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Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg)

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Abstract

To provide necessary standardization of procedures for cryopreservation of sperm, a spectrophotomeric method was developed to determine the sperm concentration of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. Wavelengths of 380, 550, 581 and 780 nm were compared, and 550 and 581 nm were found to be the most sensitive and reliable. A linear relationship between sperm concentration and photometric absorbance was observed for sperm concentrations between 2×10^7 and 2×10^9 cells mL^{-1}. The regression equation for the standard curve at 550 nm for sperm of diploid oysters was $Y = -8.528 + 1.165 \log X$. The equation for sperm of tetraploid oysters was $Y = -8.844 + 1.236 \log X$. The equation at 581 nm for sperm of diploid oysters was $Y = -8.07 + 1.104 \log X$. The equation at 581 nm for sperm of tetraploid oysters was $Y = -8.331 + 1.167 \log X$. Comparisons derived from the standard curves at 581 nm between observed values and the predicted values indicated good agreement for sperm from diploid (coefficient of determination, $r^2 = 0.983$) and tetraploid ($r^2 = 0.980$) oysters.

Keywords: *Crassostrea gigas*, sperm concentration, tetraploid

Introduction

At present, the cryopreservation of sperm from diploid oysters is limited to research reports (e.g. Hughes 1973; Usuki, Hamaguchi & Ishioka 1997; Paniagua-Chavez & Tiersch 2001), and cryopreservation of sperm from tetraploid oysters is unexplored. Moreover, most previous reports (Lannan 1971; Zell, Bamford & Hidu 1979; Kurokura, Namba & Ishikawa 1990; Yankson & Moyse 1991) for diploid oysters failed to identify sperm concentrations, instead reporting sperm volumes, which hinders or prevents reproducibility of experimental conditions. Accurate estimations of sperm concentration are necessary for the development of standardized techniques for cryopreservation of sperm of oysters and other aquatic species. Firstly, this would allow dilution of the sperm suspension into the concentration range required for cryopreservation (Paniagua-Chavez & Tiersch 2001). Secondly, accurate estimation of concentration would facilitate toxicity studies of cryoprotectants before freezing, and fertilization studies of sperm after thawing. Thirdly, it would enable standardization of the 'freezing unit' (number of sperm per freezing container) for future commercial-scale production. Finally, it would improve the utility of cryopreservation through establishment of optimal sperm-to-egg ratios for fertilization (Zell *et al.* 1979).

There are several methods to determine sperm concentration. Haemacytometer counts provide a basic method that can give an unbiased estimation if done properly, but they are tedious and time consuming (Salisbury, Beck, Elliott & Willett 1943), attributes not suitable for cryopreservation, which often requires rapid handling between sperm collection and freezing. Automated cell counters such as Coulter counters and flow cytometers offer high precision,

but can be inaccurate (Foote 1968; Foote, Arriola & Wall 1978). Furthermore, they are not routinely available because of the high cost associated with their purchase and maintenance, and the need for trained technicians to operate them. Spectrometry, however, is a reliable, rapid, simple and inexpensive method to estimate sperm concentration given that an accurate initial calibration is established. This method has been used to estimate semen concentration for use in artificial spawning of fish (Suquet, Omnes, Normant & Fauvel 1992; Piereszko & Dabrowski 1993), and has widely been used for sperm cryopreservation in the dairy industry (Foote *et al.* 1978). Application of this technique to oyster sperm would help to standardize freezing protocols, especially for commercial-scale cryopreservation of sperm from tetraploid Pacific oysters. Induced triploid oysters are useful in aquaculture because the reduced gamete output improves meat quality and growth (Allen & Downing 1991; Wang, Guo & Allen 2002). Tetraploid oysters were developed to efficiently produce triploids by crossing the gametes with normal diploids (Guo & Allen 1994; Guo, Debrosse & Allen 1996; Wang, Guo, Allen & Wang 1999; Eudeline, Allen & Guo 2000a, b). Refrigerated and frozen storage of tetraploid oyster sperm in a commercial scale will effectively expand the market of triploid seed worldwide.

