Theriogenology 135 (2019) 138–151

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Development of germplasm repositories to assist conservation of endangered fishes: Examples from small-bodied livebearing fishes

Yue Liu [a, b], Harvey Blackburn [c], Sabrina S. Taylor [d], Terrence R. Tiersch [a, *]

[a] Aquatic Germplasm and Genetic Resources Center, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA, USA
[b] Department of Biological and Agricultural Engineering, Louisiana State University Agricultural Center, Baton Rouge, LA, USA
[c] National Animal Germplasm Program, United States Department of Agriculture, Agricultural Research Service, Fort Collins, CO, USA
[d] School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA, USA

ARTICLE INFO

Article history:
Received 20 May 2019
Accepted 26 May 2019
Available online 28 May 2019

Keywords:
Germplasm repositories
Sperm cryopreservation
Live-bearing fishes
Endangered aquatic species
Conservation

ABSTRACT

Germplasm repositories are a necessary tool for comprehensive conservation programs to fully preserve valuable genetic resources of imperiled animals. Cryopreserved germplasm can be used in the future to produce live young for integration into other conservation projects, such as habitat restoration, captive breeding, and translocations; thus compensating for genetic losses or negative changes that would otherwise be permanent. Although hundreds of cryopreservation protocols for various aquatic species have been published, there are great difficulties in moving such research forward into applied conservation projects. Successful freezing of sperm in laboratories for research does not guarantee successful management and incorporation of genetic resources into conservation programs in reality. The goal of the present review is to provide insights and practical strategies to apply germplasm repositories as a real-world tool to assist conservation of imperiled aquatic species. Live-bearing (viviparous) fishes are used as models herein to help explain concepts because they are good examples for aquatic species in general, especially small-bodied fishes. Small live-bearing fishes are among the most at-risk fish groups in the world, and need urgent conservation attention. However, development of germplasm repositories for small live-bearing fishes is challenged by their unusual reproductive characteristics, such as formation of sperm bundles, initiation of spermatozoa motility in an isotonic environment, internal fertilization and gestation, and the bearing of live young. The development of germplasm repositories for goodeids and *Xiphophorus* species can provide examples for addressing these challenges. Germplasm repositories must contain multiple basic components, including frozen samples, genetic assessment and information systems. Standardization and process generalization are important strategies to help develop reliable and efficient repositories. An ideal conservation or recovery program for imperiled species should include a comprehensive approach, that combines major concerns such as habitat (by restoration projects), population propagation and maintenance (by captive breeding or translocation projects), and preservation of genetic diversity (by repository projects). In this context, strong collaboration among different sectors and people with different expertise is a key to the success of such comprehensive programs.

1. Introduction

Effective animal conservation programs for imperiled animals require a combination of activities and capabilities. Germplasm repositories are a necessary tool for comprehensive conservation programs for endangered animals to fully preserve genetic resources. Germplasm repositories have been used to assist genetic management in comprehensive recovery programs for endangered animals such as the giant panda (*Ailuropoda melanoleuca*) [1], the black-footed ferret (*Mustela nigripes*) [2], and pheasants (subfamily Phasianinae) [3]. For aquatic species, repositories of cryopreserved sperm have been established in the United States to support programs for aquaculture such as hybrid catfish [4],

* Corresponding author. Aquatic Germplasm and Genetic Resources Center, Louisiana State University Agricultural Center, School of Renewable and Natural Resources, 2288 Gourrier Avenue, Baton Rouge, LA, 70820, USA.
E-mail address: ttiersch@agcenter.lsu.edu (T.R. Tiersch).

https://doi.org/10.1016/j.theriogenology.2019.05.020
0093-691X/

Pacific oysters (*Crassostrea gigas*) [5], and Atlantic salmon (*Salmo salar*) [6], and for biomedical research models such as zebrafish (*Danio rerio*) [7], Medaka (*Oryzias latipes*) [8], and *Xiphophorus* species [9,10]. Other programs have been initiated for conservation of endangered fishes such as pallid sturgeon (*Scaphirhynchus albus*) [11] by the U.S. Fish and Wildlife Services (personal communication with William Wayman, Director, USFWS Warm Springs Fish Technology Center).

Research of cryopreservation protocols is only a first step in repository development. Although research of hundreds of cryopreservation protocols for aquatic species have been published, there are great difficulties in moving such research forward into applied conservation projects [12]. Most research and publications on fish sperm cryopreservation address development of cryopreservation protocols rather than approaches for practical implementation within germplasm repositories. It is a misconception to assume that successful application of germplasm repositories will directly result from developing freezing-thawing protocols that yield acceptable post-thaw spermatozoa motility, fertilization rates, or hatching rates. Successful freezing of sperm in laboratories for research will not by itself enable successful management and incorporation of genetic resources into conservation programs in the real world. Research can optimize extender diluents, cryoprotectants, concentrations, and cooling rates to yield relatively high post-thaw spermatozoa motility; however, the lack of methodological standardization can make it extremely difficult to replicate such protocols and obtain consistent results elsewhere. For example, if a researcher fails to report all critical details of a protocol in a standard fashion or the protocol is produced with an unstandardized home-made device, it is almost impossible for other individuals to reproduce such methods. Consequently, the quality of preserved germplasm can be unreliable for future use. In addition, research-level efforts typically do not address factors such as throughput, costs, quality control, labelling, or database requirements. Thus, many factors besides cryopreservation itself need to be considered for application within germplasm repositories, such as standardization and harmonization, quality management (including quality control, quality assurance, and quality assessment), generalization of technology, mobile facilities, high-throughput production, genetic calculations, database development and management, and effective collaboration among groups and agencies.

