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Addressing Reproducibility in Cryopreservation, and Considerations Necessary for Commercialization and Community Development in Support of Genetic Resources of Aquatic Species

Leticia Torres and Terrence R. Tiersch[1]

Aquatic Germplasm and Genetic Resources Center, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70820, USA

Abstract

For the past six decades a repeated cycle of developing new cryopreservation protocols or simply reinventing them to counteract a lack of reproducibility has led to hundreds of published studies that have offered little to the establishment of a genetic resources community for aquatic species. This has hampered repository development and inhibited industrial application. Most protocols were developed without standardized approaches, leading to irreproducible studies and questionable or meaningless comparisons. Thus cryopreservation of germplasm in aquatic species would greatly benefit from strategies to facilitate reproducibility. Our objectives were to: (1) identify major sources of irreproducibility across research, small-scale, repository, and commercial-scale development levels; (2) provide recommendations to address reproducibility challenges; and (3) offer suggestions on how researchers can directly influence commercial development and application of cryopreservation research. Sources of irreproducibility include lack of standardized procedural approaches, lack of standardized terminology, and lack of reporting guidelines. To address these challenges, we propose implementation of standard operating procedures, support of stock centers and internet content for development of training programs, and strengthening of the role of scientific journals and reviewers in reducing the frequency of irreproducible outcomes. Reproducibility is the foundation for quality management programs and product reliability, and therefore, standardization is necessary to assure efficient transition to commercial-scale application and repository development. Progress can only be possible through community-based approaches focused on coalescence and consensus of disparate groups involved in aquatic species cryopreservation and management of genetic resources.

KEYWORDS

cryopreservation, genetic resources, reproducibility

Since the 1950s hundreds of scientists around the world have devoted their careers to research leading to the commercialization of cryopreserved sperm from livestock. This technology development was spurred by large investments from public and private sectors and required years of research to develop the platforms currently in use for the multibillion-dollar global markets that exist for germplasm and genetic resources of livestock (Herman 1981). Genetic resources, which provide the basis for genetic improvement, come to us as the legacy of millions of years of evolution and as the product of millions of dollars of research and investment in selective breeding. They provide a fundamental basis for wealth in our society, and the prices we pay in the grocery store for food are directly linked to the genetic improvement that drives the value of agriculture. They have value in providing global markets for germplasm. They are society's insurance policy to protect agriculture against massive collapse due to disease epidemics. They are the basic tools of human disease research, and they are the storehouse for management and conservation of wild and endangered species. These concepts are long recognized in livestock as well as for crop plants (e.g., Byrne et al. 2018).

[1] Correspondence to: ttiersch@agctr.lsu.edu

Germplasm is also a form of wealth and functions as an exchange currency, allowing creation, maintenance, and distribution of the many values of genetic resources. Despite this, the great utility of germplasm is vastly underexploited for aquatic species because the banking mechanism, cryopreservation, requires significant improvement. Thus, aquaculture does not utilize cryopreservation despite its proven feasibility and is forced to collect, develop, maintain, and distribute genetic improvement in the form of live animals (fish and shellfish) at daily risk of disease, predation, and catastrophic loss due to disasters of natural (e.g., hurricanes) and nonnatural (e.g., oil spills) origin. This is tremendously expensive, risky, inefficient, and unnecessary. Because of this, many millions of dollars of public-sector (e.g., grant funding for genetics) and private-sector investment (e.g., fish farms) is continually at risk. Aquaculture researchers and producers are aware of this (e.g., fish diseases cost millions each year), but application efforts for cryopreserved germplasm and repository development are prevented by the lack of standardized procedural approaches, standardized terminology, and effective reporting guidelines.

Replication of experimental findings and the ability to make meaningful comparisons among studies are basic principles required for the advancement of science. In recent years, however, the reproducibility of scientific studies has begun to receive increased scrutiny due to problems, for example, in replicating biomedical research (Begley and Ellis 2012). For work to be reproducible, experiments should be replicable (able to be repeated exactly by independent groups and the original laboratory), and the outcomes should support the conclusions of the original study (Freedman et al. 2015). A lack of reproducibility has recently triggered the development and enforcement of new guidelines to perform and report the methodologies and outcomes of scientific work (e.g., https://www.nih.gov/research-training/rigor-reproducibility, accessed on January 5, 2018).

Cryopreservation of germplasm in aquatic species is a field that would greatly benefit from strategies to facilitate the reproducibility of studies. Since its origins, around 65 yr ago, hundreds of protocols have been developed for hundreds of species (Cabrita et al. 2009; Tiersch 2011). But due to a lack of community consensus and the broad variation in training and goals, most protocols were – and still are – developed without standardized approaches, terminology, or reporting guidelines, leading to irreproducible studies and questionable or meaningless comparisons (Torres et al. 2016). There are several recent reviews in the area of aquatic cryopreservation, including overview of multiple cell types (e.g., sperm, oocytes, embryos, somatic cells, and primordial germ cells) and the status of cryobanking efforts around the world (e.g., Martinez-Paramo et al. 2016). However, most work remain rooted in potential applications for research-level activities without consideration of the needs for high throughput (e.g., Magnotti et al. 2018) and have a primary focus on protocols (e.g., Cabrita et al. 2009). The need for standardized approaches is recognized in some publications but not dealt with beyond research scale (e.g., Asturiano et al. 2017). Many of these reviews also focus on direct comparisons of protocol details (e.g., extender and cryoprotectant) and results (e.g., motility, fertility, and cell viability) (e.g., Cabrita et al. 2010; Xin et al. 2017) despite the tremendous differences in practice, terminology, and reporting that disallow or weaken such comparisons.

Our goal in the present work was to raise awareness of the lack of standardized procedures, terminology, and reporting guidelines commonly observed in the aquatic species cryopreservation literature and to make recommendations using concrete examples that would help standardize or harmonize the current knowledge in the field, supporting research efforts and contributing to the eventual emergence of aquatic communities utilizing commercial markets in germplasm and genetic resources. Our specific objectives were to: (1) identify major sources of irreproducibility across research, small-scale, repository, and commercial-scale development levels; (2) provide recommendations to address reproducibility challenges; fabsence and (3) offer suggestions on how researchers can directly influence commercial development and the application of cryopreservation research.

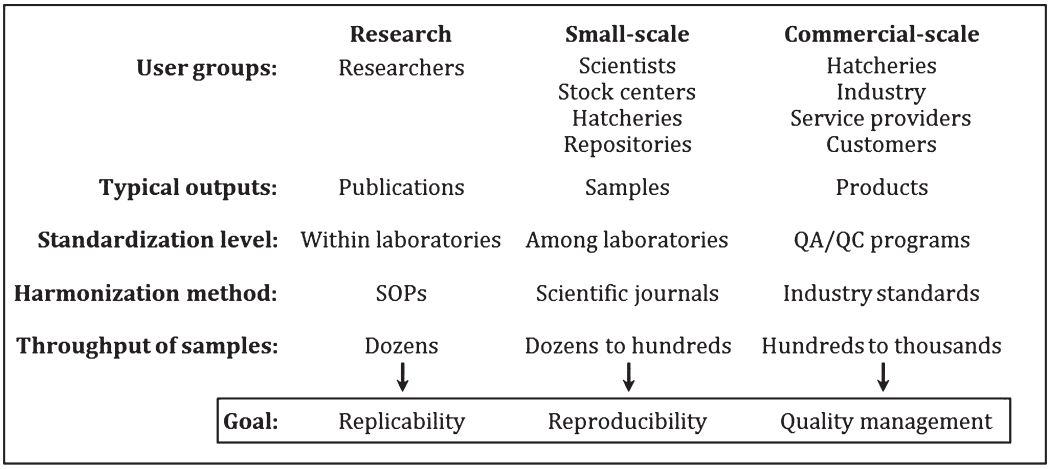

FIGURE 1. *Comparison of the attributes of three scales of cryopreservation activities. The past 65 yr of aquatic cryopreservation work has arrested primarily at the research level. Transition to higher levels of application will require changes in behavior and goals, including consideration of cost analyses. Industrial scale represents an extension of commercial-scale application and would require production levels in the millions of samples.*

Identifying Sources of Irreproducibility in Cryopreservation

In response to the great diversity of aquatic species, many hundreds of cryopreservation protocols have been developed (e.g., Cabrita et al. 2009; Tiersch and Green 2011). Differences among protocols range typically from the absence of or, at best, different methods to estimate sperm concentration, to the non-standardized addition of various types and concentrations of buffers (e.g., extender solutions) and cryoprotectants, use of various rates and modalities of cooling, and use of numerous methods to evaluate various aspects of sperm quality. As such, the sources of irreproducibility are multiple, and to identify each of them specifically would require detailed examination of each step in the cryopreservation pathway which would go beyond the intended scope of this paper. Instead, herein we will focus on major aspects responsible for irreproducible outcomes in germplasm cryopreservation of aquatic species, by relating these general problems to successive levels of use spanning research, small-scale, repository, and commercial-scale applications in terms of reproducibility (Fig. 1).

