the ultrastructure of fresh and cryopreserved spermatozoa of sharpsnout seabream, two sections of bent or coiled axonemes inside the tail vesicle were reported as indicating the presence of a double sperm tail (see Fig. 4g in Taddei et al. 2001). There was conflicting evidence for this interpretation from the appearance of two axonemes showing opposite directionality (based on the dynein arms) with the top one having a clockwise orientation, and the bottom one a counterclockwise orientation. If a double tail originated from the centriole, the two axonemes should show the same orientation instead of the opposite (Jaspers et al. 1976). Of note, the same cross-sectional view of double axonemes might derive from the cross section of the vesicle in the same report (see Fig. 4f in Taddei et al. 2001). In addition, Taddei et al. (2001) also stated that "these above types of aberrations were observed in all samples examined including fresh semen", although higher frequencies were observed with thawed samples. The consistent appearance

of these aberrations was more likely to have been attributable to the fixation procedures (hypotonic conditions) as indicated in the present study.

The ruptured plasma membranes, loss of acrosomes, and detached flagella observed in the present study with fresh spermatozoa are similar to those observed for cryopreserved spermatozoa of diploid Pacific oysters (Li et al. 2002). Although fixative of high osmolality (2,010 mOsm/kg; Karnovsky 1965) has been applied successfully in tissue sections and has been adopted (Yao et al. 2000) or modified (Taddei et al. 2001) in the fixation of spermatozoa of marine species (Table 5), greater shrinkage is expected when this fixative is applied to free-floating cells (Karnovsky 1965). Of especial importance, approximately half (44%) of the studies available on aquatic species in the literature that we have reviewed (Table 5) state that the morphological changes observed for cryopreserved sperm are also seen in fresh controls, but at a lower frequency and with a lower degree of severity. Sperm after freezing and thawing might be more sensitive to disruptions including fixation-induced effects than are fresh sperm. Future studies in this area should include reports of the osmolality of fixative to indicate whether samples have been properly preserved (e.g., under isotonic conditions) or should involve the development of optimized fixation procedures specific to cryopreserved sperm. Adequate quantitative statistical analysis should also be employed before any conclusions are drawn with respect to the effects of cryopreservation on sperm.

The results from the present study are in agreement with previous reports (Drevius and Eriksson 1966; Billard 1978, 1983) that spermatozoa are highly sensitive to changes in osmotic pressure, and that osmotic changes occur rapidly in the presence of strong fixatives. In addition, even with fresh sperm, a variety of ultrastructural changes can be produced simply by altering the osmotic pressure of the fixation solutions. In addition to bull spermatozoa in previous studies and the sperm of the rainbow trout (*Oncorhynchus mykiss*; Billard 1978) and oysters in the present study, the presence of multiple axoneme cross sections within a single cell membrane has been reported for spermatozoa of sea urchin (*Psammechinus miliaris*; Afzelius 1959), Atlantic croaker (*Micropogonias undulates*; Gwo and Arnold 1992), and sharpsnout seabream (Taddei et al. 2001), although these latter studies do not correlate the relationship between tail vesicle formation and axoneme coiling. Indeed, the mechanism of axoneme coiling within tail vesicles remains difficult to resolve. The conventional fixative of 2% glutaraldehyde in 0.1 M CB (364 mOsm/kg) is close to isotonic for cells and tissues from freshwater species (~300 mOsm/kg), whereas the same fixative, when applied to marine species (~1,000 mOsm/kg for full strength seawater), would induce hypotonic shock. These findings indicate that caution is required in the interpretation of previously published results addressing ultrastructural changes in relation to cryopreservation. Future studies should ensure that samples are prepared with isotonic fixation, especially in marine species, and that the formation of tail vesicles be scrutinized objectively before claims are made linking them to treatment effects (e.g., cryopreservation) or biological variation (e.g., double tails).

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