As such, the goal of the present review is not to discuss the steps for development of cryopreservation protocols, but to provide insights and practical strategies in the larger context to apply germplasm repositories as a real-world tool to assist conservation of imperiled fishes. Small-bodied live-bearing fishes (viviparous fishes) are used as models to help explain concepts in the present review for three reasons. Firstly, they provide good examples for conservation in general of imperiled aquatic species. Secondly, small-sized fishes are more threatened (in numbers) compared with large-bodies species because large fishes generally receive more attention due to their commercial value as fisheries, and cultured fishes [13]. Thirdly, live-bearing fishes employ unusual reproductive adaptations distinct from egg-laying species, which pose greater challenges for development of germplasm repositories, and thus small live-bearing fishes can be useful models for conservation of larger live-bearing fishes. The specific objectives of this review were to: 1) introduce the conservation concerns of live-bearing fishes; 2) describe their reproductive characteristics in terms of spermatology; 3) review potential and existing programs for application of germplasm repositories in conservation of imperiled aquatic animals, and 4) provide strategies for application of germplasm repositories in comprehensive conservation and recovery efforts.

2. Small live-bearing fishes

2.1. Background

Live-bearing is unusual among fishes, and thus is not well studied. Only about 5% of fishes (2% of bony fishes) in the world are live-bearing, whereas the vast majority of fishes are egg-laying (oviparous) [14]. Viviparity has been proposed to have evolved in fishes as early as the Devonian period (approximately 380 million years ago) among placoderms [15,16], and later emerged again among amphibians [17], and reptiles [18], and subsequently became the defining evolutionary strategy for mammals [19]. Viviparity in fishes has repeatedly evolved independently, and is currently represented in about 40 families of chondrichthyans, one monotypic family of coelacanths, and 13 families of teleosts [14]. Among these, species from five families inhabit freshwater, including three families within Cyprinodontiformes (Goodeidae, Poeciliidae, and Anablepidae), one family in Beloniformes (Hemiramphidae), and one family in Scorpaeniformes (Comephoridae) [20].

The evolution of viviparity results in a number of reproductive adaptations that are different from oviparous fishes, such as direct delivery of live young [21], maternal-fetal nutrient transfer [14], and internal fertilization [22]. Moreover, evolution of viviparity has been repeated, sporadic, and independent among fishes [19,23], which makes the shared reproductive characteristics of viviparity analogous (not homologous), with great variation among taxa in anatomical, physiological, genetic, and molecular features. This review will address both the shared and distinct reproductive characteristics among fishes in the family Goodeidae and Poeciliidae as models for the development of germplasm repositories for other small-sized fishes, live-bearing fishes, and aquatic species in general.

Live-bearing fishes are also important research models. For example, *Xiphophorus* species have provided a valuable biomedical model to investigate the genetics underlying spontaneous and induced tumorigenesis for more than 85 years, resulting in the development and maintenance of more than 50 pedigreed *Xiphophorus* lines [24,25]. Guppies (*Poecilia reticulata*, family Poeciliidae) have been used widely as models to study topics such as sperm competition [26,27]. Live-bearing goodeids are important models to study palaeohydrological hypotheses regarding formation of the Mexican Plateau, for which hydrological interpretation is difficult due to the complex volcanic and tectonic geological history [28]. Moreover, goodeids have been of great interest as models to investigate topics such as evolution [23,29], phylogeography [30,31], behavior [32] and ecotoxicology [33]. In addition to their research value, live-bearing fishes provide economic value as well. Guppies, mollies (*Poecilia* species), and *Xiphophorus* species are among the most popular ornamental fishes kept in households in the United States [34]. In the last 25 years, goodeids have also become popular among aquarium hobbyists of Central and North America, and Europe [35], and mosquitofish (*Gambusia affinis*) have been distributed around the world for decades for mosquito control enabling other research [36].

2.2. Conservation status and activities

Unfortunately, small live-bearing fishes are considered to be among the most at-risk taxa in the world [37]. Poeciliidae is the largest freshwater live-bearing family comprising more than 200 live-bearing species [38], with 38 species listed as threatened among the 116 that have been evaluated by the International Union for Conservation of Nature (IUCN) for the Red List of Threatened Species (referred as 'the Red List') [39]. Most live-bearing goodeids

are on the verge of extirpation or extinction [40]. The family Goodeidae includes two subfamilies: Empetrichthyinae and Goodeinae. Empetrichthyinae comprises 3 extant oviparous (egg-laying) species that inhabit small-volume springs of the southwestern Great Basin of the United States [31]. This review focuses on the subfamily Goodeinae, comprising about 45 viviparous species within 18 genera inhabiting shallow fresh waters within the Central Mexican Plateau [31]. Currently, 17 of these species have been assessed by the Red List [39], of these, 12 are listed as threatened, and 3 as extinct or extinct in the wild [39]. Another assessment [40] based on field research and scientific literature surveys [41,42] evaluated 40 live-bearing goodeids, suggesting that about 32 species were threatened, 5 species were extinct or extinct in the wild, and only 3 were at lower risk levels as of 2011. The rapid decline of small-bodied live-bearing fishes has been attributed to the heavy disturbance of aquatic ecosystems resulting from water pollution, reductions in levels of ground and surface waters, basin deforestation, habitat destruction, and introduction of exotic species [43–45].