Research-scale Activities

The goal of research is to explore and develop new approaches, procedures, and evaluation tools that can contribute to the development of a field. It typically involves tens to dozens of samples per study. As the aquatic scientific community has begun realizing the benefits of germplasm cryopreservation in repository development for custodial activities of the world's biodiversity with potential for commercial application, the number of scientific papers in cryopreservation of aquatic species has increased steadily throughout the past 50 yr (Martinez-Paramo et al. 2016; Tiersch 2011). However, the number of papers generated has been overshadowed by the ubiquity of irreproducible results (Daly and Tiersch 2012; Dong et al. 2005). Reasons for irreproducible outcomes can be broadly grouped in three categories: lack of standardized procedural approaches, lack of standardized terminology, and lack of reporting guidelines.

In general terms, standardization implies normalization to a particular commonality established by consensus (Greenberg 2014). This is critical to reduce variability, assuring consistent results. However, any given

cryopreservation protocol encompasses numerous individual steps, each with its own sources of variability (Leibo 2011). The evaluation of sperm motility can serve as an example. When consistently evaluated at multiple key steps during the cryopreservation process, it can provide valuable information about sample quality, cryoprotectant toxicity, cooling rate adequacy, and thawing method (Torres et al. 2016). Despite this being the most readily available procedure to evaluate sperm quality in cryopreservation research, it is typically not performed, or only performed after thawing. When performed, the methods used to estimate sperm motility are not standardized. For example, the visual estimation of motility (also called "naked eye") is still one of the most common methods used, and yet it is the most subjective. Observations are highly dependent on the experience of the observer, the methodology and classification system used, and the time after activation when the observations are made (which is rarely standardized).

The time after activation is a critical variable considering the ephemeral motility span of most fish sperm (i.e., 60 sec or less). In addition, factors such as sample volume, activating solution, viewing chamber, use of cover slip or not, and even the definition of "motile" and the descriptive scales used are not standardized. More objective methods, such as those based on computer software (e.g., computer-assisted sperm analysis [CASA]), have been implemented in recent decades (Amann and Waberski 2014), but standardized procedures to handle samples, activate sperm, and report results are rarely addressed beyond individual laboratories (Scherr et al. 2012).

It is generally accepted that standardized terminology facilitates communication. When a variable is undefined, meaningful comparisons among studies can be problematic (if not impossible) to establish (Martinez-Paramo et al. 2016). Returning to the example of sperm motility, it has been defined variously using numeric scales or percentages with descriptors such as "rapid" or "vigorous" and includes terms such as "total motility", or the proportion of cells that move versus the proportion of cells that do not move. This is in addition to terms such as "progressive motility," or the proportion of sperm cells that move in a forward direction versus the proportion of cells that do not move forward plus those that do not move at all (Douglas-Hamilton et al. 2011).

With the use of CASA, multiple kinetic characteristics can be simultaneously analyzed for individual sperm cells (Fig. 2), and motility definitions can be specified by setting a minimum velocity under which a sperm cell is considered as nonmotile. Even these readings cannot be compared, however, if the instrument settings are different or not reported (Boryshpolets et al. 2013; Yang and Tiersch 2011). In addition, these diverse motility concepts are not officially defined, and terms are commonly used interchangeably for different meanings. Thus, standardizing the terminology in cryopreservation research would not only enable meaningful comparisons among studies, but would facilitate reproducibility of protocols and results. This would save time and money, reduce opportunity costs and increase confidence in expanded use of the technology. Standardization of terminology could proceed from a draft glossary (e.g., Shaw and Jones 2003) relevant to aquatic species that can be evaluated by members of a broad genetic resources community to ensure consensus and avoid repetitive (potentially conflicting) efforts.

It is important to make a distinction between the terms "replicability" and "reproducibility" when addressing the research level. Replicability poses the higher standard and is based on the concept that scientific results need to be exactly repeatable when tested under the same conditions. Reproducibility has emerged as a minimal acceptable standard for research given the practical problems involved in repeating work exactly in different locations (Casadevall and Fang 2010). Standardization of procedures within a given laboratory would certainly benefit replication of results within that laboratory, but it would benefit the rest of the scientific community only when other laboratories have access to that information. Sharing of information is fundamental for advancement and therefore requires major attention, whether it is used for standardization (based on commonality of methods) or harmonization (based on commonality of

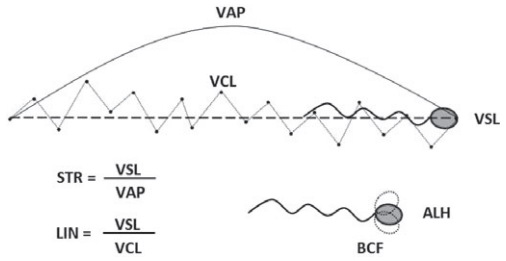

FIGURE 2. *Common kinetic features identified by computer-assisted sperm analysis (CASA) software. ALH = amplitude of lateral head displacement (corresponds to the mean width of the head oscillation as the sperm swims); BCF = beat cross frequency (frequency of sperm head crossing the sperm average path in either direction); LIN = linearity (measures the departure of the cell track from a straight line); STR = straightness (measures the departure of the smoothed cell path from a straight line); VAP = average path velocity (average velocity of the smoothed cell path); VCL = curvilinear velocity (average velocity measured over the actual point-to-point track followed by the cell); VSL = straight line velocity (average velocity measured in a straight line from the beginning to the end of track) (based on Douglas-Hamilton et al. 2011).*

results) of various approaches. At the moment, scientific journals accepting cryopreservation research for peer review do not provide structured guidelines of the types of information that should be collected and reported to facilitate the replicability or reproducibility of outcomes. We will discuss this subject in more detail below.

Small-scale Application

The goal of small-scale application is to ensure reproducibility after the focus shifts from fundamental discovery in a single research laboratory to a reduction of variability among produced samples to yield a useful technology (Benson et al. 2013). It typically involves the production of dozens to hundreds of samples. Application at a small scale can currently benefit researchers, stock centers, hatcheries, and repositories. However, the same pitfalls that affect reproducibility in individual research settings also affect small-scale applications, although at higher levels.

As pointed out above, standardization of procedures in research would benefit the replicability of outcomes in a given laboratory, but at the small-scale application level the benefits spread among other involved facilities. For example, sperm samples of a particular species or population can be deposited in a repository accepting material from multiple laboratories (e.g., Purdy 2008). Without the implementation of standardized or harmonized procedures, the quality of samples from the different source laboratories could vary greatly, perhaps more than what would be expected by biological variability alone. In addition, the procedures for thawing and use of samples could vary considerably, especially when different storage containers (e.g., straws or tubes) are used.

For example, as indicated earlier, sperm concentration can vary considerably depending on the methodology used, and is typically not estimated or controlled (Dong et al. 2007). Even assuming that all laboratories in this example used the same method (e.g., a particular sperm counting chamber) to evaluate the concentration of "fresh samples" (a term itself not typically defined), different handling and dilution approaches can lead to different apparent sperm concentrations (Torres et al. 2017; Yang et al. 2016a), different sperm-to-egg ratios, and variable fertilization success. In other words, these samples would be noncomparable in terms of sperm concentration as well as numerous other essential factors, due to the lack of standardized approaches. This example can be extended to standardization of terminology and reporting. Small-scale applications would include the participation of two or more facilities; therefore, adopting comparable terminology and reporting formats are critical for effective communication among user groups, especially those employing community-based resource centers.

