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Review

# Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development

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**Abstract.** Cryopreservation in aquatic species in general has been constrained to research activities for more than 60 years. Although the need for application and commercialisation pathways has become clear, the lack of comprehensive quality assurance and quality control programs has impeded the progress of the field, delaying the establishment of germplasm repositories and commercial-scale applications. In this review we focus on the opportunities for standardisation in the practices involved in the four main stages of the cryopreservation process: (1) source, housing and conditioning of fish; (2) sample collection and preparation; (3) freezing and cryogenic storage of samples; and (4) egg collection and use of thawed sperm samples. In addition, we introduce some key factors that would assist the transition to commercial-scale, high-throughput application.

**Additional keywords:** commercial scale, quality, spermatozoa.

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## Introduction

Cryopreservation in aquatic species goes back 65 years and began about the same time as similar research was performed in livestock (Blaxter 2011). Since then, large global industries have become established for the development, maintenance and distribution of genetic resources of livestock species, whereas activities for aquatic species have largely been limited to research efforts in protocol development for cryopreservation, with large-scale repository development just beginning. There are several factors that favoured the widespread application of cryopreservation technologies in livestock that did not exist for fish, such as the existence of breeders clubs and the general commercial acceptance of genetic gain made through selective breeding with prized sires. The application of cryopreservation in aquatic species has additionally been constrained by a lack of standardisation in terminology, methods and reporting. For example, the practitioners are of widely divergent training, backgrounds and skill levels, and results are reported across a wide range of outlets. As such, there are numerous factors that need to be standardised or harmonised for this field to progress. Accordingly, several of these have been organised based on process sequence and are identified in this review. In addition, key factors that are needed to assist the transition to commercial-scale application are presented. Research on the cryopreservation of fish oocytes and embryos remains controversial and is still in its initial stages. As such, this review focuses on the cryopreservation of spermatozoa. However, this emerging research field in oocytes and embryos would also benefit from the reasoning behind the pathways used for quality assurance and quality control presented herein. To date, the spermatozoa of more than 200 species of fish have been cryopreserved (for a comprehensive review, see Tiersch and Green 2011); however, in common practice, research on cryopreservation of spermatozoa is limited only to freezing itself, leaving aside a great number of variables that may directly or indirectly influence the outcome of the cryopreservation process and the overall goal of reliable production of fertilisation and viable offspring. To address this, we analyse, in stepwise fashion along the cryopreservation pathway, the different factors that can add variability to the process. We also identify quality assurance and quality control points that can help reduce variability among samples.

Quality assurance (QA) and quality control (QC) activities provide the foundation for operations in any industrial setting. QA comprises a series of activities that are 'process' oriented and addresses defect prevention by detecting and analysing potential human errors, with the goal of improving development and test processes to avoid defects while materials are being handled. Therefore, QA systematically uses inspection of an

operation to ensure that it conforms to a pre-established standard. Conversely, QC includes activities that are 'material' oriented and focused on defect identification (and correction) of materials at each operational step of a production line (Montgomery 2008). During the cryopreservation process, each major step can be represented by a respective material. For example, in the initial steps, the material is the fish itself, whereas further down the pathway the sperm suspension becomes the material of interest (Hu *et al.* 2013). In sperm cryopreservation of aquatic species, some QA–QC activities have been implemented mostly incidentally to provide a source of data in experiments, without the conceptual basis of a pre-established QA–QC program. For example, experimental emphasis has been placed on the evaluation of sperm quality (e.g. sperm motility), but not enough has been done to establish guidelines and standardised procedures for individual steps of the cryopreservation process (e.g. standardised cooling rates generally are not calculated, verified or reported). In addition, the activities that collect data to implement QC have their own QA procedures. For example, to evaluate sperm motility (QC), different methods can be used (e.g. visual estimation with counting chambers, computer software) and each needs to be standardised to assure accuracy and precision (through QA).

Standardisation, as defined by the dictionary, means to cause something to conform or be normalised to a particular standard, whereas harmonisation means to develop agreement. In a laboratory context, new methods need to be validated for accuracy, repeatability and precision before being standardised and, when standards are developed for each method, different procedures can then be harmonised in relation to results (Cuevas-Uribe and Tiersch 2011). These concepts are the basis for comparison and replicability within and among laboratories and institutions, and these concepts are the foundation for QA–QC programs. Consequently, they constitute a key component in the development of germplasm repositories. Some of the most wide-reaching factors (currently absent from the aquatic species cryopreservation literature) that would greatly benefit development of the field are guidelines and standardisation of reporting, uniformity of terms and agreement on the variables that need to be included in peer-reviewed publications. Therefore, throughout this review, we call attention to information that is currently missing in peer-reviewed studies.

In this review we also address the evaluation tools commonly used in cryopreservation, emphasising the areas that deserve extra attention. For this purpose, we focus the discussion around four main stages in the cryopreservation process: (1) source, housing and conditioning of fish; (2) sample collection and preparation; (3) freezing and cryogenic storage of samples; and (4) egg collection and use of thawed sperm samples. These stages encompass all the major activities relevant to cryopreservation programs (Fig. 1).

## Source, housing and fish conditioning

It should be emphasised that the quality of thawed spermatozoa is directly and indirectly linked to a variety of biological characteristics that can determine the overall quality of fresh spermatozoa. Consequently, it is fair to say that successful cryopreservation of fish spermatozoa begins with evaluation of the donor fish and their environment; however, if this information is not appropriately recorded and reported, then an incomplete profile of the sperm donor (i.e. the fish) will become increasingly problematic the longer the samples are held in frozen storage before thawing and use. Husbandry conditions, such as water quality (Paterson *et al.* 2003), light cycle (Iigo and Aida 1995), diet (Asturiano *et al.* 2001; Izquierdo *et al.* 2001), conditioning regimen (Seifi *et al.* 2011) and fish density (Liu *et al.* 2015; Newman *et al.* 2015), should be recorded because they play an inherent role in the well being of the fish and affect reproductive health and sperm quality. In addition, in field studies, key pieces of information, such as the location and characteristics of the collection site, are rarely reported. For example, coastal area captures are often associated with varied salinities, which could be critical for male maturity and optimal sperm conditions. Knowing the location of capture can also help monitoring and early detection of fluctuations in fish populations, which can provide important information to be considered when cryopreservation is used as a tool for conservation and restoration efforts.

When fish are transported to different locations for sperm collection, additional measures are necessary, such as the monitoring of transport conditions and the development of biosecurity and quarantine protocols. The transport of fish and their acclimation to new housing conditions is stressful, and this can cause detrimental effects to overall fish health, lowering the immune system response and thus leading to an increased incidence of disease (Small *et al.* 2008). Therefore, ensuring that transport conditions are suitable (e.g. water quality, transportation time) and placing the fish in isolated areas for several days (e.g. 2 weeks) before collecting spermatozoa can help minimise and identify disease outbreaks (Hadfield and Clayton 2011), increasing the odds of obtaining high-quality, uncontaminated spermatozoa. When the number of fish available is higher than needed or when there is no need to collect spermatozoa from all captured fish, selection criteria should be pre-established to avoid over-representation in the repository collection of particular segments of the overall fish population (Food and Agriculture Organization of the United Nations (FAO) 2011). Similarly, selection criteria should be in place for cases where spermatozoa are collected from dead fish, as in offshore fishing samples and long distance shipping of dead fish (Riley *et al.* 2008).

