New Chapter

Estimation of Fish Sperm Concentration by Use of Spectrophotometry

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Towards Harmonization: Some Lessons From Human Andrology Laboratories

A lack of standardization, wide variation among laboratories, and an urgent need for quality control led andrology laboratories worldwide to unify and developed standardized techniques and practices for semen analysis which were published by the World Health Organization (WHO) (Keel et al. 2002). The purpose of the WHO manual was to minimize variability among laboratories and to enable comparison of semen analysis results among laboratories. Standardized laboratory protocols and quality control are essential for meaningful comparisons of semen quality data from multiple sites (Brazil et al. 2004). Although the WHO manual is recognized globally as the "gold standard" for semen analysis, its acceptance has been less than universal. For example, most laboratories that participate in the German external quality control program for semen analysis do not use WHO-recommended methods (Cooper et al. 2007). Most inter-laboratory variation is attributed to the use of different techniques, and there is considerable disagreement regarding the relative accuracy and precision of various techniques (Brazil et al. 2004). Although improvements can be made in existing guidelines, protocols, and quality control systems, these current systems provide better tools than other non-standardized procedures (BjoRndahl et al. 2004).

New methods need to be validated for accuracy, repeatability, and precision before moving into standardization, which is a component of harmonization. To achieve harmonization, standards need to be developed for each method. For example, despite several efforts to standardize methods of semen analysis, sperm count is known to be subject to large inter-laboratory differences. Most variation is introduced through the use of different techniques (Jonckheere et al. 2005). Currently there is disparity in the equipment and procedural steps used for concentration measurements. This is because the standardization necessary for development of guidelines does not exist. After methods have been standardized, intercalibration comes into play. There is nothing inherently wrong in using different techniques as long as the results are accurate and consistent. Comparison by intercalibration is used to verify that values of a particular technique are correct. Thus the purpose of the intercalibration is not to harmonize the assessment method, but only the results (Buffagni and Furse 2006). Sometimes to ensure that the results of one technique are consistent with those of another, a transformation factor is applied to normalize the data (Poikane 2009). Criteria must be established to define the reliability of data for total allowable error specifications, and the extent of corrective measures that are acceptable. These criteria will define the procedures used for adjusting the data and compensation factors.

To minimize errors routine quality control needs to be established. Evaluation of an internal quality standard is essential to maintain accuracy, precision, and competence (Auger et al. 2000). Estimates of imprecision can be obtained from the internal quality control system. Imprecision can be reduced by regular training of personnel and by adopting best management practices. Workshops on standardization have been used to train andrology laboratory technicians (Toft et al. 2005). External quality control can also provide regular standardization

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checks and agreement among laboratories. External quality control programs should be directed at tangible elements (e.g., staff, instrumentation, equipment, and supplies) and at intangible elements (protocols and techniques) (Castilla et al. 2010). Adherence to the same standard procedures and criteria for each method will allow separate laboratories to work in unison. Harmonization results in making the outcomes comparable, not necessarily in making every laboratory do things in an identical fashion (van Nieuwerburgh et al. 2007). Harmonization allows choices between alternatives, out of which one or several can be adopted depending on the given circumstances (Figure 1).

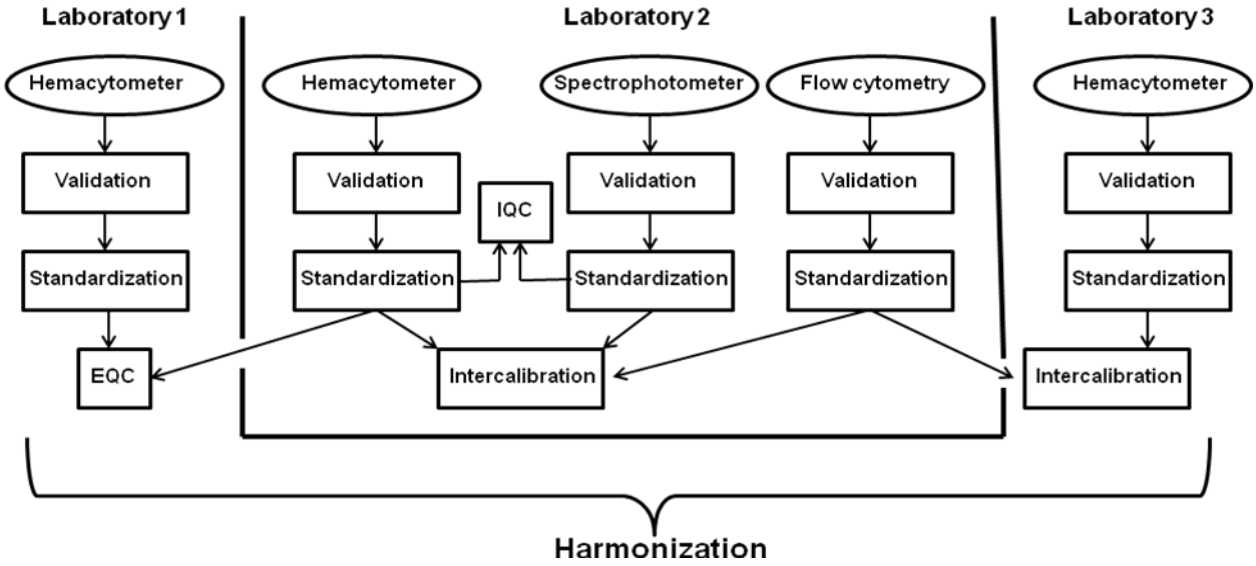

Figure 1. Schematic overview of a harmonization process (rectangles) for sperm counting methods (ovals) across three laboratories. Method validation is the first step to ensure that results are reliable. Standardization follows and allows development of guidelines for uniformity of response (a "top-down" approach). Many laboratories have more than one instrument that can perform the same function which enables internal quality control (IQC). IQC and external quality control (EQC) are needed to test variability within and among technicians, and among laboratories. Intercalibration seeks consistency in classification of results of different methods, and is used to determine if results from methods are accurate, credible, and comparable. Following intercalibration a method can be trusted and can move toward harmonization, the process of making different standards compatible and providing choices among methods. Harmonization bridges existing variation to provide a state of comparability, consistency, and similarity.

The Current Status for Sperm Quality Analysis in Aquatic Species

Currently, the single largest problem for sperm quality analysis (and standardization in general) in aquatic species is the lack of control and reporting of sperm concentration (Dong et al. 2007a). We place this problem foremost because sperm concentration will directly affect analysis results even for assays that are otherwise standardized (Tiersch et al. 2007). The first step in dealing with this problem is to recognize the importance in controlling and reporting sperm concentration, and to adopt and ultimately harmonize methods to estimate concentration. A variety of methods exist, each with advantages and disadvantages, and only a few of these have established methods. As stated above, after choosing a method, validation is necessary to move into standardization (Figure 1) which allows development of guidelines or standardized

protocols for each method. After standardization, the next step is comparison and intercalibration between and among laboratories to ensure that values are precise and accurate. Consistency, accuracy, and comparability of different methods are the keys for intercalibration (Poikane 2009). Harmonization can be addressed after the development of standardized protocols and establishment of an intercalibration process. At present, as stated above, there is a lack of standardization in the performance and reporting of sperm analyses in aquatic species. The study of sperm quality would greatly benefit from a standardization of analytical methods and tools (Fauvel et al. 2010). This would facilitate collaboration among laboratories with the aim to develop uniform (standardized) procedures and to derive similar accuracies through intercalibration exercises (Rosenthal et al. 2010) and eventual harmoniation. To facilitate making the first step in this process for aquatic species, the focus of this chapter will be on turbidimetric estimation of sperm concentration by use of spectrophotometry. This is not meant to suggest that this is the best or only technique available to measure sperm concentration, but it is widely accessible, has been widely applied for a variety of species and applications, and can serve as an overall representative model of the opportunities and problems inherent in other aspects of gamete quality analysis in aquatic species.

Principles of Turbidimetric Analysis

Whenever light strikes an object, the light can be scattered (reflected), absorbed, or passed through the object (refracted). The extent of light loss can be determined by measuring the amount scattered or reflected (nephelometry) or the amount of light transmitted (turbidimetry) (Csuros and Csuros 1999). In a turbidity measurement a spectrophotometer is used to measure the absorbance of light as a function of wavelength as it passes through a sample. The measurement of absolute absorbance depends in the separation of scattered light from the attenuation of light (absorbance). The sensitivity to measure light loss varies depending on the type, number, and position of detectors. For example, the sensitivity to measure absorbance increases if a detector is positioned far from the cuvette (sample container) (Figure 2). For this reason there are differences in the accuracy of various instruments. This is why several authors avoid use of the term "absorbance" and refer to spectrophotometric output as "apparent absorbance", "optical density", or "turbidity" (Poole and Kalnenieks 2000).

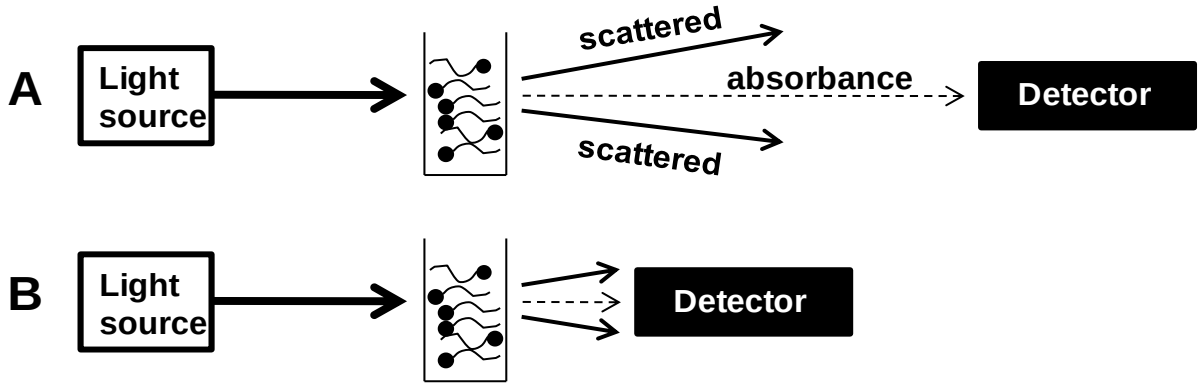

Figure 2. The measurement of absorbance varies depending on position of the detector. For accurate measurement it is necessary to eliminate or minimize the effect of light scattering. A) Positioning of the detector far from the sample enhances sensitivity because it will detect less scattered light. B) When the detector is close to the sample, scattered light will cause an artifactual reduction of absorbance (modified from Poole and Kalnenieks 2000).

Turbidity is routinely used as a measure of biomass concentration because the amount of light lost or scattered is inversely proportional to the cell concentration or directly proportional to the absorbance (Csuros and Csuros 1999). The apparent absorbance of a cell suspension depends on the wavelength used, the pathlength, and the cell concentration of the suspension. When nephelometry is used, a wavelength is chosen to optimize sensitivity and dynamic range, and to minimize the effects of light absorption by the cells or the medium components. By choosing a wavelength where no light absorption is recorded (e.g., 320-800 nm for proteins), the light striking the detector will be result only from light scattering. As a rule, choosing lower wavelengths will provide greater sensitivity of optical density measurements, but longer wavelengths will provide greater linearity over the same range of cell suspensions (Poole and Kalnenieks 2000). When turbidimetry is used, the wavelength selected is based on the maximal absorbance peak (e.g., 260 nm for nucleic acids). Sperm concentration is directly proportional to the absorbance and indirectly proportional to percentage transmittance (Csuros and Csuros 1999). To quantify an absorbance reading, a quantitative enumeration must be made (e.g. hemacytometer and coulter counter) to correlate with the apparent absorbance.

Estimation of Sperm Concentration in Livestock

The importance of reporting sperm concentration due to individual male variation and to allow comparison of studies was recognized early as being essential by the bull sperm industry. This industry next focused on development of rapid methods for accurate estimation of sperm concentration which continues today (Table 1). Different methods have been developed, ranging from comparison of sperm suspensions with opacity standards (Burbank 1935) to flow cytometric methods (Evenson et al. 1993). These methods vary in precision (Table 1).

Table 1. Coefficient of variation (repeatability) of different methods for estimation of sperm concentration reported in representative studies (arranged chronologically).

Animal studied	Hemacy-tometer	Sper-matocrit	Spectro-photometry	Coulter counter	Flow cytometry	Reference
Chicken	6.4	3.8	3.7	—	—	Taneja and Gowe 1961
Chicken	12.8	8.3	4.8	—	—	Taneja and Gowe 1961
Turkey	41.1	—	26.6	30.1	—	Brown et al. 1970
Chicken	17.9	9.9	2.2	1.6	—	Brillard and McDaniel 1985
Boar	12.3	—	2.9	2.3	—	Paulenz et al. 1995
Boar	7.1	—	10.4	—	2.7	Hansen et al. 2006
Bull	7.8	—	4.1	—	2.3	Prathalingam et al. 2006

The first record of using turbidity to measure sperm concentration in livestock dates back 70 yr (Comstock and Green 1939). The National Association of Animal Breeders has developed guidelines for measurement of sperm concentration by turbidity (Foote 1972, Foote et al. 1978). A survey among laboratories was used to identify the sources of variation including the spectrophotometer used, wavelength used, method of calibration, type of diluents, and dilution rate used (Foote et al. 1978). From the laboratories interviewed, 80% were using the same spectrophotometer and most of them were using the same wavelength (550 nm). It was concluded that the accuracy of estimating sperm concentration using spectrophotometer was

dependent on the calibration and standard curve used to correlate absorbance values with hemacytometer sperm counts.

The Importance of Estimating Sperm Concentration in Aquatic Species

The urgent need to standardize sperm studies has been emphasized recently in a workshop (Rosenthal 2008), symposium (10^{th} International Symposium on Spermatology 2006,Tiersch et al. 2007), and publications (Dong et al. 2005a, Dong et al. 2007a). Standardization is required to reproduce and optimize reports and protocols. A lack of standardization of sperm concentration has led to variability in results and reporting of studies (Dong et al. 2005a, Tiersch et al. 2007). Accurate estimation of concentration is necessary for a wide variety of topics including standardization of cryopreservation (Dong et al. 2007a), determination of optimal sperm-to-egg ratios (Suquet et al. 1995), calibration of ultraviolet irradiation to induce gynogenesis (Mims et al. 1997), assessment of spermiation following hormonal stimulation (Miranda et al. 2005), assessment of sperm production following sex manipulation (Fitzpatrick et al. 2005), nutritional studies (Rinchard et al. 2003), estimation of sperm motility (Cosson 2008), optimization of staining with fluorescent dyes (Paniagua-Chavez et al. 2006), and reproductive toxicology (Aravindakshan et al. 2004).

Uncontrolled variation in sperm concentration is one of the main reasons for the inconsistency observed among various studies associated with cryopreservation and fertilization (Dong et al. 2007a), and as such determination of concentration should be considered as an essential parameter in the assessment of sperm quality (Aas et al. 1991, Cabrita et al. 2009). Sperm volume and concentration vary among species (Piironen and Hyvarinen 1983) and individuals within the same species, with reported concentrations ranging between 2×10^6 to 6.5×10^{10} cells/ml (Leung and Jamieson 1991, Vuthiphandchai and Zohar 1999, Alavi et al. 2008). These differences are due to factors such as the stage of spawning season (Munkittrick and Moccia 1987), seasonal variation (Alavi et al. 2008), strain and genetic backgrounds (Scott and Baynes 1980, Tiersch 2001), diet (Ciereszko and Dabrowski 2000), physicochemical and social environment (Fitzpatrick and Liley 2008), disease (Rurangwa et al. 2004), and age (Poole and Dillane 1998).

The time, effort, and expense involved in rearing or capturing mature fish requires efficient use of sperm samples, especially for imperiled species (Tiersch et al. 1994). Common hatchery practices, if described in reports, typically include the addition of volumetric measures of gametes (e.g. 20 mL of sperm per 2 - 3 L of salmonid eggs; Willoughby 1999) without estimating the concentration or motility of the sperm (Aas et al. 1991). Commercial success of hatchery effort depends upon efficient utilization of available gametes (Erdahl and Graham 1987) and sperm can be in short supply due to limited numbers of broodstock or due to the small body sizes of aquarium fish used as biomedical models such as zebrafish *Danio rerio* (Tiersch 2001, Tan et al. 2010). In addition, the success or failure of cryopreservation protocols can be dictated by sperm concentration (Dong et al. 2007a). For these and other reasons it is therefore essential to routinely adopt a rapid, efficient, and accurate method for estimation of sperm concentration in aquatic species.