Commercial-scale Application

The goal of commercial-scale application is to establish product features based on recognition of customer needs. It typically involves the production of hundreds to thousands (or in the case of established markets, millions) of samples. Introduction of products to markets requires establishment of standards and guidelines (Hu et al. 2013). Processes need to provide high throughput, and products need

to be reliable, delivering reproducible satisfactory performance (i.e., to meet established standards) within a certain acceptable range (Hu et al. 2014). This performance can be mediated through a variety of means ranging from voluntary practices to regulations by an independent entity to assure customer safety and satisfaction (Tiersch 2011). Reliability and reproducibility are interconnected at this level of application. Reproducibility, therefore, needs to be guaranteed through quality assurance programs that would ensure quality management of products in each step through the production line (e.g., Yang et al. 2016b).

In research and small-scale applications, the generation of samples through standardized procedures can encourage reproducibility, but within limited ranges. For example, some level of standardization would benefit reproducibility within one or a few laboratories, hatcheries, or repositories, but there would not be a common (or minimum) standard to which all establishments generating cryopreserved samples would comply. Addressing all aspects of reproducibility at a higher level, incorporating quality, time, and costs for technology utilization within a recognized community of providers and users such as aquaculture would require significantly greater thought and coordination (Engle 2017; Yang et al. 2017), and would fall under industrial approaches used to address quality management (Hu et al. 2013, 2015).

Industry standards are an approach to address this need, and are defined as "a set of criteria within an industry relating to the standard functioning and carrying out of operations in their respective fields of production" (http://definitions.uslegal.com/i/industrial-standards/, accessed on January 5, 2018). A simple example of an industrial standard for cryopreservation would be the use of specific calibration beads for instrument setup to estimate sperm concentration. Evaluating sperm concentration at key steps of the cryopreservation process is fundamental to quality assurance and quality control (QA/QC) (Dong et al. 2007; Hu et al. 2013; Torres et al. 2016). Therefore, the adoption of specific calibration beads that can be used with a specific counting chamber or CASA are beneficial examples. There are numerous types and brands of calibration beads on the market. There are not yet, however, efforts within an established aquatic community to specify universal calibration of fish sperm concentration methods. Thus, the problem is not necessarily a need for *de novo* development (such as research), and manufacturing of a new type of beads (although possible), but instead a problem of developing community consensus on the use of defined motility as a QC parameter and the use of a particular type of calibration method at defined steps during processing. Use of a commercial model such as industry standards could be employed within nascent aquatic cryopreservation communities to ensure product quality. It is also important for cryopreservation work to include relevant cost analysis to allow it to proceed beyond research to commercial-scale application (e.g., Caffey and Tiersch 2000, 2011; Hu et al. 2015).

Recommendations for Standardization and Harmonization

When working with biological entities, inevitable variation among samples is expected due to inherent genotypic, phenotypic, and environmental variability. Therefore, measures to control variation among outcomes – increasing reproducibility – are critical. In this section, we will explore some strategies, offering specific examples that would help increase reproducibility of cryopreservation procedures, and hopefully provide perspectives to stimulate discussion necessary to support community development.

Standardizing Procedures and Recording Methods

In a laboratory setting, the development of standard operating procedures (SOPs) is a common practice especially when dealing with specialized equipment (Schmid 2012). However, when new procedures are being developed in research, the main focus is typically to obtain results for publication, and often little initial attention is paid to SOP development. This in addition with other factors, including

replacement of personnel, can lead to shifts in procedures that once were validated. The development of SOPs not only assures that a procedure will be replicated properly, but that problems can be identified easily, and that outcomes can be compared over time.

A given cryopreservation protocol can encompass several SOPs (Table 1). Writing of SOPs is often considered as being mundane and time-consuming. However, "reinventing the wheel" each time new personnel are hired, or when unexpected outcomes occur, can significantly set back research and deadlines. Writing of SOPs requires a basic level of understanding of the process intended to be described, and a particular set of writing skills. Given their relevance, writing of SOPs should be part of basic training in laboratory settings. Fortunately, there are several guides available that can help with the development and organization of SOPs (e.g., http://www.wikihow.com/Write-a-Standard-Operating-Procedure, accessed on January 5, 2018). The basic characteristics of SOPs include clarity and conciseness. All of the materials needed (with catalog numbers), and personal protective equipment should be listed, and procedures detailed, keeping in mind that these are fundamental tools for training of new students or employees. Researchers not directly familiar with the particular task outlined in a SOP should be part of the review process because their objective comments are critical to assure clarity. Approaches such as these, although generally regarded as being commonplace by most scientists, are being instituted in force by efforts within the US National Institutes of Health to ensure that public funding of research is not wasted by production of nonreproducible research (https://www.nih.gov/research-training/rigor-reproducibility, accessed on January 5, 2018). In the future, community consensus on the particular basic SOPs that should be developed and shared will be needed when working with aquatic species to facilitate communication among laboratories (Table 1).

The greatest value of cryopreserved germplasm arises from the information it encompasses. Besides the genetic information contained in the cell itself and the relevant phenotypic observations (e.g., growth rates or dressing percentages), additional essential information for repository use derives from its source (i.e., the fish), and environment at the time of collection (Hu et al. 2013; Torres et al. 2016). This type of information is not always collected or archived. In the context of germplasm repositories (where information is critical), this contributes to irreproducible results, and perhaps

TABLE 1. *List of procedures organized in four stages within the cryopreservation process for which standard operating procedures could be developed and standardized (harmonized) within an aquatic germplasm community.*[a]

Stage in process	Procedure
1. Source, housing, conditioning, and transport	Reproductive condition screening
	Transport conditions
2. Sample collection and preparation	Disease screening
	Crushing of dissected testis
	Sperm collected by stripping
	Visual motility evaluation
	CASA motility evaluation
	Sperm concentration
	Storage method and time
	Sample transport
3. Freezing and cryogenic storage of samples	Sperm concentration
	Cryoprotectant addition
	Preequilibration motility
	Postequilibration motility
	Freezing: dry ice
	Freezing: controlled rate
	Cooling rate: microprocessor
	Cooling rate: dry ice
	Freezing termination steps
	Handling of frozen samples
	Transport of samples
4. Egg collection, thawing, and use of thawed sperm samples	Egg collection method
	Pooling of eggs
	Biosecurity precautions
	Thawing of samples
	Postthaw centrifugation
	Pooling of thawed samples
	Fertilization method
	Sperm-to-egg ratio
	Toxicity evaluation (control)
	Postthaw sperm quality
	Postthaw motility
	Egg quality assessment
	Fertilization estimation
	Hatching estimation

CASA = computer-assisted sperm analysis.

[a]Information of this type is often not included in published reports, weakening reproducibility and the ability to compare findings.

more importantly, leads to an incomplete portrayal of samples from a given organism. Therefore, the recording and reporting of relevant information are essential. Numerous kinds of data are routinely missing from research reports in the cryopreservation literature (Table 1).

To facilitate data recording, standardized forms can be used. Here, we provide an example of a form (Fig. 3) that was designed to compile information concerning the animal, sperm quality at the time of collection, dilution factors, and sperm quality after the addition of cryoprotectant (i.e., equilibration time). The form also includes important identification information (e.g., species, line, strain) as well as location information (inventory and repository number) relevant to the cryopreserved sample. This form was designed to collect relevant information for transcription to a database, and is an example of how data recording can be facilitated during sperm collection for routine association with specific samples in a repository database.

A similar approach could be developed to record water quality, conditioning regime or sample transport information as needed. Such forms can be custom printed on waterproof paper and be bound into notebooks for laboratory or hatchery use. There is no doubt that forms of this type are familiar to researchers in individual laboratories, but they are not typically coordinated for use among facilities. With consensus on content and format, this approach could be adopted and harmonized across user communities for small-scale, commercial, and repository uses. A prime example of a comprehensive repository database is provided by the Animal GRIN System (https://nrrc.ars.usda.gov/A-GRIN/main_webpage_dev/ars?record_source=US) of the USDA-ARS National Animal Germplasm Program (NAGP) located in Ft. Collins, Colorado, USA. This searchable public database compiles various types of information on samples including sources, inventory status, processing data, genetic analyses, and relevant phenotypes.