Our goal was to develop standardized protocols for commercial-scale cryopreservation of sperm from Pacific oysters, *Crassostrea gigas*. The specific objectives of this study were to: (1) identify appropriate wavelengths for spectrophotometric estimation of sperm concentration of Pacific oysters; (2) establish standard curves for spectrophotometric estimation of sperm concentration of diploid and tetraploid Pacific oysters; (3) validate the adequacy of the regression models and (4) investigate the applicability of the standard curves in practice. Based on preliminary studies, we chose to measure sperm turbidity at four wavelengths (380, 550, 581 and 780 nm) within the visible range and measurements made at 550 and 581 nm were found to be the most sensitive and reliable.

Materials and methods

Tetraploid Pacific oysters ($\sim$ 3 years old) were collected from Totten Inlet (Puget Sound, 47°09′017″N, 122°57′908″W) and diploids ($\sim$ 2 years old) were from Willapa Bay (46°29′885″N, 124°01′810″W) in Washington State. Oysters were shipped chilled by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS) from June to August 2002. Sperm samples were collected by dry stripping of the gonad (Allen & Bushek 1992) and were weighed to the nearest 0.01 g. Calcium-free Hanks' balanced salt solution (C-F HBSS) (Paniagua-Chavez, Buchanam & Tiersch 1998) was used as an extender at 1000 mOsmol kg^{-1} based on previous work (Dong, Eudeline, Allen & Tiersch 2002). All chemicals used for preparation of solutions were of reagent grade (Sigma Chemical, St Louis, MO, USA). A dilution of 1:20 (weight:volume; g:mL) of sperm to extender was used for initial suspension. Sperm suspensions were filtered through a 40-μm nylon screen. A second dilution of 10:1000 (v/v) of sperm:extender was used for haemacytometer counts (Hausser Scientific, Horsham, PA, USA). To facilitate counting, sperm were immobilized by using extender at a low osmolality of 200 mOsmol kg^{-1}. The spectrophotometer used was a single-beam instrument (Genesys^{TM} 20, Thermo Spectronic, Rochester, NY, USA). Calcium-free HBSS was used as the blank standard when obtaining the readings of absorbance.

For calibration, oysters were received on 11 June, 16 July and 30 July 2002. Four wavelengths within the visible range (380, 550, 581 and 780 nm) were chosen for comparison. Concentrations of sperm suspensions of diploid oysters were estimated by haemacytometer counts, and serial dilutions were prepared of 2×10^9, 1×10^9, 8×10^8, 6×10^8, 4×10^8, 2×10^8, 1×10^8, 8×10^7, 6×10^7, 4×10^7, 2×10^7, 1×10^7, 8×10^6, 6×10^6, 4×10^6, 2×10^6, 1×10^6 and 1×10^5 cells mL^{-1}. Four readings of the four different wavelengths were obtained for the same sample of each concentration, and were replicated eight times with sperm from eight oysters.

For sperm suspensions of tetraploid oysters, the same concentrations were used except the highest, 2×10^9 cells mL^{-1}, because the tetraploid oysters provided less sperm than did diploids. Readings were also obtained at four wavelengths for each concentration, but seven replicates from seven oysters were used for readings at 380, 550 and 780 nm, and eight replicates were used for readings at 581 nm. For testing of the standard curves at 581 nm, oysters were received on 4 June, 11 June, 3 July, 10 July, 16 July, 23 July, 30 July, 13 August and 20 August 2002. A total of 137 sperm dilutions from 22 diploid oysters and 127 sperm dilutions from 33 tetraploid oysters were used for haemacytometer counts and for spectrophotometric readings.

Basic parameters such as body wet weight, shell height and gonad wet weight of oysters received and used for experiments during June and August 2002 are listed in Table 1. For diploids, only oysters with ripe gonads were used for experiments, and ripe gonads were determined by the presence of prominent genital canals. All tetraploids were used except for those with little or no gonadal tissue, or spent gonads.