Methods Used to Estimate Sperm Concentration

There are several techniques used to estimate sperm concentration as part of the process to estimate sperm quality (Figure 3). Each technique has advantages and disadvantages, but no matter which technique is chosen it is essential to control, record, and report sperm concentration.

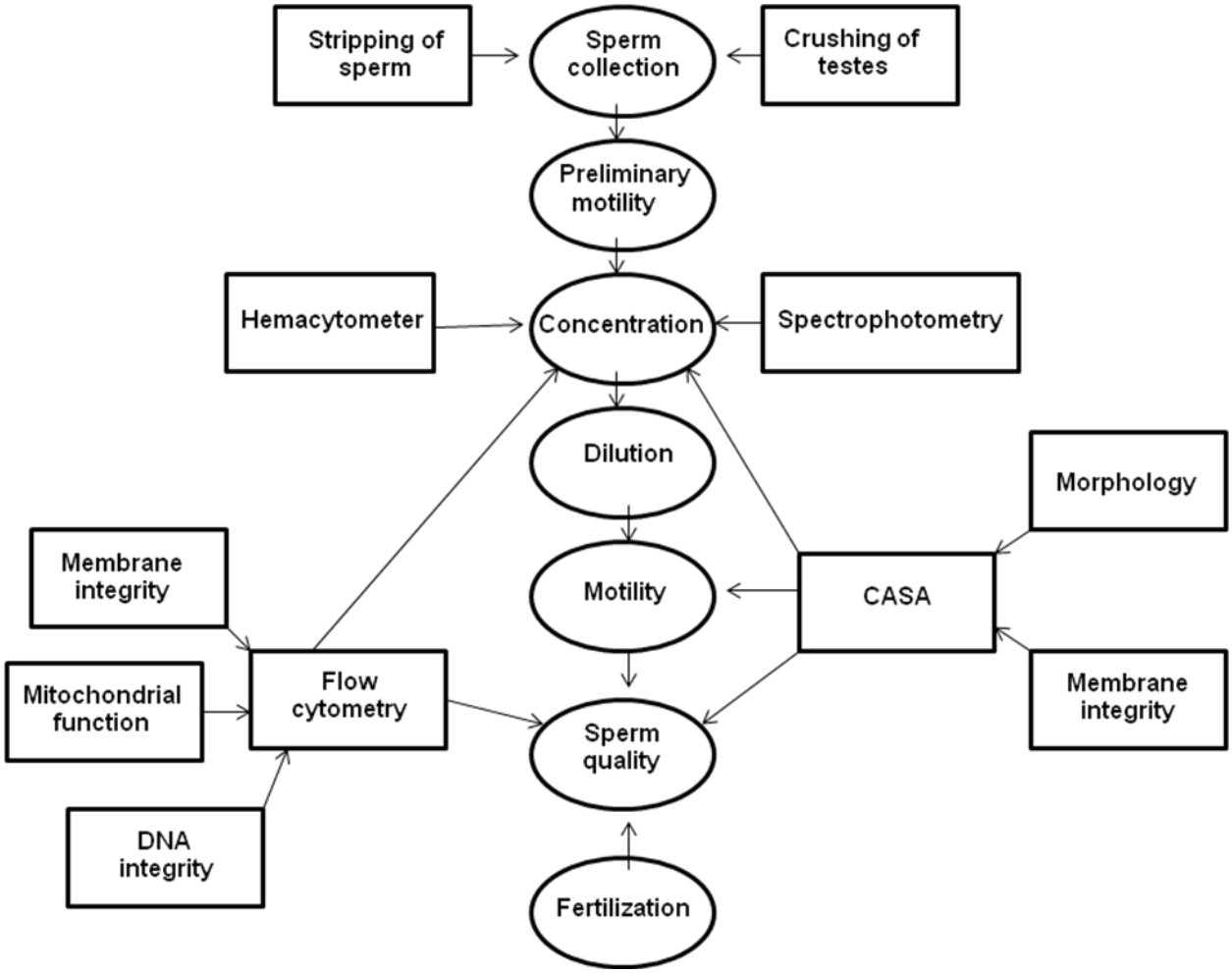

Figure 3. Relevant steps (ovals) for sperm quality assessments. Traditional sperm quality parameters (rectangles) have included motility, morphology, concentration, and membrane integrity (viability), with fertilization providing an ultimate assessment. More than one instrument can perform different tests. For example, concentration can be measured by use of computer-assisted sperm analysis (CASA), flow cytometry, hemacytometer, or spectrophotometry.

Fish sperm concentration has been assessed by three main techniques: counting in a hemacytometer chamber, estimation of spermatocrit, and turbidity evaluation (for more details see reviews by Rurangwa et al. 2004, Alavi et al. 2008, and Fauvel et al. 2010). Hemacytometer counting is precise and reliable for fish sperm, but is time consuming and thus cumbersome for fertilization protocols involving precise timing or many males (Fauvel et al. 1999, Alavi et al. 2008, Cabrita et al. 2009). Spermatocrit determination, although widely used, requires

centrifugation of the milt and only provides a relative measure expressed as the ratio of packed sperm volume to total volume of sample instead of the number of cells per mL. Because the handling time for samples should be minimized, establishment of a rapid and reliable method for sperm concentration estimation is required. Spectrophotometric determination of turbidity is an efficient and inexpensive method to estimate cell concentrations, given that an accurate initial calibration is established (Foote et al. 1978, Dong et al. 2005a). The advantages of using photometric measurement of sperm concentration in aquatic species has been known at least since 1949 (Rothschild 1950) and the application of this method to fish can be dated back at least to 1971 (Billard et al. 1971). Since then, the direct relationship between sperm concentration and absorbance has been established in approximately 41 species of fish (most within the past 10 yr). Despite or because of this diversity of use, the application of turbidity to estimate sperm concentration has not been collectively studied, and remains unstandardized and variable in methods and reporting. By reviewing the literature addressing use of spectrophotometer to measure sperm concentration in fish (described below) we found that the majority (65%) of the studies did not describe how they established the absorbance-concentration standard curve, and just one-third of the studies (35%) gave a description of the calibration curve equations, or other spectrophotometric measurement protocols.

From this review it appeared that the necessity of developing separate calibration curves for each species or population was a significant hindrance to wider utilization of this method to determine concentration. Therefore, based on a previous study done in livestock which compared sperm concentrations from bull, boar, and stallion as determined by the use of a single calibration curve (Rondeau and Rouleau 1981), we decided to evaluate the feasibility of using a single calibration to measure sperm concentration across a range of fish species.

Thus, this chapter addresses two goals, the first was to review the literature on previous estimations on fish sperm concentration by the use of spectrophotometer. With this review our objectives were to: (1) provide an overview of the different types of uses for spectrophotometric analyses, and (2) highlight the sources of variations in the technology. The second goal was to evaluate the general utility of turbidity in determining sperm concentration in fish species. The research objectives were: (1) wavelength identification for sperm concentration assessment, (2) development of standard curves for turbidity estimations in seven species, (3) validation of regression models in estimations, (4) determination of the relationship of standard curves across various species, and (5) evaluation of the effects of other cell types such as blood on turbidity measurement of sperm concentration. To our knowledge this is the first review of this topic area and the first report that demonstrates the feasibility of a general curve (instrument-specific) that can be used to measure concentration in fishes where sperm is collected by stripping, and with further procedural modification could apply to aquatic species in which the testes are crushed.

Materials and Methods

Goal One: Literature Review

We sought to use the literature review to form a database and to describe the previous uses and application of turbidity to estimate the sperm concentration in fishes. The first problem that we encountered was the difficulty of identifying scientific publications that used spectroscopy to measure sperm concentration. A simple search using the search terms “sperm concentration” and “fish spectrophotometer” in Google scholar (*scholar.google.com*) resulted in 296 publications. While compiling the publications that specifically used spectrophotometer to

estimate the sperm concentration in fish, two truly influential articles were identified (Suquet et al. 1992a, Ciereszko and Dabrowski 1993) that most of the studies using this technology made reference to. Based on the citations listed for these articles and using ISI Web of Knowledge℠, we found 52 citations for Suquet et al. (1992a) and 90 citations for Ciereszko and Dabrowski (1993). Overall between the yr 1971 and 2009 we found a total of 71 articles (in 32 journals), 9 meeting abstracts, and 2 dissertations that specified use of spectrophotometer to measure sperm concentration. The two journals publishing the most articles were *Aquaculture* (Elsevier: *www.elsevier.com)* with 13 articles and *Aquaculture Research* (Wiley-Blackwell: *www.wiley.com/WileyCDA/*) with 11 articles. The categories within the database constructed were: country of research (based on address of first author), species of fish, purpose of the study, description of the turbidity method, wavelengths tested for maximal absorbance, optimal wavelength used, absolute determination method for the generation of standard curves, correlations between methods, and concentration ranges tested.

Goal Two: General Curve Development

Sperm Collection

The scientific name, common name, sources of fish, and collection methods were listed for the seven species studied (Table 2). The studies were performed for tilapia during January to February of 2003 and the rest during March to August of 2004 at the Aquaculture Research Station of the Louisiana State University Agricultural Center in Baton Rouge.

Table 2. Scientific and common names (arranged by phylogenetic order), sources, sperm collection methods, and published work reporting other results obtained for the same fish.

Common name	Species	Source	Collection method	Dilution ratio[a]	Extender solution	Reference
Paddlefish	*Polyodon spathula*	Forest Hill, LA[b]	Stripped	2:1	HBSS200[c]	Mims and Shelton 2005
Blue catfish	*Ictalurus furcatus*	Inverness, MS[d]	Crushed testis	10:1	HBSS300	Lang et al. 2003
Channel catfish	*Ictalurus punctatus*	Baton Rouge, LA[e]	Crushed testis	10:1	HBSS300	Bates et al. 1996
			Sliced testis	none	HBSS300	Riley et al. 2004
Striped bass	*Morone saxatilis*	San Diego, CA[f]	Stripped	5:1	C-F HBSS300[g]	Thirumala et al. 2006
White bass	*Morone chrysops*	San Diego, CA[f]	Stripped	5:1	C-F HBSS300	Thirumala et al. 2006
Red drum	*Sciaenops ocellatus*	Gulf of Mexico, LA	Stripped	5:1	C-F HBSS200	Wayman et al. 1998
Tilapia[h]	*Oreochromis* sp.	Tiltech Aquafarm, LA	Stripped	32	HBSS300	Segovia et al. 2000

a sperm : extender
b Louisiana Department of Wildlife and Fisheries, Booker Fowler Fish Hatchery
c Hanks' balanced salt solution at 200 or 300 mOsmol/kg
d Harvest Select Farms
e Louisiana State University Agricultural Center, Aquaculture Research Station
f Kent Sea Tech, now Kent BioEnergy Corporation
g C-F HBSS: calcium-free HBSS
h Nile tilapia O. niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

The fish were anesthetized using tricaine methane sulfonate (MS-222, Western Chemical Inc., Ferndale, WA, USA) at 100-150 mg/l (Coyle et al. 2004) prior to stripping. They were removed from anesthesia and the genital papilla was dried with paper towels to avoid activation or contamination of the sperm by water. Samples were stripped carefully, to minimize contamination with urine or feces, and were diluted in Hanks' balanced salt solution (HBSS) or

calcium-free HBSS (C-F HBSS) of appropriate osmotic strength (Table 2). Because sperm cannot be stripped from ictalurid catfishes, testes were removed surgically and crushed in 300 mOsmol/Kg HBSS at a ratio of 1:10 (g testis:mL HBSS) to release sperm. This method yielded a mixture of cell types including mature and immature sperm cells, and somatic cells such as erythrocytes. Channel catfish testes were also sliced and squeezed to directly collect a relatively pure sample of sperm for research purposes.

Hemacytometer Counts and Dilution Preparation

An aliquot of each sperm sample was diluted before counts were made with a hemacytometer (Reichert bright-line, Hausser Scientific, Horsham, PA, USA). A 1:32 dilution (sperm:HBSS) was used for all species, except for red drum (1:500) and white bass (1:1000) which produce highly concentrated sperm samples (> 10^{10} cells per ml). Sperm concentrations were calculated using the average of four replicate hemacytometer counts with the following equation:

(Mean of quadruplet counts x dilution factor) x 50,000 = cells/ml

After the initial sperm concentrations were calculated, the solutions were diluted to contain 10^9, 10^8, 10^7 and 10^6 sperm cells/mL, and these concentrations were validated again by hemacytometer counts.

Spectrophotometer Readings

A Spectronic 20 Genesys™ (Thermo Spectronic, Rochester, NY, USA) was used to obtain the absorbance measurements except where otherwise stated. Disposable 1.5-mL polystyrene cuvets (Semimicro, Fisher Scientific, Pittsburg, PA, USA) with a 10-mm pathlength were used for each sample. Blanks were set using 1.5 mL of the extender used for each species. Diluted sperm samples (1.5 mL) from all seven species were measured at five wavelengths (400, 450, 500, 550 and 600 nm). To determine the wavelength of maximum absorbance, a sperm concentration of 2.5 x 10^8 cells/mL was used in all fishes except for red drum for which 1 x 10^9 cells/mL was used.

Effects of Other Cells Types

To evaluate the influence of blood cells on photometric measurements, we collected blood, sperm from crushed testes, and a relatively pure sperm sample collected by pipet from sliced testes from three channel catfish. Three different concentrations were used (1 x 10^8, 1 x 10^7, and 1 x 10^6 sperm cells/mL) for the sperm samples collected directly from the testes. Blood was collected by caudal puncture using sodium heparin as an anticoagulant (from Becton Dickinson Vacutainer™, Franklin Lakes, NJ, USA). In each of these sperm concentrations, whole blood (on the order of 10^9 blood cells per mL) was added to yield five different final volumetric proportions of blood and sperm (0.125%, 0.25%, 0.375%, 0.5%, and 1%). For example, to prepare one ml of sperm sample with 1% of blood, 10 µL of blood was added to 990 µL of sperm sample. The absorbance of these samples was measured using a spectrophotometer (Spectronic 20) at five wavelengths (400, 450, 500, 550 and 600 nm). The absorbance values were measured by using a scanning microspectrophotometer (Nanodrop® ND-1000 Wilmington, DE, USA) across wavelengths from 220 to 748 nm at 2-nm intervals.

Statistical Analysis

Simple linear regression was used for testing the correlation between absorbance and sperm concentration for each species at the five wavelengths. Data for sperm concentrations were logarithmically transformed (natural logarithm) prior to regression analysis (Berman et al. 1996). To test for significant differences ($P < 0.05$) among linear models, multisource regression with analysis of covariance was used. To test for correlation among species, and among blood percentages, multiple regression was used. Because R^2 values increase with the addition of new variables, the adjusted R^2 (Neter et al. 1996) was used to compensate for added explanatory variables. After determining that there were no differences among them ($P > 0.05$), the observations from Nile tilapia, blue tilapia, Mississippi commercial strain and Florida red tilapia were pooled to strengthen the sampling for tilapia species. The software used for all analyses was SAS® 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Literature Review:

Overview of Uses for Turbidity Analyses

The 82 publications collected represented 18 countries. This reflected a wide diversity of research types and a wide variety of study purposes utilizing spectrophotometer (Table 3). Estimations of sperm concentration by turbidity have been established in at least 41 species of fish and 3 species of mollusks. About 60% of the studies were done in the past 10 yr.

Sources of Variation in the Reports

Approximately 65% of the publications did not report how they standardized the technique, which included the wavelengths tested, wavelengths selected, and sperm concentration ranges tested (Table 3). Wavelengths between 260 and 660 nm have been used to determine sperm concentrations in fish. The most frequently used (20%) wavelength was 505 nm (Figure 4). The most cited reference of technique (50%) was by Ciereszko and Dabrowski (1993), and Ciereszko was an author for 25% of these publications. Another source of variation was the type of spectrophotometer used, as less than half (31 of 75) of the studies named the model of the spectrophotometer. Of these, total of 17 different models were reported.

Establishment of General Curve:

Wavelength Identification

The absorbance spectra of sperm from the seven species were stable within the wavelengths tested (Figure 5); there were no absorption peaks or discrete wavelengths of maximum absorbance within the range tested (400 – 600 nm). Therefore, any visible wavelength could be appropriate for use to generate a standard curve.