Stock Centers as Training Facilities

After a protocol is validated, sharing the news through a publication only begins the path to application. As mentioned earlier, at present the reproduction of a particular cryopreservation protocol from published reports can often be challenging. Specific processing details typically not included in publications can make large differences in outcomes. Also, necessary alterations in the protocol (e.g., switching sample containers from cryotubes to straws) or differences in equipment may require a deeper understanding of the principles of cryobiology beyond what is presented in a research publication to yield reproducible results (Benson et al. 2013). This is because cryopreservation encompasses a complex interplay of numerous factors including physical, biological, and practical. Physical factors would include glass transition temperature, surface-area-to-volume ratios, and heat transfer properties of materials. Biological factors would include cell toxicity of cryoprotectants, salt and pH (solution) effects, and dehydration (Mazur 2004). Practical factors would include cell concentrations relative to sperm production, sperm-to-egg-ratios, and the number of eggs per female. These represent a subset of the simultaneously interacting variables that would need to be balanced against factors such as unit cost per male or sample (Caffey and Tiersch 2000, 2011), and the requirements for labeling, storage, shipping, thawing, and use.

The typical response to addressing these difficulties is to test various promising cryoprotectants or cooling rates until a satisfactory outcome can be obtained. This empirical survey approach greatly hinders research by limiting experimental options for study of main effects (e.g., testing across a range of values for single or multiple variables), and only allows single comparisons of "one-off" approaches or methods, constraining development of a mechanistic understanding of relevant phenomena. This "hit or miss" approach is inefficient and decreases confidence in this research area as a legitimate field of scientific study. To overcome these potential difficulties, some form of comprehensive training is necessary.

Due to often limited funding available for research, few laboratories would be able (or willing) to develop and offer routine training to outsiders. Stock centers or other forms of community resource centers, may be better suited to

Project_____ Date_____ Notebook no._____
Start Time_____ Continued From Page_____

Incoming order# _____ Date_____ Technician_____

Species _____ Strain_____ Phenotype_____

Pedigree(s) _____ Tank_____

Dates of birth _____ Start time_____

Repository number _____

Animal ID _____

Inventory number _____

absorbance 400 (nm)	absorbance 600 (nm)

Notes:

Male #	Length (mm)	Weight (g)	Testis Weight (g)
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
		Total testis weight(mg)	

Straw label: X 30

Volume (ul) of HBSS to a _____

End Time_____

# Motile	# Non-Motile		Concentration	Motility
		Total		

# Motile	# Non-Motile		Concentration	Motility
		Total		

# Motile	# Non-Motile		Concentration	Motility
		Total		

Intial Cryoprotectant (%)_____

Total sperm volume _____

Volume of Cryoprotectant added (ul)_____

	Concentration	**Motility**
Average		

Final Cryoprotectant (%)_____

# of straws_____ Inventory location (#) _____

Entered in database_____

End Time_____ Continued on page _____

Signed _____ Date _____ 1 Signed _____ Date _____

FIGURE 3. *An example of a standardized data sheet used for collection of sperm samples. It includes information about the animals processed (origin, identification, and anatomy), sperm quality, sample preparation, and identification. This type of standardized data collection (Benson et al. 2013) would facilitate systematic entry of relevant data into repository databases and would be routine when adopted for use in small-scale and commercial-scale activities (Caffey and Tiersch 2011)*

provide cryopreservation training to particular research or user communities, and to adapt this training in response to stages in community development. Indeed, some stock centers (e.g., the Zebrafish International Resource Center [ZIRC]), Eugene, OR, USA) already have training programs and materials in place for fish husbandry (Westerfield 2007; https://zfin.org/zf_info/zfbook/zfbk.html), and some level of cryopreservation training (e.g., Matthews et al. 2018) could be included. Also, in addition to hands-on training at a stock center, strategies for remote training can be developed using online training videos and webinars. The centralization of training into community-based entities (e.g., stock centers) could largely facilitate the reproducibility of validated procedures across cryopreservation user groups (e.g., protocol section of the ZIRC website, http://zebrafish.org/documents/protocols.php). Another venue could be stand-alone workshops or workshop trainings held at widely attended meetings for user communities.

Stock centers also present opportunities for coordinating the testing and utilization of specialized devices that can be used to standardize cryopreservation. Certain uncontrolled or variable activities in the cryopreservation process, such as producing reproducible cooling rates across hundreds of separate users (e.g., Hu et al. 2017), can be addressed by widespread adoption of standardized devices in combination with accessible training (e.g., Scherr et al. 2015; Tiersch and Tiersch 2017). Such devices would follow a development pathway from innovation to customization to standardization. Innovation requires prototyping and feasibility (alpha) testing. Customization can occur within specific user communities to suit their needs and can be accomplished during performance (beta) testing or after dispersion of the technology. After suitable designs become adopted they can be coalesced into standardized, cheap, and widely available versions. New technologies such as three-dimensional (3D) printing can be used in all three of these developmental phases (Tiersch et al. 2018; Tiersch and Monroe 2016) to provide inexpensive, customizable, and widely available devices by electronic sharing of design files. Stock centers would be effective central locations to develop, test, and promulgate such devices to improve reproducibility, coordinate overall cryopreservation programs including training and web-based resources, and strengthen community identity and activities (more on this is presented below). Stock centers are well integrated among biomedical user communities (e.g., zebrafish), but will require development or establishment of analogous facilities for other communities such as aquaculture which may need to rely on state or federal hatcheries or specific laboratories to reliably provide these services.

Journals as Harmonizing Entities

Publishing is a proven method to share experiences and outcomes within the scientific community. In recent decades with development of the internet, studies are reviewed and published more rapidly, allowing scientists to disseminate new knowledge and develop new hypotheses, and bring these advances into application at a faster pace than ever before. Cryopreservation of aquatic species, however, has not yet experienced the same growth in application as other scientific fields. Despite the increased number of cryopreservation reports published in recent decades, breakthrough discoveries and actual transition to commercial application have been slow to occur. A substantial part of the problem is the difficulty in interpreting, comparing, and reproducing studies. As described above, much of this resides in incomplete descriptions of cryopreservation studies and frequent use of ineffective terminology.

Scientific journals can act as harmonizing entities in this regard for effective dissemination of scientific findings. They could, for example, provide guidelines that would assist evaluation of study validity and reproducibility, facilitating communication across the cryopreservation audience. This type of approach has been adopted by several biomedical research journals (McNutt 2014). Editors, funding agencies, and scientific leaders of biomedical fields gathered to discuss the role of scientific journals in addressing reproducibility of biomedical studies

in an initiative sponsored by the US National Institutes of Health (McNutt 2014). A product of that meeting was the generation of a common set of Principles and Guidelines in Reporting Preclinical Research (www.nih.gov/about/reportingpreclinical-research.htm) accessed on January 5, 2018), including the suggested use of checklists to ensure that essential experimental information is included in published papers.

The introduction of a checklist by leading journals that publish aquatic cryopreservation results would create awareness of the importance of information that tends to be excluded, helping researchers to plan experiments accordingly and would assist reviewers in assessing scientific merit. Cryopreservation protocols can vary greatly, but a simple backbone structure could yield significant improvement in reproducibility: samples are collected and their quality is evaluated, sperm concentration is estimated and adjusted, cryoprotectant is added and its toxicity assessed, samples are frozen, thawed, and their quality assessed. To stimulate discussion, we have assembled a tentative checklist that includes most of the variables to be considered in a basic cryopreservation pathway (e.g., Benson et al. 2013; Hu and Tiersch 2011) for any aquatic species (Table 2). Using such a checklist would help researchers to plan ahead to include essential variables in a study, while helping reviewers to keep track in a standardized manner of the key information required to reinforce study reproducibility. A checklist system would likely require phasing in by successive stages to allow researchers time to adjust their practices, including the incorporation of relevant cost analyses in their projects (e.g., Caffey and Tiersch 2011; Hu et al. 2015).