Some conservation efforts have been initiated to protect small-bodied live-bearing fishes from extinction. A stock center for live-bearing goodeids in Michoacán University of San Nicolás de Hidalgo (UMSNH), Mexico, established in 1997 by Dr. Omar Domínguez-Domínguez and colleagues, has maintained more than 40 species originally collected from natural habitats [41,42]. In addition, the Goodeid Working Group (GWG, a non-profit international organization) [46] organizes collections of wild goodeids, and distribution and exchange among aquarists, hobbyists, and volunteers who breed and maintain imperiled species with their own aquariums. These efforts represent maintenance of genetic resources as living populations.

Germplasm repositories are a critical and complementary component of species conservation that can mitigate some of the limitations associated with long-term habitat protection and short-term captive breeding approaches. Cryopreservation of sperm is the most efficient and widely used method in establishment of germplasm repositories for aquatic species [47], especially for live-bearing fishes because of the inherent difficulties in manipulation of oocytes and embryos. A germplasm repository has been established for preserving pedigreed research lines of *Xiphophorus* species at the USDA National Animal Germplasm Program (USDA-NAGP) in Fort Collins, CO, and approaches are currently being developed to address conservation needs for goodeids at the Aquatic Germplasm and Genetic Resources Center (AGGRC) of the Louisiana State University Agricultural Center in Baton Rouge, Louisiana (Table 1). Development of germplasm repositories for live-bearing species is challenging because of unusual reproductive adaptations (which are distinct from oviparous species), such as sperm bundles, initiation of spermatozoa motility in an isotonic aquatic environment, internal fertilization, and bearing of live young.

3. Spermatology of live-bearing fishes

3.1. Sperm bundles

Sperm bundles pose difficulties for development of protocols and sperm repositories, because most established methods have focused on sperm without bundles. Freshwater live-bearers usually pack sperm into un-encapsulated bundles, which are usually referred to “spermatozeugmata” (Fig. 1A–B). Encapsulated bundles (referred to as “spermatophores”) have not been documented in fish) [48,49]. Sperm bundles (un-encapsulated and encapsulated) have been identified elsewhere in invertebrates including nematodes [50], annelids [51], arthropods [52], and molluscs [53], and in vertebrates including amphibians [54], chondrichthyans [55], and teleosts [48,49].

Evolution of sperm bundles could be an advantageous for adaptation of internal fertilization. In these fishes, male gametes are delivered to the female by transfer with copulatory organs,

Table 1
Summaries of cryopreservation protocols in research and repository development of freshwater live-bearing fishes.

Species	Extender	Fresh motility	Cryoprotectant and equilibration[a]	Cooling	Thawing	Post-thaw motility	Fertilization rate[b]	Established repositories[c]
Poeciliids								
Xiphophorus. helleri [10]	HBSS at 310 or 500 mOsmol kg^{-1}	80–95%	14% glycerol for 15 min	20 °C/min	42 °C for 5 s	20–70%	10%	697 straws of 10 strains
X. couchianus [85]	HBSS at 500 mOsmol kg^{-1}	60–90%	14% glycerol for 15 min	20 °C/min	40 °C for 5 s	1–70%	8%	95 straws of 1 strain
X. maculatus [88]	HBSS at 500 mOsmol kg^{-1}	60–90%	14% glycerol for 15 min	20 °C/min	40 °C for 5 s	1–70%	18%	1126 straws of 16 strains
X. variatus [9]	HBSS at 500 mOsmol kg^{-1}	40–80%	14% glycerol for 15 min	20 °C/min	40 °C for 5 s	5–70%	10%	298 straws of 3 strains
Another 20 *Xiphophorus spp.*[d]	HBSS at 300 mOsmol kg^{-1}	0–95%	14% glycerol for 20 min	20 °C/min	40 °C for 7 s	0–58%	Not evaluated	1604 straws of 28 strains
Poecilia. reticulata [81]	HBSS at 300 mOsmol kg^{-1}	50–80%	14% glycerol for 10 min	20 °C/min	40 °C for 7 s	~40%	50%	Unknown
P. latipinna [81]	HBSS at 300 mOsmol kg^{-1}	~80%	14% glycerol for 10 min	20 °C/min	40 °C for 7 s	~40%	20%	Unknown
Goodeids								
Xenotoca. eiseni [90]	HBSS at 300 mOsmol kg^{-1}	43–79%	15% DMSO for 20 min	10 °C/min	40 °C for 7 s	7–9%	2%	None
Goodea atripinni [64]	HBSS at 300 mOsmol kg^{-1}	>90%[e]	10% DMSO for 20 min	10 °C/min	40 °C for 7 s	~90%[e]	Not evaluated	None
Ataeniobius toweri [64]	HBSS at 300 v	>90%[e]	10% DMSO for 20 min	10 °C/min	40 °C for 7 s	~65%[e]	Not evaluated	None

[a] The method used to mix cryoprotectants and calculate equilibration time may vary. Please refer to original publications for details.
[b] The fertilization rate herein was defined as number of females fertilized/number of females inseminated.
[c] Samples that have been preserved in the repository at the USDA-NAGP. To get access to detailed data, visit: https://nrrc.ars.usda.gov/A-GRIN/tax_inv_drilldown_page_dev?language=EN&record_source=US.
[d] Unpublished data. Sperm of these species were processed and cryopreserved at the LSU-AGGRC and transported to the USDA-NAGP for long-term preservation.
[e] Sperm were cryopreserved in the form of sperm bundles, and thus “motility” referred to the percentage dissociable bundles.