### *QC checkpoints*

Up to this point of the process the fish has been the focus of the QC. An evaluation of fish age, size, health condition and reproductive maturity (e.g. secondary sex characteristics, gonadosomatic index, testis colouration) is needed (but many times neglected) given the potential association of these variables with sperm quality (e.g. Aliniya *et al.* 2013; Chatakondi and Davis 2013). Male-to-male variability in terms of sperm quality has been reported in many fish species, including the northern pike (Babiak *et al.* 1997), Atlantic herring (Geffen 1999), Atlantic halibut (Ottesen *et al.* 2009) and *Xiphophorus couchianus* (Yang *et al.* 2009); strain-to-strain or population-to-population variability has also been reported (e.g. channel catfish; Li *et al.* 2001, 2004; Peterson *et al.* 2004). Given this

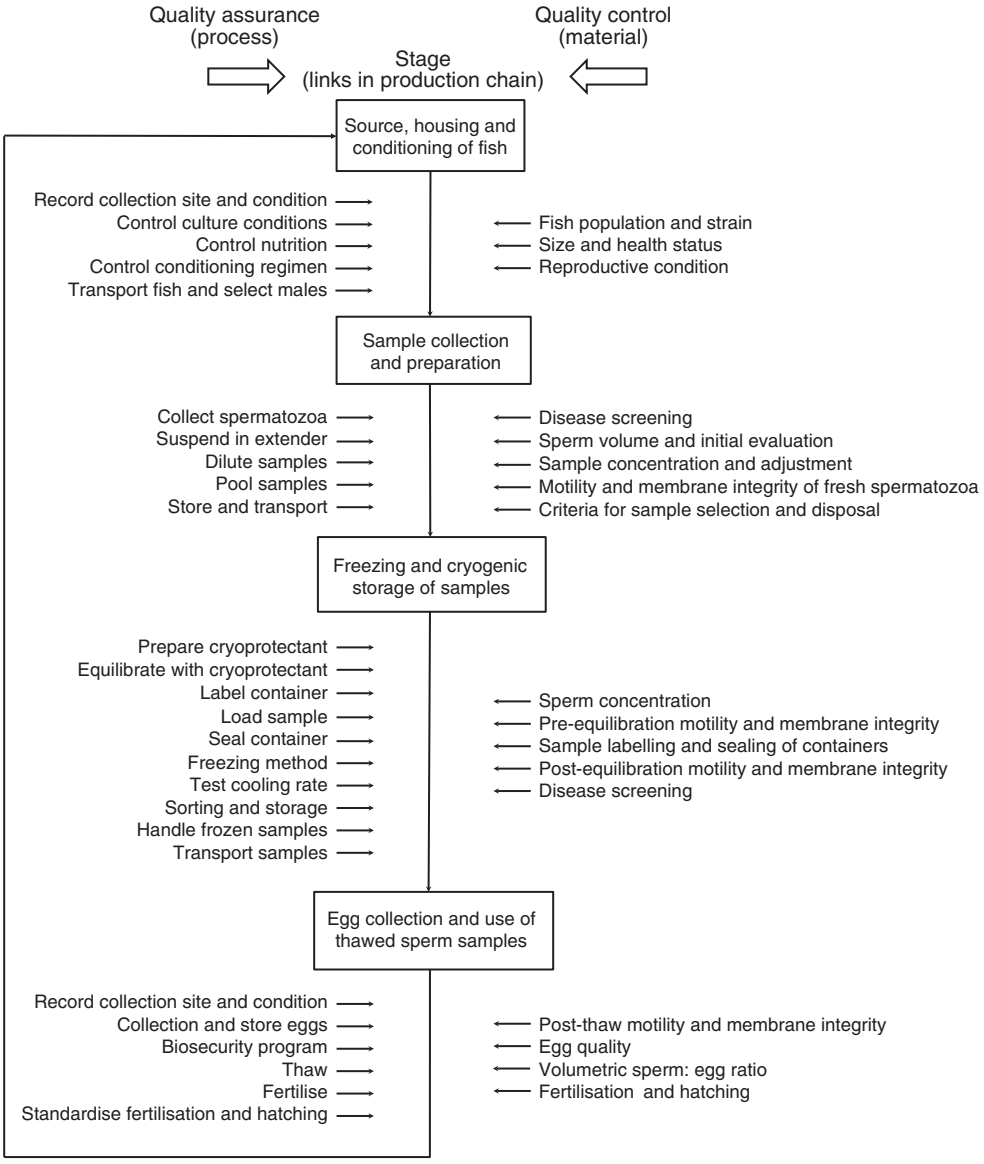


**Fig. 1.** Key quality assurance (left) and quality control (right) activities that take place along the four main stages of the cryopreservation process.

variation, these quality characteristics should be noted as part of the initial examination. An initial assessment of each male may determine whether it is worth the effort (i.e. meets minimum standards) and resources for sperm collection before continuing to the next stages in the cryopreservation process (Fig. 1). Early detection of QC defects (i.e. a fish showing symptoms of disease) prevents the waste of resources.

## Sample collection and preparation

Spermatozoa are generally obtained by two methods: testis dissection and stripping. Each method has downsides in terms of sample purity. During dissection, for example, tissue contamination (i.e. collection of cells different from spermatozoa) is likely to occur, whereas during stripping, urine contamination is common. Therefore, containment actions should be in place for the particular method of sperm collection. Tissue contamination can be decreased by filtering the sperm suspension (Hu *et al.* 2014). In addition, in most freshwater fishes, urine contamination can be counteracted by mixing the spermatozoa upon collection with an extender with an osmolality higher than that of the fish plasma (Yang *et al.* 2007*a*).

Extenders, defined as a solution of salts with certain osmolality and pH, are used to dilute spermatozoa while keeping them immotile, lengthening their usable lifetime (Renard *et al.* 1994; Strüssmann *et al.* 1994). Extender osmolality is critical because this is one of the main factors triggering sperm activation (Cosson 2004; Alavi and Cosson 2006) and is the reason why

extenders of various osmolalities should be tested to choose the best fit with a particular species. In addition, storage duration and temperature (e.g. refrigerated storage) should be chosen to ensure the quality of stored and shipped spermatozoa (Glenn *et al.* 2011). Despite the importance of extender choice and refrigerated storage, extender composition, temperature and duration of storage are not often reported.

### *QC checkpoints*

At this point of the process the sperm suspension has become the focus of the QC. Ideally, specific pathogens or contaminants should have been screened in the recently collected spermatozoa, and measurements to avoid cross-contamination among samples should be in place during sperm collection. The volume of spermatozoa (or mass of testis) collected may be associated with the reproductive status of the fish (Chatakondi and Davis 2013), and therefore should be measured and reported.

An initial assessment of sperm concentration, motility and other quality characteristics (e.g. sperm membrane integrity, morphology, mitochondrial activity, ATP content) should take place in the freshly collected sample. We discuss the most common sperm quality characteristics (concentration, motility and membrane integrity) below, but it should be noted that later along the process these end-points need to be re-evaluated to identify potential problems associated with the cryopreservation protocol (Fig. 1).

#### *Sperm concentration*

Estimating sperm concentration in freshly collected samples provides important information about the reproductive health of the fish (e.g. a fish that is not ready to spawn may have dilute or immature spermatozoa), and avoids the introduction of uncontrolled male-to-male variation (Tan *et al.* 2010). Sperm concentration should also be estimated and adjusted to a specific value before freezing because it reinforces the cell number : cryoprotectant ratio and ensures that the cells will be protected during freezing, and estimated again after thawing to account for the potential reduction in sperm numbers as a consequence of damage caused by freezing and thawing procedures. Therefore, knowledge of the sperm concentration allows for a better understanding of cryopreservation as a whole (Tan *et al.* 2010). Unfortunately, this step is many times avoided or poorly documented. Despite being a straightforward concept (the number of spermatozoa per millilitre of sperm suspension), the methodology behind the estimation of sperm concentration is more complex.