Creating an Involved Community

Consensus is necessary for any recommendations toward standardization, harmonization, and improved reproducibility to take effect. Only by general agreement would establishment of standardized protocols, reference standards, or calibration standards be possible. An agreement can only be achieved by coalescence and strengthening of awareness within the cryopreservation and genetic resource communities. To date, researchers working on germplasm cryopreservation of aquatic species are widely dispersed across disciplines, and have diverse training and backgrounds. For example, people working with commercial species may be most likely found in aquaculture, those working with natural populations may focus on resource management issues, those working with endangered species may be part of conservation groups, and those working with biomedical species may restrict their attention to health sciences.

Despite their common needs and interests, and the generalizability of the cryopreservation technology involved (Torres et al. 2016), a structured community where those working in all sectors of aquatic germplasm cryopreservation can interact does not yet exist. Part of the problem may be that the importance for germplasm cryopreservation in aquatic species is yet to be fully recognized, and that it is viewed primarily as a stand-alone technology. Another part of the problem beyond the nonoverlap of these communities is the simple lack of a single organizing theme or models for standardization to bring them together.

Despite the fact that cryopreservation research started simultaneously in mammals and fish, germplasm cryopreservation in livestock species has advanced considerably to the point that now it has become common practice in multiple species, including humans. It is safe to say that its success was directly proportional to the strength of its user communities. Looking back at its early beginnings, germplasm cryopreservation in mammals (specifically livestock) was welcomed as a valuable commercial tool that directly built upon existing breeding systems (e.g., stud registries), and now constitutes US$ multibillion global markets (Chandler and Godke 2011; Foote 2002). It was recognized that the cost of animal maintenance (e.g., space, food, medicine) was far higher than the costs to cryopreserve sperm, which protected and enabled easy distribution of valuable genetic resources, propelling the development and global expansion of the livestock germplasm cryopreservation industry. Thereafter, cryopreservation was used as a tool, to not only protect resources, but also

TABLE 2. *Checklist approach used to improve reproducibility of studies by journals that publish cryopreservation results.*[a]

Source, housing, conditioning, and transport	
[1]	Specify **strain** and source of organisms
[1]	Specify culture conditions, including water quality, **photoperiod**, and stocking density
[1]	Provide description (e.g., water quality), and GPS coordinates of collection site
[1]	Provide descriptive statistics for body size, **condition factor**, and estimated age or **reproductive stage**
[1]	Specify if collection took place during the breeding period, and describe the presence of **secondary sex characteristics**
[1]	Provide information describing reproductive condition (e.g., **sperm volume** and **GSI**)
[1]	Specify conditioning regime, including diet, photoperiod, or hormone treatments
[2]	Specify disease screening information for organisms and populations
[1]	Specify how long organisms were in transit, and the water quality and temperature changes during transport
[2]	Specify disease screening information for sperm
Sperm collection, freezing, and cryogenic storage	
[1]	Provide **male selection criteria**
[1]	Specify method of sperm collection (e.g., were the fish dissected or stripped?)
[1]	Specify volume of sperm sample collected per male
[1]	Provide protocol and data on **initial sperm quality analyses**
[1]	Specify composition, osmotic pressure, and pH of any extenders or buffers
[1]	Provide **sample quality selection criteria**
[1]	Specify if sperm samples were **pooled** or not at collection
[1]	Specify **sample dilution ratios**
[1]	Provide protocol for sperm concentration estimation
[1]	Provide **initial** and **adjusted sperm concentrations**
[1]	Specify storage method and storage duration if freezing is not immediate
[2]	Provide details about sample transport (e.g., transit time and temperature at arrival)
[1]	Specify if samples were pooled before freezing
[1]	Provide cryoprotectant **final concentration**
[1]	Specify cryoprotectant addition method
[1]	Provide protocol and data on **preequilibration** sperm quality analyses
[1]	Specify and define **equilibration time** (time after addition of cryoprotectant, before freezing)
[2]	Specify packaging container (e.g., catalog number), sample labeling method, sample loading volume, and container sealing method
[1]	Provide protocol and data on **postequilibration** sperm quality analyses
[1]	Provide standardized **thermal mass value** (number of samples frozen per batch)
[1]	Specify placement of containers in the freezing chamber
[1]	Provide freezing protocol when using dry ice
[1]	Provide controlled-rate freezer manufacturer and model
[1]	Provide **definition of cooling rate**, including **freezing curve** (specified time vs. temperature), and sample, chamber and programmed cooling rates
[1]	Specify freezing termination steps
[1]	Specify methods for sample sorting, storage, and storage duration
[1]	Specify protocol for handling of frozen samples
[2]	Specify disease screening information for frozen samples
[1]	Specify transport method for frozen samples
Egg collection, thawing, and use	
[2]	Provide information on conditioning regime, health status, and reproductive condition of females
[1]	Specify egg collection method
[1]	Specify **egg storage duration** and conditions
[1]	Specify if eggs were **pooled**
[2]	Provide information on biosecurity precautions for fertilization trials
[1]	Specify time intervals before and after thawing and thawing method
[1]	Specify postthaw centrifugation method, if used
[1]	Specify if thawed samples were **pooled**
[1]	Specify **interval before fertilization** (i.e., transfer time of samples after thawing before gamete activation)
[1]	Provide fertilization protocol, **total fertilization volume**, and **sperm-to-egg ratio**

TABLE 2. *Continued.*

Egg collection, thawing, and use	
[1]	Specify final **working concentration** of sperm (e.g., number of motile sperm per milliliter)
[1]	Provide protocol for water hardening step
[2]	Provide data on toxicity evaluation of cryoprotectants to gametes and early life stages
[2]	Provide protocol and data on **postthaw sperm quality analyses**
[2]	Provide **egg quality assessment** data (and **control treatments**)
[1]	Specify experimental design
[1]	Provide definitions of **fertilization** and **hatching**

GPS = global positioning system; GSI = gonadosomatic index.

[a]A checklist would probably best be implemented in phases (indicated in brackets) to allow gradual changes in practices. For example, Phase 1 could include data and practices required in every publication to be phased in within a 1–2 yr period; Phase 2 could include data and practices required in every publication within a 5-yr period. The following list is intended only to stimulate discussion. Terms requiring specific definition in publications are indicated in bold.

to generate genetic improvement through intensive breeding programs, and to generate profits beyond the food value of the animals involved: the gametes and associated genetic resources provided substantial forms of value themselves.

As mentioned earlier, standards and practices need to be satisfied and enforced for an industry to develop. Consensus in this regard is critical, as has been exemplified by the scope and purpose of the National Association of Animal Breeders (NAAB) whose goal is "...to unite those individuals and organizations engaged in the artificial insemination of cattle and other livestock into an affiliated federation operating under self-imposed standards of performance and to conduct and promote the mutual interest and ideals of its members" (http://www.naab-css.org/about/naab.html, accessed on January 5, 2018). The livestock cryopreservation community has benefited from NAAB activities, and utilizes the guidelines and standards promulgated by Certified Semen Services, Inc. (CSS), a subsidiary of NAAB. The CSS performs inspections, establishes rules and minimum standards for certification to assure semen quality, and is a source for standard procedures for handling, use, and labeling of products. Industry members are free to adopt these standards and participate in animal agriculture improvement programs through a certification program (https://www.naab-css.org/participation-agreement, accessed on January 5, 2018). As such, CSS is a means for the national animal breeding industry (livestock germplasm community) to employ self-regulation of its industry without direct governmental involvement.

The needs for sperm cryopreservation for aquatic species are even more profound in an era of rampant habitat loss and climate change. After 65 yr of research, it is now time to engage in the building of a genetic resource community focused on the final emergence of a commercial-scale approach to repository development and utilization of cryopreservation technologies in aquatic species. Earlier we discussed recommendations and the needed consensus that would facilitate reproducibility in terms of research and small- and large-scale applications. Here, we will focus on the next step: the role that research efforts would play in the establishment of a larger community, and the subsequent role of that community to actively establish a basis for a transition to commercial-scale applications.

