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Workshop report: Cryopreservation of aquatic biomedical models

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ABSTRACT

The genetic resources of aquatic biomedical model organisms are the products of millions of years of evolution, decades of scientific development, and hundreds of millions of dollars of research funding investment. Genetic resources (e.g., specific alleles, transgenes, or combinations) of each model organism can be considered a form of scientific wealth that can be accumulated and exchanged, typically in the form of live animals or germplasm. Large-scale maintenance of live aquatic organisms that carry these genetic resources is inefficient, costly, and risky. *In situ* maintenance may be substantially enhanced and backed up by combining cryopreserved germplasm repositories and genetic information systems with live animal culture. Unfortunately, cryopreservation has not advanced much beyond the status of an exploratory research for most aquatic species, lacks widespread application, and methods for successful cryopreservation remain poorly defined. For most aquatic species biological materials other than sperm or somatic cells are not comprehensively banked to represent and preserve a broad range of genetic diversity for each species. Therefore, new approaches and standardization are needed for repository-level application to ensure reproducible recovery of cryopreserved materials. Additionally, development of new technologies is needed to address preservation of novel biological materials, such as eggs and embryos of aquatic species. To address these goals, the Office of Research Infrastructure Programs (ORIP) of the National Institutes of Health (NIH) hosted the Cryopreservation of Aquatic Biomedical Models Workshop on January 7 to 8, 2017, in conjunction with the 8th Aquatic Animal Models of Human Disease Conference in Birmingham, Alabama. The goals of the workshop were to assess the status of germplasm cryopreservation in various biomedical aquatic models and allow representatives of the scientific community to develop and prioritize a consensus of specific actionable recommendations that will move the field of cryopreservation of aquatic resources forward. This workshop included sessions devoted to new approaches for cryopreservation of aquatic species, discussion of current efforts and approaches in preservation of aquatic model germplasm, consideration of needs for standardization of methods to support reproducibility, and enhancement of repository development by establishment of scalable high-throughput technologies. The following three broad recommendations were forwarded from workshop attendees:

1: Establish a comprehensive, centralized unit ("hub") to programmatically develop training for and documentation of cryopreservation methods for aquatic model systems. This would include development of species-specific protocols and approaches, outreach programs, community development and standardization, freezing services and training of the next generation of experts in aquatic cryopreservation.

2: Provide mechanisms to support innovative technical advancements that will increase the reliability, reproducibility, simplicity, throughput, and efficiency of the cryopreservation process, including vitrification and pipelines for sperm, oocytes, eggs, embryos, larvae, stem cells, and somatic cells of all aquatic species. This recommendation encompasses basic cryopreservation knowledge and engineering technology, such as microfluidics and automated processing technologies.

3: Implement mechanisms that allow the various aquatic model stock centers to increase their planning, personnel, ability to secure genetic resources and to promote interaction within an integrated, comprehensive repository network for aquatic model species repositories.

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1. Introduction

During the past century, aquatic animal species have emerged as powerful models for studying topics such as human development, behavior, genetics, and disease. Aquatic animals such as zebrafish [46], medaka [21,59], *Xiphophorus* [26,36,37,41] and *Xenopus* [7] are increasingly valuable to biomedical researchers because they provide critical insights to the mechanisms that underlie human health and disease. The ability to produce transgenic, knockout, and mutant lines of aquatic species has provided investigators with a variety of tools for translational research. Despite the significant cost to generate these lines, routine, reliable and cost-effective approaches for long-term preservation of these scientific resources are still lacking. Although cryopreservation of sperm is the sole and proven method for the long-term maintenance in many aquatic models, other approaches—such as additional germplasm preservation formats (oocyte, embryo, ovarian tissue, testicular tissue, larvae, or embryonic stem cells) or reproductive engineering technologies—are not currently available to aquatic researchers [28].

To address this need, the National Institutes of Health (NIH) Office of Research Infrastructure Programs (ORIP) sponsored a Cryopreservation of Aquatic Biomedical Models Workshop in conjunction with the 8th Aquatic Animal Models of Human Disease Conference, in Birmingham, Alabama. The objectives of the workshop, held on January 7–8, 2017, were to: (1) assess the status of germplasm cryopreservation in various aquatic models; (2) identify the obstacles, opportunities, and priorities that may address the need for improved methods, and (3) evaluate novel and emerging research and technologies that might lead to the reliable preservation of other germplasm formats.

Dr. Mary Hagedorn welcomed the participants and provided a cautionary tale about securing genetic resources. Coral is the most restricted reproductive species on the planet, with species and populations reproducing only 2 nights per year. More than 400 species of coral exist in the Australian Great Barrier Reef, but only 14 species have been banked, and until recently the Great Barrier Reef Marine Park Authority (who has supervisory oversight) has not funded cryopreservation work. Given the recent trends in coral bleaching, with more frequent and larger die-offs, species preservation is particularly critical. All opportunities to preserve aquatic resources must be taken, and cryopreservation banks may facilitate these efforts. Given this as a background, the experts assembled at the workshop were tasked to provide avenues for securing genetic resources of biomedical species, which can be used as models for wild populations and food resources. Given that NIH is open and willing to support the preservation of genetic diversity of many types of model aquatic species related to human disease, this was a timely and significant opportunity that warranted serious consideration.

Dr. Miguel Contreras acknowledged the efforts of the workshop organizing committee and provided an overview of ORIP, which was established in 2011 when the National Center for Research Resources was dissolved and several of its programs reassigned to the Office of the Director. The ORIP supports multiple NIH missions by providing essential resources to drive research discoveries related to human health. This workshop was sponsored by ORIP to provide an overview of the current status of aquatic cryopreservation among aquatic biomedical models, identify barriers that need to be addressed to move the field forward, discuss new opportunities and available technologies, and provide actionable recommendations to inform the office where to best place its resources.

2. Summary of presentations and discussions

2.1. Session 1: opportunities for cryopreservation of aquatic species: eggs, embryos, reproductive tissues and methods (Michael Chang, Moderator)

2.1.1. Development of a universal platform for aquatic germplasm cryopreservation (John Bischof)

Since the inception of modern cryopreservation techniques, successful cryopreservation of lower vertebrate embryos, including zebrafish, has remained an elusive goal for the past 67 years [20]. Multiple barriers exist for the cryopreservation of zebrafish embryos, including their large size, the presence of multiple internal compartments, low membrane permeability to cryoprotectants, and chill sensitivity. Microinjection of cryoprotectants and rapid cooling were used to overcome the permeability barrier. The relatively large size of the embryo, however, encourages the formation of lethal intracellular ice upon warming, even under the most rapid convective conditions [10–12].

This has necessitated a search for new ways to rapidly and uniformly warm these embryos. Laser irradiation of gold nano-rods (GNRs) was used to warm zebrafish embryos that had been frozen and stored in liquid nitrogen. The researchers developed protocols for micro-injection of a cryoprotectant (propylene glycol) and gold nano-rods into zebrafish embryos followed by rapid and uniform cooling at approximately 90,000 °C/min. This was followed by ultra-rapid warming at rates of 1.3×10^7 °C/min produced using a 1064-nm laser pulse (approximately 1 msec) that heated the GNRs and surrounding embryo, thereby outpacing intracellular ice formation (recrystallization) [20]. The approach appears physically scalable for germplasm as large as 1.4 mm. This ultrafast laser warming technology has the potential to transform banking of fish systems while also establishing a platform for conserving the germplasm of other oviparous vertebrate and non-vertebrate egg and embryo systems. Further experimentation, optimization and automation are planned.

2.1.1.1. Discussion. In response to a question regarding whether thermal conductivity changes with the gold nano-rods, Dr. Bischof indicated that it does not change. The participants discussed the use of dimethyl sulfoxide versus propylene glycol as cryoprotectants, noting that although dimethyl sulfoxide may be able to enter the cells of organisms that are less permeable, the yolk of the zebrafish and perhaps many other aquatic species, may be impermeable to most cryoprotectants [10–12]. Because a uniform distribution of gold nano-rods is necessary, the researchers are in the process of demonstrating uniformity through optical microscopy. The gold nano-rods do not need to be in every embryonic cell but should be uniformly distributed. In response to a question about whether the method would work in other fish species, Dr. Bischof thought that it would.