Table 3 Previous studies that estimated sperm concentration of aquatic species by spectrophotometry (presented in chronological order). Standarization of the technique include: wavelength tested, absolute determination, coefficient of determination, and concentration range of the standard curve.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Rainbow trout	*Oncorhynchus mykiss*	Spermatogenesis	Yes	200 - 600	410	hemacytometer	0.99	1×10^{10} to 2.8×10^{10}	Billard et al. 1971
Rainbow trout	*Oncorhynchus mykiss*	Spermiation	ND*	ND	410	ND	ND	ND	Billard 1974
Common carp	*Cyprinus carpio*	Spermiation	ND	ND	410	ND	ND	ND	Takashima et al. 1984
Turbot	*Psetta maxima*	Sperm concentration	Yes	300 - 750	420	hemacytometer	0.94	5×10^{9} to 8×10^{10}	Suquet et al. 1992a
Turbot	*Psetta maxima*	Spermiation	ND	ND	420	ND	ND	ND	Suquet et al. 1992b
Rainbow trout	*Oncorhynchus mykiss*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.95	1.9×10^{9} to 2.1×10^{10}	Ciereszko & Dabrowski 1993
Lake whitefish	*Coregonus clupeaformis*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.71	3.4×10^{9} to 1.4×10^{10}	Ciereszko & Dabrowski 1993
Yellow perch	*Perca flavescens*	Sperm concentration	Yes	400 - 700	505	hemacytometer	0.94	3.7×10^{10} to 4.7×10^{10}	Ciereszko & Dabrowski 1993
Turbot	*Psetta maxima*	Sperm characterization	ND	ND	420	ND	ND	ND	Suquet et al. 1993
Eastern oyster	*Crassostrea virginica*	Fertilization trials	ND	ND	650	hemacytometer	ND	ND	Gaffney et al. 1993
Lake whitefish	*Coregonus clupeaformis*	Sperm storage	ND	ND	505	ND	ND	ND	Ciereszko & Dabrowski 1994
Rainbow trout	*Oncorhynchus mykiss*	Sperm storage	ND	ND	505	ND	ND	ND	Ciereszko & Dabrowski 1994
Rainbow trout	*Oncorhynchus mykiss*	Sperm quality	ND	ND	ND	ND	ND	ND	Ciereszko & Dabrowski 1995
Eurasian perch	*Perca fluviatilis*	Sperm characterization	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1995
Turbot	*Psetta maxima*	Fertilization trials	ND	ND	420	ND	ND	ND	Suquet et al. 1995
Bleak	*Alburnus alburnus*	Sperm physiology	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1996a
Blue mussel	*Mytilus edulis*	Larval culture	Yes	200 - 800	320	hemacytometer	0.98	1.3×10^{9} to 1.3×10^{10}	Del Rio-Portilla 1996
Yellowtail flounder	*Limanda ferruginea*	Sperm physiology	Yes	300 - 900	420	hemacytometer	0.92	2.7×10^{9} to 2.7×10^{10}	Clearwater 1996
Muskellunge	*Esox masquinongy*	Sperm characterization	Yes	400 - 800	610	hemacytometer spermatocrit	0.79	7.5×10^{9} to 3×10^{10}	Lin et al. 1996a
Muskellunge	*Esox masquinongy*	Cryopreservation	ND	ND	610	ND	ND	ND	Lin et al. 1996b
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	Yes	ND	505	hemacytometer	ND	ND	Conget et al. 1996
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1996b
Rainbow trout	*Oncorhynchus mykiss*	Reproductive performance	ND	ND	ND	ND	ND	ND	Dabrowski & Ciereszko 1996
Rainbow trout	*Oncorhynchus mykiss*	Sperm storage	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1997
Shovelnose sturgeon	*Scaphirhynchus***	Gynogenesis	ND	ND	ND	ND	ND	ND	Mims et al. 1997
Shovelnose sturgeon	*Scaphirhynchus***	Gynogenesis	ND	ND	ND	ND	ND	ND	Mims & Shelton 1998
Common carp	*Cyprinus carpio*	Gynogenesis	Yes	ND	360	hemacytometer	0.78	$21.2 \pm 12.8 \times 10^{6}$	Porter 1998
Bluegill	*Lepomis macrochirus*	Gynogenesis	Yes	ND	360	hemacytometer	0.84	$6.9 \pm 2.7 \times 10^{6}$	Porter 1998
Brown trout	*Salmo trutta*	Sperm concentration	Yes	ND	505	hemacytometer	0.94	2.2×10^{9} to 2.7×10^{10}	Poole & Dillance 1998
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	hemacytometer	ND	ND	Lahnsteiner et al. 1998

Table 3. Continued.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Atlantic salmon	*Salmo salar*	Reproductive performance	ND	ND	ND	ND	ND	ND	Estay et al. 1999
Bream	*Abramis brama*	Cryopreservation	Yes	ND	530	hemacytometer	0.97	6×10^9 to 2.1×10^{10}	Glogowski et al. 1999
Muskellunge	*Esox masquinongy*	Cryopreservation	ND	ND	ND	ND	ND	ND	Ciereszko et al. 1999
European bass	*Dicentrarchus labrax*	Fertilization trials	Yes	200 - 500	260	hemacytometer	0.97	4×10^7 to 2×10^8	Fauvel et al. 1999
Paddlefish	*Polyodon spathula*	Spermiation	Yes	ND	450	hemacytometer	0.86	1×10^8 to 1.6×10^9	Linhart et al. 2000
Rainbow trout	*Oncorhynchus mykiss*	Cryopreservation	ND	ND	ND	ND	ND	ND	Glogowski et al. 2000
Common carp	*Cyprinus carpio*	Sperm characterization	Yes	ND	610	hemacytometer	ND	$1\text{-}2 \times 10^5$	Dzuba et al. 2001
Argentinian silverside	*Odontesthes bonariensis*	Spermiation	Yes	ND	600	hemacytometer	ND	ND	Miranda et al. 2001
Zebra mussel	*Dreissena polymorpha*	Sperm characterization	Yes	ND	500	hemacytometer	0.98	2.2×10^6 to 1.8×10^7	Ciereszko et al. 2001
African catfish	*Clarias gariepinus*	Spermiation	ND	ND	505	hemacytometer	0.69	5×10^8 to 9×10^9	Viveiros et al. 2001
African catfish	*Clarias gariepinus*	Spermiation	ND	ND	505	hemacytometer	0.88	ND	Viveiros et al. 2002
Sterlet	*Acipenser ruthenus*	Sperm characterization	ND	ND	530	hemacytometer	ND	ND	Piros et al. 2002
Siberian sturgeon	*Acipenser baerii*	Sperm characterization	ND	ND	530	hemacytometer	ND	ND	Piros et al. 2002
Siberian sturgeon	*Acipenser baerii*	Fertilization trials	ND	ND	ND	ND	ND	ND	Glogowski et al. 2002
Rainbow trout	*Oncorhynchus mykiss*	Gamete and embryo storage	ND	ND	ND	ND	ND	ND	Babiak & Dabrowski 2003
African catfish	*Clarias gariepinus*	Spermiation	Yes	ND	505	hemacytometer	0.85	2×10^8 to 1.2×10^{10}	Viveiros et al. 2003
African catfish	*Clarias gariepinus*	Sperm characterization	ND	ND	650	hemacytometer	ND	3×10^9 to 9×10^9	Mansour et al. 2004
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Ciereszko et al. 2004
Siberian sturgeon	*Acipenser baerii*	Cryopreservation	ND	ND	530	ND	ND	ND	Sarosiek et al. 2004
Sterlet	*Acipenser ruthenus*	Cryopreservation	ND	ND	530	ND	ND	ND	Sarosiek et al. 2004
Atlantic cod	*Gadus morhua*	Sperm quality	Yes	ND	260	hemacytometer	0.99	1×10^9 to 5×10^{10}	Suquet et al. 2005
Blue catfish	*Ictalurus furcatus*	Sperm concentration	Yes	400 - 600	600	hemacytometer	0.77	1.1×10^7 to 1×10^8	Campbell et al. 2005a
Channel catfish	*Ictalurus punctatus*	Sperm concentration	Yes	400 - 600	500	hemacytometer	0.53	1×10^6 to 3×10^8	Campbell et al. 2005b
Zebrafish	*Danio rerio*	Cryopreservation	Yes	460, 560, 660	All	hemacytometer	0.97	ND	Yang & Tiersch 2005
Argentinian silverside	*Odontesthes bonariensis*	Spermiation	ND	ND	410	ND	ND	ND	Miranda et al. 2005
Paddlefish	*Polyodon spathula*	Aquaculture	ND	ND	450	hemacytometer	ND	2×10^8 to 1.7×10^9	Mims & Shelton 2005
Brown trout	*Salmo trutta* Linnaeus	Toxicology	ND	ND	405	hemacytometer	ND	ND	Lahnsteiner et al. 2005a
Rainbow trout	*Oncorhynchus mykiss*	Toxicology	ND	ND	ND	ND	ND	ND	Lahnsteiner et al. 2005b
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2005b
Pacific oyster	*Crassostrea gigas*	Sperm concentration	Yes	380 - 780	550, 581	hemacytometer	0.99	2×10^7 to 2×10^9	Dong et al. 2005a
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2005b
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2005c
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2006
Eurasian perch	*Perca fluviatilis*	Sperm characterization	ND	ND	ND	ND	ND	ND	Krol et al. 2006
European bass	*Dicentrarchus labrax*	Spermiation	ND	ND	260	ND	ND	ND	Schiavone et al. 2006

Table 3. Continued.

Species	Scientific name	Purpose of study	Method described	Wavelength tested (nm)	Optimal wavelength	Absolute determination	R^2	Sperm concentration range tested	Citation
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	581	ND	ND	ND	Dong et al. 2007a
Pacific oyster	*Crassostrea gigas*	Cryopreservation	ND	ND	ND	ND	ND	ND	Dong et al. 2007b
Caspian brown trout	*Salmo trutta caspius*	Sperm concentration	Yes	ND	480	hemacytometer spermatocrit	0.91	7×10^8 to 6.5×10^9	Hatef et al. 2007
Brown trout	*Salmo trutta*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007a
Common carp	*Cyprinus carpio*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007a
Common carp	*Cyprinus carpio*	Sperm characterization	ND	ND	ND	ND	ND	ND	Wojtczak et al. 2007
Rainbow trout	*Oncorhynchus mykiss*	Fertilization trials	ND	ND	ND	ND	ND	ND	Dietrich et al. 2007b
Rainbow trout	*Oncorhynchus mykiss*	Spermiation	Yes	ND	410	spermatocrit	0.98	ND	Fitzpatrick & Liley 2008
Rainbow trout	*Oncorhynchus mykiss*	Fertilization trials	ND	ND	ND	ND	ND	ND	Tuset et al. 2008
Rainbow trout	*Oncorhynchus mykiss*	Sperm characterization	ND	ND	ND	ND	ND	ND	Dietrich et al. 2008
Arctic char	*Salvelinus alpinus*	Fertilization trials	ND	ND	ND	ND	ND	ND	Mansour et al. 2008a
Arctic char	*Salvelinus alpinus*	Cryopreservation	ND	ND	ND	ND	ND	ND	Mansour et al. 2008b
Blue mussel	*Mytilus edulis*	Sperm concentration	Yes	200 - 800	320	hemacytometer coulter counter	0.99	9.4×10^5 to 1.1×10^7	Del Rio Portilla & Beaumont 2008
Powan	*Coregonus lavaretus*	Cryopreservation	ND	ND	ND	ND	ND	ND	Ciereszko et al. 2008
Brook trout	*Salvelinus fontinalis*	Sperm concentration	ND	ND	ND	NucleoCounter	0.96	8.2×10^9 to 1.8×10^{10}	Nynca & Ciereszko 2009
Zebrafish	*Danio rerio*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.92	2.2×10^7 to 5.9×10^8	Tan et al. 2010
Swordtail	*Xiphophorus helleri*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.94	ND	Tan et al. 2010
Medaka	*Oryzias latipes*	Sperm concentration	Yes	200 - 780	380 - 700	hemacytometer	0.93	ND	Tan et al. 2010
Powan	*Coregonus lavaretus*	Sperm characterization	ND	ND	530	ND	ND	ND	Hliwa et al. 2010
European bass	*Dicentrarchus labrax*	Sperm quality	ND	200 - 500	260	hemacytometer	0.97	ND	Fauvel et al. 2010
Gilthead bream	*Sparus aurata*	Sperm quality	ND	200 - 500	260	hemacytometer	0.96	ND	Fauvel et al. 2010
Turbot	*Psetta maxima*	Sperm quality	ND	200 - 500	260	hemacytometer	0.95	ND	Fauvel et al. 2010
Wreckfish	*Polyprion americanus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.98	ND	Fauvel et al. 2010
Bluefin tuna	*Thunnus thynnus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.84	ND	Fauvel et al. 2010
Striped catfish	*Pangasianodon hypophthalmus*	Sperm quality	ND	200 - 500	260	hemacytometer	0.86	ND	Fauvel et al. 2010
Atlantic cod	*Gadus morhua*	Sperm quality	ND	200 - 500	260	hemacytometer	0.99	1×10^6 to 5×10^7	Fauvel et al. 2010

*ND: not described; **Scaphirhynchus platorynchus

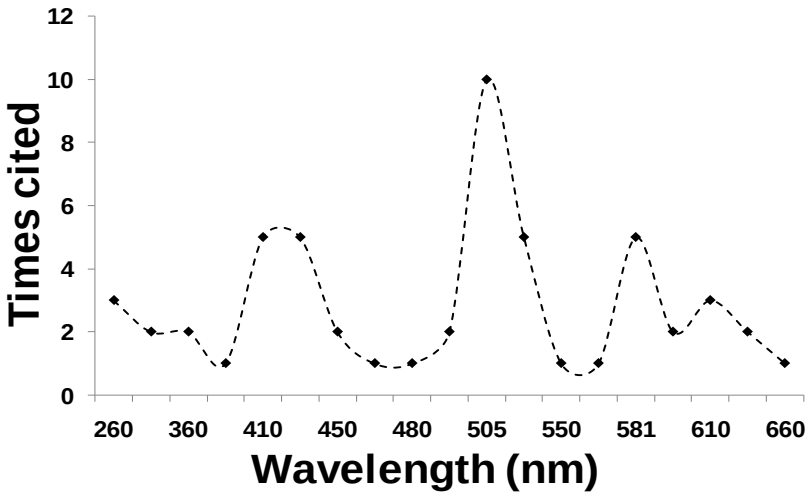

Figure 4. Frequency of use of various wavelengths evaluated in 54 previous studies for the development of standard curves to estimate sperm concentration in fishes.

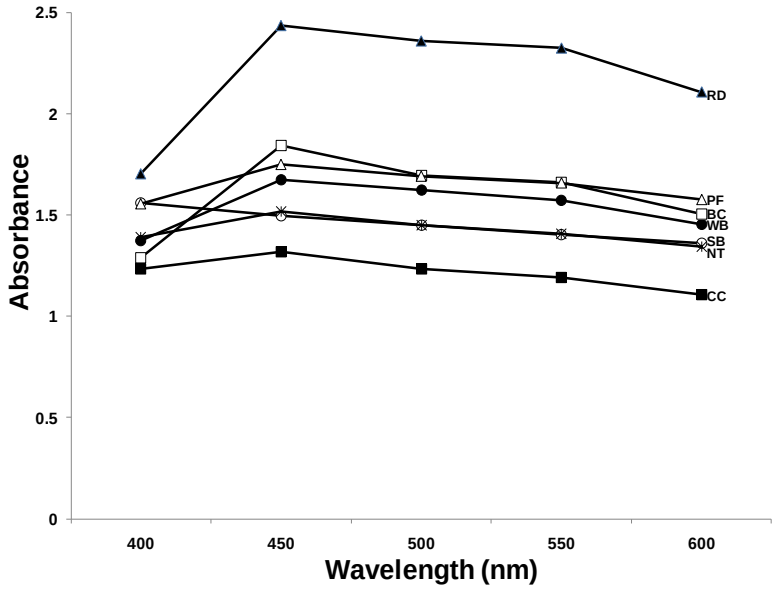

Figure 5. Absorbance spectra measured in this study for sperm of red drum (RD: filled triangles), paddlefish (PF: open triangles), blue catfish (BC: open squares), channel catfish (CC: filled squares), white bass (WB: filled circles), striped bass (SB: open circles), and Nile tilapia (NT: asterisks). Absorbance values were based on using 2.5 x 10^8 cells/mL for all species, except red drum at 1 x 10^9 cells/mL.

Development of Standard Curves

A linear relationship (R^2 values from 0.586 to 0.904) was found between the natural logarithm of sperm concentration (1 x 10^6 to 6 x 10^{10} cells/mL) assessed by hemacytometer counting and the corresponding absorbance in the different wavelengths used (Table 4). The strongest correlations at the different wavelengths remained stable for the majority of the species,

except at 400 nm for red drum (adjusted $R^2 = 0.040$). This indicated that there was a wide range of wavelengths that could be used to estimate sperm concentration.

Table 4. Standard curves, linear regression equations, and coefficient of determination from the logarithmic regression of sperm counts and absorbance at the different wavelengths tested.