As defined by the dictionary, communities arise from recognition of fellowship with others, as a result of sharing common attitudes, interests, and goals. In addition to the inherent taxonomic and physiological differences across fish and shellfish, researchers working with aquatic species have often relied on approaches (and training) derived from work with terrestrial vertebrates. This has in part hindered the emergence of a common identity and sense of belonging to an aquatic germplasm community. To address this problem, some of the early efforts in gathering cryopreservation researchers as an aquatic community included publication of the book "Action Before Extinction" (Harvey et al. 1998)

and the first and second editions of the book "Cryopreservation in Aquatic Species" (Tiersch and Green 2011; Tiersch and Mazik 2000).

Communication is critical to strengthen community identity, and scientific societies and journals that solely focus on aquatic community development in germplasm and genetic resources have not yet emerged. Today, however, new technologies and the internet offer opportunity to develop and strengthen communities, while offering global accessibility. Take for example the rapidly emerging 3D printing community of users and developers mentioned earlier. This is a growing online community where members benefit from available designs, as well as contribute with suggestions or new designs. These designs are collected on open sites (e.g., https://www.thingiverse.com/) and distributed as open-source files to be printed by anyone, facilitating sharing while assuring reproducibility of results (Tiersch and Monroe 2016). Traditional scientific societies and online communities are built under the same premises of identity and collaboration, but online communities also offer broader access and instant feedback. It is, therefore, worth consideration of online communities as a potential tool that can benefit aquatic species cryopreservation in terms of community development and reproducibility. A 3D printing community is especially relevant to cryopreservation due to the ability to rapidly prototype and test cryogenic devices made especially for aquatic species (e.g., Hu et al. 2017; Tiersch et al. 2018).

Undeniably, there truly is great societal need for aquatic germplasm repositories and their markets. But there are reasons why some markets thrive and others do not. Markets for livestock flourished rapidly because of preexisting demand and organization for use of improved genetics. Townspeople would pool their financial resources in Breeders Clubs to purchase superior bulls to service their cows and improve the local genetic pool. They were happy to switch to frozen semen when it became available through technology development and was commercialized with reliable quality, despite the additional expense for artificial insemination. Because this is a well-established industry, decades old, it may be difficult to see the direct linkage between it and industries such as aquaculture that do not currently utilize germplasm repositories. A more striking example is the rapid development of lucrative germplasm markets when technology was commercialized for deer and elk semen to supply improved genetics for breeders and hunters of trophy animals (Barnes et al. 2016). Captive deer breeding in the USA is a billion-dollar industry, and shows that when technology and reliability become available, commercialization can quickly follow.

The ultimate goal for aquatic species cryopreservation is to conserve genetic diversity regardless of the target species (e.g., endangered, natural fisheries, cultured, or biomedical). This goal resonates with the mission of germplasm repositories, placing them at the center of any cryopreservation community. Repositories can, therefore, influence community development directly by facilitating the exchange of research advances through scientific meetings, virtual communities, and by laying out recommendations to help smooth the transition to commercial-applications.

Recognizing commonalties among community members is the first step. The development of a community is, however, an active and continuous process that requires collective action by its membership. Different approaches can be used: some might be focused on processes and others on outcomes (Table 3). In any case, examples of key aspects that need to be addressed within aquatic species as the basis for industrial application include:

1. Establishment of minimum standards for evaluation and preservation of gamete quality.
2. Consensus on storage containers and labeling.
3. Consensus on the use of standardized protocols to evaluate sperm and egg quality.
4. Development, implementation, and reinforcement of biosecurity practices.
5. Consideration of certification entities designed to play a role comparable to CSS within the aquatic species cryopreservation community.

TABLE 3. *Approaches on priority issues based on processes and outcomes that need to be addressed at the core of community development to assure reproducibility in germplasm cryopreservation at three levels of application.*

	Community development approaches	
Community needs	Process	Outcome
Research activities		
Standard operating procedure (SOP) development	Workshops and training	SOP database access
Standardized terminology	Glossary development	Universal terminology
Reporting guidelines	Identify required information	Journals enforcing guidelines
Quality assessment	Gain recognition of importance	Journals enforcing specific endpoints
Small-scale application		
Standardization of protocols	Identify key steps in procedures	Adoption of standardized procedures
Standardization of terminology	Encourage use of key terminology	Adoption of standardized terminology
Reporting guidelines	Identify required information	Journals enforcing guidelines
Quality control (QC)	QC program development	QC program adoption
Commercial-scale application		
Industrial standards	Development of minimum standards	Certification processes
Quality management	QA/QC program development	QA/QC program adoption
Biosecurity	Guidelines, practices and regulations	Biosecurity adoption and enforcement

6. Development of comprehensive training programs.
7. Establishment of market-driven approaches to pricing for products and services.

Addressing these basic points as a community (leading to consensus) would support existing industries such as aquaculture (e.g., hatchery and selective breeding operations), and facilitate the emergence of new industries based on the market value of genetic resources similar to the multibillion dollar global markets existing for germplasm of livestock.

Connecting Research and Application

To complete this look to the future, it would be useful here to explore the concepts of "commercial scale" and "industrial scale" as they relate to aquatic cryopreservation. Commercial-scale activity would require high-quality automated processing on the order of thousands (i.e., tens to hundreds) of samples per year, bringing us to true high-throughput levels. This could satisfy the needs of several commercial farms, or a segment of a particular industry, for example, production of hybrid catfish (channel catfish, *Ictalurus punctatus*, female × blue catfish, *Ictalurus furcatus*, male; Hu et al. 2014, 2015). This could also accommodate systematic backup of reference and high-producing lines in archival storage at a repository such as the USDA-NAGP to safeguard future breeding. Industrial scale would go further to address the needs of entire or multiple industries and would require routine high-throughput processing of millions of samples per year. At present, automated systems available to process and store these numbers of samples are based on the plastic straws (e.g., 0.25-mL or 0.5-mL French straws) used for packaging of livestock semen. Automated equipment such as this is expensive (ca. US$90–100,000) and requires skilled users and maintenance. Commercial freezers are also expensive (ca. US$30–40,000), as are the large storage dewars ("cryogenic freezers") that would safely store approximately 200,000 samples each (ca. US$35,000).

It is important to reflect on the magnitude of disconnect between these high-throughput numbers and the practical reality of most aquatic cryopreservation researchers. The entire global production of aquatic cryopreservation researchers over the past 35 yr may be on the order of a million samples (and would be interesting to formally estimate). That estimate is substantially increased by inclusion of the few researchers addressing high-throughput methodologies (rather than protocol development), and

most researchers only produce hundreds or thousands of samples over an entire career. As such, commercial or industrial application is outside the day-to-day approach of most researchers. For example, they would more likely be interested in developing new assays to evaluate gamete quality rather than in utilizing gamete quality as a QC parameter in a production pathway as part of an overall quality management program adhering to industrial standards.

This is a fundamental concept. If scalability beyond research is not considered during protocol establishment, the systems developed will likely not be cost effective, efficient, or reliable at higher production scales. For example, consider the labor and inefficiency involved in filling, sealing, or labeling a million straws by hand. The need for automated processing is not an example of "pie in the sky" wishful thinking. Commercial and industrial levels of production are possible today as illustrated in the following examples. A single automated processing system (such as the Quattro, Minitube International, https://www.minitube.com/en/) can fill, seal, and label 15,000 straws per hour (there are two such high-throughput systems at the LSUAC Aquatic Germplasm and Genetic Resources Center). If we were to package only 20,000 straws per week, we would handle 1 million samples per year. Indeed the time bottleneck is not in the processing, but in the sorting, grouping, and inventorying of the samples before they go into storage. Other barriers include the cost of acquiring additional storage dewars and keeping track of individual customer needs and accounts.