2.1.2. Production of viable trout offspring derived from frozen testis via germ cell transplantation (Goro Yoshizaki)

Many salmonid species have recently become at risk of extinction. For fish species with eggs that cannot be cryopreserved, establishment of techniques to preserve genetic resources such as eggs and embryos is imperative. To help overcome this challenge, studies on transplantation and cryopreservation of testicular stem cells have been performed during the last few years [24,25,63]. Specifically, spermatogonia from male trout were transplanted into the peritoneal cavity of newly hatched sterile triploid salmon. Transplanted trout spermatogonia created new donor gonads in the sterile salmon recipients. In male recipients, transplanted spermatogonia underwent spermatogenesis. Likewise, in female recipients, transplanted spermatogonia underwent oogenesis. At 2 to 3 years following transplantation, triploid salmon recipients produced only donor-derived trout sperm and eggs. Using artificial insemination with the sperm and eggs obtained from the triploid salmon recipients, only donor-derived trout offspring were produced. Combined with cryopreservation of spermatogonia, the present technique

makes it possible to preserve fish genetic resources and to revive extinct species when necessary. The researchers recently confirmed that this technology is also applicable to zebrafish and medaka.

2.1.2.1. Discussion. In response to a question regarding evolution, Dr. Yoshizaki explained that if the fish belong to the same genus, the host transplantation success rate is often high. When the fish belong to different genera, inducing egg generation is more difficult than sperm. Regarding the size of the testes in the samples, Dr. Yoshizaki indicated that although it depended on the maturity, the approximate length was 3 to 5 cm, and the diameter was ~2 mm. In response to a question about the rate of freezing, Dr. Yoshizaki stated that the sample was frozen at 1 °C/min and maintained at −80 °C for 90 min, followed by transfer and immersion in liquid nitrogen. Regarding whether the researchers had tried to dissociate the cells first, he explained that the laboratory had and it works, but the focus is on the protection of endangered species, so whole testis freezing is much easier. The survival rates between dissociated cells and whole testis were similar. Dr. Yoshizaki indicated that when whole testis or ovarian cells were transplanted; germ cell purification was not necessary. Stem cells can migrate to the recipient gonad.

2.1.3. Lyopreservation of sperm (Sankha Bhowmick)

The need for simple preservation technologies is becoming increasingly urgent as biomedical science and biotechnology develop a complex array of cell-based and tissue-based products. Desiccation preservation of nucleated mammalian cells offers an attractive alternative to liquid nitrogen cryopreservation protocols by potentially allowing space-efficient ambient-temperature storage. Inspiration for this comes from nature, where anhydrobiotic organisms survive extreme drought by moving to metabolic stasis, followed by resumption of life when water becomes available. Expression of intracellular non-reducing saccharides like trehalose seems to be the adaptive pathway taken by most of these organisms. A similar strategy has been adopted for desiccation preservation of mammalian cells; however, sophisticated techniques are required for delivering intracellular disaccharides.

During the past decade, researchers have focused on murine and bovine sperm as important models for desiccation preservation. Murine sperm preservation techniques require new strategies because of an exponentially increasing pool of transgenic mice. In this research, desiccation preservation strategies focused on recovering intact DNA that was used to fertilize eggs through intracytoplasmic sperm injection. The use of trehalose and the calcium chelator, EGTA, allowed long-term preservation of murine sperm DNA. For bull sperm, the requirements are more stringent where motility recovery is essential for the dairy industry. Using combinatorial methods of intracellular trehalose along with iron chelators (e.g., desferel) and osmolytes (e.g., sorbitol), desiccation tolerance has been improved in bovine sperm [45]. These results indicate that improving osmotic tolerance is a first important step in moving toward desiccation tolerance in mammalian cells. Further studies elucidating the dynamic transport events that lead to cellular desiccation stresses are underway.

2.1.4. Practical applications of somatic cell nuclear transfer in zebrafish (Jose Cibelli)

The use of zebrafish as an animal model for human disease is growing exponentially. New gene editing tools such as CRISPr/Cas are contributing to the increase in the number of zebrafish mutant strains created. Currently, the only method available to conserve germplasm of zebrafish lines is sperm cryopreservation, and while this process has recently been shown to be reproducible, it only preserves a haploid genome. The development of a germplasm conservation method that could preserve diploid genomes is desirable. Somatic cell nuclear transfer (SCNT) is a method that uses a diploid cell as nucleus donor, and an enucleated oocyte as the recipient cytosol. Once the donor is delivered into the enucleated egg, the cell cycle resumes, and a new animal is created.

This technique has been tested in zebrafish using the nuclei of multiple different cell types [42,43]. It was hypothesized that by finding a cryopreservation protocol for zebrafish somatic cells, such cells could be thawed and used as nuclei donors for SCNT, thereby developing a new germplasm conservation method for diploid genomes. Multiple freezing protocols and factors were evaluated, including commercially available growth substrates (e.g., Matrigel® and Geltrex®), culture medium and cryoprotectants, insulin-like growth factor (IGF), cell density, and the addition of a proprietary cell death inhibitor for cryopreservation and thawing. Freezing and thawing was successful for zebrafish somatic cells from the Tübingen, AB and Casper strains. Preliminary results indicated that thawed cells could also generate cloned fish. Future studies are aimed at optimizing the SCNT protocol and testing cloned fish for the capacity to generate offspring by fertilization. Other work includes oocyte activation methods using high-throughput compound screening, in collaboration with chemical engineers at Michigan State University.

2.1.4.1. Discussion. In response to a question regarding the method used for dissociating embryos for culture, Dr. Cibelli indicated that the protocol involved homogenous mixing and pipetting. He also explained that mitochondria were derived from the eggs, and the most useful eggs were those that were produced from the F_1 cross of Tubingen and AB lines.

2.1.5. A practical method for cryopreservation of medaka sperm and its application to other aquatic models (Kiyoshi Naruse)

A practical method was established for sperm cryopreservation in medaka based on a combination of results from two publications [2,22]. It was found that 10% dimethylformamide-fetal calf serum was an efficient cryopreservation medium that prevented premature initiation of sperm movement and increased viability after thawing. Use of glass capillary tubes allowed multiple *in vitro* fertilizations from a single male. Approximately 100 eggs could be fertilized with sperm from a single capillary tube. A 15-ml Falcon tube embedded in crushed dry-ice served as an insulator to induce a slow freezing rate. These data suggest that this method is applicable to sperm cryopreservation of stickleback (family *Gasterosteidae*), as well. An inexpensive and convenient method for sperm quality control and quantification utilized a phase-contrast microscope to produce mp4 movies of sperm motility for analysis using Adobe Photoshop and ImageJ [40].

2.1.5.1. Discussion. In response to a question regarding whether the method was suitable for *Xenopus*, Dr. Naruse explained that sperm quality is the most important factor in determining success. The trigger for activating *Xenopus* sperm is the decease of osmolarity [16]. Thus, this method could be effective for cryopreservation for *Xenopus* sperm. In terms of the freezing rate, the researchers quickly cooled the glass capillary tubes from room temperature to 4 °C using ice. A participant asked whether a quantified cutoff has been determined for quality control? Dr. Naruse explained that concentration was examined visually using video images.

2.1.6. Panel discussion

A participant noted that freezing of zebrafish embryos is essentially freezing of the chorion and the yolk sac. Some of the frozen yolk sacs can be thawed and survive to 24 h. The cells may not be viable after 24 h for many reasons (e.g., chorion hardening, oxygen issues). Hagedorn and Bischof have preliminary results with gold nano-rods, and other opportunities exist to explore this area [20]. A participant noted that uniformity is important because it would enable study of specific biological issues relevant to the different reasons for why some embryos die, and others survive. The researchers indicated that barriers to survival must be identified and addressed. A participant noted that freezing *per se* is not the bottleneck; the bottleneck is moving from 2 to

3% efficiency to 20–30% efficiency. At present, automation may not provide an advantage, as an experienced technician can process multiple samples. Also, a comprehensive gene bank must have the ability to utilize different approaches (e.g., cryopreservation, lyopreservation) and cell types. After researchers determine whether and what genes are activated by freezing and thawing, one approach could be to co-inject gold nano-rods with mRNA from the identified genes to suppress apoptotic pathways. Researchers must ask the right questions if they are going to be able to coax embryos to adulthood. Recent reports suggest that the methods have improved and a small subset of embryos that survive cryopreservation can reach maturity and produce normal offspring (Khosla, personal communication).