Several methods have been used to estimate sperm concentration, including counting chambers (e.g. haemocytometer; Makler®, Selfi-Medical Instruments, Haifa, Israel), spectrophotometry, computer-assisted sperm analysis (CASA) and flow cytometry. Counting chambers are a widely used inexpensive method. Despite being common, there is a lack of consensus regarding the information to be reported in terms of the methodology used to count and calculate sperm concentration (Cuevas-Uribe and Tiersch 2011). Each method has disadvantages. Some counting chambers, like the haemocytometer, require relatively large volumes of sample ($>10\,\mu L$), which is a problem when dealing with small fish where the total volume obtained is in the range 2–5 μL (Yang *et al.* 2007*a*, 2012). In addition, this method is time consuming, requiring 10–15 min per sample, which may not be suitable for high-throughput applications (Tan *et al.* 2010).

Spectrophotometry has been used to estimate sperm concentration (e.g. Suquet *et al.* 1992; Ciereszko and Dabrowski 1993; Dong *et al.* 2005). It is fast and some specialised models (e.g. Nanodrop; Thermo Scientific, Wilmington, DE, USA) require volumes of $\leq 2\,\mu L$ spermatozoa, which makes spectrophotometry suitable for use with small fish (Tan *et al.* 2010). However, this method is far from being standardised. In a comprehensive analysis of estimation of fish sperm concentration, more than 80 publications that used a spectrophotometer were reviewed and, of those, approximately 65% did not report how methods were standardised (i.e. wavelengths, calibration curves and equations generated) and <50% of the studies even reported the model of the spectrophotometer used (Cuevas-Uribe and Tiersch 2011).

CASA systems are used to estimate sperm motility (see below), but these powerful instruments can also be used to estimate sperm concentration and, when both features are measured simultaneously, this method is highly time effective. Several models of CASA systems are available, and it is important to report the model and version of the software, the settings used for cell identification, the type of viewing chamber used, the dilution factor and the microscope and camera settings to obtain consistent results across studies. However, CASA may not be suitable for all species. For example, in some cases of dissected testes, as in some strains of the genus *Xiphophorus*, the spermatozoa can be easily misidentified as debris particles because of their small size and elongated shape. Unless staining of the sperm nucleus is performed before analysis, this hinders the ability of the software to specifically collect data from spermatozoa. In such cases, spectrophotometry or a counting chamber may be more appropriate.

Flow cytometry allows the analysis of different traits in individual cells. For example, membrane integrity is commonly analysed in spermatozoa (see below). Some flow cytometry models (e.g. BD Accuri; BD Biosciences, San Jose, CA, USA; see http://www.bdbiosciences.com, accessed 1 October 2015) are designed to measure the volume of sample collected, allowing the calculation of cell concentration without the addition of counting beads. Thus, although the purpose of a flow cytometer may not be to measure cell concentration, this feature can be used as an additional method to verify sperm concentration at the time another particular cell trait is being analysed. Unfortunately, as we have seen elsewhere for other technologies, the lack of standardisation in flow cytometry protocols impairs reliable comparisons across studies.

Given the variety in species, goals (e.g. research scale vs high-throughput production) and available budgets, it is unreasonable to expect adoption of one universal method to estimate sperm concentration. However, the standardisation of individual protocols and the harmonisation of different methodologies are necessities, and need to begin with the reporting of calibration techniques, settings and detailed protocols.

In addition, it should be emphasised that after estimating concentration, adjustment to a proper working concentration is

often species and container dependent. Studies should be planned to balance the sperm concentration with QC (e.g. sufficient cryoprotectant) in relation to costs and utilisation. For example, the difference in working with cell concentrations of $10^8$ versus $10^9$ cells $mL^{-1}$ would involve a production difference of a factor of 10 in straw numbers (e.g. 500 straws for $10^9$ cells $mL^{-1}$ vs 5000 straws for $10^8$ cells $mL^{-1}$) for the same volume of sperm sample. This has significant implications for planning, cost estimates and implementation of high-throughput processing.

### *Sperm motility*

Regardless of the species, motility is currently considered to be the most practical indicator of sperm quality. In fish with external fertilisation, spermatozoa are activated during spawning generally because of an osmotic or ionic change upon contact with water (Cosson 2004; Alavi and Cosson 2006). Once activated, the duration of motility is limited, ranging from a few seconds to few minutes depending on the species. During the stages of the cryopreservation process, motility should be evaluated at several times: in freshly collected samples, after sample storage and transport, before and after the addition of the cryoprotectant and at the time of thawing when the spermatozoa are ready to be used. The most common methods used to evaluate sperm motility are visual estimation ('naked eye'), counting chambers and computer software, such as used by CASA (i.e. motion analysis). Regardless of the method of choice, an important factor when estimating sperm motility is the timing of analysis. Motility estimated 10 s after activation may not be the same as that estimated after 30 or 40 s in most fish species; therefore, this timing should be carefully controlled and the information should be clearly reported.

Visual estimation of sperm motility has been the most widely used method, but it is the most subjective because it relies entirely on the ability and the experience of the person making the estimation and, unless standardised in some way, it cannot be reliably compared from person to person (Amann and Waberski 2014; Lu *et al.* 2014). In addition, there is no clear established methodology, because people may or may not use, for example, a coverslip to do the observations. The use of counting chambers is a step forward to standardisation. Specialised commercially available counting chambers such as the Makler® chamber (http://www.sefimedical.com, accessed 1 October 2015) have a grid and a fixed depth (e.g. 10 μm), which creates a visual monolayer of spermatozoa, making the counting of motile and static spermatozoa more accurate. Disadvantages of the counting chambers include that they rely on the ability of the observer to distinguish and count motile spermatozoa and they do not have archivable capabilities such as those offered by computer software.

Conversely, CASA systems offer detailed information about the kinetic characteristics of individual spermatozoa and the ability to store a record of the observations in frame-by-frame files. However, because currently there are no standardised protocols and no associated detection standards (Lu *et al.* 2014), CASA results can vary widely, jeopardising the original goal of replicability. Some of the factors that can significantly alter CASA results are recording quality, frame rate (Boryshpolets *et al.* 2013), hardware and instrument settings (Amann and Waberski 2014), variations in counting chambers (Lu *et al.* 2014) and cell media (Amann and Waberski 2014). In addition, specific settings should be developed to account for different sperm behaviour before and after freezing, and for inter-species differences. Once validated, CASA can provide important information about the quality of spermatozoa in terms of overall motility and speed, as well as providing researchers the opportunity to understand sperm population heterogeneity and responses to changes in microenvironments (Amann and Waberski 2014). Despite these advantages, CASA may not always be suitable for all species. As mentioned earlier, in some cases the software is not able to discriminate spermatozoa from surrounding debris; in other cases, such as species from the family Goodeidae, spermatozoa are encapsulated into bundles (i.e. spermatozeugmata), and semiquantitative estimation techniques may be necessary to characterise motility (Y. Liu, unpubl. data).

### *Sperm membrane integrity*

Membrane integrity, or the proportion of membrane-intact cells in populations, is a useful indicator of sperm quality during the cryopreservation process given that the sperm plasma membrane is one of the main structures that can be damaged during processing (He and Woods 2004). Assessment of membrane integrity is one of the most common sperm quality assays and it is ideally performed in conjunction with motility. As stated above, multiple assessments are needed: in the freshly collected sample, after sample storage and transport, before and after the addition of the cryoprotectant and at the time of thawing when the spermatozoa are ready to be used.