To determine whether there was variation in gonad development during the normal spawning season (June to August 2002), the sperm concentration per gram of gonad wet weight (sperm g^{-1} gonad) was approximated by assuming a sperm density of 1.0 g mL^{-1}. Net gonad wet weight was approximated by subtraction of the mass of digestive gland from the gross wet weight of the gonad after dissection from other tissues. Thus, after obtaining the net wet weight (W) of gonad tissue and suspension with extender (V) at 1:20 (w/v) of sperm to extender, and estimating the sperm concentration (C) in the solution by a haemacytometer, the sperm g^{-1} gonad were obtained using the following formula:

$$\text{sperm/g gonad} = \frac{C \times (V + W/D)}{W}$$

where C is the sperm concentration (cell mL^{-1}), V the extender volume (mL), W the gonad wet weight (g) and D the sperm density (1.0 g mL^{-1}).

The values obtained from the spectrophotometer were single readings of absorbance, and for the haemacytometer were the average of duplicate counts. Correlation and simple linear regression (SLR) were used for data analysis (SPSS 10.0 for Windows 1999, SPSS, Chicago, IL, USA). Data for sperm concentrations were logarithmic (common logarithm) transformed prior to SLR analysis. The average of the absorbance readings was used to construct the standard curves. Two-way ANOVA was used to test the difference of sperm g^{-1} gonad between diploid and tetraploids, and for differences among different shipments.

Results

Within the concentration range of 1×10^5 to 2×10^9 cells mL^{-1}, higher correlations were found at higher wavelengths between the haemacytometer counts and absorbance readings for sperm suspensions from diploid and tetraploid oysters (Table 2). High correlations were also observed within the four wavelengths, especially those between 550 and 780 nm (correlation coefficient, $r = 0.999$), 581 and 780 nm ($r = 0.999$), 550 and 581 nm ($r = 1.000$). Readings at 380 nm were found to be least correlated with the haemacytometer counts and readings at other wavelengths (Table 2).

Table 1 Basic parameters of diploid (2C) and tetraploid (4C) Pacific oysters received and used for experiments during June and August 2002

	Sample size		Body wet weight (g)		Shell height (mm)		Gonad net wet weight (g)*	
Ploidy	Received	Used	Received	Used	Received	Used	Received	Used
2C	34	22	52.4 ± 21.5	58.0 ± 23.6	93.1 ± 14.8	95.6 ± 15.0	3.62 ± 2.03	4.10 ± 2.23
4C	38	25	138.6 ± 107.0	100.1 ± 92.5	105.6 ± 21.9	98.2 ± 19.0	5.31 ± 3.96	4.15 ± 3.60

*Net gonad wet weight was approximated by subtraction of the mass of digestive gland from the gross wet weight of the gonad after dissection from other tissues.

Table 2 Correlations among estimates of sperm cell concentrations from diploid (2C) and tetraploid (4C) Pacific oysters performed by spectrophotometric analysis at four wavelengths or by haemacytometer counts

	Correlations with other estimates							
	380 nm		550 nm		581 nm		780 nm	
Method (wavelength)	2C	4C	2C	4C	2C	4C	2C	4C
Haemacytometer	0.869	0.810	0.940	0.914	0.938	0.908	0.950	0.921
Spectrophotometer* (380 nm)	–	–	0.976	0.976	0.979	0.979	0.969	0.970
Spectrophotometer (550 nm)	–	–	–	–	1.000	1.000	0.999	0.999
Spectrophotometer (581 nm)	–	–	–	–	–	–	0.999	0.999

*Spectronic 20.

The standard curves showed a curvilinear relationship within the tested concentration range for readings obtained at all four wavelengths for sperm from diploid oysters (Fig. 1). A linear relationship was observed between the log of sperm counts and absorbance when sperm concentrations were between 2×10^7 and 2×10^9 cells mL^{-1} (4×10^6 to 2×10^9 cells mL^{-1} for readings at 380 nm), and the regression equations for the standard curve were calculated (Fig. 1). Concentrations below 2×10^7 cells mL^{-1} were not reliably measured by spectrophotometry, and concentrations above 2×10^9 cells mL^{-1} were at the upper limit of the photometric range of the instrument. The standard curve at 550 nm had the same coefficient of determination ($r^2 = 0.988$) as that of 581 nm ($r^2 = 0.988$). Sperm concentrations between 4×10^6 and 1×10^9 cells mL^{-1} were used to generate the standard curve at 380 nm, which showed a lower coefficient of determination ($r^2 = 0.975$) than did analysis at 550, 581 and 780 nm.