2.2. Session 2: reproducibility and standardization for repository development and throughput/scalability technologies (John Bischof, Moderator)

2.2.1. Reproducibility, quality control, and standardization (Terrence Tiersch)

Laboratories around the world have produced tens of thousands of mutant and transgenic zebrafish lines. Within the past decade, sperm cryopreservation has steadily improved to accommodate these lines, and cryopreservation is becoming applied for repository development. Despite this success, cryopreservation remains problematic for most aquatic models, and results are characterized by a pronounced lack of reproducibility and standardization [49]. Basic factors necessary for reproducibility (e.g., measuring and adjusting sperm concentration) are not employed, and large uncontrolled variation is an intrinsic condition that greatly reduces overall success and efficiency [50]. In addition, the primary quality control assay (motility) is either not utilized or used insufficiently, and when this assay is used for initial characterization of samples, it is not always predictive of post-thaw performance. These problems exist because current methods for assaying necessary factors, such as concentration and motility, are typically viewed as being as unnecessary, time-consuming and difficult to perform, and when performed, are plagued with the problems of lack of standardization and repeatability [51]. Continuation of the current practices for freezing and sharing of samples includes at least three levels of problematic consequences:

1. Small laboratories do not typically have access to reproducible technology, and in-house backup of lines can be difficult to perform or fail entirely. This lack of reproducibility creates doubt and requires effort and expense to freeze additional samples, or worse, it can require regeneration of lost lines, further increasing the effort and cost for research. As such, laboratories may have to submit lines repeatedly to resource centers with the accompanying recordkeeping.
2. Resource centers and repositories also suffer from the consequences of poor reproducibility. Because of a lack of standardization, resource centers must accommodate a wide diversity of sample containers and labeling, which causes problems for physical storage and recovery. They also must deal with multiple idiosyncratic protocols, with extra effort required to discern thawing and fertilization conditions. Additionally, when samples and lines have to be submitted repeatedly (requiring multiple thawing and fertilization attempts) the work load and costs increase. This can lead to thawing of all available samples of a line to ensure fertilization, thus wasting samples and leading to the risk of losing lines that were considered to be secure. Overall, this can impair the reputation of the resource center to protect or recover lines, despite the fact that samples were obtained from a submitting laboratory. This may prompt groups to generate mutants without utilizing resource centers, ultimately slowing research and weakening the connection of the centers with their research communities. To address this, the resource center must receive animals and be capable of performing freezing for laboratories that lack the capability.
3. Users of lines from resource centers also experience negative consequences. They depend on timely reconstitution of frozen lines, and if there are problems in regenerating frozen material delaying availability, the recipient laboratories lose valuable time. Alternatively, if lines are not recovered, users are charged a recovery (thawing) fee for material that they did not receive. This erodes trust in the resource center, and the affected laboratory may have to generate the particular mutants, further stalling projects and resulting in negative effects on performance of funded research.

These problems constitute an enormous unnecessary inefficiency, especially if multiplied across the numbers of males (and females) needed to back up tens of thousands of research lines that require freezing for storage. With the inclusion of relevant quality control steps (such as cell concentration and motility assessment), however, the likelihood of failure for properly handled samples decreases, and ultimately, the variability in use of thawed sperm could be largely confined to female conditioning and egg quality. These efforts can be assisted by custom prototyping and fabrication of devices and platforms that can be used for on-site cryopreservation by use of technologies such as 3-D printing [15,48] and microfluidics [38].

2.2.1.1. Discussion. When asked for examples of models that can address these problems Dr. Tiersch indicated that current thinking within such groups as the National Association of Animal Breeders and the biobanking community can be useful. The National Animal Germplasm Program headed by Dr. Harvey Blackburn of the U.S. Department of Agriculture (USDA) is an especially good resource. Dr. Tiersch also noted that the private sector has devoted resources to rapid genetic improvement of bovines. Although the federal government is also involved, the private sector has driven innovation. A participant noted that interagency collaboration would be needed to explore innovations for species other than bovines. Efforts to facilitate communication will be important because organizations and institutions have different interests and languages. Dr. Tiersch commented that not performing quality control does not save time and money because it only shifts the costs to later in the process (i.e., thawing and use). Within biomedical models, wild fisheries, imperiled organisms, and aquaculture each of hundreds of species has its own user communities and it would be beneficial for these communities to find ways to collaborate and generalize technology and approaches [47]. It will also be necessary to develop mechanisms for different goals and scales to be addressed comprehensively.

2.2.2. Microfabricated devices for standardization, reproducibility, and throughput of sperm cryopreservation (Todd Monroe)

Central repositories are refining protocols of cryopreservation to maintain and protect the genetic resources of thousands of lines of aquatic model species. This process is hindered, however, in individual research laboratories where freezing and analysis of gametes is problematic because of the lack of reproducibility and standardization. For example, sperm motility assessment lacks standardization across and within laboratories because manual methods are subject to human variation. Computer-assisted sperm analysis (CASA) has improved reproducibility over manual estimation but lacks control over the small sample volumes and short motility timeframes in many aquatic species.

These challenges can be addressed with microfluidic platforms in microfabricated systems that have been used to shorten analysis times, reduce volumes of reagents, and enable new discoveries in cell biology. These microfluidic “laboratory-on-a-chip” devices have been utilized in studies of gamete and embryo physiology, where microchannels have been used for reliable delivery of sperm to oocytes, gamete and embryo isolation and culture, and sperm sorting and separation. Achieving sperm activation in freshwater species for CASA requires mixing of the sperm sample with water which is difficult at the microscale because of

the low Reynold's number, where laminar fluid flow streams will not mix unless unique microchannel geometries are utilized. These micromixers are capable of activating zebrafish sperm more rapidly and reproducibly than manual mixing [33]. The goals of this effort are to utilize computational fluid dynamics simulations, computer-aided design, and 3-D printing and soft lithography microfabrication technologies to enable simulation, design, and fabrication of microfabricated laboratory-on-a-chip prototype devices to improve consistency in handling and analysis of small samples [38,39]. The use of microfluidic devices for activation of aquatic sperm cells can overcome several hurdles in activation studies [3]. These have the potential to improve cryopreservation protocols by allowing high-throughput testing of different cryopreserved samples under controlled conditions. This work also serves as a potential for much-needed standardization. Microfluidics methods are robust and can be used to reduce the variability within and across aquatic sperm research laboratories and repositories.

2.2.2.1. Discussion. Dr. Monroe explained that a silanization process can reduce cell adhesion, making it possible to reuse the chips, and that the most expensive part of production is making the master transparency (which costs approximately $30). It is possible that the material costs eventually could be reduced to $0.05-$0.10 per chip.

2.2.3. Can microfluidic platforms for droplets and bubbles enable high-throughput cryopreservation (Cari Dutcher)?

Microfluidic flow addresses geometries with at least one length scale less than ~300 μm. Microfluidics can be applied to biomedical and manufacturing processes and have increased convenience because of small size, lower cost, and the capability of providing multiple processes. Polydimethylsiloxane (PDMS) microfluidic devices, which require less than a day to fabricate after the master wafer has been made, can be used for as long as 1 yr, and fluid flow can be driven by pressure gradients or electric fields. Droplet microfluidic experiments can be non-contact and high throughput, with “on-the-fly” changes to chemical composition and thermal conditions possible. Droplet deformation can be used to measure such properties as surface tension [30]. Examples of the biomedical applications of microfluidics have been highlighted [5,29]. Droplets have been used to encapsulate mouse cells along with a fluorescent probe which, when bound by antibodies, produced a fluorescent signal. Droplet sorting has been performed with dielectrophoresis. Microfluidics can be used for single-cell high-throughput screening. Two-phase droplet microfluidics also may have a role in addressing current challenges of cryopreservation, such as finding a method to rapidly transport embryos to and from freezing devices. Microfluidics can provide a monodisperse, high-throughput droplet method for germplasm manipulation and may assist in several cryopreservation steps (e.g., cryoprotectant addition, cooling, storage, and warming). Microfluidics may also provide a platform to link, improve, or replace steps in the cryopreservation process.