Common name	N[1]	Obs[2]	Wavelengths (nm)				
			400	450	500	550	600
Blue catfish	15	72	y= -2.994 + 0.215 lnX Adj R^2 = 0.810	y= -3.418 + 0.243 lnX Adj R^2 = 0.739	y= -3.130 + 0.222 lnX Adj R^2 = 0.730	y= -2.977 + 0.212 lnX Adj R^2 = 0.694	y= -2.678 + 0.190 lnX Adj R^2 = 0.703
Channel catfish	47	202	y= -2.067 + 0.162 lnX Adj R^2 = 0.587	y= -2.450 + 0.185 lnX Adj R^2 = 0.601	y= -2.348 + 0.176 lnX Adj R^2 = 0.603	y= -2.233 + 0.167 lnX Adj R^2 = 0.586	y= -2.118 + 0.158 lnX Adj R^2 = 0.592
Paddlefish	4	11	y= -6.799 + 0.446 lnX Adj R^2 = 0.901	y= -8.663 + 0.553 lnX Adj R^2 = 0.889	y= -8.567 + 0.544 lnX Adj R^2 = 0.885	y= -8.442 + 0.535 lnX Adj R^2 = 0.881	y= -8.106 + 0.513 lnX Adj R^2 = 0.877
Red drum	5	14	y= 1.495 + 0.010 lnX Adj R^2 = 0.040	y= -2.702 + 0.243 lnX Adj R^2 = 0.777	y= -3.246 + 0.265 lnX Adj R^2 = 0.805	y= -3.040 + 0.253 lnX Adj R^2 = 0.784	y= -1.228 + 0.157 lnX Adj R^2 = 0.791
Striped bass	12	45	y= -5.626 + 0.349 lnX Adj R^2 = 0.850	y= -6.457 + 0.397 lnX Adj R^2 = 0.860	y= -6.285 + 0.386 lnX Adj R^2 = 0.853	y= -6.129 + 0.376 lnX Adj R^2 = 0.846	y= -5.914 + 0.362 lnX Adj R^2 = 0.840
Tilapia[3]	69	114	y= -5.207 + 0.336 lnX Adj R^2 = 0.843	y= -6.523 + 0.410 lnX Adj R^2 = 0.820	y= -6.379 + 0.400 lnX Adj R^2 = 0.816	y= -6.214 + 0.389 lnX Adj R^2 = 0.812	y= -5.895 + 0.369 lnX Adj R^2 = 0.814
White bass	21	75	y= -4.476 + 0.289 lnX Adj R^2 = 0.871	y= -6.588 + 0.411 lnX Adj R^2 = 0.904	y= -6.525 + 0.406 lnX Adj R^2 = 0.902	y= -6.476 + 0.402 lnX Adj R^2 = 0.901	y= -5.879 + 0.366 lnX Adj R^2 = 0.903

1N = number of fish
2Obs = number of observations, each observation is a different concentration
3 Nile tilapia Oreochromis niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

Validation of Regression Models

When validating the equation generated with the concentrations provided by hemacytometer counts, there were no significant differences between the observed values by counts and the predicted values of the standard curves, except when comparing the predicted concentrations from the general curve of all species (combining the data for crushed testis and stripped sperm) ($P = 0.001$).

Relationship of Standard Curves Among Species

When comparing the regression curves across species within individual genera (i.e. *Ictalurus, Morone,* and *Oreochromis),* there were no differences, except for blue and channel catfish at 400 and 450 nm, and white and striped bass at 400 nm (Table 5). When comparing the curves across all species (independent of the genus), there were significant differences for all the wavelengths ($P < 0.001$). However, when catfishes were removed from the model, there was no difference in the curves of paddlefish, red drum, striped bass, and tilapias for all the wavelengths except 400 nm (Table 5). Overall, the linear relationship of catfishes followed a pattern different from the values of the other species studied (Figure 6). When the absorbance values of catfishes were compared with those for other fishes within the absorbance range of 0.1 to 2.5 at concentrations of lower than 1×10^9 cells/mL, there was an overestimation of absorbance for the catfishes at any given concentration. This difference was likely because suspensions from crushed testes were contaminated with somatic cells such as erythrocytes which increased the

absorbance values. Overestimation was greater at lower sperm concentrations due to the higher relative proportion of somatic cells in relation to sperm cells (Figure 6).

Table 5. Comparison of standard curves among species. The curves were compared by an analysis of covariance (ANCOVA) at each wavelenght. Multiple regression was used to correlate among the species the absorbance, and natural logarithmic of sperm concentration.

Common name	N[1]	Obs[2]	Wavelengths (nm)				
			400	450	500	550	600
Blue & channel catfish	2	274	p-value = 0.022	p-value = 0.028	p-value = 0.061	p-value = 0.068	p-value = 0.149
White & striped bass	2	120	p-value = 0.019	p-value = 0.633	p-value = 0.497	p-value = 0.379	p-value = 0.889
Tilapia[3]	4	114	p-value = 0.057	p-value = 0.296	p-value = 0.252	p-value = 0.248	p-value = 0.239
All species	8	533	R^2 = 0.774 p-value = <.001	R^2 = 0.806 p-value = <.001	R^2 = 0.811 p-value = <.001	R^2 = 0.805 p-value = <.001	R^2 = 0.805 p-value = <.001
All species without catfish	6	259	R^2 = 0.875 p-value = <.001	R^2 = 0.899 p-value = 0.358	R^2 = 0.896 p-value = 0.401	R^2 = 0.895 p-value = 0.334	R^2 = 0.890 p-value = 0.183
All species without blood	8	282	R^2 = 0.879 p-value = <.001	Adj R^2 = 0.899 p-value = 0.079	Adj R^2 = 0.896 p-value = 0.041	Adj R^2 = 0.894 p-value = 0.023	Adj R^2 = 0.900 p-value = 0.013
Blood	1	108	p-value = <.001	p-value = 0.208	p-value = 0.067	p-value = 0.188	p-value = 0.149

1N = number of species
2Obs = number of observations, each observation is a different concentration
3 Nile tilapia O. niloticus, blue tilapia O. aureus, Mississippi commercial strain, Florida red tilapia

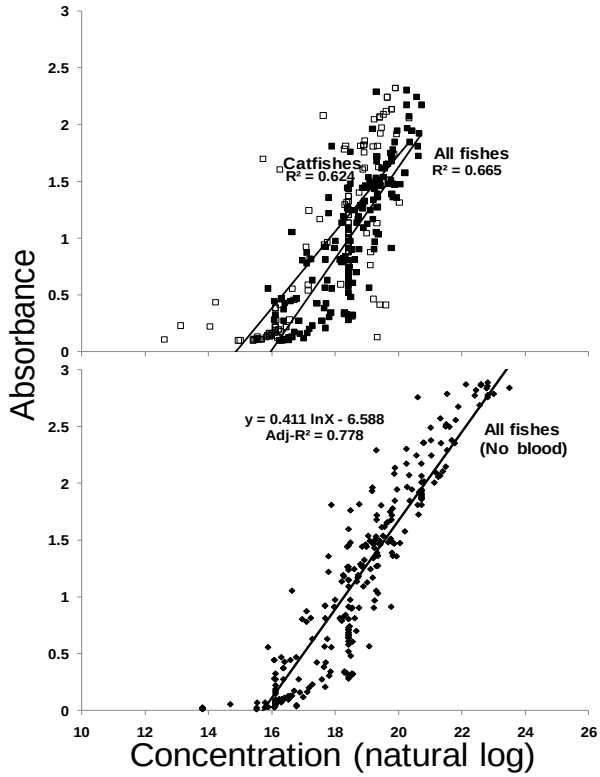

Figure 6. Relationship between absorbance at 450 nm and sperm counts by hemacytometer. Upper panel: regression lines for all fishes without catfishes, and catfishes only. The absorbance range was 0.1 to 2.5, and the maximum sperm concentration was 1 x 10^9 cells/ml. Lower panel: standard curve for all fishes, including catfish, with no blood.

Subsequent plotting of data for catfish sperm collected without blood (relatively pure samples) were combined with the data for paddlefish, red drum, tilapia, white bass and striped bass, and there was no significant difference for each wavelength except for 400 nm (Table 5). Also, the estimated values from the standard curve of all fish samples without blood had no significant differences (P = 0.181 at 450 nm) when compared with the hemacytometer counts. Moreover, plotting of the observed values of all species without blood against the standard curve generated from readings at 450 nm indicated a strong relationship (R^2 = 0.778) (Figure 6).

Effect of Blood

The interaction of the different blood percentages was significant for the 400 nm wavelength (Table 5). As the volumetric proportions of blood increased, the absorbance values also increased (Figure 7).

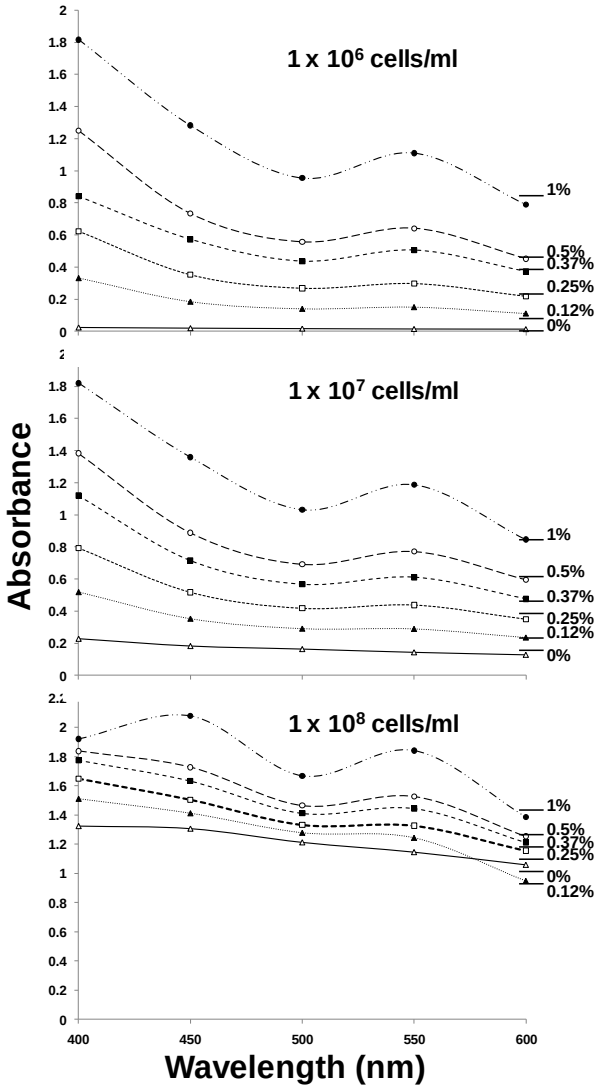

Figure 7. Absorbance values measured at wavelengths between 400 nm and 600 nm for three concentrations of channel catfish sperm with different volumetric proportions of blood. Upper panel: absorbance values using 1 x 10^6 sperm cells/mL with different percentages of blood; middle panel: 1 x 10^7 cells/mL; lower panel: 1 x 10^8 cells/mL.

A broad absorbance peak did not occur at low volumetric proportions of blood but the peak increased with higher proportions of blood. This is consistent with the absorbance spectra for pure blood samples. When the absorbance of blood was measured at different wavelengths, there was an increase in absorbance at 450 nm, with a maximum at 500 to 550 nm (Figure 8).

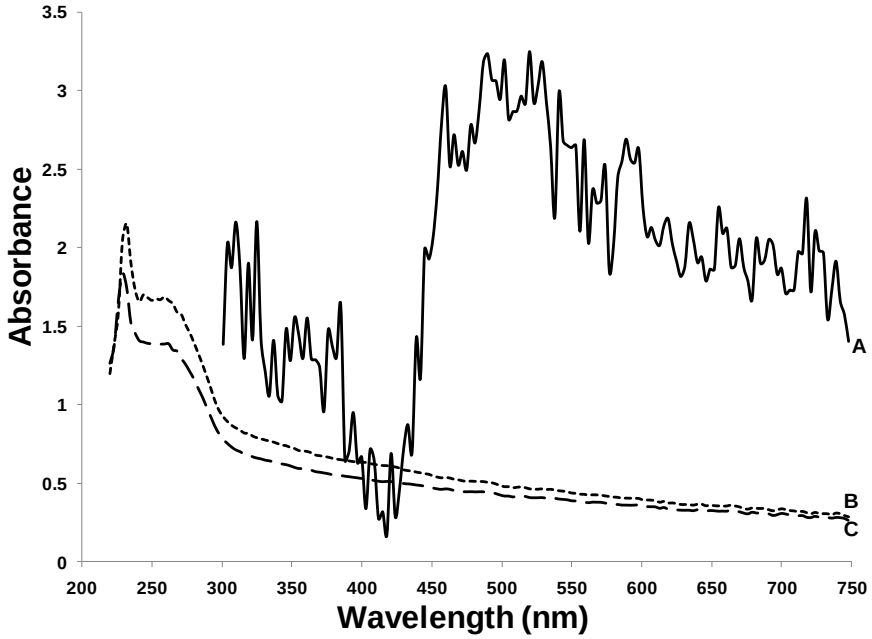

Figure 8. Absorbance values measured at wavelengths between 220 nm to 748 nm for: A) channel catfish blood, B) sperm collected from crushed testis, and C) sperm without blood. Absorbance values were the average of three males with a sperm concentration of 1 x 10^8 cells/mL. There was no maximal absorbance peak within the visible spectrum for the samples. The maximal absorption of blood was between 500 to 550 nm. Absorbance values of samples below 300 nm were variable.

Absorbance values of blood at wavelengths lower than 300 nm were inconsistent. However, the absorbance of sperm from crushed testes and sperm without blood remained constant. Although a maximum absorbance was detected at 230 nm, the absorbance values (greater than 1.2) yielded high variability (Figure 8). Within the visible wavelengths (390 – 750 nm) there was no absorbance peak. In general there was a gradual linear reduction in absorbance as the wavelength increased. Therefore, at low sperm concentrations (< 1 x 10^6 cells/mL) the absorbance values were influenced more by the presence of blood. As the sperm concentrations increased (> 1 x 10^7 cells/mL), there was an increase of absorbance caused by the sperm. When no blood was added the absorbance values were directly related to the sperm concentration (Figure 7).

Discussion

Sperm concentration is rarely reported during studies of aquatic species, and typically when reported does not include a description of the methods used. This leads to problems for comparison and reproducibility of published studies especially for activities such as cryopreservation and fertilization trials. The estimation of sperm concentration is essential for a range of activities including standardization of cryopreservation, optimization of fertilization, calibration of ultraviolet irradiation to induce gynogenesis, and study of spermiation following

hormonal stimulation. Despite measurements in more than 40 species, currently there is a lack of specific information regarding sperm concentration determination and how it relates to cryopreservation and fertilization in essentially all aquatic species (Tiersch et al. 2007). Traditionally in livestock species, sperm concentrations have been estimated by the use of cell counting devices such as the hemacytometer or other specialized counting chambers (Foote et al. 1978, Prathalingam et al. 2006). Although by observing the sperm using a microscope, other parameters such as morphology could be determined. But counting chambers are time consuming, and require the use of microscopes and trained technicians typically not available in hatchery settings. As such, most aquaculture work does not include sperm concentrations (Campbell et al. 2005a, Dong et al. 2005a).

In the search for faster and more practical ways to estimate relative or absolute sperm concentration, centrifugation (to determinate spermatocrit) and spectrophotometer (to determine turbidity) have been used. Because spermatocrit and absorbance are easy to measure, the choice of methods has generally been based on access to equipment (Tvedt et al. 2001). Spermatocrit is an indirect method which is expressed as the volume of sperm in relation to the total volume of sample (packed cell volume divided by total sample volume). One of the common problems in spermatocrit estimation is the lack of a clear separation between the packed sperm cells and the seminal fluid; this can lead to false estimations of spermatocrit. To avoid this problem, prolonged centrifugation times are needed, usually more than 10 min, but as long as 45 min in species with dense sperm samples such as striped bass (Vuthiphandchai and Zohar 1999). An additional problem is the relatively large volume (at least 0.1 ml) needed (Lin et al. 1996b).

Sperm evaluation should be rapid and effective so that samples can be processed efficiently to preserve initial quality and fertility (Foote 1980). Spectrophotometric determination of turbidity is recognized as a reliable, efficient, and rapid technique to estimate the concentration within semen samples in farm animals (Brillard and McDaniel 1985). From the previous work that used spectrophotometer to determine sperm concentration in aquatic species, there has been no attempt to evaluate the feasibility of generating a general calibration curve.