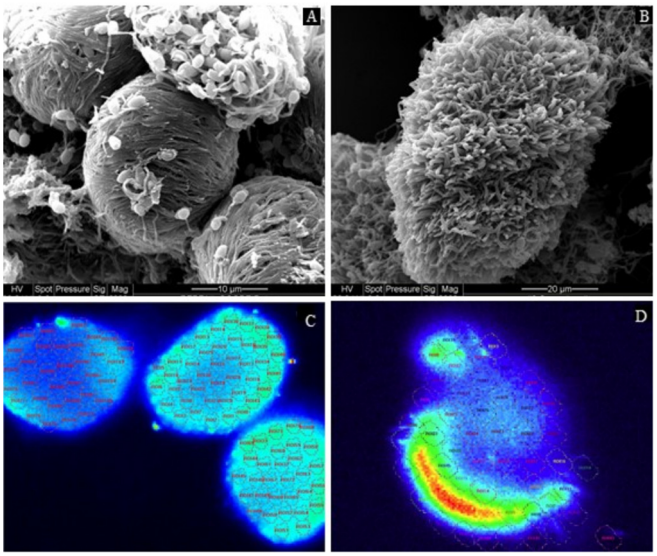

Fig. 1. Sperm bundles (spermatozeugmata) of live-bearing fishes. The organization of sperm bundles (scanning electron micrographs) are different between (A) goodeids (*Xenotoca eiseni*) and (B) poeciliids (*Xiphophorus helleri*). The bundle periphery is composed of spermatozoa tails in goodeids but with spermatozoa heads in poeciliids. This is an example of variation in shared traits arising from the independent evolution of viviparity. Intracellular signaling of spermatozoa within bundles of (C) *X. eiseni* and (D) *Xiphophorus maculatus* were evaluated with cell imaging techniques [80]. Using fluorescence microscopy, concentrations of intracellular Ca^{2+} were indicated with colors and quantified by use of a CCD camera and associated software. Spermatozoa within bundles were loaded with the dye Fura-2 AM and multiple regions of interest were selected within bundles. Each region of interest represented multiple spermatozoa. The levels of intercellular Ca2+ were evaluated by fluorescence intensity (green and yellow colorations indicate elevations in intracellular Ca2+ whereas blue coloration indicate baseline levels. The colorations were produced by a cell imaging program for imaging purposes, and thus were not equivalent to actual colors of fluorescent emissions).

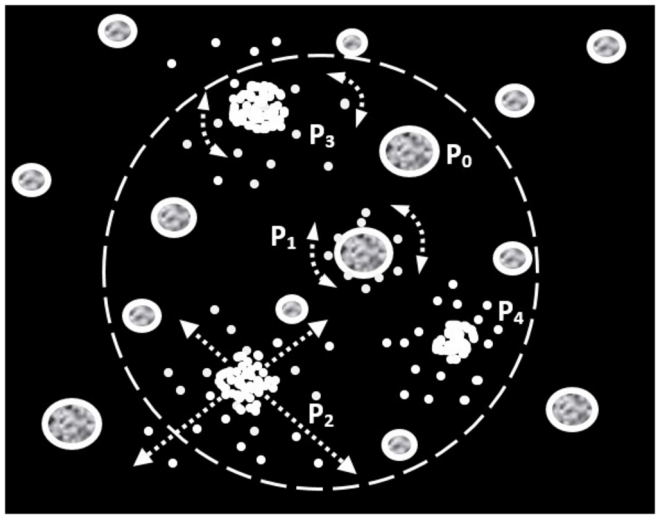

Fig. 2. Evaluation scheme for the five activation phases of spermatozoa within bundles by categorizing sperm bundles distributed in a viewing area (within the dashed circle) [62]. The dashed straight arrows indicate free-swimming spermatozoa released from a sperm bundle and the curved double-arrows indicate sperm vibrating in place but not swimming. In the dashed circle, there are ten sperm bundles including six at P_0, one at P_1, one at P_2, one at P_3, and one at P_4, thus the frequency of activation phases (FAPs) are estimated as 60% FAP_0, 10% FAP_1, 10% FAP_2, 10% FAP_3, and 10% FAP_4. For demonstration purposes, the sizes of spermatozeugmata, spermatozoa, and viewing area do not reflect actual scale. The FAP can be used for evaluation of quality of spermatozoa within bundles [64].

which are usually modified pelvic or anal fins, such as claspers in Chondrichthyes [56], gonopodia in poeciliids, or andropodia (split anal fin) in goodeids [22]. Some forms of male copulatory organs may not deliver the gametes fully into the female reproductive tract to ensure residence. For example, in goodeids, the andropodium does not function as an intromittent organ to deliver male gametes into females, but acts as a copulation device to form a pocket around the female urogenital opening, assisting transfer of the gametes [57]. Thus, the aqueous environment can disturb the transfer process by flushing free gametes away from the female. As such, spermatozeugmata are believed to be adaptations to facilitate efficient transfer of male gametes into the female [58,59], possibly by resisting flushing effects. In addition, sperm bundles among different taxa have different structures. For example, spermatozoa tails are oriented toward the interior in goodeids (Fig. 1A) but oriented toward the periphery in poeciliids (Fig. 1B), reflecting the independent evolutionary history of viviparity in these groups.