2.2.3.1. Discussion. Participants discussed the logistics of using microfluidics for cryopreservation. Current platforms can attain dry ice temperatures, so the next challenge would be in attaining liquid nitrogen temperatures. Previous laboratory studies have shown that a biological unit (e.g., mammalian embryo) has survived microfluidic processes. Many different microfluidic systems exist, and researchers are beginning to examine questions associated with cryopreservation. Microfluidics may help to address obtaining fast freezing rates and could be better than, or used in conjunction with, conventional devices (e.g., the Cryotop® used for vitrification of embryos). A participant thought that examining the thermal mass of tiny droplets could be a modeling exercise and was curious about the rate of heat loss. Another participant stated that the process is similar to flow cytometry, noting the difficulty in finding an aperture that would accommodate a zebrafish embryo. Development of microfluidics to sort fluorescently labeled eggs of transgenic zebrafish, medaka, or other species would be a benefit to allow high-throughput analysis of breeding experiments. The participants agreed that combining microfluidic technology with other technologies (e.g., laser) would be beneficial.

2.2.4. Genetic resource preservation and management at the zebrafish international resource center (Zoltan Varga)

The Zebrafish International Resource Center (ZIRC) serves as a global central genetic repository for wild-type, mutant, and transgenic zebrafish lines. In the past 18 years, ZIRC has imported more than 40,000 alleles representing over 11,000 fish lines. To effectively manage these resources, ZIRC has improved its outdated cryopreservation method [27]; rewritten large parts of its inventory database, and implemented flexible strategies for the importation, maintenance, re-derivation, amplification, and distribution of its resources. The improved cryopreservation protocol plays a pivotal role in all of these processes. The new protocol is flexible, and can be adapted (scaled) for small, intermediate, and large laboratory operations, as well as for resource centers. In addition, it provides a basis for cryopreservation standards for the zebrafish research community. A collaborative approach, which ZIRC engaged in and established with NIH ORIP support, can also serve as a model to develop cryopreservation capabilities for other aquatic resource centers.

The goals of ZIRC are to become more efficient in managing its genetic inventory, to increase the number and types of genetic lines it cryopreserves, and to add services to its portfolio that support the research community. Wild-type lines for example continue to be regularly generated and molecularly characterized for specific research purposes [14,23,60]. However, these cannot be preserved by freezing haploid (sperm) cells and must be maintained as live populations, which places disproportionate demands on ZIRC operations for relatively few lines. The ZIRC is therefore particularly interested to adopt novel cryopreservation methods combined with reproductive technologies such as embryo cryopreservation [20] or somatic cell nuclear transfer [44]. These methods would provide important tools for ZIRC to achieve its repository goals. For example, by implementing embryo cryopreservation, somatic cell culture and cryopreservation, and SCNT, future services could include genetic preservation and distribution of sequenced wild-type lines, rescue of valuable research lines that are in danger of extinction, generation and distribution of clonal lines, or generation of pathogen-free fish.

2.2.4.1. Discussion. Dr. Varga explained that European Zebrafish Resource Center (EZRC) could coordinate better with ZIRC regarding protocols. The ZIRC protocol has been published [27] and in the interim between the conference and the publication of this report EZRC has begun using the protocol (personal communication, Dr.Varga). In response to a question regarding egg characteristics that potentially decrease the success of *in vitro* fertilization, Dr. Varga noted that animal husbandry and the condition of the male and female are critical. Unfortunately, it is not possible to reliably assess egg condition until after fertilization has been attempted. The assumption is that fertilization success is more dependent on egg quality than on sperm quality or quantity.

A participant asked about the number of full-time equivalents and the amount of funding the center devotes to cryopreservation. Dr. Varga explained that, on a weekly basis, about 75% of ZIRC effort is focused on thawing and shipping; the remaining 25% is focused on breeding and shipping. The specific costs to freeze and maintain a line have not been calculated by ZIRC because of the difficulties in identifying all of the components of the cost calculation. Popular lines may be requested five to six times per year, whereas other lines may be requested only once every other year. Dr. Varga noted that it is challenging to determine how many lines have been generated in the research community but have not yet been submitted to ZIRC. This may be due to reluctance to submit unpublished lines, because once submitted, they become part of the public domain. Based on the ZFIN database (www.

zfin.org), his guess was that 15,000 to 20,000 additional lines not currently maintained at ZIRC exist within the research community.

A participant noted that cryopreservation versus long-term maintenance of live animals is extremely cost-effective and suggested that ZIRC provide cryopreserved sperm for purchase. Harmonization of the various cryopreservation techniques used across the research community into a common platform would be a powerful approach to coalesce the activities of different groups given that the results are linked to quality control. The ZIRC protocol can be used as a model to help other communities. In addition, transportation of cryopreserved sperm can be less challenging than shipping of live animals. Cryogenic transport makes sense with bulk shipments, and ZIRC has used frozen sperm shipments to import large numbers of samples from large-scale genetic screens. However, shipping of only a few lines to laboratories is probably at present more efficient with live animals.

2.3. Perspectives from resource centers on current cryopreservation status and roadblocks (Terrence Tiersch, Moderator, substituting for Harvey Blackburn)

Dr. Tiersch described the three questions that directors of aquatic resource centers were asked to focus on during their presentations:

1. What are the realities of using cryopreservation at the center level?
2. What is needed to increase the dependability of cryopreserved germplasm?
3. Why is increased success of cryopreserved gametes important?

2.3.1. Implementing cryopreservation in Xenopus for the National Xenopus Resource (Marko Horb)

Cryopreservation in *Xenopus* is not standardized, and only in the last 10 years have a few published methods been tested in this genus [16,34,35]. Few laboratories utilize cryopreservation, with most maintaining live animals. With larger numbers of *Xenopus* lines being produced, the National *Xenopus* Resource (NXR) must improve cryopreservation for storage and to increase the use of lines by researchers. Even with current methods, however, frozen sperm preparations are inconsistent, and NXR frequently receives frozen sperm that cannot be used to produce viable embryos. The NXR is working closely with the European *Xenopus* Resource Center to improve cryopreservation methods and address these issues. When cryopreservation methods were tested in *X. laevis* and *X. tropicalis*, a large variation was observed, and efficiency was dependent on the females used. Efficiency, however, has improved over time. Dr. Horb reiterated the need to improve current inconsistent cryopreservation methods in *Xenopus* because of the increased numbers of transgenic and mutant frog lines being produced. This inconsistency is also increased as a result of rapid staff turnover, limited staff numbers, and inexperience at the NXR. Compounding these issues has been the reduction in NXR grant funding since 2011. Additionally, different *Xenopus* stock centers and individual researchers do not use a uniform cryopreservation method. The use of cryopreserved sperm within the *Xenopus* community would enhance research by expanding the number of different transgenic lines available for study. Most research in *Xenopus* is focused on the first 3 d of embryonic development; as such it is essential that cryopreserved sperm reliably produce hundreds of embryos at each use.

2.3.1.1. Discussion. Regarding training, Dr. Horb explained that NXR hosts two 1-wk workshops annually and a principal investigator meeting every other year. Another program allows laboratories to use NXR resources for a fee. Dr. Horb clarified that the NXR maintains 70 cryopreserved samples; no other center maintains cryopreserved *Xenopus* species. It is difficult for NXR to hire and maintain knowledgeable technicians based on geography and cost of living. Dr. Varga noted that technician performance was crucial when cryopreservation was in its infancy at ZIRC, but as the protocols have improved technician performance is becoming less critical. In coming years, it should be easier to teach replacement technicians the protocol.

2.3.2. Cryopreservation status and needs for the axolotl (Randal Voss)

The *Ambystoma* Genetic Stock Center (AGSC) at the University of Kentucky maintains a historically significant collection of Mexican axolotls *(Ambystoma mexicanum)* and provides living materials from this resource in support of biomedical research nationally and internationally [54]. Most notably, axolotls are studied because they are unique among vertebrates in being able to regenerate numerous tissues and body parts [53]. The AGSC currently is not cryopreserving germplasm to preserve or re-derive lines and mutants because a reliable cryopreservation method has not been established for any salamander species. Cryopreservation methods and an overall cryopreservation strategy were identified as critical needs for the salamander community at a recent meeting (Salamander Models in Cross-Disciplinary Biological Research, Vienna, Austria, 2018). A cryopreservation method is needed because the AGSC can accommodate only a fraction of community-generated living stocks, including knockouts and transgenics.