The objective of membrane integrity assays is to distinguish between cells with impaired and intact cell membranes by using a combination of dyes. This can be done in a variety of ways. Some are cheaper (e.g. eosin–nigrosin) but less effective (Chalah and Brillard 1998). The most commonly used fluorescent dyes are propidium iodide (PI) and SYBR-14, each targeting the sperm DNA. PI can only enter cells with impaired membranes, rendering them red (when exposed to excitation wavelengths), whereas SYBR-14 will enter cells with intact membranes and accumulate in the nucleus, rendering them fluorescent green (Garner *et al.* 1994). The proportion of cells with impaired versus intact membranes can be calculated using a fluorescence microscope or a flow cytometer. The use of a flow cytometer can be an expensive alternative; however, it is advantageous because it allows the individual analysis of thousands of cells in few seconds.

One long-standing problem is that the use of nucleic acid dyes demands a prompt analysis because the dyes tend to quickly leak from the cells (Perfetto *et al.* 2010). Therefore, they are not suitable for use when a flow cytometer is not readily available, as typically occurs during sperm collection (e.g. at a hatchery). Fortunately, there are new alternatives, such as the use of dyes that allow for cell fixation (Perfetto *et al.* 2010), to enable collection of membrane integrity information when a flow cytometer is not immediately available.

Regardless of the advantages that flow cytometry may confer to sperm analyses, there is considerable variability in the reporting of sample preparation, staining and flow cytometry protocols that makes direct comparisons difficult among laboratories. Some of the information that needs to be reported includes staining conditions (i.e. final dye concentrations and volume, incubation time and temperature), collection parameters (i.e. number of events or volume collected and flow rate), gating parameters (i.e. identification of the sperm population and the method for the exclusion of debris from analyses) and reporting of results (concentration of intact cells and the basis for calculation of percentage data; Daly and Tiersch 2011).

## Freezing and cryogenic storage of samples

There is a wide diversity of cryoprotectants used with fish spermatozoa, such as methanol, dimethyl sulfoxide (DMSO), sucrose and glycerol. Some cryoprotectants, like methanol, will permeate cells, whereas others with larger molecular weights, like sucrose, generally do not pass through the cell membrane (Meryman 1966). Although cryoprotectants help reduce damage from the formation of ice crystals during freezing, most of these chemicals are toxic to the cell. Therefore, acute toxicity tests (using sperm motility, membrane integrity and sperm concentration as end-points) are critical to help elucidate the cryoprotectant concentration and the exposure time period yielding the least adverse effects (i.e. establishing the cryoprotectant equilibration time). In addition, it is important to take into account that spermatozoa from different species may react differently to a particular cryoprotectant and, as such, it is recommended to test several cryoprotectants at different concentrations to select a cryoprotectant, concentration and appropriate equilibration time. The equilibration time and temperature are critical variables that may determine the success and replicability of the cryopreservation process, which are reasons why they should be measured, controlled and reported.

Freezing is a physical process that implies the changing of a substance from liquid to solid state by loss of heat. Freezing of cells in a solution is not a straightforward concept. Freezing should be uniform and the cooling rate (the rate at which cells are cooled during freezing) should be controlled. Various cooling rates should be tested every time with a new species and after the cooling rate that causes the least (or acceptable) damage to the cell is found for the given set of conditions (e.g. specific container, sample volume, extender), replicability should be the most essential feature. However, depending on the freezing media, storage container and sample loading volume, cooling rates can vary greatly and can be hard to measure (Yang and Tiersch 2009). For example, dry ice is one of the most common freezing media used with aquatic species. Dry ice is a heterogeneous matrix that includes empty spaces of different sizes that are randomly distributed. Grinding of dry ice or mixing it with alcohols can yield a more homogeneous medium (Sargent and Mohun 2005); however, accurate measurement of the cooling rate of a sample is still difficult (Yasui *et al.* 2008). In this regard, computer-controlled freezers offer advantages, whereby the temperature is uniform within a chamber and can be lowered steadily at a desired rate, not only controlling cell damage, but also allowing for practical replication. Different storage containers, such as French straws and cryovials, have different volumes and physical properties (i.e. surface-to-volume ratio), that can alter the cooling rates. Similarly, fluctuation in the sample loading volume can translate into inconsistent cooling rates. For all these reasons, reporting of the freezing media, storage containers and sample loading volumes is necessary to ensure replicability and reliable comparisons across studies, and would provide a basis for harmonisation efforts.

The selection of the storage container has several implications: (1) the container, when loaded at consistent volume, should be able to achieve a replicable cooling rate; (2) it should be sealable to avoid cross-contamination among samples; (3) it should be durable and able to hold a permanent identification label; and (4) it should be easy to store and retrieve efficiently. These characteristics are a requirement to guarantee the integrity of samples and the biological and genetic information they represent.

It should be understood that the inherent value of germplasm repositories is to store information, not only the genetic information contained in the sample, but also the other relevant variables associated with that particular sample (e.g. fish age, size, location, environmental variables). The amount of information necessary to adequately characterise a single sample in a repository can be intimidating. Therefore, the development of interactive databases that allow the storage and retrieval of information is as important as the development of reliable frozen storage.

In general, frozen samples have to be transported, either to a repository or to a laboratory or hatchery for fertilisation trials. The handling and transportation protocols of frozen samples are generally not addressed in the literature, despite their critical importance (Tiersch 2011). A well-cryopreserved sample is of no value if it is ruined by poor handling or shipping. Protocols for handling frozen samples (e.g. sorting them under liquid nitrogen) should be established and reported accordingly. Unfortunately, most of the time the transport of samples is done through a third-party shipping company and no control exists over the care that they may have with a shipment. For this reason, it is advisable to try different shipping companies and ship replicate or unimportant samples to different locations to become familiar with the different types of damage that can occur, and thus take preventive measures (Tiersch 2011). A proactive approach to improve the current shipping methods is also needed. For example, the inclusion of temperature monitors (e.g. http://www.cryoguard.com, accessed 1 October 2015; http://www.monnit.com, accessed 1 October 2015) in shipping dewars can provide a temperature profile during shipment that can be included in QA and QC programs.

### *QC checkpoints*

At this point, the sperm suspension and the frozen samples have become the focus of QC. As mentioned above, freshly collected sperm suspensions often need to be transported to a different location for freezing. Thus, at the point of arrival, sperm concentration, motility and membrane integrity should be evaluated to account for any cell damage resulting from the extender,

temperature and time after collection. Likewise, to later evaluate the efficiency of cryoprotectant choice, these quality characteristics should be re-evaluated again before and after the addition of the cryoprotectant (i.e. at the end of the equilibration period; Fig. 1).

After the sample is loaded in the container of choice, its identity should be cross-verified with the label assigned to the container. The long-term durability of the labelling method should be verified and the information encoded has to be easily tracked back to a database (identifiers such as barcodes are effective). Equally important is to verify that containers are filled with the proper volume and properly sealed before freezing. They should remain sealed and intact after freezing to avoid any potential source of sperm or pathogen cross-contamination during storage or use. As noted above, when sperm samples are frozen, there is a possibility that additional, unwanted pathogenic organisms can be frozen too. Indeed, it is not uncommon to find bacteria in sperm samples, regardless of the biosecurity policies in place (Jenkins 2011; Oplinger and Wagner 2015). Therefore, it is important to screen sperm samples before freezing for the most common pathogens because, with this information, further decisions regarding the use (or disposal) and destination of samples can be made, avoiding, for example, the introduction of disease in unexposed populations.