Literature Review:

Previous studies that estimated sperm concentration by spectrophotometry in fishes are characterized by a lack of description of the methodology used (Table 3). This failure of reporting and in defining procedures limits reproducibility, weakens results, and makes direct comparisons among studies problematic or impossible. There is a pressing need for development of standardized protocols. Less than 20% of the studies in our literature review tested different wavelengths to identify the wavelength of maximum absorbance (this may or may not be a large problem depending on the instrument used). Our review also found that one third of the studies used wavelengths between 500 to 550 nm which can be affected by the presence of blood in the samples (Figure 7). The reference cited most (18 of 54, 33%) in the methods sections of published papers was by Ciereszko and Dabrowski (1993). This publication addressed comparison of three methods for sperm concentration determination (i.e., spectrophotomer, hemacytometer, and spermatocrit). Almost all of these publications (16 of 18) did not state which of these three methods were actually used (e.g., Rinchard et al. 2001, Kowalski et al. 2006). In an attempt to compare previous studies with the present study, we estimated the sperm concentration from the equations described in six studies and plotted them (Figure 9). The differences among studies can be explained by the difference in instruments (see below) and the ranges of sperm concentration tested.

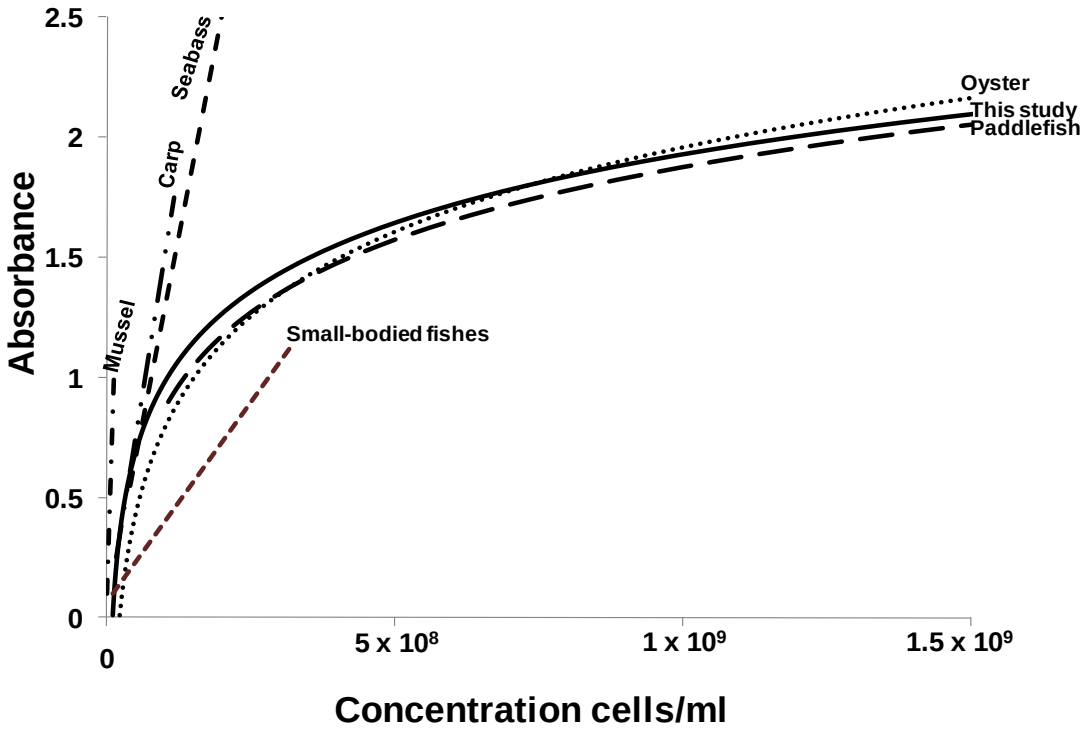

Figure 9. Relationships between absorbance and sperm concentration in previous studies: blue mussel (Del Rio-Portilla and Beaumont 2008), common carp (Takashima et al. 1984), seabass (Fauvel et al. 1999), Pacific oyster (Dong et al.), paddlefish (Linhart et al.), small-bodied fishes (zebrafish, medaka, and green swordtail) (Tan et al. 2010), and a multi-species curve (this study).

A spectrophotometer is generally composed of one or more light sources, a wavelength selector, sample container, detector, signal processor, and readout devices. In a turbidity measurement a spectrophotometer is used to measure the attenuation of light as a function of wavelength as it passes through a sample. Because previous studies used different wavelengths and different spectrophotometers, the instrument characteristics (such as light transmission properties of the sample cell, aperture size, distance between sample cell and detector, and pathlengths) were different. For example, cuvettes made of plastic or glass can be used to measure within the visible spectrum, while a cuvette made of quartz glass or fused silica should be used for the UV region (i.e., below 350 nm). In addition, there are several types of detectors such as photovoltaic cells, vacuum photodiodes, photomultiplier tubes, and silicon photodiodes (Cole and Levine 2003). Each detector has a differential sensitivity of wavelength range (Cole and Levine 2003). All of these components and configurations are instrument specific and constitute some of the known and unknown sources of variation in previous studies.

Previous studies in livestock compared the estimation of sperm concentration using different spectrophotometers. Comparison of duplicate instruments yielded nearly identical results in bull sperm (Foote 1972, Foote et al. 1978) while the use of different models of spectrophotometers resulted in different readings for the same sperm samples from boar (Knox et al. 2002, Knox 2004). It is important to note that one study compared three spectrophotometers for the sperm of boar, bull, and stallion (Rondeau and Rouleau 1981). Two of the instruments yielded no difference in standard curves, and the authors concluded that if spectrophotometers have the same characteristics in terms of spectral bandwidth, the calibration curves need not be statistically different. Even so, variable maintenance and calibration of instruments, different

types and quality of cuvettes, and the lifespan of instrument lamps could introduce differences in responsivity and accuracy across time for a single instrument or among duplicate instruments.

Feasibility of establishing a general curve:

Determination of an absorption spectrum for analyzing a given sample material is the first step of turbidimetry analysis to identify the maximum sensitivity for measurements. This is typically done by plotting the absorbance measurement as a function of wavelength (Dong et al. 2005a, Tan et al. 2010). Wavelengths in the range of 260-660 nm have been used to determine sperm concentrations in fishes. The livestock industry has used wavelengths in the range of 275 to 630 nm (Foote et al. 1978). Wavelengths in the range of 550 to 576 nm appear most sensitive for white suspensions (color induced absorbance) such as for sperm, and a wavelength of 550 nm is recommended and mostly used (Foote et al. 1978, Knox 2004). In the present study, we tested five wavelengths (400, 450, 500, 550 and 600 nm) with seven species. There was no single wavelength that yielded maximum absorbance. This indicates that any of these wavelengths could be appropriate to generate a standard curve.

Other studies found that lower wavelengths yield greater variation. For example, for zebrafish the maximum absorbance of sperm was around 265 nm but the absorbance profiles at wavelengths below 380 nm (UV) varied widely for single samples and the use of wavelengths above 400 nm was recommended (Tan et al. 2010). In a different study with blue mussels, the maximum absorbance was at 216 nm but the absorbance values had a large variation and the use of wavelengths above 320 nm was recommended (Del Rio-Portilla and Beaumont 2008). In contrast, another study recommended the use of 260 nm based on the hypothesis that differences in optical density using this wavelength in different fishes were due to the DNA content. This report compared the light absorption of sperm of turbot *Psetta maxima*, seabream *Sparus auratus*, and seabass *Dicentrarchus labrax* but without a standardized concentration (Fauvel et al. 2010). This hypothesis and other claims such as that volume changes in sperm can be tracked by their absorbance (Dzuba and Kopeika 2002), should be addressed in future research. In general, lower wavelengths yield higher transmittance values and thus have been recommended for use (e.g., Del Rio-Portilla and Beaumont 2008). Absorbance values below 0.1 and above 1.0 represent 10% and 20% transmittance, respectively, while absorbance values above 2.0 represent ≤1% transmittance. In earlier spectrophotometers, this low transmittance could result in inaccurate readings, although this is not normally a problem with current instruments (Mantle and Harris 2000). Earlier spectrophotometers used vacuum photodiodes while current spectrophotometers use silicon photodiodes and optical filters with higher resolution.

Overall, there are three main types of spectrophotometers: 1) Visible spectrophotometers that have inexpensive glass components, use tungsten lamps as the light source, and operate across a range of 325 to 1,000 nm. Older instruments of this type rely on blue- and red-sensitive phototubes. 2) Ultraviolet-Visible spectrophotometers that measure absorbance in the 200 to 1,000 nm range. For instruments of this type the most common source of radiation is the hydrogen-discharge lamp, but if more intensity is desired (3-5 times) a deuterium-discharge lamp is used. 3) Infrared spectrophotometers that use a heat source (i.e. Globar and Nernst glower) and the spectra result from molecular vibrational transitions in the range of 750–15,000 nm (Csuros 1997). These have been applied for multi-component analyses of fish meat (Elvingson and Sjaunja 1992) but have not been used for estimation of sperm concentrations.

In this study, we evaluated wavelengths within the visible spectrum (390–750 nm). This is the first study that attempted to evaluate a general standard curve to measure sperm

concentration for fish species. Although there is a publication in which a single formula was used to measure sperm concentration in the Siberian sturgeon *Acipenser baerii* and sterlet *A. ruthenus*, there was no explanation of the reasoning behind this usage, or if both species were validated for that equation (Sarosiek et al. 2004). It has been stated as a common belief that different calibration curves are required for different species because of the specificities of the sperm (Foote et al. 1978). However, as stated above, a general standard curve has been established for bulls, boars, and stallions (Rondeau and Rouleau 1981), and there were no differences among the slopes of the calibration curves for these livestock species. Whether for mammals or for fish, seminal samples consist of seminal plasma (or seminal fluid) and spermatozoa. Fish seminal plasma contains mainly mineral compounds and low concentrations of organic substances (Ciereszko et al. 2000a). The absorbance of seminal plasma (< 0.1) was measured in seabass and yellowtail flounder *Limanda ferruginea*, which led to the conclusion that seminal plasma did not interfere within the wavelength range (200–900 nm) tested (Clearwater 1996, Fauvel et al. 1999). In fact, the effect of light scattering and light absorption by spermatozoa has been shown to dominate the effect of light absorption by seminal plasma (Rothschild 1950, Taneja and Gowe 1961). Therefore, in the present study there was little justification to remove the seminal plasma before estimating the concentration by the use of spectrophotometer. We hypothesized that a single calibration curve could be used to determine the concentration of spermatozoa for most fish species.

In this study, after comparing the data of all fishes, the correlation coefficient of the standard curve at all wavelengths was higher than 0.77. But the validations of the observed values against the spectrophotometric estimation were significantly different. This was resolved however when the collection methods (stripped and crushed) were separated. The correlation coefficients for all species without catfish were higher ($R^2 = 0.87$) and there was no statistical difference between observed and estimated values at wavelengths higher than 450 nm. Differences in absorbance between sperm collection methods (stripped and crushed) have been reported in zebrafish and green swordtail *Xiphophorus helleri* (Tan et al. 2010), although a robust applicability was reported in that study among all of the curves generated across species (zebrafish, swordtail, and medaka *Oryzias latipes*), and different collection techniques (stripped and crushed). For relatively pure, homogenous sperm samples, if the sperm size and shape among species are similar, the changes in light absorbed among samples will primarily be due to a difference in sperm cell concentration (Rondeau and Rouleau 1981). In addition, studies have shown that dilute suspensions of most bacteria have nearly the same absorbance per unit of dry weight concentration, regardless of the variation of cell size and shape (Omstead 1990). Therefore, based on our results, we postulate that a general standard curve for any single instrument should be able to measure concentration for most fishes from which pure samples of sperm can be collected.

To evaluate the effect of other cell types such as those in whole blood, sperm with no overt blood contamination was collected in catfishes. When these absorbance values were combined with the data for all species without blood, the resulting correlation value was the same as that observed for all fishes collected by stripping. When there was no addition of blood to the samples, the absorbance was directly related to the sperm concentration. Therefore blood cells and other cell types that are mixed with sperm during crushing of the testis can interfere with accurate estimation of sperm concentration. Depending on the timing in relation to the spawning season, the ratio of somatic cells and germ cells can vary considerably. Crushing of the testes can release a mixture of cells types such as spermatogonia, spermatocytes, spermatids,

spermatozoa, and Sertoli-like cells (Viveiros 2003). Failure to properly clean the testes before crushing could also contaminate samples with connective tissues that contain Leydig-like cells, nerve fibers, fibroblasts, collagen fibers, smooth muscle cells, and endothelial cells (Grier and Uribe 2009). In addition, the cytoplasm of epithelial cells of the main testicular ducts and spermatic ducts contains lipid vacuoles, and the seminal fluids also contain lipids during interspawning periods (Lahnsteiner and Patzner 2009). Based on our observations contamination of this sort can lead to a systematic overestimation of sperm concentration in direct relationship to the volume of blood or other contaminants present as described above (Figure 7). Other studies found similar effect, when debris present in the raw semen, such as cytoplasmic droplets, affected the accuracy of the spectrophotometric method (Christensen et al. 2004). Thus, depending on spawning condition the correlation between absorbance and sperm concentration could be affected by somatic contamination, and more work needs to be done to evaluate measurements of samples collected from crushed testes at different times of the year. It should be noted that microscopic observations can be used to assess the level of contamination of samples before measuring the absorbance (Figure 3).

This is not the first publication to mention that other cell types such as blood can disturb absorbance measurements. In fact, the presence of other cells in the sperm of the landlocked sea lamprey *Petromyzon marinus* disrupted the use of spectrophotometry (Ciereszko et al. 2000b). Stripped samples of the African catfish *Clarias gariepinus* were contaminated with blood and the turbidity estimation for sperm concentration could not be applied (Viveiros et al. 2003). And, in African catfish two types of sperm samples, "white" and "grey", were collected by dissection and stripping of the testes. The white samples had a higher sperm cell concentration and absorbance values (650 nm) than did grey samples (Mansour et al. 2004). The white samples were characterized by high sperm densities and a low number of spermatids, while the grey samples contained numerous germinal cysts with spermatids in addition to sperm. In another study, different levels of blood contamination of rainbow trout *Oncorhynchus mykiss* milt were obtained in relation to sampling period and method of milt collection (Ciereszko et al. 2004). In an attempt to measure the influence of blood in sperm samples, blood was added to a pure sample (final volumetric proportion of blood was 0.2%) of rainbow trout sperm (sperm concentration 9×10^9 to 1.4×10^{10} cells/mL) (Ciereszko et al. 2004). Sperm quality parameters (osmolality, protein concentration, lactate dehydrogenase activity) were not affected by the contribution from this small amount of blood and although this study used turbidity methods it did not report any influence of blood on the estimation of sperm concentration (we presume due to the high proportion of sperm cells in relation to the erythrocytes).

In the present study, five different volumetric proportions of blood were tested with three concentrations of sperm. Although the absorbance values for the different volumes of blood were different, there was no statistical difference in the standard curves for wavelengths higher than 450 nm. This corresponded with the sperm collected by crushed testes in which higher correlations were obtained at wavelengths from 500 to 600 nm. Contamination with as much as 1% blood, did not affect the standard curve for concentrations as low as 1×10^6 cells/mL. This indicates that blood contamination might not be a major factor of concern when measuring concentration by spectrophotometer in samples with a high proportion of sperm cells in relation to blood cells (Figure 7, lower panel). It could however be expected that higher proportions of blood to sperm (> 1%) at low sperm concentrations would influence the absorbance, especially at the wavelengths of maximum absorbance of blood (> 450 nm) which corresponds to the peaks of maximum absorbance of oxyhemoglobin (540 and 575 nm) (Stryer 1995).

The effects caused by crushing of testes with respect to contamination of the samples are not simple or straightforward. Errors in absorbance or sperm concentration can result in overestimations or underestimations. For example, use of a calibration curve developed during the peak of the spawning season using pure sperm when applied to crushed testis samples could yield overestimations of concentration (based on the inflated absorbance values of the samples). Or, conversely use of a curve developed early in the spawning season when testes did not contain large volumes of mature sperm would result in an upward-shifted curve (based on higher somatic absorbances) and could yield underestimates in concentration later in the spawning season when sperm production peaks. Thus, the types of errors are affected by variations of cell types in relation (proportion) to one another and this relationship varies over time due to reproductive seasonality. As such, the observed effects can sometimes be small between crushed testis and pure sperm samples (e.g., Figure 8), but the potential contribution of cells such as erythrocytes to absorbance should not be overlooked. It is also important to note that the patterns observed in the absorbance profiles generated by blood addition (Figure 7) do not exactly match the absorbance profile of pure blood (Figure 8) likely due to the relative contributions of the various components in contaminated samples (as described above) to the aggregate profile. Hemoglobin itself can exist in a variety of forms with different absorbance profiles based on interactions with atmospheric gases (Stryer 1995).