Similar findings were observed for the standard curves for sperm from tetraploid oysters (Fig. 2). Linear regressions were constructed between sperm concentrations of 2×10^7 and 1×10^9 cells mL^{-1} at wavelengths of 550, 581 and 780 nm. The standard curve at 550 nm had a similar coefficient of determination ($r^2 = 0.997$) as that of 580 nm ($r^2 = 0.998$). The standard curve at 380 nm also could be applied to a wider range (4×10^6 to 1×10^9 cells mL^{-1}), but had a smaller coefficient of determination ($r^2 = 0.981$).

Plotting the observed values against the standard curves generated from readings at 581 nm indicated good agreement for concentration estimates of sperm suspensions from diploid and tetraploid Pacific oysters (Fig. 3). Models constructed with the observed values showed coefficients of determination similar to those of standard curves for sperm from diploid ($r^2 = 0.983$) and tetraploid ($r^2 = 0.980$) Pacific oysters.

The overall average of sperm g^{-1} gonad for oysters received during the normal spawning season was

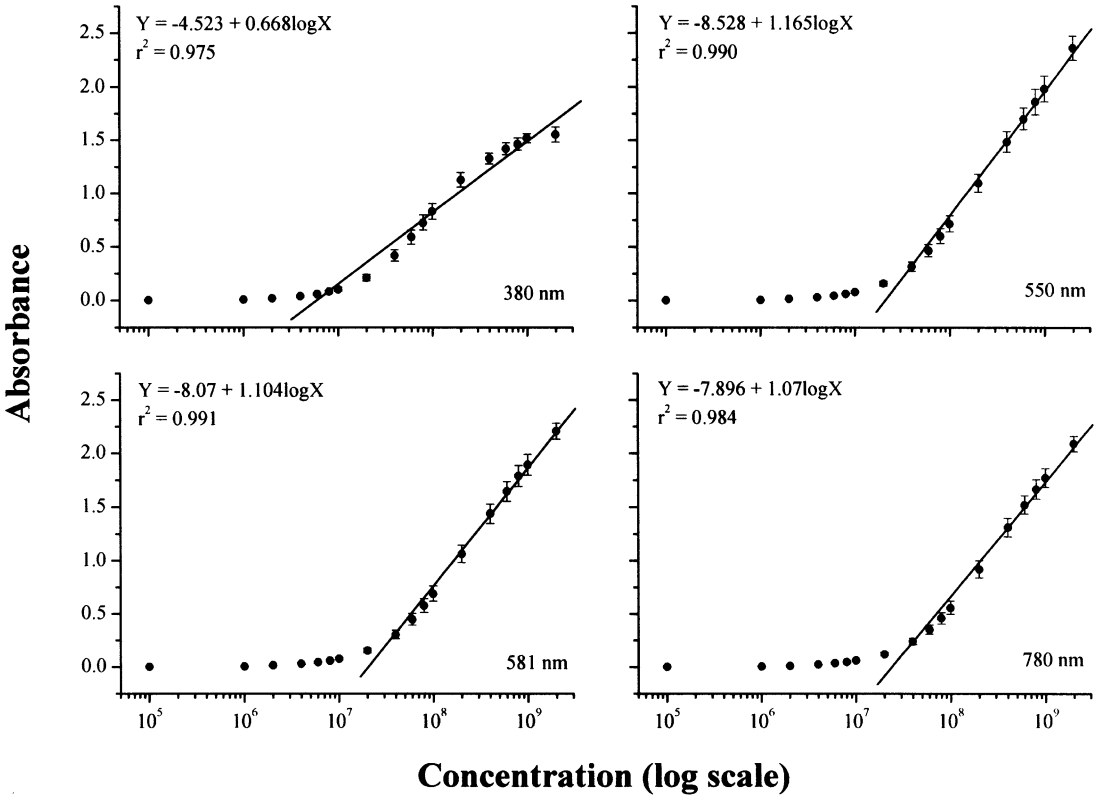

Figure 1 Standard curves for sperm suspensions from diploid Pacific oysters. Each point represents the mean of eight replicates of sperm from eight diploid oysters. Error bars indicate standard deviations. Linear regressions were constructed for concentrations between 4×10^6 and 2×10^9 cells mL^{-1} for readings at 380 nm, and between 2×10^7 and 2×10^9 cells mL^{-1} for readings at 550, 581 and 780 nm.