## Egg collection and use of thawed sperm samples

Although fertilisation and the production of viable offspring are the ultimate goals behind the cryopreservation process, little emphasis has been placed on the standardisation of egg collection and fertilisation protocols. For example, the criteria for female selection (e.g. age, size and reproductive traits) are rarely reported. Similarly, as suggested above with males, a screening method for specific pathogens or contaminants is not always followed or reported. Additional sources of variation that may have an effect on egg quality include different female conditioning protocols (including the use of gonadotropic hormones), egg collection methods, protocols for egg storage before fertilisation (e.g. extender used, time of storage, oxygen level; Donaldson *et al.* 2011) and the pooling of eggs from several females (vs using eggs from individual females). All these procedures should not only be standardised and controlled, but also appropriately reported to facilitate replicability among fertilisation trials.

The thawing of sperm samples is a critical step that, if not done properly, will waste the effort of the entire cryopreservation process. Important steps during thawing include any time lapse between sample retrieval from the liquid nitrogen and the start of the thawing protocol, thawing temperature (e.g. water bath), thawing duration (this will vary depending on the storage container) and the final temperature of the sample after thawing. Once thawed, it is often essential to control the time before fertilisation, and any additional procedures or amendments, such as sperm centrifugation (to remove cryoprotectant and concentrate the sample), should be reported in detail.

Artificial external fertilisation is generally viewed as a simple process, although in fact it can vary widely. Variables such as timing, gamete activation steps, volumes used and osmotic pressure are not always measured, controlled or reported. The total fluid volume, for example, can significantly alter the sperm–egg contact, changing the probability of fertilisation (Hu 2012). Similarly, the sperm : egg ratio and the number of motile spermatozoa in the final fertilisation volume are variables that, once standardised, improve the fertilisation outcome and significantly reduce sperm waste (Suquet *et al.* 1995; Butts *et al.* 2009, 2014; Babiak *et al.* 2012); however, these parameters are rarely calculated, controlled or reported.

Artificial internal fertilisation in live-bearing fish is even more complex because it involves additional variables that are often difficult to control. Obtaining virgin females, for example, can be difficult but necessary to be able to verify paternity because, in many species (e.g. in the genus *Xiphophorus*) females are able to store spermatozoa for long periods of time after mating (Tavolga 1949). In addition, it is recommended for fertilisation testing to use spermatozoa of a different species to produce hybrids with distinguishable phenotypes to verify protocols, so the paternity can be readily verified (Yang *et al.* 2007*b*, 2012). Furthermore, improper handling of the female during insemination can cause permanent injury or kill the fish. In terms of thawed spermatozoa, centrifugation has been necessary to remove cryoprotectant by washing and to concentrate the number of spermatozoa in an appropriate volume for insemination. In some species, the rate of pregnancy with AI is low even using fresh spermatozoa (Yang *et al.* 2012); therefore, the number of replicates to test cryopreserved spermatozoa should be large enough to account for the normal pregnancy incidence (Yang *et al.* 2007*b*) and should be factored into the number of units of frozen spermatozoa that are collected and used.

As mentioned above, cryoprotectants are toxic to spermatozoa above a species-specific concentration threshold. After the spermatozoa are thawed, the cryoprotectant could also be toxic to the eggs. For this reason, it is advisable to perform acute toxicity tests to evaluate the potential damage to eggs and embryos resulting from cryoprotectant exposure. If there is an indication of detrimental effects, additional measures to ensure egg quality can be taken, such as changing the cryoprotectant, washing thawed sperm suspensions before fertilisation or washing eggs after fertilisation to remove most of the cryoprotectant.

### *QC checkpoints*

At this point the thawed sperm suspension, oocytes and fertilised eggs have become the focus of QC. After the sperm sample is thawed, a re-evaluation of sperm concentration, motility and membrane integrity (preferable before pooling of thawed samples, if that is needed) is necessary to evaluate the outcome of the freezing process.

The evaluation of sperm quality at each stage of the cryopreservation process aims to preserve fertilisation capacity. It is therefore a challenge to adequately evaluate the success of the process when the quality characteristics of the other vital components in the fertilisation equation (i.e. the egg) are missing. That is, it is difficult (if not impossible) to obtain viable offspring when egg quality is not optimal, regardless of the quality of well-cryopreserved spermatozoa. Thus, establishment

of QA and QC protocols for egg quality should be a priority in fertilisation activities. Indicators of egg quality include egg size, oil droplet size, total lipid content and lipid classes (Hauville *et al.* 2015). In addition, if there are suspicions of water quality problems, a toxicological profile of contaminants in the eggs would be important given that lipid-soluble toxicants can be transferred into oocytes (Ostrach *et al.* 2008; Stefansson *et al.* 2014; Sühring *et al.* 2015; Wang *et al.* 2015). Likewise, it has been observed that the female diet (Hauville *et al.* 2015), body size (Carter *et al.* 2015), age and maturity level (Valdebenito *et al.* 2015), female-to-female variation (Kjesbu *et al.* 1992; Brooks *et al.* 1997), exposure to predation and stress (Åberg Andersson *et al.* 2011; Giesing *et al.* 2011) and time of egg collection within the spawning season (Galo *et al.* 2015) can affect egg quality. Because most of these factors are generally neglected, not collected or not reported concerning fertilisation trials, fertilisation and hatching success often do not reflect the quality of thawed spermatozoa. For this reason, the incorporation of controls, using fresh spermatozoa, is essential. Indeed establishment of a robust QA–QC program for spermatozoa can, when used with a dose concept, eliminate or greatly reduce functional male (spermatozoa) variability. This can be used to identify problems with eggs or females (Hu and Tiersch 2011).

Another long-standing problem is that the definition of 'per cent fertilisation' varies greatly across the literature, with usage varying from first mitotic division to post-hatching development stages, and using absolute values or values relative to controls (Tiersch 2011). It is essential to standardise reporting of how fertilisation and hatching are defined to be able to compare outcomes across studies. At present, most direct comparisons of 'per cent fertilisation' reported in published studies of aquatic species are problematic, useless or downright misleading.

## Commercial-scale applications

The various aspects of sperm cryopreservation discussed above need to be assessed with a different perspective when moving forward from research into commercial-scale application. This change of scenario requires a different set of tools (or disciplines) to eventually allow the delivery of products to a market. Our purpose below is to put forward a few key factors that could assist the transition to commercial-scale application:

### *Manufacturing*

The establishment of a production line with a defined number of steps, each evaluated by comprehensive QC checkpoints, is the key behind a reliable product. A well-established QA–QC program should be open, transparent and periodically revised, and should include training programs to ensure that personnel comply with the set standards (Montgomery 2008).

After QA–QC programs are adopted in each production facility, the provision of guidelines should follow. For example, in the livestock semen industry, Certified Semen Services, Inc. (CSS; http://www.naab-css.org, accessed 1 October 2015) is a self-regulating entity that provides inspection services, establishes minimum standards to ensure sperm quality, develops a uniform labelling system of semen containers, and provides training for the producers and users.

Development, standardisation and overseeing biosecurity programs within and among cryopreservation facilities would be a priority to avoid genetic or pathogenic cross-contamination. The World Organisation for Animal Health (OIE; http://www.oie.int, accessed 1 October 2015) offers standards and guidelines for animal welfare and notifications and control of animal diseases. Adopting such guidelines would lower the risk of introducing diseases and pathogens, and would facilitate the exchange of samples within and among countries.