The testes of ictalurid catfishes in particular present an even more complicated picture with regard to contamination caused by crushing. The testes possess two recognizable portions: anterior and posterior (Figure 10) (Sneed and Clemens 1963). These two portions are characterized by different cells types, and their relative sizes and color vary depending on the spawning period. The anterior portion is considered to be spermatogenic in function and is small, flat and transparent outside of the spawning season, but expands dramatically, turning white with finger-like extensions, and composing 2/3 of the testis, as sperm production fills the tubules and lumen (Guest et al. 1976). This portion contains the majority of available sperm and is sometimes the only portion of the testis to be harvested while the posterior portion is discarded (Tiersch et al. 1994). The posterior portion has been reported to function as an accessory glandular organ that secretes a mucopolysaccharide-protein-lipid-rich fluid (seminal vesicular fluid) believed to contribute to the seminal volume and participate in prolongation and stabilization of sperm viability (Chowdhury and Joy 2007). The posterior testis is composed of interstitial cells, fibroblasts, blood capillaries, and nerve elements. During spawning the epithelial cells of the posterior portion contain an abundance of rough endoplasmic reticulum, Golgi apparatus, secretory vacuoles, and electron-dense secretion products (Chowdhury and Joy 2007). The role of the posterior portion is most likely involved in maturation and nutrition of sperm, although it possesses a similar sperm concentration (based on cells per wet weight of tissue) as the anterior (Guest et al. 1976, Jaspers et al. 1978). The size and color of the posterior portion is more variable than the anterior portion (our unpublished observation) and it can be larger or smaller than the anterior portion, although it is almost darker (pink to light red to brown).

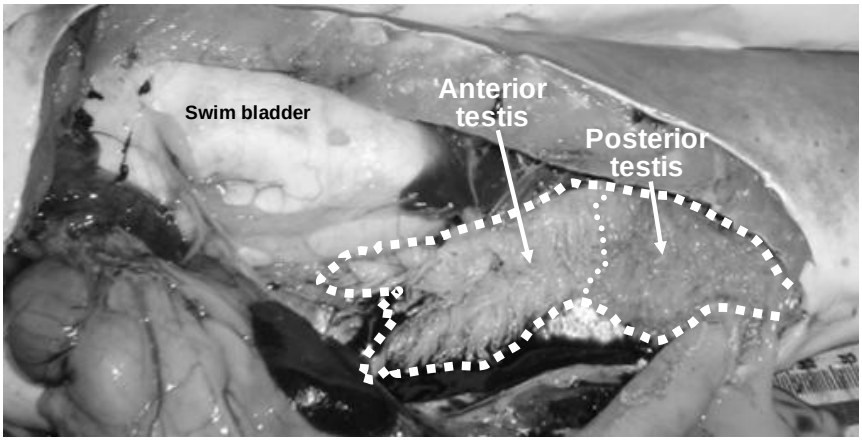

Figure 10. View of channel catfish testes within the body cavity during the spawning period (April to May in Southern Louisiana). The anterior portion is considered to be spermatogenic and the posterior portion to serve glandular functions. Dissection ahynd crushing of the testis to collect sperm can involve both portions or only the anterior portion, and can contribute a variety of somatic cells and other contaminants to samples

As indicated above, sperm collection can proceed by crushing of the anterior portion alone, or as an admixture with the posterior portion yielding a complex collection of cell types and compounds within the sample with potential for considerable effects on the aggregate absorbance values. In this study, we chose to crush and mix both portions of the testes to fully capture the variation encountered in different protocols. From the previous discussion it should be evident that development of calibration curves from either portion or their combination would present considerable variation throughout reproductive seasonality and offers great opportunity for standardization of protocols and reporting to reduce or minimize variation within and among studies and hatchery operations. Future studies are needed to more fully evaluate these portions of the ictalurid testis in terms of biological function and the methods appropriate for their use in practical spawning protocols (including cryopreservation).

Protocols describing methodology to use turbidity to measure sperm concentrations have been published previously for livestock (Foote 1972, Foote et al. 1978), and a recent study was performed to illustrate development and standardization of photometric measurement of sperm concentration in Pacific oysters *Crassostrea gigas* (Dong et al. 2005a). Procedurally, care should be taken when collecting sperm, because contamination with other substances such as urine could affect calibration and concentration estimates (Clearwater 1996). The viscosity of the sperm often makes it difficult to obtain a homogeneous dilution of the sample, and depending on the pipette and tips used for sample handing, different values could be obtained from the same sample. Therefore thorough mixing of sperm suspensions before measurement is essential for accurate readings (Cabrita et al. 2009). The presence or absence of small aggregations of spermatozoa in an aliquot of dilution could affect the accuracy and precision of sperm concentration estimates (Rakitin et al. 1999). These types of errors are magnified when working with the limited sperm samples (2-4 µl) collected from small-bodied fish such as zebrafish (Tan et al. 2010). Standard curves should be established for each spectrophotometer and regular calibrations are needed to ensure accurate estimation of sperm concentration (Knox et al. 2002). In the present study, standard curves generated at wavelengths from 450 to 550 nm, within the range of 1×10^6 to 6×10^{10} cells/mL were effective for determination of the concentration of sperm from paddlefish, red drum, tilapias, white bass, and striped bass, and would likely be

useful for other fish species. Data for sperm concentration should be logarithmically transformed prior to application of simple linear regression. Such log transformation has been suggested previously (Berman et al. 1996, Handelsman 2002) and applied in Pacific oysters (Dong et al. 2005a), yellowtail flounder (Clearwater 1996), paddlefish (Linhart et al. 2000), and blue catfish (Campbell et al. 2005a). It is important to note that the generation of a standard curve will be specific for each type of spectrophotometer or instrument and even different laboratories, because not all conditions can be expected to be the same among locations (Knox et al. 2002). Accurate and comprehensive reporting of methods is thus necessary to accurately evaluate and compare studies.

Although the hemacytometer is considered as the "gold standard" for measurement of sperm concentration, there are variations among different designs and operators (Seaman et al. 1996, Christensen et al. 2005). Estimation of sperm concentration from a spectrophotometric determination of turbidity is routinely used in artificial insemination of mammals because it is fast and precise (Prathalingam et al. 2006). The precision of turbidity estimation derives from standard curves produced from multiple hemacytometer counts. Although some have considered spectrophotometer to be costly and time consuming (Powell 2002), there is little evidence to support this observation. Compared to a hemacytometer there is a higher initial cost for the spectrophotometer (Table 6). However, the same spectrophotometer can be used for other purposes such as measuring water quality or general laboratory analyses. There are portable spectrophotometers that are used in the daily routine of fish farming, and could provide an inexpensive and rapid method for sperm concentration determination in field conditions (Dietrich et al. 2005a, Dong et al. 2005a). The use of a portable spectrophotometer (Eppendorf, Germany) has been reported to estimate the sperm concentration in whitefish (Ciereszko et al. 2008). Other options are microspectrophotometers that work with microliter sample volumes. These microspectrophotometers can be essential to measure sperm concentration for small-bodied fishes such as zebrafish that only yield 2-4 µL total of sperm sample (Tan et al. 2010).

Other techniques available to estimate sperm concentration include computer-assisted sperm analysis (CASA) instruments (e.g., Hamilton Thorne, Beverly, MA, USA; SQA-V Medical Electronic Systems, Los Angeles, CA, USA), flow cytometry, and fluorescence microscopy, and Coulter counter. These techniques require specialized instruments that are prohibitively expensive if they are used only to measure sperm concentration. A disadvantage for using CASA is that it often requires a specific disposable chamber in which non-uniform distribution of the spermatozoa can lead to false estimations (Lu et al. 2007). There is no replacement for a direct observation of the sperm to detect other sperm quality parameters such as morphology and the presence of clumps or other types of contaminants. There is image analysis software available for free downloading distributed by the National Institutes of Health (*rsbweb.nih.gov/ij*) that has been used to estimate sperm concentration in Atlantic bluefin tuna *Thunnus thynnus thynnus* (Mylonas et al. 2007).

Table 6. Comparison of price, volume requirements, and wavelengths available for different spectrophotometers.

Spectrophotometer	Cost (US$)	Volume needed	Wavelength range (nm)	Web address
Ultrospec 10, GE HealthCare[1]	742	1.5 ml	600	www.gelifesciences.com
Biowave, Biochrom WPA[1]	762	10 µl	190 - 900	www.biochrom.co.uk
YSI 9300[1]	807	10 ml	450 - 650	www.ysi.com
Smart2, LaMotte[1]	909	10 ml	350 - 1000	www.lamotte.com
DR890 Colorimeter, Hach[1]	1,177	2.5 ml	420 - 610	www.hach.com
Genesys 20, Thermo Scientific[1]	1,862	1 ml	325 - 1100	www.thermo.com
590b Densimeter, ARS[2]	1,895	200 µl	ND[3]	www.arssales.com
BioPhotometer, Eppendorf[1]	4,585	50 µl	230 - 650	www.eppendorf.com
GeneQuant, BioChrom[1]	5,071	7 µl	190 - 900	www.gelifesciences.com
Epoch, BioTek[1]	8,950	2 µl	200 - 999	www.biotek.com
NanoDrop, Thermo Scientific[1]	8,950	0.5 µl	190 - 840	www.nanodrop.com

The use of flow cytometry to measure sperm concentration has yielded variable results (Lu et al. 2007, Anzar et al. 2009) and depends on how the counts are performed (i.e., typically in relation to a known concentration of a fluorescent bead internal standard), the concentration range tested, and whether the emphasis is on precision or accuracy (Haugen 2007). Future research needs to be conducted to compare newer designs of flow cytometers (such as the Accuri C6®, Ann Arbor, MI, USA) which measure the actual volume that is pulled from the sample and can directly estimate cell counts without the need of fluorescent bead standards. Another new flow cytometry device (S-FCM, Kobe, Japan) was developed to measure sperm concentration. This device has been reported to be suitable for measurement of human sperm concentration (Tsuji et al. 2002). A recent publication demonstrated that computer-aided fluorescent microscopy (NucleoCounter SP-100, Denmark) could be used to measure sperm concentration in brook trout *Salvelinus fontinalis* (Nynca and Ciereszko 2009). Coulter counters have long been accepted as a reliable technique for particle sizing and counting (Brillard and McDaniel 1985) and are available in some fish hatcheries for ploidy determination (Wattendorf 1986), but are expensive for sperm counting only. Other techniques such as cell-UV chambers, and packed cell volumes (e.g., VoluPAC, Sartorius, Germany) need to be evaluated for aquatic species. Potentially, a general standard curve for fishes could be incorporated into analysis-specific spectrophotometers (e.g., 590B Densimeter, Animal Reproduction System, Chino, CA, USA). Such densimeters are designed to measure sperm concentrations of stallions, bulls, boars, canines, roosters, and turkeys.

Conclusions

We conclude that estimation of sperm concentration is essential for many studies in aquatic species and that reports using sperm estimations from spectrophotometric determination of turbidity should include at a minimum: the spectrophotometer model and type, cuvette description, wavelengths used, absolute determination method (e.g., hemacytometer) used, range

of sperm concentrations tested, absorbance range tested, the standard curve, linear regression equation, and the coefficient of determination. In this study, a single general (instrument-specific) standard curve generated at wavelengths of 450 to 600 nm within the range of 1×10^6 to 6×10^{10} cells/mL was developed for determining the concentration of sperm from seven different fish species where sperm was collected by stripping. With further study or procedural modifications this could apply to fishes for which the testes are crushed. This would have broad applicability in reproductive studies and is essential for standardization of cryopreservation.

The importance of sperm concentration determination in livestock has been recognized since 1939 (Comstock and Green 1939). Sperm concentration is vital in artificial insemination because the number of sperm determines how many females can be inseminated (Foote 1972). The livestock industry sells packaged semen for artificial insemination of livestock in "frozen doses" that supply a specific number of sperm. This requires a high level of strict quality control and standardization for all of the parameters involved in the dose, including sperm concentration. Standardization and reproducibility are key factors for the success of this industry for livestock (Thibier and Wagner 2002).

Sperm cryopreservation is a proven technique for developing, maintaining, and distributing genetic improvement in livestock, and provides great unexploited potential for fish breeding. In addition, the availability of frozen sperm allows the creation of genetic resource repositories and conservation programs to increase the potential breeding population size to ensure that proper genetic combinations are produced in breeding of endangered species (Tiersch 2008). The future development and utility of technologies such as cryopreservation will rely on standardization and control of major variables such as sperm concentration. The results presented in this chapter call attention to the need for standardization and suggest that variation in sperm concentration results can be influenced more by the instrument used than the species studied.

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References

Aas, G. H., T. Refstie and B. Gjerde. 1991. Evaluation of milt quality of Atlantic salmon. Aquaculture 95:125-132.

Alavi, S. M. H., O. Linhart, K. Coward and M. Rodina. 2008. Fish spermatology: implications for aquaculture management. *In*: Fish spermatology. S. M. H. Alavi, J. Cosson, K. Coward and G. Rafiee, editors. Alpha Science International Ltd., Oxford, United Kingdom. Pp. 397-460.

Anzar, M., T. Kroetsch and M. M. Buhr. 2009. Comparison of different methods for assessment of sperm concentration and membrane integrity with bull semen. Journal of Andrology 30:661-668.

Aravindakshan, J., V. Paquet, M. Gregory, J. Dufresne, M. Fournier, D. J. Marcogliese and D. G. Cyr. 2004. Consequences of xenoestrogen exposure on male reproductive function in spottail shiners (*Notropis hudsonius*). Toxicological Sciences 78:156-165.

Auger, J., F. Eustache, B. Ducot, T. Blandin, M. Daudin, I. Diaz, S. E. Matribi, B. Gony, L. Keskes, M. Kolbezen, A. Lamarte, J. Lornage, N. Nomal, G. Pitaval, O. Simon, I. Virant-Klun, A. Spira and P. Jouannet. 2000. Intra- and inter-individual variability in human sperm concentration, motility and vitality assessment during a workshop involving ten laboratories. Human Reproduction 15:2360-2368.

Babiak, I. and K. Dabrowski. 2003. Refrigeration of rainbow trout gametes and embryos. Journal of Experimental Zoology Part A: Comparative Experimental Biology 300A:140-151.

Bates, M. C., W. R. Wayman and T. R. Tiersch. 1996. Effect of osmotic pressure on the activation and storage of channel catfish sperm. Transactions of the American Fisheries Society 125:798-802.

Berman, N. G., C. Wang and C. A. Paulsen. 1996. Methodological issues in the analysis of human sperm concentration data. Journal of Andrology 17:68-73.

Billard, R. 1974. La production spermatogénétique de la truite arc-en-ciel au cours du premier cycle reproducteur. Bulletin Français de Pisciculture 253:139-149.

Billard, R., B. Breton and B. Jalabert. 1971. La production spermatogénétique chez la truite. Annales de Biologie Animale Biochimie Biophysique 11:199-212.

BjoRndahl, L., M. Tomlinson and C. L. R. Barratt. 2004. Raising standards in semen analysis: professional and personal responsibility. Journal of Andrology 25:862-863.

Brazil, C., S. H. Swan, C. R. Tollner, C. Treece, E. Z. Drobnis, C. Wang, J. B. Redmon and J. W. Overstreet. 2004. Quality control of laboratory methods for semen evaluation in a multicenter research study. Journal of Andrology 25:645-656.

Brillard, J. P. and G. R. McDaniel. 1985. The reliability and efficiency of various methods for estimating spermatozoa concentration. Poultry Science 64:155-158.

Brown, K. I., K. E. Nestor and M. Topscher. 1970. A comparison of various methods of estimating spermatozoa concentration of turkey semen. Pultry Science 49:1267-1271.

Buffagni, A. and M. Furse. 2006. Intercalibration and comparison – major results and conclusions from the STAR project. Hydrobiologia 566:357-364.

Burbank, R. C. 1935. The quantitative standardization of sperm suspensions by means of opacity. Quarterly Journal of Experimental Physiology 25:393-397.

Cabrita, E., V. Robles and P. Herraez. 2009. Sperm quality assessment. *In*: Methods in reproductive aquaculture: marine and freshwater species. E. Cabrita, V. Robles and P. Herraez, editors. CRC Press, Boca Raton, Florida, USA. Pp. 93-147.

Campbell, W. T., A. M. Guitreau and T. R. Tiersch 2005a. Spectrophotometric estimation of sperm concentration in blue catfish. Book of Abstracts, Louisiana Chapter of the American Fisheries Society meeting. Baton Rouge, Louisiana, USA. P. 12.