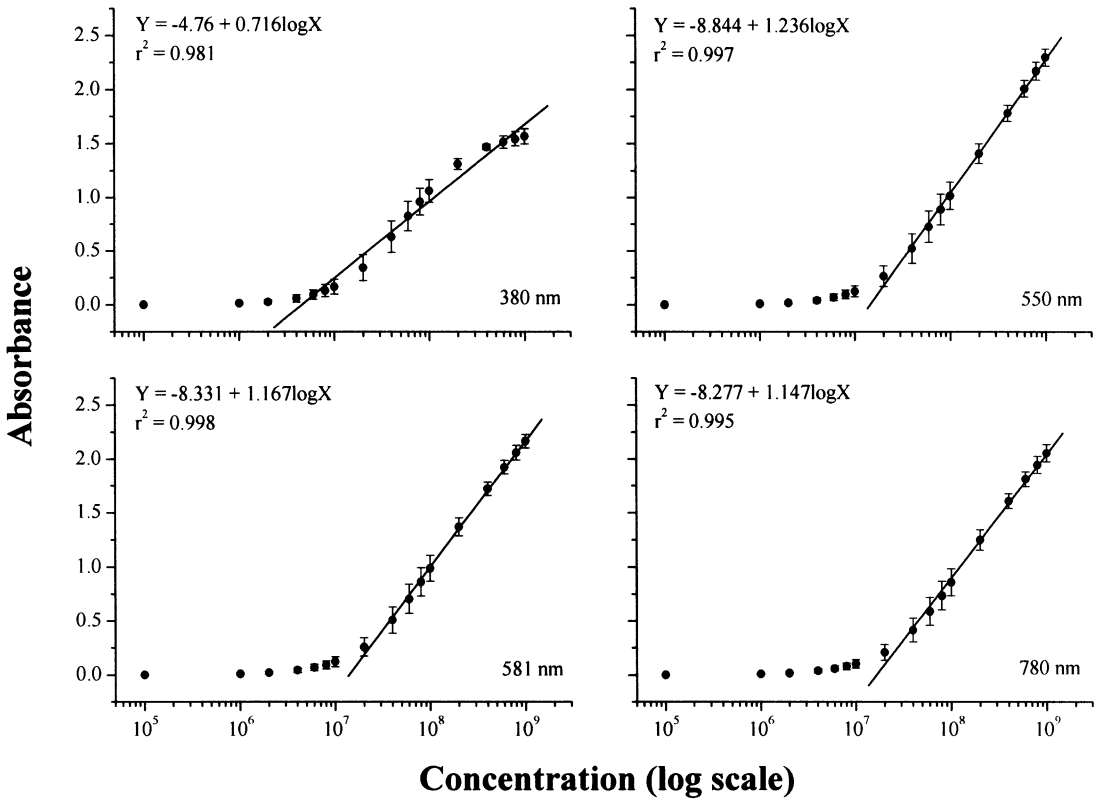

Figure 2 Standard curves for sperm suspension from tetraploid Pacific oysters. Each point represents the mean of seven replicates (eight replicates at 581 nm) of sperm from seven (or eight) tetraploid oysters. Error bars indicate standard deviations. Linear regressions were constructed for concentrations between 4×10^6 and 1×10^9 cells mL^{-1} for readings at 380 nm, and between 2×10^7 and 1×10^9 cells mL^{-1} for readings at 550, 581 and 780 nm.

$(5.4 \pm 0.9) \times 10^{10}$ for diploids, and $(2.7 \pm 0.5) \times 10^{10}$ for tetraploids. A significant difference of sperm g^{-1} gonad was observed between the ploidy levels ($P < 0.0001$), but there was no significant difference in the values for sperm g^{-1} gonad among shipments within diploids ($P = 0.27$) and within tetraploids ($P = 0.0669$) (Fig. 4).