3.2. Spermatozoa motility assessment

Sperm quality evaluation is the initial step to develop cryopreservation protocols and provides a basis for establishment of quality management programs for comprehensive germplasm repositories. Sperm bundles present difficulties for standardized assessment of male gametes. For example, upon activation by suitable media, the motility of free spermatozoa from most externally fertilized fishes can be roughly estimated by naked-eye methods, or rapidly and accurately counted by computer-assisted sperm analysis (CASA) systems [60]. However, such methods cannot be directly applied to sperm within bundles, and most existing assays for estimation of spermatozoa motility are based on characterizing movement patterns of free spermatozoa rather than bundles.

Sperm bundles of most poeciliids can be simply dissociated into free spermatozoa by crushing the testis or vortexing sperm bundle suspension with suitable activation solution (a video of guppy sperm bundle dissociation can be accessed via https://vimeo.com/312590774), and thus common spermatozoa quality parameters, such as concentration, motility, and membrane integrity can be analyzed for free single spermatozoon [9,61]. However, such methodologies are not suitable for study of spermatozoa quality within un-dissociated bundles, and not suitable for bundles that cannot be dissociated by physical crushing and vortexing such as bundles from most goodeids [62]. Using livebearing goodeids and poeciliids, standardized assays to qualitatively evaluate activation of spermatozoa within bundles were established by classifying activity patterns into five phases and calculating the frequency of each phase at specific time intervals [62]. During the activation of spermatozoa within bundles of redtail splitfin (*Xenotoca eiseni*), five activation phases were presented in a chronological order of $P_0 \rightarrow P_1 \rightarrow P_2 \rightarrow P_3 \rightarrow P_4$, representing the general activation sequencing of single spermatozon motility: quiescence → beginning of motility → highest motility → diminishing motility → end of motility (Fig. 2) [62]. This classification method can be used to evaluate activation status of sperm within un-dissociated bundles.

3.3. Spermatozoa concentration

Knowledge of spermatozoa concentration is essential for reproducible sperm cryopreservation [63,64]. Among the popular methods for rapid estimation of sperm concentration, counting chambers, such as Makler® chambers and hemocytometers, are relatively low-cost and portable. However, when counting chambers are used for sperm bundles, the bundle sizes from different

species must be considered. For example, the Makler® chamber overestimated bundle concentration from live-bearing goodeids, whereas hemocytometer estimations more accurately reflected the actual concentration [62]. The difference in concentrations observed among different methods may be related to the bundle size. The height between cover slip and bottom glass of the counting volume is 10 μm for the Makler® counting chamber and 100 μm for the hemocytometer, and the diameter of sperm bundles of goodeids is about 31 μm. As such, when the cover glasses were placed, sperm bundles were compressed to the maximum depth of the space between cover glass and base of counting devices, resulting in underestimation of fluid volume containing these sperm bundles.

3.4. Spermatozoa motility activation

Motility activation is the most widely used indicators for evaluation of sperm quality in aquatic species [65]. Fish spermatozoa are typically immotile within the testes and the male reproductive tract. Spermatozoa motility is essential for reproduction because spermatozoa must travel to, bind, and penetrate oocytes to initiate fertilization [66]. During the process of natural spawning, spermatozoa become motile upon discharge into the aqueous environment for externally fertilized species, or into the female reproductive tract for internally fertilized species [67]. Several physicochemical factors play important roles in inducing spermatozoa motility activation in fishes. For most egg-laying species, spermatozoa motility can be induced by hypotonic solutions for freshwater fishes, or by hypertonic solutions for marine fishes (Fig. 3) [68,69]. In some species, in addition to or as an alternative to osmotic pressure, concentrations of ions are critical to regulate initiation of spermatozoa motility. For example, influxes of Ca^{2+} and the ensuing signaling initiates sperm motility of common carp (*Cyprinus carpio*) [70]. In salmonids, a relatively high K^+ concentration is a major inhibitor of sperm motility prior to spawning, whereas Ca^{2+} is antagonistic of this inhibitory effect and intracellular Ca^{2+} were are prerequisite for the initiation of sperm motility [68,70].

Methods to induce spermatozoa motility activation for oviparous fish species have been well established, however, such methods for live-bearing fishes are rarely reported. Live-bearing fishes are internally fertilized, requiring unique conditions such as isotonic activation (Fig. 3), and their sperm have relatively long motility duration compared to oviparous species (hr rather than min) [71]. Different from oviparous species, many live-bearing fishes can transfer sperm from males to females presumably without shifts of osmotic pressure in the fluidic environment surrounding the spermatozoa. Thus, theoretically sperm must be activated by factors other than the changes in osmotic pressure while remaining isotonic. Due to the complex environment of the female reproductive tract, specific ions, pH, or other physiochemical factors may have greater effects on spermatozoa motility activation than observed for externally fertilized species [72,73] (Fig. 4). In addition, sperm bundles complicate the induction of spermatozoa motility, which involves two separate but interacting processes: dissociation of the bundles and activation of free spermatozoa (Fig. 4). In *Xiphophorus* species, free spermatozoa (dissociated from bundles) were observed to remain active for 10 d in electrolyte solutions such Hanks' balanced salt solution (HBSS) with highest motilities occurring at near isotonic conditions (250–350 mOsmol kg^{-1}) [71]. This pattern is consistent with studies on other poeciliids, such as guppy [74] and mosquitofish, [75]. In goodeids, free sperm could be activated in Ca^{2+}-free Hanks' balanced salt solution at 81–516 mOsmol kg^{-1} with the highest motility at 305 mOsmol kg^{-1} and remain active for 84 h. Factors such as $CaCl_2$ and pH had prominent effects in dissociation of sperm bundles [72].