2.3.2.1. Discussion. In response to a question about whether the lines are strictly inbred, Dr. Voss explained that the center maintains 11 different mutant lines with some genetic variation. He clarified that the AGSC does not have the resources to develop a specific cryopreservation protocol for axolotls, but could perform cryopreservation on-site if a protocol existed. Cryopreservation would protect the existence of research lines which have been selected to breed and survive well in captivity over the past 100 yr. If the lines were lost, it would not be possible to duplicate them without cryopreserved samples because the necessary wild-type axolotls are no longer available from nature.

Dr. Voss answered questions related to spawning. On average, each spawn produces from 1 to 10 spermatophores and 200–600 eggs. Thus, spermatophores are readily available for cryopreservation. If cryopreservation of spermatophores or sperm could be achieved, *in vitro* fertilization could be performed to re-derive lines. Females can be induced to lay unfertilized eggs by intraperitoneal injection of human chorionic gonadotrophin. The *in vitro* fertilization process itself is highly efficient but time-constrained, as eggs and sperm must be mixed within 10 min of egg collection, and egg laying is a protracted process that takes several hours to complete. However, if only a few eggs are needed to re-derive a line, 50 to 100 eggs could be collected in 1 to 2 hours for this purpose.

2.3.3. Augmenting the preservation of genetic resources (Zoltan Varga)

The development of an improved sperm cryopreservation protocol has benefitted ZIRC operations and proven to be crucial for efficient import, management, and distribution of resources. Several areas, however, require continued attention and development of community-wide standards and methods. For example, potentially dangerous pathogens can be cryopreserved along with genetic resources [32]. Hence, biosecurity protocols need to be developed that address the potential propagation of pathogens when cryopreserved resources are re-activated. Second, virtually no protocols exist specifically for the cryopreservation of zebrafish somatic-, stem-, or cancer cell lines. Current protocols rely on methods derived from cryopreservation protocols for bacteria or for cells from non-aquatic vertebrate species.

Lastly, sperm cryopreservation and re-derivation of lines necessitate the maintenance of live wild-type reference strains, which are needed to produce eggs for fertilization after sperm sample thawing. Thus, 50% of the reconstituted genome will contain unmodified sequences from cryopreserved sperm, whereas the other 50% can drift over multiple generations by being subjected to intended and unintended genetic selection, the accumulation of background mutations, and progressive loss of genetic diversity [52]. Zebrafish embryo or oocyte cryopreservation is urgently needed to conserve and restore published

sequences when reference strains are reactivated in the future.

2.3.3.1. Discussion. Dr. Varga explained that for the reactivation of frozen sperm samples, wild-type strains must be maintained live to provide females that can be stripped for eggs for *in vitro* fertilization with thawed sperm. If it was feasible to also preserve this "female-derived" portion of the genome, there would not be a discrepancy between the live wild-type line accumulating sequence polymorphisms, variation, and drift away from the established (cryopreserved) sequence. If large quantities of eggs or embryos could be preserved, it would be possible to directly go back to the reference strain in the future. A participant noted that the current method to avoid this discrepancy is to back cross with thawed sperm, and that sperm contains essentially the entire genome (providing 50% of the genetic material in any cross, but representing almost all of the available loci). For example, it is possible to apply hormones and change the sex of the fish and generate lines with complete genomes. However, recent studies showed that the sex-determining loci were lost in some, but not all laboratory reference strains [60]. Thus, hormone-induced sex-reversal strategies using only sperm may not conserve the entire genome of some reference strains that are in use today. In addition, it is more difficult to generate homozygous diploid androgenic fish [4] than to generate parthenogenetic haploids and gynogenetic diploid offspring from eggs and UV-inactivated sperm [55]. Participants discussed the different definitions of "drift" and agreed that resurrecting the genome from cryopreserved embryos would be considerably faster and more efficient than back breeding several generations with sperm.

2.3.4. Cryopreservation in viviparous fishes (Xiphophorus spp.) (Ronald Walter)

Xiphophorus backcross hybrids were first developed as an experimentally tractable model for melanoma research in 1927. Since these early studies other *Xiphophorus* tumor models have been characterized and published [26,41,56–58]. In addition to cancer etiology, fishes in this genus are also used to explore sexual selection, behavior genetics, gene interactions after interspecies hybridization, pigment cell biology and speciation. The *Xiphophorus* Genetic Stock Center was established in New York in 1930 and operated there until moving to Texas State University in 1992. The center is an NIH-supported national scientific resource, with 24 of 26 known *Xiphophorus* species available for the research community. The stock center maintains 53 pedigreed lines and has produced 29 distinct interspecies hybrid cross models. *Xiphophorus* is a New World genus comprised of species distributed from northern Mexico and south along the Sierra Madre uplift into Belize. As live-bearing fishes with intricate mate selection and specialized breeding adaptations, *Xiphophorus* sperm look different than the sperm of other species [62]. In 2007 the first successful report of insemination and brood production using frozen sperm was published [61]. Currently, about 20 lines have enough cryopreserved sperm (i.e., at least 80 0.25-ml straws) to be considered appropriately backed up. These straws are held at the USDA National Animal Germplasm Program in Fort Collins, CO under the direction of Dr. Harvey Blackburn. Thirty-three additional fish lines are currently being processed for sperm cryopreservation.

Cryopreservation of about 1/3 of the lines in the *Xiphophorus* Genetic Stock Center would allow the facility to save noticeable space and labor because these lines are being maintained for perpetuation of single chromosomes that could easily be regenerated from cryopreserved sperm. In addition, cryopreservation of germplasm from these species is also considered a conservation effort due to destruction of natural site locales by urban expansion. At least one species held in the Center *(X. couchianus)* is considered to be extinct in the wild and four others are severely threatened. The ability of these fishes to produce fertile offspring makes cryopreservation of sperm a viable mechanism to maintain the genetic heritage of these valuable species should they become extinct in the wild.

2.3.4.1. Discussion. Dr. Walter explained that the XGSC has not attempted to apply a surrogate system to embryos because *Xiphophorus* are live-bearing where internal fertilization occurs within the female. Answering another question, he stated that the XGSC has maintained several inbred lines (via brother-sister mating) through more than 100 generations with no apparent problems for fish health, at least within the culture conditions used at XGSC.

2.3.5. Cryopreservation challenges for the national resource for Aplysia (Michael Schmale)

Aplysia californica, the California sea hare, is an ideal system for studies of neural circuits, the cellular basis of memory and learning, and changes associated with aging in an annual animal. The significance of the *Aplysia* model system derives from several features of its biology: (1) the simplicity of the nervous system — the *Aplysia* nervous system consists of only about 20,000 cells as compared to $\sim 10^9$ cells for mouse and $\sim 10^{12}$ cells in the human brain [17]; (2) the ability of *Aplysia* to exhibit simple forms of learning including habituation as well as more complex learning, such as classical and operant conditioning, context conditioning, taste aversion and conditioned 'fear' [6]; (3) the large size of the neurons (up to 1 mm cell body diameter for the largest cells in the abdominal ganglion); (4) the ease of relocating individual neurons from animal to animal, and (5) the ability to directly and reliably map neural networks responsible for simple behaviors [1]. In addition, a number of studies have shown that *Aplysia*, as a member of the Lophotrochozoa, has a much more conserved genome with more similarities to vertebrates than Ecdysozoa, which include such common animal models as *Drosophila* and *C. elegans* [8,31]. Recently these annual animals have also been shown to be excellent models of aging and particularly of the impact of aging on correlated changes in behavior, neurophysiology and neural transcriptomes [9,18,19].