### *Research and development*

With the standardisation and harmonisation of protocols across commercial-scale facilities, a new niche can be created for equipment development, supplies and reagents specific for cryopreservation of aquatic species. This would provide alternatives to adapting technologies designed for livestock semen, and the development of equipment able to address the specific needs presented by aquatic species.

### *Product positioning*

A high level of QC and standardisation would guarantee reproducibility to the point where each frozen unit (i.e. 0.5-mL straw) would contain a specific number of viable spermatozoa able to fertilise a specific number of eggs. That is, each straw will become a dose able to fertilise a predetermined number of eggs (Hu 2012). The establishment of the dose concept for each species is critical because it increases efficiency and significantly reduces costs (Hu 2012), especially by avoiding unnecessary waste. The dose can be set for specific applications or for species by the number of eggs routinely used for fertilisation events. For example, zebrafish, which are small bodied ($<$5 cm), produce approximately 100 eggs per female; however, channel catfish are larger ($\geq$60 cm) and produce approximately 10 000 eggs per female. The dose contained within a single straw would provide sufficient high-quality spermatozoa to reliably fertilise a known number of eggs relevant to each of those species. This would avoid waste of spermatozoa and eggs.

### *Market assessment*

Regardless of the advantages that cryopreservation offers, the relationship of cost versus value is still poorly understood. It is often believed that cryopreserving and storing frozen spermatozoa is too expensive (Caffey and Tiersch 2000). However, when the costs and risks of animal husbandry (e.g. food, space, time) are taken into consideration, cryopreservation can become cost-effective. At a commercial scale, efficient cryopreservation can equal or surpass traditional husbandry operations in cost-effectiveness.

### *Appearance design*

Important product features that should be addressed include (but are not limited to) high biosecurity containers, labelling that allows the recording (and retrieval) of detailed information and provision of access to improved genetics. Multiple types of containers are commercially available and the market will determine which is best for specific activities. Our opinion is that French straws will be most adequate for commercial-scale

applications because they offer a highly controlled microenvironment because of their thin walls and small internal radius, high biosecurity features (they can be plugged or sealed at both ends), large surface area to allow for a descriptive label, a wide assortment of colours, easy and effective storage systems, low cost and decades of previous use for livestock semen. In addition, a variety of automated straw fillers and printers (originally developed for the livestock industry) are currently available and can be adapted for use with aquatic species.

### *Policy and regulations*

Mechanisms addressing establishment of gametic value and price, as well as the creation of appropriate material transfer agreements, intellectual property protections, treaties and regulatory frameworks, should be addressed to assist commercial-scale development of applications for aquatic species.

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## References

Åberg Andersson, M., Silva, P. I. M., Steffensen, J. F., and Höglund, E. (2011). Effects of maternal stress coping style on offspring characteristics in rainbow trout (*Oncorhynchus mykiss*). *Horm. Behav.* **60**, 699–705. doi:10.1016/J.YHBEH.2011.09.008

Alavi, S. M. H., and Cosson, J. (2006). Sperm motility in fishes. II. Effects of ions and osmolality: a review. *Cell Biol. Int.* **30**, 1–14. doi:10.1016/J.CELLBI.2005.06.004

Aliniya, M., Khara, H., Noveiri, S. B., and Dadras, H. (2013). Influence of age of common carp (*Cyprinus carpio*) broodstock on reproductive traits and fertilization. *Turk. J. Fish. Aquat. Sci.* **13**, 19–25. doi:10.4194/1303-2712-V13_1_03

Amann, R. P., and Waberski, D. (2014). Computer-assisted sperm analysis (CASA): capabilities and potential developments. *Theriogenology* **81**, 5–17.e3. doi:10.1016/J.THERIOGENOLOGY.2013.09.004

Asturiano, J. F., Sorbera, L. A., Carrillo, M., Zanuy, S., Ramos, J., Navarro, J. C., and Bromage, N. (2001). Reproductive performance in male European sea bass (*Dicentrarchus labrax*, L.) fed two PUFA-enriched experimental diets: a comparison with males fed a wet diet. *Aquaculture* **194**, 173–190. doi:10.1016/S0044-8486(00)00515-9

Babiak, I., Glogowski, J., Luczynski, M. J., and Luczynski, M. (1997). Effect of individual male variability on cryopreservation of northern pike, *Esox lucius* L., sperm. *Aquacult. Res.* **28**, 191–197. doi:10.1111/J.1365-2109.1997.TB01032.X

Babiak, I., Marschháuser, V., Ottesen, O., Rudolfsen, G., Eggen, B., and Babiak, J. (2012). Effects of extender, storage and sperm-to-egg ratio on cryopreservation success of Atlantic cod (*Gadus morhua* L.) sperm. *J. Appl. Ichthyol.* **28**, 941–947. doi:10.1111/JAI.12063

Blaxter, J. H. S. (2011). Seminal studies in the cryopreservation of fish sperm. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green.) pp. 886–889. (World Aquaculture Society: Baton Rouge, LA.)

Boryshpolets, S., Kowalski, R. K., Dietrich, G. J., Dzyuba, B., and Ciereszko, A. (2013). Different computer-assisted sperm analysis (CASA) systems highly influence sperm motility parameters. *Theriogenology* **80**, 758–765. doi:10.1016/J.THERIOGENOLOGY.2013.06.019

Brooks, S., Tyler, C. R., and Sumpter, J. P. (1997). Egg quality in fish: what makes a good egg? *Rev. Fish Biol. Fish.* **7**, 387–416. doi:10.1023/A:1018400130692

Butts, I. A. E., Trippel, E. A., and Litvak, M. K. (2009). The effect of sperm to egg ratio and gamete contact time on fertilization success in Atlantic cod *Gadus morhua* L. *Aquaculture* **286**, 89–94. doi:10.1016/J.AQUACULTURE.2008.09.005

Butts, I. A. E., Sørensen, S. R., Politis, S. N., Pitcher, T. E., and Tomkiewicz, J. (2014). Standardization of fertilization protocols for the European eel, *Anguilla anguilla*. *Aquaculture* **426–427**, 9–13. doi:10.1016/J.AQUACULTURE.2014.01.020

Caffey, R. H., and Tiersch, T. R. (2000). Cost analysis for integrating cryopreservation into an existing fish hatchery. *J. World Aquac. Soc.* **31**, 51–58. doi:10.1111/J.1749-7345.2000.TB00697.X

Carter, A. B., Carton, A. G., McCormick, M. I., Tobin, A. J., and Williams, A. J. (2015). Maternal size, not age, influences egg quality of a wild, protogynous coral reef fish *Plectropomus leopardus*. *Mar. Ecol. Prog. Ser.* **529**, 249–263. doi:10.3354/MEPS11277

Chalah, T., and Brillard, J. P. (1998). Comparisson of assessment of fowl sperm viability by eosin–nigrosin and dual fluorescence (SYBR-14/PI). *Theriogenology* **50**, 487–493. doi:10.1016/S0093-691X(98)00155-1

Chatakondi, N. G., and Davis, K. B. (2013). Maturity stage and plasma testosterone levels are related to sperm production in blue catfish, *Ictalurus furcatus*. *Aquacult. Res.* **44**, 161–166. doi:10.1111/J.1365-2109.2011.03019.X

Ciereszko, A., and Dabrowski, K. (1993). Estimation of sperm concentration of rainbow trout, whitefish and yellow perch using a spectrophotometric technique. *Aquaculture* **109**, 367–373. doi:10.1016/0044-8486(93)90175-X

Cosson, J. (2004). The ionic and osmotic factors controlling motility of fish spermatozoa. *Aquac. Int.* **12**, 69–85. doi:10.1023/B:AQUI.0000017189.44263.BC

Cuevas-Uribe, R., and Tiersch, T. R. (2011). Estimation of fish sperm concentration by use of spectrophotometry. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green.) pp. 162–200. (World Aquaculture Society: Baton Rouge, LA.)