Discussion

There is a lack of standardization in protocols and reporting of results for cryopreservation in aquatic species. Researchers new to the field are often confronted with successful protocols that cannot be repeated, unsuccessful experiments that cannot be interpreted and contradictory findings even within a single species (Tiersch 2000). A lack of standardized sperm concentrations is a major source of this uncertainty within studies. For example, when specific volumes or percentages of cryoprotectant are applied to different sperm concentrations, the observed variation is usually attributed to genetic or environmental differences among males. The use of sperm or gonadal volumes instead of sperm concentrations makes experiments difficult to repeat even for the same population. In aquaculture, when there is a need to expand sperm cryopreservation from research to commercial production, as in the case of sperm from tetraploid Pacific oysters, standardization of sperm concentration would reduce quality variation and improve consistency after thawing. Such standardized practices have been incorporated for decades with sperm cryopreservation in the dairy industry, for example (Foote *et al.* 1978).

Wavelengths in the range of 375–630 nm have been used to determine semen concentrations in the dairy industry (Foote *et al.* 1978). In the present study, we tested four wavelengths (380, 550, 581 and 780 nm), representing four different portions of the vision range, and wavelengths of 550 and 581 nm appeared to be the most suitable because of the highest

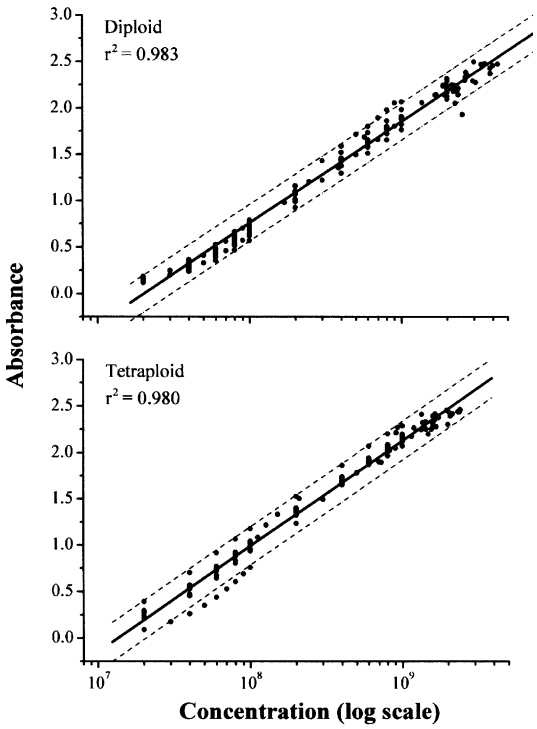

Figure 3 Plot of observed values against the standard curves at 581 nm for sperm from diploid and tetraploid Pacific oysters. Upper panel: data for 137 sperm dilutions collected from 22 diploid oysters; lower panel: data for 127 sperm dilutions collected from 33 tetraploid oysters. Dashed lines indicate the 95% confidence intervals for predicted individual points.

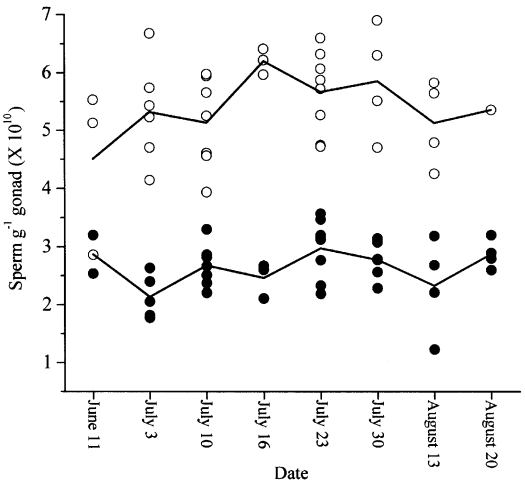

Figure 4 Mean sperm concentration per gram of wet gonad weight (sperm g^{-1} gonad) of diploid ($n = 36$, open circles) and tetraploid ($n = 39$, filled circles) oysters received during shipments in June and August 2002. Each circle represents the number of sperm g^{-1} of gonad of a single oyster. The lines represent the average values for each shipment.

coefficients of determination. In addition, for the same series of standard solutions, absorbances of 550 and 581 nm had wider reading ranges than did 380 and 780 nm. Thus, the change in absorbance at 550 and 581 nm for a given concentration change was greater, leading to enhanced sensitivity and accuracy in measurement.