The National Resource for *Aplysia* breeds, rears, and ships approximately 15,000 *Aplysia* per year at all life stages to laboratories in the U.S. and worldwide. It is the only facility of its kind and the only source of animals in early life stages. Cryopreservation would be advantageous for this species to create an archive of specific egg batch cohorts for use over multiple years, save inbred lines for genome studies, and assist future efforts to create transgenic lines. However, several challenges are associated with cryopreservation. *Aplysia* are simultaneous hermaphrodites (non-self-fertilizing) with internal fertilization. They breed on a daily basis yielding thousands of eggs which are encased in a double-layered egg capsule in addition to a chorion before being released in a continuous egg strand. These egg cases are tough and easy to handle but prevent easy access to the embryos. The complex process of encapsulation of newly fertilized eggs required for normal development will likely prevent successful *in vitro* fertilization (i.e., preventing the use of frozen eggs or sperm to preserve lines). Thus, cryopreservation will likely depend on preservation of embryos or newly released veliger larvae which emerge from the egg capsule approximately 7 days after fertilization. This will require methods to manipulate the embryos or larvae within the capsule, viably remove them, or to cryopreserve newly hatched larvae.

2.3.5.1. Discussion. Dr. Schmale explained that *Aplysia* are gastropod mollusks and although there are other gastropod models for neurobiology, *Aplysia* is by far the most widely used. Cryopreservation of embryos could be attempted at several stages; the trochophore stage which develops at 2 to 3 days after fertilization [13], veliger stages which develop during days 4 to 7, or newly released veliger larvae. The choice of approach would likely be a trade-off between ease of cryopreservation and likelihood of successful rearing after thawing. These stages are roughly 100 to 140 μm in diameter with veligers developing shells that are complete at hatching. Shell formation might interfere with cryopreservation, but more advanced developmental stages could be more likely to be successfully reared.

3. Summary and recommendations (Mary Hagedorn and Ronald Walter, Moderators)

3.1. Panel discussion

Based on the previous presentation and discussions, the participants discussed possible areas in which to provide specific, actionable recommendations to ORIP, identifying three clusters dealing with scope, scale, and approach. The participants agreed that addressing only one of these would not be enough; all three must be addressed to be successful. The three clusters were as follows:

1. Technology development in cryopreservation of samples
   a. Cryopreservation of embryonic and larval stages
   b. Cryopreservation of other materials (e.g., stem cells, sperm)
2. Improvement in cryopreservation mechanics (i.e., engineering technology and solutions)
   a. Improvements in microfluidic handling of embryos
   b. Improvements in cryopreservation efficiency and pipelines (e.g., automation, smaller sample volumes)
3. Establishment of a centralized center of cryopreservation expertise
   a. Fellowships and training in cryopreservation
   b. Dedicated expertise in developing aquatic models, protocols, and pipelines
   c. Development of customized protocols and pipelines
   d. Standardized digital record keeping, working back-up samples, long-term storage, and so forth

Prior to the meeting, participants were asked to respond to a detailed survey. Dr. Varga presented an overview of the survey questions and results (Appendix C). He commented that the current (23-question) survey could be reduced to ten questions for use with a wider audience, and it should request suggestions for short-term and long-term actions. The goal of the discussion was to understand the current state of the art, and how to address existing challenges; some systems may be more easily addressed than others, and each aquatic model has specific needs. Although most of the previous workshop discussions focused on cryopreservation more than on viability after thawing, the question arose whether animals grow and live a normal life following thawing. The participants agreed that revival must be considered, and post-fertilization animals must be evaluated. Cryopreservation of somatic cells also must be considered. Some zoos are cryopreserving somatic cells, and even cryopreserved blood cells may be useful. The ability to cryopreserve oocytes and eggs is at present a technical issue related to protocol development. Eggs should be preserved even if *in vitro* fertilization is not currently successful.

The participants discussed harmonization and standardization across and within communities, with priority being to identify resources to start this process. This will be especially helpful for laboratories that are not able to themselves justify the cost of investing in cryopreservation equipment. It was agreed that "tactical implementation" that allows sharing across communities and access to multiple laboratories would be important. This could include establishing a center (e.g., "hub") that allows laboratory personnel to visit and use resources or supporting visits by center personnel to laboratories that can provide training and expertise. The four tactical strategies for laboratories were to: (1) acquire and use the technology in-house; (2) invite personnel from a center to the laboratory to perform cryopreservation or training; (3) send material to a center for cryopreservation, or (4) send personnel to a center to receive training in protocols that do not require the laboratory to purchase special equipment or technologies. Community-based resources should be established that are centralized or distributed, and a culture of collaboration must be fostered.

A centralized unit where all aquatic model system communities can go to acquire expertise is desired. This unit should consistently apply standards to all aquatic resources and harmonize protocols. Storage would occur at individual resources centers, many of which would distribute their aquatic resources. Continued programmatic development is critical. The centralized unit also may need to possess husbandry capabilities so that professionals being trained at the center can work on their specific model rather than learning in a theoretical manner. A board of directors with expertise in this area should advise the centralized unit. Knowledge loss is a concern, so it would be useful to develop video and internet resources to ensure that the knowledge and expertise remain available over time despite retirement and turnover. Training must be specific to each species and model. The community also must consider and plan for future technical, staff, and knowledge needs. The ability to network at workshops and meetings also is critical.

The group discussed establishing this genetic resource workshop as an annual event. Dr. Michael Chang (ORIP, NIH) commented that a follow-up workshop potentially could be planned in 2 years to examine the status of the effort and recalibrate as necessary. Participants noted other meeting opportunities. A cryopreservation session could be added to the Aquatic Animal Models of Human Disease Conference, and workshop participants could attend the Annual Meeting of the Society for Cryobiology, which is held in the summer. The participants agreed that it would be helpful to develop a calendar of important, advantageous meetings.

3.2. Summary of "protection of essential genetic resources: survey for resource centers and research community"

As indicated above, a survey was conducted among workshop participants and Aquatic Resource Center Directors to assess the status and the needs of the research community for researching and developing novel cryopreservation methodologies, beyond sperm cryopreservation. The ten respondents used a variety of aquatic species, including Mexican axolotl (*Ambystoma mexicanum*), Fugu (*T. rubripes*), Gar (*Lepisosteus oculatus*), Killifish, Medaka (*Oryzias latipes*), *Xiphophorus* species, Zebrafish (*Danio rerio*) and other *Danio* species, Icefish (*Chaenocephalus aceratus*), Arctic charr (*Salvelinus alpinus*), and *Xenopus* species. Several aquatic species that are frequently used in aquatic research such as Goldfish (*Carassius auratus*) or Stickleback (*Gasterosteus aculeatus*) were not represented in this survey.

All respondents worked with wild-type and specialized (mutant, transgenic) fish lines in their facilities and preserved these genetic resources as live stocks or frozen sperm. Additional, less frequently used methods for genetic storage included embryo cryopreservation (1 laboratory), testicular stem cell preservation (1), or cryopreservation of whole testes (4 laboratories). All ten facilities indicated that it was important to develop more protection for their genetic resources and that they needed practical cryopreservation technology to be available for use in their repository or laboratories. The majority (90%) of respondents indicated that their research would benefit (significantly) from increased reproducibility if reliable cryopreserved genetic resources were routinely available and 80% thought that the role of particular model organisms would advance in biomedical research or benefit otherwise, if better cryopreservation methodologies were available to them. Several advanced supporting technologies, including web-based model organism database development and access, microinjection, intracytoplasmic sperm injection, somatic cell nuclear transfer, vitrification, laser warming/thawing, or 3-D printing would add particular value to their research (100%). At least half (50% or more) felt that reproducibility was the most important value criterion, followed by reliability, time-, and cost-efficiency.

During the closing session of the workshop it was discussed again that the relatively long and detailed survey provided to center directors (Appendix C.1) might not be effective for a wider audience, and that a shorter, more concise version of the survey with ten essential questions (Appendix C.1, in italics) could be circulated to the various aquatic research communities. The detailed results of the long version survey

are presented in a summary table (Appendix C.2).