3.5. Signaling mechanisms of spermatozoa motility activation

Intracellular signaling regulates the molecular pathways of spermatozoa motility activation, therefore understanding signaling mechanisms can help develop standard and effective buffer and activation solutions for cryopreservation. Cellular signaling pathways of spermatozoa activation are basically understood for oviparous species. Using the common carp as a model, studies have

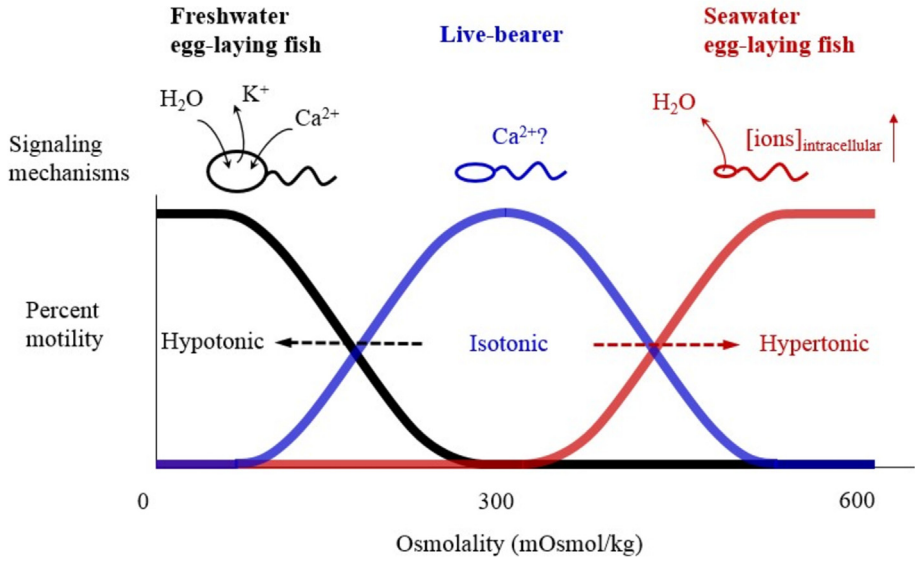

Fig. 3. Representative mechanisms for spermatozoa motility activation in fishes with different spawning strategies. Freshwater egg-laying (oviparous) fishes release sperm into hypotonic environments, which induce water influx and K^+ efflux, leading to Ca^{2+} influx as an intracellular signal that triggers spermatozoa motility activation. Seawater egg-laying fishes release sperm into hypertonic environments, which induce water efflux, leading to increases in intracellular ions that trigger spermatozoa motility activation. Live-bearing fishes (such as goodeids and poeciliids) transfer sperm from males into females across isotonic environments.

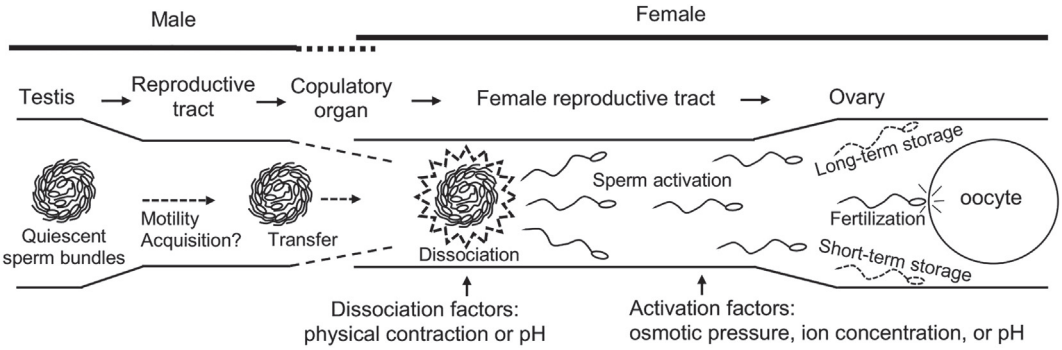

Fig. 4. Schematic of the major steps involved in the journey of male gametes from testis to ovary of internally fertilized fish in the Cyprinodontiformes [72]. Sperm bundles are intact and spermatozoa are immotile in testes. It is unclear whether spermatozoa within bundles acquire motility potential during their passage through the male reproductive tract. Bundles are transferred into females by copulatory organs, such as gonopodia (the intromittent type) of poeciliids or andropodia (the non-intromittent type) of goodeids. Upon the arrival within the female reproductive tract, the dissociation of bundles may result from physical pressure, or dissolution by elevated pH of presumptive substances that bind spermatozoa within bundles. The dissociation could also be triggered by activation of the spermatozoa within bundles. Activation of spermatozoa can be affected by osmotic pressure, ion concentration or pH. After activation, free spermatozoa need to travel through the tract before fertilizing oocytes in the ovary. However, sometimes there are no available oocytes to fertilize immediately. Thus, before fertilization free spermatozoa may persist for a short term (several d) as in goodeids or longer term (several months) as in poeciliids. The long motility duration (several d) of spermatozoa from internally fertilized species is likely required for efficient transit and fertilization.

indicated that release of spermatozoa into a hypo-osmotic environment with decreased extracellular K^+ triggers a transient shift of membrane potential, which removes inactivation of Ca^{2+} channels, resulting in the influx of Ca^{2+} as an important signaling mechanism to initiate spermatozoa motility (Fig. 3) [70]. Alternatively, sperm from saltwater species are released into hypertonic conditions. The sudden rise of external osmolality immediately leads to water efflux and an increase of internal ionic concentrations in spermatozoa, which reaches optimal values for activation of dynein motors in flagella [65]. Such mechanisms of osmotic-shock induced signaling for oviparous fishes are not applicable for live-bearing fishes which activate spermatozoa motility within an isotonic environment.