Another real-world example of scale comes from the USDA-NAGP, which since its inception in 2000 has catalogued more than a million samples in its archival collection from more than 50,000 animals (including aquatic species) with a robust publicly accessible database (Animal GRIN). A final example of large-scale application for aquatic species comes from the ZIRC, which has around 100,000 samples (Z. Varga, Director, pers. comm.) representing more than 34,000 different genetic lines held in frozen storage (http://zebrafish.org/fish/lineAll.php), with most of these accessions coming in the past 5 yr (Murray et al. 2016).

Therefore, if only a single concept was to be taken from this article, it would hopefully be the realization that establishment of high throughput is a real-world concern that should be built into research intended to lead to large-scale application or commercialization of aquatic genetic resources. At present, scalability to high throughput has one primary path: the well-equipped central facility that has the equipment, trained personnel, and other resources necessary to routinely work at that level. A secondary path would be development of a mobile capability to bring true high-throughput processing to on-site locations. This would also require expensive equipment and trained personnel but could address situations where broodstock or samples cannot be shipped or would lose quality if transported. A mobile laboratory of this type has been developed and described, including cost analyses for construction and use (Childress et al. 2018).

A third path which should receive increased attention in the future would be the concept of "aggregate throughput." This is the embodiment of standardization (based on methodology), or harmonization (based on results), and would entail development of robust approaches that could place reliable, inexpensive, simple devices, with relevant methods and training, into the hands of multiple users mediated through internet websites or smartphone apps. The scale here would be hundreds to thousands of separate users, presumably organized into user communities. An example would be individual zebrafish research groups. Worldwide there are more than 1,200 registered zebrafish research groups (www.ZFIN.org). If most of these would provide 100 backup samples to a central repository or stock center, this would aggregate approximately 100,000 samples per year. This kind of production system clearly illustrates the need for community acceptance of standardization and quality assurance.

Working at these scales brings up another problem deriving from a persistent disconnect between research and application. The pricing of products and services is very sensitive to

production costs. What seems like an acceptable cost for a research study that utilizes less than 50 samples, can be excessively costly for application. For example, a recent study compared commercially available devices for vitrification (ultra-rapid freezing) of fish sperm (Kása et al. 2017). The device that provided the best results given the study parameters was one designed for medical use with human embryos that held a 2-µL sample and cost more than US$20 each (the "cryotop"; https://www.kitazato-dibimed.com/cryotop-vitrification/). No consideration was given in that study to comparison of performance and cost which has been recognized as an important factor for mammalian embryo vitrification (e.g., Marco-Jimenez et al. 2016). A different device in that study provided less satisfactory performance but with a larger volume (10 µL) and a much lower cost of US$0.1 each (allowing purchase of 200 for the price of one cryotop, and allowing freezing of 2,000 µL instead of 2 µL for the same cost). This latter device (a bacteriological inoculation loop), which has been used to vitrify sperm and produce live young in a live-bearing fish species (Cuevas-Uribe et al. 2011) clearly offers more promise for applied use and would warrant further study to improve its performance. Even better would be research addressing the customized production and testing of 3D printed devices for vitrification at a material cost of around US$0.01 each (Tiersch et al. 2018).

Given the points raised earlier, large-scale application and commercialization will not proceed directly from the current approaches to research. To express this bluntly, if research samples are packaged or labeled by hand, in containers that cannot be used in automated processing, or that cannot be reliably stored or retrieved from frozen storage, the value of that research is greatly diminished with respect to industrial relevance. Any findings would have to be reevaluated in the context of platforms supporting high throughput. Another blunt expression of this would be that any research that is not based on the use of specific (controlled) sperm concentrations for quality analysis, packaging, freezing, and use for fertilization is not relevant to high-throughput processing and commercialization because of incompatibility with the necessary QA/QC practices, product reliability (including fertilization dose concepts; Hu et al. 2014) and market pricing. And, as indicated earlier, research without relevant cost analysis does not lend itself in any way to large-scale application.

Conclusions

Basic research is fundamental to scientific progress and provides a foundation for discoveries to be brought into use by society. Applied research can, in its early phases, be similar to basic research when preliminary systems and platforms are prototyped and tested. However, with time this work should become increasingly concerned with the needs specific for application. With respect to aquatic cryopreservation and repository development this report has laid out a progression from early applied research, to small-scale application, to commercialization, and eventually industrial-scale activities. This transitional pathway is based on the need for high-throughput processing and the ability to reliably and efficiently handle samples along a gradient of scale (from tens of samples to millions). This progression for aquatic resources has been constrained during the past six decades for the most part by arresting at the boundary of basic research and early applied research. This illustrates the difficulties in bridging the gap between cryobiology research and its applications that has been observed previously within the broader biobanking and biorepository communities (Benson et al. 2013). The reasons for this lack of progress arise from multiple sources, but the need for reproducibility, standardization, and harmonization is a clear, correctable situation. Herein we recommend addressing the sources of irreproducibility that constrain research and application by use of standardized procedural approaches (including SOP development and training programs), standardized terminology (including community-based glossaries and clear definitions of terms in publications), and reporting guidelines (including checklists of necessary information and data) to strengthen the resources for scientific

journals and reviewers to reinforce reproducible outcomes and quality management. In addition, a clear understanding of the goals of applied research is necessary for community development to progress to include stakeholders from the private sector, and to offer solutions to real-world problems (e.g., QA-QC programs, and cost analyses). This would enable development of a larger, broader community supporting the availability and protection of germplasm of aquatic species, and greatly facilitate development and expansion of comprehensive repositories and establishment of lucrative international markets based on aquatic genetic resources.

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Literature Cited

Amann, R. P. and D. Waberski. 2014. Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology 81:5–17.

Asturiano, F., E. Cabrita, and A. Horvath. 2017. Progress, challenges and perspectives on fish gamete cryopreservation: a mini-review. General and Comparative Endocrinology 245:69–76.

Barnes, K. R., F. D. Mills, L. A. Wolfskill, S. S. Nair, J. R. Franken, and D. R. Ullrich. 2016. White-tailed deer semen auction data: a hedonic analysis. Texas Journal of Agriculture and Natural Resources 29:39–51.

Begley, C. G. and L. M. Ellis. 2012. Drug development: raise standards for preclinical cancer research. Nature 483:531–533.

Benson, E. E., F. Betsou, B. J. Fuller, K. Harding, and O. Kofanova. 2013. Translating cryobiology concepts into trans-disciplinary storage guidelines for biorepositories and biobanks: a concept paper. CryoLetters 34:277–312.

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

Byrne, P. F., G. M. Volk, C. Gardner, M. A. Gore, P. W. Simon, and S. Smith. 2018. Sustaining the future of plant breeding: the critical role of the USDA-ARS National Plant Germplasm System. Crop Science 58:451–468.

Cabrita, E., V. Robles, and P. Herráez. 2009. Methods in reproductive aquaculture: marine and freshwater species. CRC Press, Boca Raton, Florida, USA.

Cabrita, E., C. Sarasquete, S. Martinez-Paramo, V. Robles, S. Perez-Cerezales, and M. P. Herraez. 2010. Cryopreservation of fish sperm: applications and perspectives. Journal of Applied Ichthyology 26:623–635.

Caffey, R. H. and T. R. Tiersch. 2000. Cost analysis for integration of sperm cryopreservation into an existing fish hatchery. Journal of the World Aquaculture Society 31:51–58.

Caffey, R. H. and T. R. Tiersch. 2011. Economics and marketing of cryopreserved fish sperm. Pages 896–916 *in* T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Casadevall, A. and F. C. Fang. 2010. Reproducible science. Infection and Immunity 78:4972–4975.

Chandler, J. E. and R. A. Godke. 2011. Cryopreservation of bull sperm. Pages 291–297 *in* T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Childress, W., R. Caffey, and T. R. Tiersch. 2018. Design and cost analysis of a self-contained mobile laboratory for commercial-scale aquatic species cryopreservation. Journal of the World Aquaculture Society https://doi.org/10.1111/jwas.12525.

Cuevas-Uribe, R., H. Yang, J. Daly, M. G. Savage, R. B. Walter, and T. R. Tiersch. 2011. Production of F_1 offspring with vitrified sperm from a live-bearing fish, the green swordtail *Xiphophorus helleri*. Zebrafish 8:167–179.