3.3. Recommendations forwarded to ORIP by the workshop to improve cryopreservation in aquatic models utilized in biomedical research technologies

The following three broad recommendations are forwarded from workshop attendees:

1. Establish a comprehensive, centralized unit ("hub") to programmatically develop training for and documentation of cryopreservation methods for aquatic model systems. This would include development of species-specific protocols and approaches, outreach programs, community development and standardization, freezing services and training of the next generation of experts in aquatic cryopreservation.
2. Provide mechanisms to support innovative technical advancements that will increase the reliability, reproducibility, simplicity, throughput, and efficiency of the cryopreservation process, including vitrification and pipelines for sperm, oocytes, eggs, embryos, larvae, stem cells, and somatic cells of all aquatic species. This recommendation encompasses basic cryopreservation knowledge and engineering technology, such as microfluidics and automated processing technologies.
3. Implement mechanisms that allow the various aquatic model stock centers to increase their planning, personnel, ability to secure genetic resources and to promote interaction within an integrated, comprehensive repository network for aquatic model species repositories.

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Appendix A. Supplementary data

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References

[1] T.W. Abrams, Studies on *Aplysia* neurons suggest treatments for chronic human disorders, Curr. Biol. 22 (2012) R705–R711.
[2] K. Aoki, M. Okamoto, K. Tatsumi, Y. Ishikawa, Cryopreservation of medaka spermatozoa, Zool. Sci. 14 (1997) 641–644.
[3] J. Beckham, F. Alam, V. Omojola, T. Scherr, A. Guitreau, A. Melvin, D.S. Park, J.W. Choi, T.R. Tiersch, W. Todd Monroe, A microfluidic device for motility and osmolality analysis of zebrafish sperm, Biomed. Microdevices 20 (2018) 67.
[4] B.P. Brandhorst, G.E. Corley-Smith, Production of haploid and diploid androgenetic zebrafish, Methods Mol. Biol. 254 (2004) 255–270.
[5] E. Brouzes, M. Medkova, N. Savenelli, D. Marran, M. Twardowski, J.B. Hutchison, J.M. Rothberg, D.R. Link, N. Perrimon, M.L. Samuels, Droplet microfluidic technology for single-cell high-throughput screening, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14195–14200.
[6] T.J. Carew, Cellular and molecular advances in the study of learning in *Aplysia*, in: J.P. Changeaux, M. Konishi (Eds.), Neural and Molecular Basis of Learning, John Wiley, Berlin, 1987, pp. 177–204.
[7] R.M. Grainger, *Xenopus tropicalis* as a model organism for genetics and genomics: past, present and future, Methods Mol. Biol. 917 (2012) 3–15.
[8] J.B. Greer, S. Khuri, L.A. Fieber, Phylogenetic analysis of ionotropic L-glutamate receptor genes in the Bilateria, with special notes on *Aplysia californica*, BMC Evol. Biol. 17 (2017) 11.
[9] J.B. Greer, M.C. Schmale, L.A. Fieber, Whole-transcriptome changes in gene expression accompany aging of sensory neurons in *Aplysia californica*, BMC Genomics 19 (2018) 529.
[10] M. Hagedorn, E.W. Hsu, U. Pilatus, D.E. Wildt, W.R. Rall, S.J. Blackband, Magnetic resonance microscopy and spectroscopy reveal kinetics of cryoprotectant permeation in a multicompartmental biological system, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7454–7459.
[11] M. Hagedorn, F.W. Kleinhans, D. Artemov, U. Pilatus, Characterization of a major permeability barrier in the zebrafish embryo, Biol. Reprod. 59 (1998) 1240–1250.
[12] M. Hagedorn, F.W. Kleinhans, R. Freitas, J. Liu, E.W. Hsu, D.E. Wildt, W.F. Rall, Water distribution and permeability of zebrafish embryos, *Brachydanio rerio*, J. Exp. Zool. 278 (1997) 356–371.
[13] A. Heyland, Z. Vue, C.R. Voolstra, M. Medina, L.L. Moroz, Developmental transcriptome of *Aplysia californica*, J. Exp. Zool. B Mol. Dev. Evol. 316b (2011) 113–134.
[14] K. Howe, M.D. Clark, C.F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J.E. Collins, S. Humphray, K. McLaren, L. Matthews, S. McLaren, I. Sealy, M. Caccamo, C. Churcher, C. Scott, J.C. Barrett, R. Koch, G.J. Rauch, S. White, W. Chow, B. Kilian, L.T. Quintais, J.A. Guerra-Assuncao, Y. Zhou, Y. Gu, J. Yen, J.H. Vogel, T. Eyre, S. Redmond, R. Banerjee, J. Chi, B. Fu, E. Langley, S.F. Maguire, G.K. Laird, D. Lloyd, E. Kenyon, S. Donaldson, H. Sehra, J. Almeida-King, J. Loveland, S. Trevanion, M. Jones, M. Quail, D. Willey, A. Hunt, J. Burton, S. Sims, K. McLay, B. Plumb, J. Davis, C. Clee, K. Oliver, R. Clark, C. Riddle, D. Elliot, G. Threadgold, G. Harden, D. Ware, S. Begum, B. Mortimore, G. Kerry, P. Heath, B. Phillimore, A. Tracey, N. Corby, M. Dunn, C. Johnson, J. Wood, S. Clark, S. Pelan, G. Griffiths, M. Smith, R. Glithero, P. Howden, N. Barker, C. Lloyd, C. Stevens, J. Harley, K. Holt, G. Panagiotidis, J. Lovell, H. Beasley, C. Henderson, D. Gordon, K. Auger, D. Wright, J. Collins, C. Raisen, L. Dyer, K. Leung, L. Robertson, K. Ambridge, D. Leongamornlert, S. McGuire, R. Gilderthorp, C. Griffiths, D. Manthravadi, S. Nichol, G. Barker, et al., The zebrafish reference genome sequence and its relationship to the human genome, Nature 496 (2013) 498–503.
[15] E. Hu, W. Childress, T.R. Tiersch, 3-D printing provides a novel approach for standardization and reproducibility of freezing devices, Cryobiology 76 (2017) 34–40.
[16] T. Inoda, M. Morisawa, Effect of osmolality on the initiation of sperm motility in *Xenopus laevis*, Comp. Biochem. Physiol. A Comp. Physiol. 88 (1987) 539–542.
[17] E.R. Kandel, Cellular Basis of Behavior, Freeman and Co, San Fransico, 1976.
[18] A.T. Kempsell, L.A. Fieber, Aging in sensory and motor neurons results in learning failure in *Aplysia californica*, PLoS One 10 (2015) e0127056.
[19] A.T. Kempsell, L.A. Fieber, Habituation in the tail withdrawal reflex circuit is impaired during aging in *Aplysia californica*, Front. Aging Neurosci. 8 (2016) 24.
[20] K. Khosla, Y. Wang, M. Hagedorn, Z. Qin, J. Bischof, Gold nanorod induced warming of embryos from the cryogenic state enhances viability, ACS Nano 11 (2017) 7869–7878.
[21] S. Kirchmaier, K. Naruse, J. Wittbrodt, F. Loosli, The genomic and genetic toolbox of the teleost medaka (*Oryzias latipes*), Genetics 199 (2015) 905–918.
[22] A. Krone, J. Wittbrodt, A simple and reliable protocol for cryopreservation of Medaka (*Oryzias latipes*) spermatozoa, The Fish Biology Journal Medaka 9 (1997) 47-4F.
[23] M.C. LaFave, G.K. Varshney, M. Vemulapalli, J.C. Mullikin, S.M. Burgess, A defined zebrafish line for high-throughput genetics and genomics: NHGRI-1, Genetics 198 (2014) 167–170.
[24] S. Lee, Y. Iwasaki, S. Shikina, G. Yoshizaki, Generation of functional eggs and sperm from cryopreserved whole testes, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 1640–1645.
[25] S. Lee, G. Yoshizaki, Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*), Cryobiology 72 (2016) 165–168.
[26] B. Malitschek, D. Förnzler, M. Schartl, Melanoma formation in *Xiphophorus*: a model system for the role of receptor tyrosine kinases in tumorigenesis, Bioessays 17 (1995) 1017–1023.
[27] J.L. Matthews, J.M. Murphy, C. Carmichael, H. Yang, T. Tiersch, M. Westerfield, Z.M. Varga, Changes to extender, cryoprotective medium, and *in vitro* fertilization improve zebrafish sperm cryopreservation, Zebrafish 15 (2018) 279–290.
[28] P. Mazur, S.P. Leibo, G.E. Seidel Jr., Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions, Biol. Reprod. 78 (2008) 2–12.
[29] L. Mazutis, J. Gilbert, W.L. Ung, D.A. Weitz, A.D. Griffiths, J.A. Heyman, Single-cell analysis and sorting using droplet-based microfluidics, Nat. Protoc. 8 (2013) 870–891.
[30] A.R. Metcalf, H.C. Boyer, C.S. Dutcher, Interfacial tensions of aged organic aerosol particle mimics using a biphasic microfluidic platform, Environ. Sci. Technol. 50 (2016) 1251–1259.