Signaling pathways of spermatozoa motility activation from live-bearing species have rarely been reported. A variety of methods has been used to study intracellular signaling of free spermatozoa, such as flow cytometry [76], cell imaging [70,77], and fluorescence spectrophotometry [78]. However, the presence of sperm bundles represents an obstacle for these methods. For example, flow cytometers require individual passage of cells through a laser beam [79], which is not feasible for intact bundles. Using live-bearing goodeids and poeciliids as a model, initial methods were established to detect intracellular Ca^{2+} signals from spermatozoa within bundles by detecting Fura-2 AM fluorescence with cell imaging techniques, with >20 regions of interest (each region representing multiple cells) selected for each intact bundle for a total of 2–3 bundles (Fig. 1C–D) [80]. Using this method, intracellular Ca^{2+} signals arising within sperm bundles were detected for the first time from a live-bearing fish (the redtail splitfin, *Xenotoca eiseni*) [80], suggesting a novel mechanism for motility regulation of fish spermatozoa: an alkaline environment within the female reproductive tract opens Ca^{2+} channels in the plasma membrane without osmotic shock, and subsequent increases in intracellular Ca^{2+} function as a second messenger to activate motor proteins controlling flagella movement (the activated sperm within bundles subsequently caused bundle dissociation). This method can be used to study intracellular signaling of other live-bearing fishes and animals that produce sperm bundles.

3.6. Internal fertilization and artificial insemination

Internal fertilization makes it difficult to study sperm cryopreservation of live-bearing fishes. Typically, sperm are transferred into the female reproductive tract by artificial insemination [81]. After transfer, sperm bundles must dissociate and spermatozoa must be active to traverse the female reproductive tract. In some species, such as *Xiphophorus* fishes and guppy, spermatozoa can be stored for weeks or months inside females for future use [82], whereas in species such as goodeids, spermatozoa released from bundles fertilize mature oocytes within days without extended storage in the female [59]. In addition, hatching rates and the quality of spermatozoa and eggs of externally fertilized species can be readily evaluated [83,84]. Internal fertilization currently makes it impossible to evaluate oocyte quality without dissection of females. For example, live young were harvested from about 10% of females inseminated with fresh or thawed spermatozoa (motility > 70%) from *Xiphophorus* species [9,10,85–88]. In these studies, it was difficult to confirm whether the 90% observed infertility was caused by quality problems of spermatozoa or eggs because ovarian development could not be evaluated. In addition, it is difficult to assess the fertilization rate for live-bearing fishes. For cryopreservation of externally fertilized species, fertilization rates can be estimated after mixing of eggs and sperm by direct observation of embryonic development at specific checkpoints [89]. Such a method is impossible for live-bearing fishes because embryonic development is retained within females.

For live-bearing fishes, production of live young from cryopreserved sperm has only been reported in one species (redtail splitfin) [90] within the family Goodeidae, and six species within two genera within the family Poeciliidae: green swordtail [10], Monterrey platyfish [85], Southern platyfish [88], and variable platyfish [9] within the genus *Xiphophorus*, guppy and black molly [81] within the genus *Poecilia*.

4. Germplasm repositories as a tool for conservation

4.1. Necessity of germplasm repositories in conservation of endangered fishes

An effective animal conservation program for endangered animals requires a “tool box” that combines different methodologies as conservation tools. The ultimate goal for conservation of endangered species is for target species to be self-sustaining with healthy population sizes and genetic diversity in the wild. The most important tool for achieving this goal is habitat restoration.

Practices that cause habitat degradation, such as drainage and modification of streams for recreational usage, and pollution of waterways would need to be stopped or reduced. In addition, habitats may need to be remediated to restore or reintroduce the native flora and fauna. However, it is difficult to accomplish these long-term objectives, and the specific methodologies are subject to debate. For example, in a recent review of 78 habitat rehabilitation (or enhancement) projects targeting increases in riverine and wetland fish populations, just four (5% of the 55 completed projects) were able to demonstrate an increase in fish production, although 98% achieved their habitat restoration targets [91]. In addition, restoration efforts typically involve multiple stakeholders at different management levels, and large expenditures of time, money, and other resources are often required for coordination and implementation [41,91,92]. However, population sizes and genetic diversity will continue to decline until habitats are protected or fully restored.

To address limitations in habitat restoration, conservation tools such as captive breeding and translocation may be used as short-term solutions. Captive breeding is the most widely applied tool to ensure short-term survival of organisms or partial preservation of genetic diversity. Captive breeding for species of conservation concern is the act of bringing rare or endangered animals into captivity (such as hatcheries, zoos, or aquariums) with the hope of rearing sustained captive populations for eventual reintroduction into the wild [93].