Campbell, W. T., P. Pawiroredjo, A. M. Guitreau and T. R. Tiersch 2005b. Evaluating sperm concentration in channel catfish. Book of Abstracts, World Aquaculture Society meeting. New Orleans, Louisiana, USA. P. 57.

Castilla, J. A., R. R. de Assín, M. C. Gonzalvo, A. Clavero, J. P. Ramírez, F. Vergara and L. Martínez. 2010. External quality control for embryology laboratories. Reproductive BioMedicine Online 20:68-74.

Chowdhury, I. and K. Joy. 2007. Seminal vesicle and its role in the reproduction of teleosts. Fish Physiology and Biochemistry 33:383-398.

Christensen, P., J. P. Stenvang and W. L. Godfrey. 2004. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. Journal of Andrology 25:255-264.

Christensen, P., H. Stryhn and C. Hansen. 2005. Discrepancies in the determination of sperm concentration using Burker-Turk, Thoma and Makler counting chambers. Theriogenology 63:992-1003.

Ciereszko, A. and K. Dabrowski. 1993. Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. Aquaculture 109:367-373.

Ciereszko, A. and K. Dabrowski. 1994. Relationship between biochemical-constituents of fish semen and fertility - the effect of short-term storage. Fish Physiology and Biochemistry 12:357-367.

Ciereszko, A. and K. Dabrowski. 1995. Sperm quality and ascorbic-acid concentration in ranbow-trout semen are affected by dietary vitamin C - a cross-season study. Biology of Reproduction 52:982-988.

Ciereszko, A., K. Dabrowski, F. Lin, S. A. Christ and G. P. Toth. 1999. Effects of extenders and time of storage before freezing on motility and fertilization of cryopreserved muskellunge spermatozoa. Transactions of the American Fisheries Society 128:542-548.

Ciereszko, A. and K. Dabrowski. 2000. Effect of ascorbic acid supplement in vitro on rainbow trout sperm viability. Aquaculture International 8:1-8.

Ciereszko, A., Dabrowski, K., Piros, B., Kwasnik, M., Glogowski, J. . 2001. Characterization of zebra mussel (*Dreissena polymorpha*) sperm motility: duration of movement, effects of cations, pH and gossypol. Hydrobiologia 452:225-232.

Ciereszko, A., G. J. Dietrich, M. Wojtczak, M. Sobocki, P. Hliwa, H. Kuzminski, S. Dobosz, M. Slowinska and J. Nynca. 2008. Characterization and cryopreservation of whitefish (*Coregonus lavaretus* L.) semen from Lake Lebsko, Poland. Fundamental and Applied Limnology 173:59-65.

Ciereszko, A., J. Glogowski and K. Dabrowski. 2000a. Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. *In*: Cryopreservation in aquatic species. T. R. Tiersch and P. M. Mazik, editors. World Aquaculture Society, Baton Rouge, Louisiana, USA. Pp. 20-48.

Ciereszko, A., J. Glogowski and K. Dabrowski. 2000b. Fertilization in landlocked sea lamprey: storage of gametes, optimal sperm: egg ratio, and methods of assessing fertilization success. Journal of Fish Biology 56:495-505.

Ciereszko, A., T. Wlasow, S. Dobosz, K. Goryczko and J. Glogowski. 2004. Blood cells in rainbow trout *Oncorhynchus mykiss* milt: relation to milt collection method and sampling period. Theriogenology 62:1353-1364.

Clearwater, S. J. 1996. The reproductive physiology of yellowtail flounder (*Pleuronectes ferrugeneus*) with an emphasis on sperm physiology. Ph.D. thesis, Memorial University of Newfoundlad, St. John's, Canada, 199 pp.

Cole, K. and B. Levine. 2003. Spectrophotometry. *In*: Principles of Forensic Toxicology. B. Levine, editor. AACC Press, Washington, D.C., USA. Pp. 79-88.

Comstock, R. E. and W. W. Green. 1939. Methods for semen evaluation I. Density, respiration, glycolysis of semen. The American Society of Animal Production 32:213-216.

Conget, P., M. Fernandez, G. Herrera and J. J. Minguell. 1996. Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) spermatozoa using programmable freezing. Aquaculture 143:319-329.

Cooper, T. G., B. Hellenkemper and E. Nieschlag. 2007. External quality control for semen analysis in Germany (QuaDeGA) the first 5 years. Journal fur Reproduktionsmedizin und Endokrinologie 6:331-335.

Cosson, J. J. 2008. Methods to analyze the movements of fish spermatozoa and their flagella. *In*: Fish spermatology. S. M. H. Alavi, J. J. Cosson, K. Coward and G Rafiee, editors. Alpha Science International Ltd., Oxford, United Kingdom. Pp. 63-102.

Coyle, S. D., R. M. Durborow and J. H. Tidwell. 2004. Anaesthetics in Aquaculture. Southern Regional Aquaculture Center (SRAC), Publication Number 3900.

Csuros, M. 1997. Environmental sampling and analysis. CRC Press, Boca Raton, Florida, USA, 373 pp.

Csuros, M. and C. Csuros 1999. Microbial Examination of Water and Wastewater. CRC Press, Boca Raton, Florida, USA, 324 pp.

Dabrowski, K. and A. Ciereszko. 1996. Ascorbic acid protects against male infertility in a teleost fish. Experientia 52:97-100.

Del Rio-Portilla, M. A. and A. R. Beaumont. 2008. Sperm concentration in the mussel *Mytilus edulis* L.: a spectrophotometric measurement protocol. Aquaculture International 16:573-580.

Del Rio Portilla, M. A. 1996. Genetic aspects of *Mytilus edulis* in relation to spawning and hatchery culture. Ph.D. Thesis, University of Wales, Bangor, Wales. Pp. 213.

Dietrich, G. J., R. Kowalski, M. Wojtczak, S. Dobosz, K. Goryczko and A. Ciereszko. 2005a. Motility parameters of rainbow trout (*Oncorhynchus mykiss*) spermatozoa in relation to sequential collection of milt, time of post-mortem storage and anesthesia. Fish Physiology and Biochemistry 31:1-9.

Dietrich, G. J., A. Szpyrka, M. Wojtczak, S. Dobosz, K. Goryczko, L. Zakowski and A. Ciereszko. 2005b. Effects of UV irradiation and hydrogen peroxide on DNA fragmentation, motility and fertilizing ability of rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Theriogenology 64:1809-1822.

Dietrich, G. J., M. Zabowska, M. Wojtczak, M. Slowinska, D. Kucharczyk and A. Ciereszko. 2007a. Effects of different surfactants on motility and DNA integrity of brown trout (*Salmo trutta* fario) and common carp (*Cyprinus carpio*) spermatozoa. Reproductive Biology 7:127-142.

Dietrich, G. J., M. Wojtczak, M. Slowinska, S. Dobosz, H. Kuzminski and A. Ciereszko. 2007b. Broken eggs decrease pH of rainbow trout (*Oncorhynchus mykiss*) ovarian fluid. Aquaculture 273:748-751.

Dietrich, G. J., M. Wojtczak, M. Sowiska, S. Dobosz, H. Kuzminski and A. Ciereszko. 2008. Effects of ovarian fluid on motility characteristics of rainbow trout (*Oncorhynchus mykiss* Walbaum) spermatozoa. Journal of Applied Ichthyology 24:503-507.

Dong, Q. X., B. Eudeline, C. Huang and T. R. Tiersch. 2005a. Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture Research 36:86-93.

Dong, Q. X., B. Eudeline, C. J. Huang, S. K. Allen and T. R. Tiersch. 2005b. Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. Cryobiology 50:1-16.

Dong, Q. X., C. J. Huang, B. Eudeline, S. K. Allen and T. R. Tiersch. 2006. Systematic factor optimization for sperm cryopreservation of tetraploid Pacific oysters, *Crassostrea gigas*. Theriogenology 66:387-403.

Dong, Q. X., C. J. Huang, B. Eudeline and T. R. Tiersch. 2005c. Systematic factor optimization for cryopreservation of shipped sperm samples of diploid Pacific Oysters, *Crassostrea gigas*. Cryobiology 51:176-197.

Dong, Q. X., C. J. Huang and T. R. Tiersch. 2007a. Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: Evidence from sperm agglutination in oysters. Cryobiology 54:87-98.

Dong, Q. X., C. J. Huang, B. Eudeline and T. R. Tiersch. 2007b. Cryoprotectant optimization for sperm of diploid Pacific oysters by use of commercial dairy sperm freezing facilities. Aquaculture 271:537-545.

Dzuba, B. B., G. A. Bozhok and S. V. Rudenko. 2001. A study of the dynamics of volume changes during the period of active motility in carp, *Cyprinus carpio* L., spermatozoa. Aquaculture Research 32:51-56.

Dzuba, B. B. and E. F. Kopeika. 2002. Relationship between the changes in cellular volume of fish spermatozoa and their cryoresistance. Cryoletters 23:353-360.

Elvingson, P. and L. O. Sjaunja. 1992. Determination of fat, protein and dry matter content of fish by mid-infrared transmission spectroscopy. Aquaculture Research 23:453-460.

Erdahl, A. W. and E. F. Graham. 1987. Fertility of teleost semen as affected by dilution and storage in a seminal plasma-mimicking medium Aquaculture 60:311-321.

Estay, F., C. Vergara and N. F. Diaz. 1999. Reproductive performance of cultured Atlantic salmon *Salmo salar* L-1758 in Chile. Aquaculture Research 30:759-764.

Evenson, D. P., J. E. Parks, M. T. Kaproth and L. K. Jost. 1993. Rapid determination on sperm cell concentration in bovine semen by flow cytometry. Journal of Dairy Science 76:86-94.

Fauvel, C., O. Savoye, C. Dreanno, J. Cosson and M. Suquet. 1999. Characteristics of sperm of captive seabass in relation to its fertilization potential. Journal of Fish Biology 54:356-369.

Fauvel, C., M. Suquet and J. Cosson. 2010. Evaluation of fish sperm quality. Journal of Applied Ichthyology 26:636-643.

Fitzpatrick, J. L., J. C. Henry, N. R. Liley and R. H. Devlin. 2005. Sperm characteristics and fertilization success of masculinized coho salmon (*Oncorhynchus kisutch*). Aquaculture 249:459-468.

Fitzpatrick, J. L. and N. R. Liley. 2008. Ejaculate expenditure and timing of gamete release in rainbow trout *Oncorhynchus mykiss*. Journal of Fish Biology 73:262-274.

Foote, R. H. 1972. How to measure sperm cell concentration by turbidity (optical density). Proceedings of the Fourth Technical Conference on Artificial Insemination and Reproduction. National Association of Animal Breeders, NAAB. Pp. 57-61.

Foote, R. H. 1980. Artificial insemination. *In*: Reproduction in Farm Animals. E. S. E. Hafez, editor. Lea and Febiger, Philadelphia, PA, USA. Pp. 521-545.

Foote, R. H., J. Arriola and R. J. Wall 1978. Principles and procedures for photometric measurement of sperm cell concentration. Proceedings of the seventh Technical Conference on Artificial Insemination and Reproduction. National Association of Animal Breeders, Madison, Wisconsin, USA. Pp. 55-61.

Gaffney, P. M., C. M. Bernat and S. K. Allen. 1993. Gametic incompatibility in wild and cultured populations of the eastern oyster, *Crassostrea virginica* (Gmelin). Aquaculture 115:273-284.

Glogowski, J., I. Babiak, D. Kucharczyk, M. Luczynski and B. Piros. 1999. Some properties of bream *Abramis brama* L-sperm and its cryopreservation. Aquaculture Research 30:765-772.

Glogowski, J., R. Kolman, M. Szczepkowski, A. Horvath, B. Urbanyi, P. Sieczynski, A. Rzemieniecki, J. Domagala, W. Demianowicz, R. Kowalski and A. Ciereszko. 2002. Fertilization rate of Siberian sturgeon (*Acipenser baeri*, Brandt) milt cryopreserved with methanol. Aquaculture 211:367-373.

Glogowski, J., M. Kwasnik, B. Piros, K. Dabrowski, K. Goryczko, S. Dobosz, H. Kuzminski and A. Ciereszko. 2000. Characterization of rainbow trout milt collected with a catheter: semen parameters and cryopreservation success. Aquaculture Research 31:289-296.

Grier, H. J. and M. C. Uribe. 2009. The testis and spermatogenesis in teleosts. *In*: Reproductive Biology and Phylogeny of Fishes. B. G. M. Jamieson, editor. Science Publishers, Enfield, New Hampshire, USA. Pp. 119-142.

Guest, W. C., J. W. Avault and J. D. Roussel. 1976. A spermatology study of channel catfish, *Ictalurus punctatus*. Transactions of the American Fisheries Society 105:463-468.

Handelsman, D. J. 2002. Optimal power transformations for analysis of sperm concentration and other semen variables. Journal of Andrology 23:629-634.

Hansen, C., T. Vermeiden, J. P. W. Vermeiden, C. Simmet, B. C. Day and H. Feitsma. 2006. Comparison of FACSCount AF system, Improved Neubauer hemocytometer, Corning 254 photometer, SpermVision, UltiMate and NucleoCounter SP-100 for determination of sperm concentration of boar semen. Theriogenology 66:2188-2194.

Hatef, A., H. Niksirat, B. M. Amiri, S. M. H. Alavi and M. Karami. 2007. Sperm density, seminal plasma composition and their physiological relationship in the endangered Caspian brown trout (*Salmo trutta caspius*). Aquaculture Research 38:1175-1181.

Haugen, T. B. 2007. In search of an accurate and rapid method for sperm counting. Scandinavian Journal of Clinical and Laboratory Investigation 67:436-438.

Hliwa, P., G. J. Dietrich, M. Dietrich, J. Nynca, A. Martyniak, M. Sobocki, R. Stabinski and A. Ciereszko. 2010. Morphology and histology of cranial and caudal lobes of whitefish (*Coregonus lavaretus* L.) testes. Fundamental and Applied Limnology / Archiv für Hydrobiologie 176:83-88.

Jaspers, E. J., J. W. Avault and J. D. Roussel. 1978. Testicular and spermatozoal characteristics of channel catfish, *Ictalurus punctatus*, outside the spawning season. Transactions of the American Fisheries Society 107:309-315.

Jonckheere, J., N. Callewaert, A. J. Grootenhuis, W. M. Kersemaekers and T. G. Cooper. 2005. Feasibility study for the centralized measurement of sperm concentration. International Journal of Andrology 28:311-315.

Keel, B. A., T. W. Stembridge, G. Pineda and N. T. Serafy. 2002. Lack of standardization in performance of the semen analysis among laboratories in the United States. Fertility and Sterility 78:603-608.

Knox, R. V. 2004. Practicalities and pitfalls of semen evaluation. Advances in pork production 15:315-322.

Knox, R. V., S. L. Rodriguez-Zas and K. Ruggiero. 2002. Use and accuracy of instruments to estimate sperm concentration: pros, cons and economics. Proceedings, American Association of Swine Veterinarians 33rd Annual Meeting. Kansas City, Missouri, USA. Pp. 20-31.

Kowalski, R. K., P. Hliwa, A. Andronowska, J. Krol, G. J. Dietrich, M. Wojtczak, R. Stabinski and A. Ciereszko. 2006. Semen biology and stimulation of milt production in the European smelt (*Osmerus eperlanus* L.). Aquaculture 261:760-770.

Krol, J., J. Glogowski, K. Demska-Zakes and P. Hliwa. 2006. Quality of semen and histological analysis of testes in Eurasian perch *Perca fluviatilis* L. during a spawning period. Czech Journal of Animal Science 51:220-226.

Lahnsteiner, F., B. Berger, M. Kletzl and T. Weismann. 2005a. Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta* f. fario. Aquatic Toxicology 75:213-224.

Lahnsteiner, F., B. Berger, F. Grubinger and T. Weismann. 2005b. The effect of 4-nonylphenol on semen quality, viability of gametes, fertilization success, and embryo and larvae survival in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 71:297-306.

Lahnsteiner, F., B. Berger, T. Weismann and R. Patzner. 1995. Fine structure, and motility of spermatozoa and composition of the seminal plasma in the perch. Journal of Fish Biology 47:492-508.

Lahnsteiner, F., B. Berger, T. Weismann and R. A. Patzner. 1996a. Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. Fish Physiology and Biochemistry 15:167-179.

Lahnsteiner, F., B. Berger, T. Weismann and R. A. Patzner. 1996b. Physiological and biochemical determination of rainbow trout, *Oncorhynchus mykiss*, semen quality for cryopreservation. Journal of Applied Aquaculture 6:47-73.