The present study also showed high correlations among readings at 380, 550, 581 and 780 nm, indicating that all of these wavelengths were available for determining sperm concentration as long as accurate standard curves were generated. This is explainable because sperm solutions are mixtures of many components. Consequently, the observed absorption at any wavelength is the sum of the individual absorption spectra of the components, which makes it difficult to find a discrete peak in the absorption specta. However, differences were evident between the absorbance at 380 nm and at higher wavelengths. Lower correlations were observed between the absorbance values at 380 nm and the haemacytometer counts for sperm from the diploid and tetraploid oysters. Also, for the same series of standards of known sperm concentrations, absorbance values at 380 nm showed the narrowest reading range.

The standard curves were curvilinear in the concentration range from 1×10^5 to 2×10^9 cells mL^{-1} at all four wavelengths for sperm from diploid and tetraploid oysters. Although we could use a polynomial regression (a cubic model) to construct a best-fit calibration, a simple linear relationship was observed between the absorbance and log transformation of sperm concentration between 2×10^7 and 2×10^9 cells mL^{-1}, which was simpler and expedient for practical purposes. And most importantly, this was within the useful concentration range for the cryopreservation of oyster sperm, because motility is lost if concentrations are too dilute (Gray 1928; Bougrier & Rabenomanana 1986; Usuki *et al.* 1997; Paniagua-Chavez *et al.* 1998). Of the 14 studies we have found in the literature that directly address cryopreservation of oyster sperm, only four indicate an approximate sperm concentration for freezing, and these are within the range from 1×10^7 to 1×10^9 cells mL^{-1} (Staeger 1973; Usuki, Hamaguchi & Ishioka 1999; Paniagua-Chavez & Tiersch 2001; Smith, Pugh, Tervit, Roberts, Janke, Kaspar & Adams 2001).

Based on the principle of photometer operation, as long as the size, shape and opacity of the particles are similar from sample to sample, concentration estimation based on standard curves should be accurate given accurate calibration. In general, oyster sperm of the same species with the same ploidy level were quite homogenous. This was especially true for sperm collected during the normal spawning season, which was demonstrated by the constant sperm concentration per gonad wet weight during the present study. The good agreement between the predicted and observed values also indicated the general applicability of the standard curves developed at 581 nm for estimation of sperm concentration in Pacific oysters across the spawning season.

Sperm from tetraploid oysters were different from those of diploids in size, density and motility, and thus a separate calibration was necessary for the two ploidy levels. Another important factor that may influence the applicability of the standard curves was the extender solution used to suspend sperm. Sperm from tetraploid Pacific oysters were found to exhibit variable motility when suspended in different extender solutions (Dong *et al.* 2002), which may affect the absorbance readings because sperm with low motility may tend to precipitate sooner than those with higher motility.

Other factors that can affect the accuracy of spectrophotometry to estimate sperm concentration include dilution ratios (Foote *et al.* 1978), and the accuracy of sampling desired volumes for dilution (Ciereszko & Dabrowski 1993). Care should also be taken when collecting the sperm, because contamination with other substances such as gut contents would affect calibration and concentration estimates. Thorough mixing of sperm suspensions before measurement was also important for accurate readings. Overall, to use the standard curves effectively and accurately, sperm sample preparation and measurement should always follow the exact procedures used for calibration. In the present study, standard curves generated at wavelengths of 550 and 581 nm within the range of 2×10^7 and 2×10^9 cells mL^{-1} are recommended for determination of the concentration of sperm from diploid and tetraploid Pacific oysters. It is important to note that the specific standard curves developed in this study may not be applicable to all populations within the species (such as in oysters grown in different environments), and validation of standard curves should be performed when working with any aquatic species. Thus the process outlined in this report can serve as a model for establishment of widespread use and reporting of sperm concentration estimates for work in areas such as cryopreservation, genetic manipulation (such as in ultraviolet irradiation of sperm for induction of gynogenesis) and in artificial spawning.

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