[31] L.L. Moroz, A.B. Kohn, Do different neurons age differently? Direct genome-wide analysis of aging in single identified cholinergic neurons, Front. Aging Neurosci. 2 (2010).
[32] L.J. Norris, V. Watral, M.L. Kent, Survival of bacterial and parasitic pathogens from zebrafish (*Danio rerio*) after cryopreservation and thawing, Zebrafish 15 (2018) 188–201.
[33] D.S. Park, R.A. Egnatchik, H. Bordelon, T.R. Tiersch, W.T. Monroe, Microfluidic mixing for sperm activation and motility analysis of pearl Danio zebrafish, Theriogenology 78 (2012) 334–344.
[34] E. Pearl, S. Morrow, A. Noble, A. Lerebours, M. Horb, M. Guille, An optimized method for cryogenic storage of *Xenopus* sperm to maximise the effectiveness of research using genetically altered frogs, Theriogenology 92 (2017) 149–155.
[35] M.G. Sargent, T.J. Mohun, Cryopreservation of sperm of *Xenopus laevis* and *Xenopus tropicalis*, Genesis 41 (2005) 41–46.
[36] M. Schartl, R.B. Walter, Xiphophorus and medaka cancer models, Adv. Exp. Med. Biol. 916 (2016) 531–552.
[37] M. Schartl, R.B. Walter, Y. Shen, T. Garcia, J. Catchen, A. Amores, I. Braasch, D. Chalopin, J.N. Volff, K.P. Lesch, A. Bisazza, P. Minx, L. Hillier, R.K. Wilson, S. Fuerstenberg, J. Boore, S. Searle, J.H. Postlethwait, W.C. Warren, The genome of the platyfish, *Xiphophorus maculatus*, provides insights into evolutionary adaptation and several complex traits, Nat. Genet. 45 (2013) 567–572.
[38] T. Scherr, G.L. Knapp, A. Guitreau, D.S. Park, T. Tiersch, K. Nandakumar, W.T. Monroe, Microfluidics and numerical simulation as methods for standardization of zebrafish sperm cell activation, Biomed. Microdevices 17 (2015) 65.
[39] T. Scherr, C. Quitadamo, P. Tesvich, D.S. Park, T. Tiersch, D. Hayes, J.W. Choi, K. Nandakumar, W.T. Monroe, A planar microfluidic mixer based on logarithmic spirals, J. Micromech. Microeng. 22 (2012) 55019.
[40] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.
[41] Y. Shen, D. Chalopin, T. Garcia, M. Boswell, W. Boswell, S.A. Shiryev, R. Agarwala, J.N. Volff, J.H. Postlethwait, M. Schartl, P. Minx, W.C. Warren, R.B. Walter, X. couchianus, X. hellerii, Genome models provide genomic variation insight among *Xiphophorus* species, BMC Genomics 17 (2016) 37.
[42] K. Siripattarapravat, B. Pinmee, E.A. Chang, J.D. Munoz, K. Kawakami, J.B. Cibelli, The influence of donor nucleus source on the outcome of zebrafish somatic cell nuclear transfer, Int. J. Dev. Biol. 54 (2010) 1679–1683.
[43] K. Siripattarapravat, B. Pinmee, P.J. Venta, C.C. Chang, J.B. Cibelli, Somatic cell nuclear transfer in zebrafish, Nat. Methods 6 (2009) 733–735.
[44] K. Siripattarapravat, S. Prukudom, J. Cibelli, Method for somatic cell nuclear transfer in zebrafish, Methods Cell Biol. 135 (2016) 245–257.
[45] R. Sitaula, H. Elmoazzen, M. Toner, S. Bhowmick, Desiccation tolerance in bovine sperm: a study of the effect of intracellular sugars and the supplemental roles of an antioxidant and a chelator, Cryobiology 58 (2009) 322–330.
[46] B. Tavares, S. Santos Lopes, The importance of zebrafish in biomedical research, Acta Med. Port. 26 (2013) 583–592.
[47] T.R. Tiersch, C.C. Green, Introduction, Cryopreservation in Aquatic Species, second ed., World Aquaculture Society, Baton Rouge, 2011.
[48] T.R. Tiersch, W.T. Monroe, Three-dimensional printing with polylactic acid (PLA) thermoplastic offers new opportunities for cryobiology, Cryobiology 73 (2016) 396–398.
[49] L. Torres, E. Hu, T.R. Tiersch, Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development, Reprod. Fertil. Dev. 28 (2016) 1105–1115.
[50] L. Torres, Y. Liu, A. Guitreau, H. Yang, T.R. Tiersch, Challenges in development of sperm repositories for biomedical fishes: quality control in small-bodied species, Zebrafish 14 (2017) 552–560.
[51] L. Torres, T.R. Tiersch, Addressing reproducibility in cryopreservation, and considerations necessary for commercialization and community development in support of genetic resources of aquatic species, J. World Aquacult. Soc. 49 (2018) 644–663.
[52] B. Trevarrow, B. Robison, Genetic backgrounds, standard lines, and husbandry of zebrafish, Methods Cell Biol. 77 (2004) 599–616.
[53] S.R. Voss, H.H. Epperlein, E.M. Tanaka, *Ambystoma mexicanum*, the axolotl: a versatile amphibian model for regeneration, development, and evolution studies, Cold Spring Harb. Protoc. 2009 (2009) pdb.emo128.
[54] S.R. Voss, M.R. Woodcock, L. Zambrano, A tale of two axolotls, Bioscience 65 (2015) 1134–1140.
[55] C. Walker, G.S. Walsh, C. Moens, Making gynogenetic diploid zebrafish by early pressure, JoVE (2009).
[56] R.B. Walter, L. Hazlewood, S. Kazianis, The Xiphophorus Genetic Stock Center Manual, Texas State University, 2006.
[57] R.B. Walter, S. Kazianis, *Xiphophorus* interspecies hybrids as genetic models of induced neoplasia, J. Ins. Lab. Animal Res. 42 (2001) 299–322.
[58] R.B. Walter, S. Kazianis, L. Hazlewood, D. Johnston, J. Kumar, The Xiphophorus genetic stock center, in: M.C. Uribe, G. H (Eds.), Viviparous Fishes, New Life Publications, Homestead, FL, 2005, pp. 343–350.
[59] R.B. Walter, T. Obara, Workshop report: the medaka model for comparative assessment of human disease mechanisms. Comparative biochemistry and physiology, Toxicol. Pharmacol.: CB (Curr. Biol.) 178 (2015) 156–162.
[60] C.A. Wilson, S.K. High, B.M. McCluskey, A. Amores, Y.L. Yan, T.A. Titus, J.L. Anderson, P. Batzel, M.J. Carvan 3rd, M. Schartl, J.H. Postlethwait, Wild sex in zebrafish: loss of the natural sex determinant in domesticated strains, Genetics 198 (2014) 1291–1308.
[61] H. Yang, L. Hazlewood, S.J. Heater, P.A. Guerrero, R.B. Walter, T.R. Tiersch, Production of F1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the swordtail *Xiphophorus helleri*, Biol. Reprod. 76 (2007) 401–406.
[62] H. Yang, L. Hazlewood, R.B. Walter, T.R. Tiersch, Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*, Cryobiology 52 (2006) 209–218.
[63] G. Yoshizaki, K. Fujinuma, Y. Iwasaki, T. Okutsu, S. Shikina, R. Yazawa, Y. Takeuchi, Spermatogonial transplantation in fish: a novel method for the preservation of genetic resources, Comp. Biochem. Physiol. Genom. Proteonomics 6 (2011) 55–61.