Lahnsteiner, F., B. Berger, T. Weismann and R. A. Patzner. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture 163:163-181.

Lahnsteiner, F. and R. A. Patzner. 2009. Male reproductive system: spermatic duct and accessory organs of the testis. *In*: Reproductive biology and phylogeny of fishes. B. G. M. Jamieson, editor. Science Publishers, Enfield, New Hampshire, USA. Pp. 141-186.

Lahnsteiner, F., T. Weismann and R. A. Patzner. 1997. Aging processes of rainbow trout semen during storage. Progressive Fish-Culturist 59:272-279.

Lang, R. P., K. L. Riley, J. E. Chandler and T. R. Tiersch. 2003. The use of dairy protocols for sperm cryopreservation of blue catfish *Ictalurus furcatus*. Journal of the World Aquaculture Society 34:66-75.

Leung, L. K. P. and B. G. M. Jamieson. 1991. Live preservation of fish gametes. *In*: Fish Evolution and Systematics: Evidence From Spermatozoa. B. G. M. Jamieson, editor. Cambridge University Press, Cambridge, United Kingdom. Pp. 245-269.

Lin, F., A. Ciereszko and K. Dabrowski. 1996a. Sperm production and cryopreservation in muskellunge after carp pituitary extract and human chorionic gonadotropin injection. Progressive Fish-Culturist 58:32-37.

Lin, F., L. Liu and K. Dabrowski. 1996b. Characteristics of muskellunge spermatozoa .1. Ultrastructure of spermatozoa and biochemical composition of semen. Transactions of the American Fisheries Society 125:187-194.

Linhart, O., S. Mims, B. Gomelsky, A. Hiott, W. Shelton, J. Cosson, M. Rodina and D. Glea. 2000. Spermination of paddlefish (*Polyodon spathula,* Acipenseriformes) stimulated with injection of LHRH analogue and carp pituitary powder. Aquatic Living Resources 13:1-6.

Lu, J. C., F. Chen, H. R. Xu, Y. M. Wu, X. Y. Xia, Y. F. Huang and N. Q. Lu. 2007. Is flow cytometry really adapted to the determination of sperm concentration? Scandinavian Journal of Clinical and Laboratory Investigation 67:394-401.

Mansour, N., F. Lahnsteiner and B. Berger. 2004. Characterization of the testicular semen of the African catfish, *Clarias gariepinus* (Burchell, 1822), and its short-term storage. Aquaculture Research 35:232-244.

Mansour, N., F. Lahnsteiner, M. A. McNiven and G. F. Richardson. 2008a. Morphological characterization of Arctic char, *Salvelinus alpinus*, eggs subjected to rapid post-ovulatory aging at 7 ^{o}C. Aquaculture 279:204-208.

Mansour, N., G. F. Richardson and M. A. McNiven. 2008b. Effect of seminal plasma protein on postthaw viability and fertility of Arctic char spermatozoa. North American Journal of Aquaculture 70:92-97.

Mantle, T. J. and D. A. Harris. 2000. Spectrophotometric assays. *In*: Spectrophotometry and Spectrofluorimetry: A Practical Approach. M. G. Gore, editor. Oxford University Press, New York, New York, USA. Pp. 183-208.

Mims, S. D. and W. L. Shelton. 1998. Induced meiotic gynogenesis in shovelnose sturgeon. Aquaculture International 6:323-329.

Mims, S. D. and W. L. Shelton. 2005. Paddlefish. American Fisheries Society Symposium 46:227-249.

Mims, S. D., W. L. Shelton, O. Linhart and C. Z. Wang. 1997. Induced meiotic gynogenesis of paddlefish *Polyodon spathula*. Journal of the World Aquaculture Society 28:334-343.

Miranda, L. A., M. C. Cassara and G. M. Somoza. 2005. Increase in milt production by hormonal treatment in the pejerrey fish *Odontesthes bonariensis* (Valenciennes 1835). Aquaculture Research 36:1473-1479.

Miranda, L. A., Escaray, R.U., Bustingorry, J.F., Somoza, G.M. 2001. Effecto deo fotoperíodo de la administración de gonadotrofina coriónica humana (hCG) sobre al espermiación en el pejerry (Odon*testhes bonnariensis).* Revista Argentina de Producción Animal 21:95-105.

Munkittrick, K. R. and R. D. Moccia. 1987. Seasonal changes in the quality of rainbow trout (*Salmo gairdneri*) semen: Effect of a delay in stripping on spermatocrit, motility, volume and seminal plasma constituents. Aquaculture 64:147-156.

Mylonas, C. C., C. Bridges, H. Gordin, A. B. Ríos, A. García, F. De La Gándara, C. Fauvel, M. Suquet, A. Medina, M. Papadaki, G. Heinisch, G. De Metrio, A. Corriero, R. Vassallo-Agius, J.-M. Guzmán, E. Mañanos and Y. Zohar. 2007. Preparation and administration of gonadotropin-releasing hormone agonist (GnRHa) implants for the artificial control of reproductive maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*). Reviews in Fisheries Science 15:183-210.

Neter, J., M. H. Kutner, C. J. Nachtsheim and W. Wasserman 1996. Applied Linear Statistical Models, 4th edition. McGraw-Hill, Burr Ridge, Illinois, USA, 1408 pp.

Nynca, J. and A. Ciereszko. 2009. Measurement of concentration and viability of brook trout (*Salvelinus fontinalis*) spermatozoa using computer-aided fluorescent microscopy. Aquaculture 292:256-258.

Omstead, D. R. 1990. Computer Control of Fermentation Process, CRC Press, Boca Raton, Florida, USA, 360 pp.

Paniagua-Chavez, C. G., J. Jenkins, M. Segovia and T. R. Tiersch. 2006. Assessment of gamete quality for the eastern oyster (*Crassostrea virginica*) by use of fluorescent dyes. Cryobiology 53:128-138.

Paulenz, H., I. S. Grevle, A. Tverdal, P. O. Hofmo and K. A. Berg. 1995. Precision of the coulter® counter for routine assessment of boar-sperm concentration in comparison with the haemocytometer and spectrophotometer. Reproduction in Domestic Animals 30:107-111.

Piironen, J. and H. Hyvarinen. 1983. Composition of the milt of some teleost fishes. Journal of Fish Biology 22:351-361.

Piros, B., J. Glogowski, R. Kolman, A. Rzemieniecki, J. Domagala, A. Horvath, B. Urbanyi and A. Ciereszko. 2002. Biochemical characterization of Siberian sturgeon *Acipenser baeri* and sterlet *Acipenser ruthenus* milt plasma and spermatozoa. Fish Physiology and Biochemistry 26:289-295.

Poikane, S. 2009. Water Framework Directive Intercalibration Technical Report Part 2: Lakes. European Commission, Joint Research Center, Institute for Environment and Sustainability, Italy, 174 pp.

Poole, W. R. and M. G. Dillane. 1998. Estimation of sperm concentration of wild and reconditioned brown trout, *Salmo trutta* L. Aquaculture Research 29:439-445.

Poole, R. K. and U. Kalnenieks. 2000. Introduction to light absoprtion: visible and ultraviolet spectra. *In*: Spectrophotometry and Spectrofluorimetry: A Practical Approach. M. G. Gore, editor. Oxford University Press, New York, New York, USA. Pp. 1-32.

Porter, M. 1998. Calibrating ultraviolet irradiation of fish sperm: optimizing the Hertwig effect. Journal of Applied Aquaculture 8:13-26.

Powell, J. 2002. Optimizing fertilization rates - getting the best bang for your buck. Northern aquaculture 8:15.

Prathalingam, N. S., W. W. Holt, S. G. Revell, S. Jones and P. E. Watson. 2006. The precision and accuracy of six different methods to determine sperm concentration. Journal of Andrology 27:257-262.

Rakitin, A., M. M. Ferguson and E. A. Trippel. 1999. Spermatocrit and spermatozoa density in Atlantic cod (*Gadus morhua*): correlation and variation during the spawning season. Aquaculture 170:349-358.

Riley, K. L., C. G. Holladay, E. J. Chesney and T. R. Tiersch. 2004. Cryopreservation of sperm of red snapper (*Lutjanus campechanus*). Aquaculture 238:183-194.

Rinchard, J., K. Dabrowski and J. Ottobre. 2001. Sex steroids in plasma of lake whitefish *Coregonus clupeaformis* during spawning in Lake Erie. Comparative Biochemistry and Physiology, Part C, Toxicology and Pharmacology 129:65-74.

Rinchard, J., K. J. Lee, K. Dabrowski, A. Ciereszko, J. H. Blom and J. S. Ottobre. 2003. Influence of gossypol from dietary cottonseed meal on haematology, reproductive steroids and tissue gossypol enantiomer concentrations in male rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition 9:275-282.

Rondeau, M. and M. Rouleau. 1981. Effects of dilution rates, animal species and instruments on the spectrophotometric determination of sperm counts. Revue Canadienne De Biologie 40:173-180.

Rosenthal, H. 2008. On the biology of fish sperm: workshop summary and recommendations. Journal of Applied Ichthyology 24:359-361.

Rosenthal, H., J. F. Asturiano, O. Linhart and A. Horvath. 2010. On the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. Journal of Applied Ichthyology 26:621-622.

Rothschild, L. 1950. Counting spermatozoa. Journal of Experimental Biology 26:388-395.

Rurangwa, E., D. E. Kime, F. Ollevier and J. P. Nash. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234:1-28.

Sarosiek, B., A. Ciereszko, A. Rzemieniecki, J. Domagata and J. Glogowski. 2004. The influence of semen cryopreservation on the release of some enzymes from Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*) spermatozoa. Archives of Polish Fisheries 12:13-21.

Schiavone, R., L. Zilli, S. Vilella and C. Fauvel. 2006. Human chorionic gonadotropin induces spermatogenesis and spermiation in 1-year-old European sea bass (*Dicentrarchus labrax*): Assessment of sperm quality. Aquaculture 255:522-531.

Scott, A. P. and S. M. Baynes. 1980. A review of the biology, handling and storage of salmonid spermatozoa. Journal of Fish Biology 17:707-739.

Seaman, E. K., E. Goluboff, N. BarChama and H. Fisch. 1996. Accuracy of semen counting chambers as determined by the use of latex beads. Fertility and Sterility 66:662-665.

Segovia, M., J. A. Jenkins, C. Paniagua-Chavez and T. R. Tiersch. 2000. Flow cytometric evaluation of antibiotic effects on viability and mitochondrial, function of refrigerated spermatozoa of Nile tilapia. Theriogenology 53:1489-1499.

Sneed, K. E. and H. P. Clemens. 1963. The morphology of the testes and accessory reproductive glands of the catfishes (*Ictaluridae*). Copeia 1963:606-611.

Stryer, L. 1995. Biochemistry, 4th edition. W. H. Freeman & Company, New York, New York, USA, 1064 pp.

Suquet, M., R. Billard, J. Cosson, Y. Normant and C. Fauvel. 1995. Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture 133:83-90.

Suquet, M., G. Dorange, M. H. Omnes, Y. Normant, A. Leroux and C. Fauvel. 1993. Composition of the seminal fluid and ultrastructure of the spermatozoon of turbot (*Scophthalmus maximus*). Journal of Fish Biology 42:509-516.

Suquet, M., M. H. Omnes, Y. Normant and C. Fauvel. 1992a. Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). Aquaculture 101:177-185.

Suquet, M., M. H. Omnes, Y. Normant and C. Fauvel. 1992b. Influence of photoperiod, frequency of stripping and presence of females on sperm output in turbot, *Scophthalmus maximus* (L.). Aquaculture and Fisheries Management 23:217-225.

Suquet, M., Rouxel, C., Cosson, J., Severe, A., Quemener, L., Fauvel, C. 2005. Changes in Atlantic cod (*Gadus morhua*) sperm quality with time. *In*: C. I. Hendry, G. V. Stappen, M. Wille and P. Sorgeloos, editors. Fish and Shellfish Larviculture Symposium. European Aquaculture Society, Oostende, Belgium. Pp. 503-505.

Takashima, F., C. Weil, R. Billard, L. Crim and A. Fostier. 1984. Stimulation of spermination by LHRH analogue in carp. Bulletin of the Japanese Society of Scientific Fisheries 50:1323-1329.

Tan, E., H. Yang and T. R. Tiersch. 2010. Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. Zebrafish 7:233-240.

Taneja, G. C. and R. S. Gowe. 1961. Spermatozoa concentration in the semen of two breeds of fowl estimated by three different methods. Poultry Science 40:608-615.

Thibier, M. and H. G. Wagner. 2002. World statistics for artificial insemination in cattle. Livestock Production Science 74:203-212.

Thirumala, S., W. T. Campbell, M. R. Vicknair, T. R. Tiersch and R. V. Devireddy. 2006. Freezing response and optimal cooling rates for cryopreserving sperm cells of striped bass, *Morone saxatilis*. Theriogenology 66:964-973.

Tiersch, T. R. 2001. Cryopreservation in aquarium fishes. Marine Biotechnology 3:S212-S223.

Tiersch, T. R. 2008. Strategies for commercialization of cryopreserved fish semen. Revista Brasileira de Zootecnia 37:15-19.

Tiersch, T. R., C. A. Goudie and G. J. Carmichael. 1994. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm. Transactions of the American Fisheries Society 123:580-586.

Tiersch, T. R., H. Yang, J. A. Jenkins and Q. Dong. 2007. Sperm cryopreservation in fish and shellfish. *In*: Spermatology. E. Roldan and M. Gomendio, editors. Nottingham University Press, Nottingham, United Kingdom. Pp. 493-508.

Toft, G., A. Rignell-Hydbom, E. W. A. Tyrkiel, M. Shvets and A. Giwercman. 2005. Quality control workshops in standardization of sperm concentration and motility assessment in multicentre studies. International Journal of Andrology 28:144-149.

Tsuji, T., H. Okada, M. Fujisawa, Y. Hamaguchi and S. Kamidono. 2002. Automated sperm concentration analysis with a new flow cytometry-based device, S-FCM. American Journal of Clinical Pathology 117:401-408.

Tuset, V. M., G. J. Dietrich, M. Wojtczak, M. Sowiska, J. d. Monserrat and A. Ciereszko. 2008. Relationships between morphology, motility and fertilization capacity in rainbow trout (*Oncorhynchus mykiss*) spermatozoa. Journal of Applied Ichthyology 24:393-397.

Tvedt, H. B., T. J. Benfey, D. J. Martin-Robichaud and J. Power. 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 194:191-200.

Van Nieuwerburgh, L., R. Bonter and L. Heuer 2007. Results of the second transnational Interreg IIIB HARBASINS conference, National Institute for Coastal and Marine Management, The Netherlands, 36 pp.

Viveiros, A. T. M. 2003. Semen collection in catfish species, with particular emphasis on the African catfish. Animal Breeding Abstracts 71:7R-13R.

Viveiros, A. T. M., E. H. Eding and J. Komen. 2001. Effects of 17 alpha-methyltestosterone on seminal vesicle development and semen release response in the African catfish, *Clarias gariepinus*. Reproduction 122:817-827.

Viveiros, A. T. M., Y. Fessehaye, M. ter Veld, R. W. Schulz and J. Komen. 2002. Hand-stripping of semen and semen quality after maturational hormone treatments, in African catfish *Clarias gariepinus*. Aquaculture 213:373-386.

Viveiros, A. T. M., A. Jatzkowski and J. Komen. 2003. Effects of oxytocin on semen release response in African catfish (*Clarias gariepinus*). Theriogenology 59:1905-1917.

Vuthiphandchai, V. and Y. Zohar. 1999. Age-related sperm quality of captive striped bass *Morone saxatilis*. Journal of the World Aquaculture Society 30:65-72.

Wattendorf, R. J. 1986. Rapid identification of triploid grass carp with a Coulter counter and channelyzer. The Progressive Fish-Culturist 48:125-132.

Wayman, W. R., T. R. Tiersch and R. G. Thomas. 1998. Refrigerated storage and cryopreservation of sperm of red drum, *Sciaenops ocellatus* L. Aquaculture Research 29:267-273.

WHO. 2010. WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th edition. World Health Organization, Geneva, Switzerland, 271 pp.

Willoughby, S. 1999. Manual of Salmonid farming, Fishing News Books, Oxford, United Kingdom, 344 pp.

Wojtczak, M., G. J. Dietrich, I. Irnazarow, P. Jurecka, M. Slowinska and A. Ciereszko. 2007. Polymorphism of transferrin of carp seminal plasma: relationship to blood transferrin and sperm motility characteristics. Comparative Biochemistry and Physiology, Part B, Biochemistry and Molecular Biology 148:426-431.