Daly, J. and T. R. Tiersch. 2012. Sources of variation in flow cytometric analysis of aquatic species sperm: the effect of cryoprotectants on flow cytometry scatter plots and subsequent population gating. Aquaculture 370–371:179–188.

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

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

Douglas-Hamilton, D. H., K. M. Craig, and T. G. Kenny. 2011. Computer-aided semen analysis. Pages 255–260

in T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Engle, C. R. 2017. High-impact research: the critical need for strong engagement with aquaculture producers. Journal of the World Aquaculture Society 48:687–688.

Foote, R. H. 2002. The history of artificial insemination: selected notes and notables. Journal of Animal Science 80(E-Suppl._2):1–10.

Freedman, L. P., I. M. Cockburn, and T. S. Simcoe. 2015. The economics of reproducibility in preclinical research. PLoS Biology 13(6):e1002165.

Greenberg, N. 2014. Update on current concepts and meanings in laboratory medicine – standardization, traceability and harmonization. Clinica Chimica Acta 432:49–54.

Harvey, B., C. Ross, D. Greer, and J. Carolsfeld. 1998. Action before extinction: an international conference on conservation of fish genetic diversity. World Fisheries Trust, Victoria, Canada.

Herman, H. A. 1981. Improving cattle by the millions: NAAB and the development and worldwide application of artificial insemination. University of Missouri Press, Columbia, Missouri, USA.

Hu, E. and T. R. Tiersch. 2011. Development of high-throughput cryopreservation for aquatic species. Pages 995–1003 *in* T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Hu, E., T. W. Liao, and T. R. Tiersch. 2013. A quality assurance initiative for commercial-scale production in high-throughput cryopreservation of blue catfish sperm. Cryobiology 67:214–224.

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

Hu, E., T. W. Liao, and T. R. Tiersch. 2015. Simulation modeling of high-throughput cryopreservation of aquatic germplasm: a case study of blue catfish sperm processing. Aquaculture Research 46:432–445.

Hu, E., W. Childress, and T. R. Tiersch. 2017. 3-D printing provides a novel approach for standardization and reproducibility of freezing devices. Cryobiology 76:34–40.

Kása, E., G. Bernáth, T. Kollár, D. Żarski, J. Lujić, Z. Marinović, Z. Bokor, Á. Hegyi, B. Urbányi, M. Carmen Vílchez, M. Morini, D. S. Peñaranda, L. Pérez, J. F. Asturiano, and Á. Horváth. 2017. Development of sperm vitrification protocols for freshwater fish (Eurasian perch, *Perca fluviatilis*) and marine fish (European eel, *Anguilla anguilla*). General and Comparative Endocrinology 245:102–107.

Leibo, S. P. 2011. Sources of variation in cryopreservation. Pages 298–308 *in* T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Magnotti, C., V. Cerqueira, M. Lee-Estevez, J. G. Farias, I. Valdebenito, and E. Figueroa. 2018. Cryopreservation and vitrification of fish semen: a review with special emphasis on marine species. Reviews in Aquaculture 10(1):15–25.

Marco-Jimenez, F., E. Jimenez-Trigos, V. Almela-Miralles, and J. S. Vicente. 2016. Development of cheaper embryo vitrification device using the minimum volume method. PLoS One 11(2):e0148661.

Martinez-Paramo, S., A. Horváth, C. Labbé, T. Zhang, V. Robles, P. Herráez, M. Suquet, A. Serean, A. Viveiros, T. Tiersch, and E. Cabrita. 2016. Cryobanking of aquatic species. Aquaculture 472:156–177.

Matthews, J., J. M. Murphy, C. Carmichael, H. Yang, T. Tiersch, M. Westerfield, and Z. Varga. 2018. Changes to extender, cryoprotective medium, and in vitro fertilization improve zebrafish sperm cryopreservation. Zebrafish . https://doi.org/10.1089/zeb.2017.1521.

Mazur, P. 2004. Principles of cryobiology. Pages 3–65 *in* J. Fuller, N. Lane, and E. E. Benson, editors. Life in the frozen state. CRC Press, Boca Raton, Florida, USA.

McNutt, M. 2014. Journals unite for reproducibility. Science 346:679.

Murray, K. N., Z. M. Varga, and M. L. Kent. 2016. Biosecurity and health monitoring at the Zebrafish International Resource Center. Zebrafish 13 (Suppl. 1):S30–S38.

Purdy, P. H. 2008. Swine gene banking: a quality control perspective on collection, and analysis of samples for a national repository. Theriogenology 70:1304–1309.

Scherr, T., C. Quitadamo, P. Tesvich, D. Park, T. R. Tiersch, D. Hayes, J. W. Choi, K. Nandakumar, and W. T. Monroe. 2012. A planar microfluidic mixer based on logarithmic spirals. Journal of Micromechanics and Microengineering 22:055019 (1–10).

Scherr, T., G. Knapp, A. Guitreau, D. Park, T. R. Tiersch, K. Nandakumar, and T. W. Monroe. 2015. Microfluidics and numerical simulation as methods for standardization of zebrafish sperm cell activation. Biomedical Microdevices 17:65–74.

Schmid, I. 2012. How to develop a standard operating procedure for sorting unfixed cells. Methods 57:392–397.

Shaw, J. M. and G. M. Jones. 2003. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Human Reproduction Update 9:583–605.

Tiersch, T. R. 2011. Process pathways for cryopreservation research, application and commercialization. Pages 646–671 *in* T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Tiersch, T. R. and C. C. Green, editors. 2011. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Advances in World Aquaculture, Baton Rouge, Louisiana, USA.

Tiersch, T. R. and P. M. Mazik, editors. 2000. Cryopreservation in aquatic species. Advances in World Aquaculture, Volume 7. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Tiersch, T. R. and W. T. Monroe. 2016. Three-dimensional printing with polylactic acid (PLA) thermoplastic offers new opportunities for cryobiology. Cryobiology 73:396–398.

Tiersch, N. J. and T. R. Tiersch. 2017. Standardized assessment of thin-film vitrification for aquatic species. North American Journal of Aquaculture 79:283–288.

Tiersch, N. J., W. M. Childress, and T. R. Tiersch. 2018. 3-D Printing of vitrification loop prototypes for aquatic species. Zebrafish. https://doi.org/10.1089/zeb.2017.1520.

Torres, L., E. Hu, and T. R. Tiersch. 2016. Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development. Reproduction, Fertility and Development 28:1105–1115.

Torres, L., Y. Liu, A. Guitreau, H. Yang, and T. R. Tiersch. 2017. Challenges in development of sperm repositories for biomedical fishes: quality control in small-bodied species. Zebrafish 14:552–560.

Westerfield, M. 2007. The zebrafish handbook, 5th edition; a guide for the laboratory use of zebrafish (*Danio rerio*). University of Oregon Press, Eugene, Oregon, USA.

Xin, M., M. A. M. Siddique, B. Dzyuba, R. Cuevas-Uribe, A. Shaliutina-Kolesova, and O. Linhart. 2017. Progress and challenges of fish sperm vitrification: a mini review. Theriogenology 98:16–22.

Yang, H. and T. R. Tiersch. 2011. Application of computer-assisted sperm analysis (CASA) to aquatic species. Pages 240–254 *in* T. R. Tiersch and C. C. Green, editors. Cryopreservation in aquatic species, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Yang, H., J. Daly, and T. R. Tiersch. 2016a. Determination of sperm concentration using flow cytometry with simultaneous analysis of sperm plasma membrane integrity in zebrafish *Danio rerio*. Cytometry Part A 89:350–356.

Yang, H., J. Daly, C. Carmichael, J. Matthews, Z. Varga, and T. R. Tiersch. 2016b. A procedure-spanning analysis of plasma membrane integrity for assessment of cell viability in sperm cryopreservation of zebrafish *Danio rerio*. Zebrafish 13:144–151.

Yang, H., E. Hu, J. Buchanan, and T. R. Tiersch. 2017. A strategy for sperm cryopreservation of Atlantic salmon *Salmo salar* for remote commercial-scale high-throughput processing. Journal of the World Aquaculture Society 49(1):96–112. https://doi.org/10.1111/jwas.12431.