Daly, J., and Tiersch, T. R. (2011). Flow cytometry for the assessment of sperm quality in aquatic species. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green) pp. 201–207. (World Aquaculture Society, Baton Rouge, LA.)

Donaldson, E., Solar, I. I., and Harvey, B. (2011). Induced ovulation and spermiation, and factors influencing gamete quality of fishes. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green) pp. 37–45. (World Aquaculture Society, Baton Rouge, LA.)

Dong, Q., Eudeline, B., Huang, C., and Tiersch, T. R. (2005). Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquacult. Res.* **36**, 86–93. doi:10.1111/J.1365-2109.2004.01188.X

Food and Agriculture Organization of the United Nations (FAO) (2011). 'Draft Guidelines for the Cryoconservation of Animal Genetic Resources.' Available at http://www.fao.org/docrep/meeting/022/mb553e.pdf. [verified 28 September 2015].

Galo, J. M., Ribeiro, R. P. B., Streit, D. P., Albuquerque, D. M., Fornari, D. C., Roma, C. F. C., and Guerreiro, L. R. J. (2015). Oocyte quality of Tambaqui (*Colossoma macropomum*) during the reproductive season. *Braz. J. Biol.* **75**, 279–284. doi:10.1590/1519-6984.10113

Garner, D. L., Johnson, L. A., Yue, S. T., Roth, B. L., and Haugland, R. P. (1994). Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. *J. Androl.* **15**, 620–629.

Geffen, A. J. (1999). Variations in sperm motility of the Atlantic herring *Clupea harengus*. *Mar. Biol.* **134**, 637–643. doi:10.1007/S002270050579

Giesing, E. R., Suski, C. D., Warner, R. E., and Bell, A. M. (2011). Female sticklebacks transfer information via eggs: effects of maternal experience with predators on offspring. *Proc. Biol. Sci.* **278**, 1753–1759. doi:10.1098/RSPB.2010.1819

Glenn, D. W., III, Lang, R. P., and Tiersch, T. R. (2011). Evaluation of extenders for refrigerated storage of koi carp and goldfish sperm. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green.) pp. 107–124. (World Aquaculture Society: Baton Rouge, LA.)

Hadfield, C. A., and Clayton, L. A. (2011). Fish quarantine: current practices in public zoos and aquaria. *J. Zoo Wildl. Med.* **42**, 641–650. doi:10.1638/2011-0034.1

Hauville, M. R., Rhody, N. R., Resley, M. J., Bell, J. G., Main, K. L., and Migaud, H. (2015). Comparative study of lipids and fatty acids in the liver, muscle, and eggs of wild and captive common snook broodstock. *Aquaculture* **446**, 227–235. doi:10.1016/J.AQUACULTURE.2015.04.026

He, S., and Woods, L. C., III (2004). Effects of dimethyl sulfoxide and glycine on cryopreservation induced damage of plasma membranes and mitochondria to striped bass (*Morone saxatilis*) sperm. *Cryobiology* **48**, 254–262. doi:10.1016/J.CRYOBIOL.2004.01.009

Hu, E. (2012). High-throughput sperm cryopreservation of aquatic species. Ph.D. Thesis, Louisiana State University, Baton Rouge, LA, USA.

Hu, E., and Tiersch, T. R. (2011). Development of high-throughput cryopreservation for aquatic species. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green.) pp. 995–1003. (World Aquaculture Society: Baton Rouge, LA.)

Hu, E., Liao, T. W., and Tiersch, T. R. (2013). A quality assurance initiative for commercial-scale production in high-throughput cryopreservation of blue catfish sperm. *Cryobiology* **67**, 214–224. doi:10.1016/J.CRYOBIOL.2013.07.001

Hu, E., Bosworth, B., Baxter, J., and Tiersch, T. R. (2014). On-site evaluation of commercial-scale hybrid catfish production using cryopreserved blue catfish sperm. *Aquaculture* **426–427**, 88–95. doi:10.1016/J.AQUACULTURE.2014.01.024

Iigo, M., and Aida, K. (1995). Effects of season, temperature, and photoperiod on plasma melatonin rhythms in the goldfish, *Carassius auratus*. *J. Pineal Res.* **18**, 62–68. doi:10.1111/J.1600-079X.1995.TB00141.X

Izquierdo, M. S., Fernández-Palacios, H., and Tacon, A. G. J. (2001). Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture* **197**, 25–42. doi:10.1016/S0044-8486(01)00581-6

Jenkins, J. A. (2011). Minimizing microbial contamination of sperm samples. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green.) pp. 684–687. (World Aquaculture Society: Baton Rouge, LA.)

Kjesbu, O. S., Kryvi, H., Sundby, S., and Solemdal, P. (1992). Buoyancy variations in eggs of Atlantic cod (*Gadus morhua* L.) in relation to chorion thickness and egg size: theory and observations. *J. Fish Biol.* **41**, 581–599. doi:10.1111/J.1095-8649.1992.TB02685.X

Li, M. L., Manning, B. B., Robinson, E. H., Bosworth, B. G., and Wolters, W. R. (2001). Comparison of growth, processing yield, and body composition of USDA103 and Mississippi 'normal' strains of channel catfish fed diets containing three concentrations of protein. *J. World Aquac. Soc.* **32**, 402–408. doi:10.1111/J.1749-7345.2001.TB00466.X

Li, M. L., Robinson, E. H., Manning, B. B., Yant, D. R., Chatakondi, N. G., Bosworth, B. G., and Wolters, W. R. (2004). Comparison of the channel catfish, *Ictalurus punctatus* (NWAC103 Strain) and the channel × blue catfish, *I. punctatus* × *I. furcatus*, $F_1$ hybrid for growth, feed efficiency, processing yield, and body composition. *J. Appl. Aquaculture* **15**, 63–71. doi:10.1300/J028V15N03_05

Liu, B., Liu, Y., and Wang, X. (2015). The effect of stocking density on growth and seven physiological parameters with assessment of their potential as stress response indicators for the Atlantic Salmon (*Salmo salar*). *Mar. Freshwater Behav. Physiol.* **48**, 177–192. doi:10.1080/10236244.2015.1034956

Lu, J. C., Huang, Y. F., and Lü, N. Q. (2014). Computer-aided sperm analysis: past, present and future. *Andrologia* **46**, 329–338. doi:10.1111/AND.12093

Meryman, H. T. (1966). Review of biological freezing. In 'Cryobiology'. (Ed. H. T. Meryman.) pp. 1–114. (Academic Press: New York.)

Montgomery, D. C. (2008). 'Introduction to Statistical Quality Control.' (Wiley: New York.)

Newman, A. E. M., Edmunds, N. B., Ferraro, S., Heffell, Q., Merritt, G. M., Pakkala, J. J., Schilling, C. R., and Schorno, S. (2015). Using ecology to inform physiology studies: implications of high population density in the laboratory. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **308**, R449–R454.

Oplinger, R. W., and Wagner, E. J. (2015). Use of penicillin and streptomycin to reduce spread of bacterial coldwater disease I: antibiotics in sperm extenders. *J. Aquat. Anim. Health* **27**, 25–31. doi:10.1080/08997659.2014.966211

Ostrach, D. J., Low-Marchelli, J. M., Eder, K. J., Whiteman, S. J., and Zinkl, J. G. (2008). Maternal transfer of xenobiotics and effects on larval striped bass in the San Francisco estuary. *Proc. Natl Acad. Sci. USA* **105**, 19 354–19 359. doi:10.1073/PNAS.0802616105

Ottesen, O. H., Babiak, I., and Dahle, G. (2009). Sperm competition and fertilization success of Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture* **286**, 240–245. doi:10.1016/J.AQUACULTURE.2008.09.018

Paterson, B. D., Rimmer, M. A., Meikle, G. M., and Semmens, G. L. (2003). Physiological responses of the Asian sea bass, *Lates calcarifer* to water quality deterioration during simulated live transport: acidosis, red-cell swelling, and levels of ions and ammonia in the plasma. *Aquaculture* **218**, 717–728. doi:10.1016/S0044-8486(02)00564-1

Perfetto, S. P., Chattopadhyay, P. K., Lamoreaux, L., Nguyen, R., Ambrozak, D., Koup, R. A., and Roederer, M. (2010). Amine-reactive dyes for dead cell discrimination in fixed samples. *Curr. Protocol. Cytom.* **9.34**, 1–20.

Peterson, B. C., Small, B. C., and Bosworth, B. G. (2004). Effects of bovine growth hormone (Posilac®) on growth performance, body composition, and IGFBPs in two strains of channel catfish. *Aquaculture* **232**, 651–663. doi:10.1016/S0044-8486(03)00526-X

Renard, P., Strüssmann, C. A., Ling, H., and Takashima, F. (1994). Evaluation of extenders for pejerrey *Odontesthes bonariensis* sperm. *Fish. Sci.* **60**, 661–666.

Riley, K. L., Chesney, E. J., and Tiersch, T. R. (2008). Field collection, handling, and refrigerated storage of sperm of red snapper and gray snapper. *N. Am. J. Aquaculture* **70**, 356–364. doi:10.1577/A07-061.1

Sargent, M. G., and Mohun, T. J. (2005). Cryopreservation of sperm of *Xenopus laevis* and *Xenopus tropicalis*. *Genesis* **41**, 41–46. doi:10.1002/GENE.20092

Seifi, T., Imanpoor, M. R., and Golpour, A. (2011). The effect of different hormonal treatments on semen quality parameters in cultured and wild carp. *Turk. J. Fish. Aquat. Sci.* **11**, 595–602. doi:10.4194/1303-2712-V11_4_13

Small, B. C., Davis, K. B., and Peterson, B. C. (2008). Elucidating the effects of cortisol and stress on economically important traits in channel catfish. *N. Am. J. Aquaculture* **70**, 223–235. doi:10.1577/A07-031.1

Stefansson, E. S., Heyes, A., and Rowe, C. (2014). Tracing maternal transfer of methylmercury in the sheepshead minnow (*Cyprinodon variegatus*) with an enriched mercury stable isotope. *Environ. Sci. Technol.* **48**, 1957–1963. doi:10.1021/ES404325C

Strüssmann, C. A., Renard, P., Ling, H., and Takashima, F. (1994). Motility of pejerrey *Odontesthes bonariensis* spermatozoa. *Fish. Sci.* **60**, 9–13.

Sühring, R., Freese, M., Schneider, M., Schubert, S., Pohlmann, J.-D., Alaee, M., Wolschke, H., Hanel, R., Ebinghaus, R., and Marohn, L. (2015). Maternal transfer of emerging brominated and chlorinated flame retardants in European eels. *Sci. Total Environ.* **530–531**, 209–218. doi:10.1016/J.SCITOTENV.2015.05.094

Suquet, M., Omnes, M. H., Normant, Y., and Fauvel, C. (1992). Assessment of sperm concentration and motility in turbot (*Scophthalmus maximus*). *Aquaculture* **101**, 177–185. doi:10.1016/0044-8486(92)90241-C

Suquet, M., Billard, R., Cosson, J., Normant, Y., and Fauvel, C. (1995). Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. *Aquaculture* **133**, 83–90. doi:10.1016/0044-8486(94)00395-5

Tan, E., Yang, H., and Tiersch, T. R. (2010). Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. *Zebrafish* **7**, 233–240. doi:10.1089/ZEB.2010.0655

Tavolga, W. N. (1949). Embryonic development of the platyfish (*Platypoecilus*), the swordtail (*Xiphophorus*) and their hybrids. *Bull. Am. Mus. Nat. Hist.* **94**, 161–230.

Tiersch, T. R. (2011). Process pathways for cryopreservation research, application and commercialization. In 'Cryopreservation in Aquatic Species'. (Eds T. R. Tiersch and C. C. Green.) pp. 646–671. (World Aquaculture Society: Baton Rouge, LA.)

Tiersch, T. R., and Green, C. C. (2011). Cryopreservation in aquatic species. (World Aquaculture Society, Baton Rouge, LA.)

Valdebenito, I. I., Gallegos, P. C., and Effer, B. R. (2015). Gamete quality in fish: evaluation parameters and determining factors. *Zygote* **23**, 177–197. doi:10.1017/S0967199413000506

Wang, Q., Lai, N. L.-S., Wang, X., Guo, Y., Lam, P. K.-S., Lam, J. C.-W., and Zhou, B. (2015). Bioconcentration and transfer of the organophorous flame retardant 1,3-dichloro-2-propyl phosphate causes thyroid endocrine disruption and developmental neurotoxicity in zebrafish larvae. *Environ. Sci. Technol.* **49**, 5123–5132. doi:10.1021/ACS.EST.5B00558

Yang, H., and Tiersch, T. R. (2009). Current status of sperm cryopreservation in biomedical research fish models: zebrafish, medaka, and *Xiphophorus*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **149**, 224–232. doi:10.1016/J.CBPC.2008.07.005

Yang, H., Carmichael, C., Varga, Z. M., and Tiersch, T. R. (2007*a*). Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish *Danio rerio*. *Theriogenology* **68**, 128–136. doi:10.1016/J.THERIOGENOLOGY.2007.02.015

Yang, H., Hazlewood, L., Heater, S. J., Guerrero, P. A., Walter, R. B., and Tiersch, T. R. (2007*b*). Production of $F_1$ interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the swordtail *Xiphophorus helleri*. *Biol. Reprod.* **76**, 401–406. doi:10.1095/BIOLREPROD.106.056549

Yang, H., Hazlewood, L., Walter, R. B., and Tiersch, T. R. (2009). Sperm cryopreservation of a live-bearing fish, *Xiphophorus couchianus*: male-to-male variation in post-thaw motility and production of $F_1$ hybrid offspring. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **149**, 233–239. doi:10.1016/J.CBPC.2008.10.103

Yang, H., Cuevas-Uribe, R., Savage, M. G., Walter, R. B., and Terrence, T. R. (2012). Sperm cryopreservation in live-bearing *Xiphophorus* fishes: offspring production from *Xiphophorus variatus* and strategies for establishment of sperm repositories. *Zebrafish* **9**, 126–134. doi:10.1089/ZEB.2012.0737

Yasui, G. S., Arias-Rodriguez, L., Fujimoto, T., and Arai, K. (2008). Simple and inexpensive method for cryopreservation of fish sperm combining straw and powdered dry ice. *Cryo Letters* **29**, 383–390.