Although captive breeding can solve some problems, it can cause others. For example, genetic changes can occur in captive populations, reducing the ability to persist after a population is reintroduced back into a natural habitat [93]. Such changes include loss of genetic diversity, inbreeding depression, accumulation of deleterious mutations, and domestication [93,94]. In genetic studies of live-bearing goodeids, inbreeding and loss of genetic diversity have been observed in captive populations [95]. These studies suggest that a captive population should maintain more than 100 individuals (effective population size) and utilize more than 15 founders to effectively counteract inbreeding and a loss of genetic diversity. However, 67% of captive populations in zoos and aquariums in the world have populations of less than 100 individuals according to the Association of Zoos and Aquariums [96], and sufficient founders are often not available in the wild. In addition, the maintenance of large numbers of live animals presents high costs, a risk of adaptation to captivity, and risk of disease outbreaks.

Translocations are another important tool in wildlife conservation. Translocations include deliberate transfers of animals to establish new populations, re-establish extirpated populations, augment critically small populations, or to mitigate for conflicts between animals and humans [97]. Translocations have been utilized in conservation programs to alleviate the detrimental effects of inbreeding depression and demographic stochasticity of isolated populations [98]. Although translocation sites can provide relatively suitable conditions in the wild to maintain population sizes and retain genetic diversity, translocations can have negative consequences in some circumstance, including a low success rate [99], hybridization with closely related species, or reduction of fitness because of the difficulty in adaptation to new habitats and exposure to diseases [98]. Although less common, outbreeding depression with existing wild populations of the same species can occur when there are fixed chromosomal differences, the populations have been isolated for a sufficiently long period (for example, > 500 years), or the populations are adapted to different environments [100].

Germplasm repositories are an important tool for comprehensive conservation programs to fully preserve genetic resources. The major components of repositories are cryopreserved germplasm (such as germ cells, gametes, or embryos), a comprehensive database that at a minimum contains information related to the germplasm donor (such as genotype, phenotype, gamete quality, and habitat information), sample preparation (e.g. freezing methods and containers), and inventory. Cryogenic storage in liquid nitrogen can maintain the viability of cells almost indefinitely if relevant procedures are implemented appropriately, because biological reactions that require molecular motion and activation do not occur at −196 °C [101]. As such, valuable genetic resources of imperiled animals can be preserved as cryopreserved germplasm, which can be used in the future to produce live young for integration into other conservation projects, such as habitat restoration, captive breeding, and translocations, compensating for genetic losses or negative changes that would otherwise be permanent [102]. In addition, cryopreservation of germplasm can be relatively safe, low cost, fast, and efficiently managed, allowing easy transport and administration compared to the maintenance of live animals [103]. For example, cost efficiency is an important potential advantage for germplasm repositories. Although recognition of the importance of public germplasm repositories exists, limited empirical studies have been done to place actual economic value on collections of genetic resources [104]. Traditional economic theory states that “collections should be expanded so long as the expected marginal value of an additional accession exceeds the costs (with both benefits and costs appropriately discounted over time)”. In practice, the economic discussion on germplasm repository development can focus on cost comparisons between maintaining genetic resources as live populations or germplasm collections. In one of the most informative studies of this type, *in-situ* live maintenance of chicken research populations vs. germplasm collections over a 20-year time horizon were calculated, and showed that cryopreserved germplasm use was 94% cheaper than maintaining live populations [105]. This situation would be applicable to efforts intended to maintain living populations of aquatic species and suggests that germplasm collections can become quite large given their relatively low cost of development and maintenance [105].

4.2. Key components of germplasm repositories in conservation of aquatic animals

For repositories to meet a mandate of conserving aquatic species, thematic areas must include: cryobiology and reproduction, genetic assessment, and information systems. Obviously, reliable cryopreservation protocols are required for the repository to function as a store of genetic resources which can be used for a variety of purposes. Assessing the genetic aspects of the species can be performed prior to, after, or during germplasm collection based upon the status of the repository collection and can include a variety of approaches that include quantitative and molecular genetic approaches. For example, Eastern oysters (*Crassostrea virginica*)

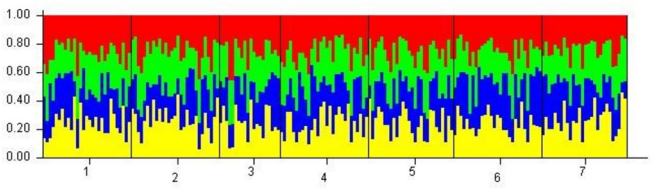

Fig. 5. Evaluation of genetic differences among seven Louisiana coastal management areas where four subpopulations (various colors) were identified using STRUCTURE software with a Bayesian model, whereas x-axis represents the seven locations sampled and the y-axis represents the proportional assignment of the four identified clusters.

were collected along the Louisiana coastline including seven recognized management areas (our unpublished data). Results of a single nucleotide polymorphisms (SNP) panel suggested that there were four subpopulations but these subpopulations were each located in all the seven areas. Therefore, representative sampling could be accomplished at a single physical location (Fig. 5). Using other genetic metrics such as effective population size and measures of heterozygosity are also useful. Of course, if specific genes are known and of interest it can be determined if subpopulations carry the alleles of interest and be sampled accordingly. This has been demonstrated as a useful approach in determining how to sample subpopulations of cattle [106]. Genetic characterization of some imperiled live-bearing fishes samples from wild habitats has been reported, which can be used as a reference for future repository development [28,30,31].

Information systems (including databases) are an extremely important element of germplasm repositories as they produce the vehicle for repository managers and stakeholders to evaluate collections and determine if there are samples of interest for use with a specific project. In essence, this information component is the glue holding the repository process together. For aquatic species, this minimally must include information such as on: animal identification (species and individual), germplasm or tissue type, phenotype, genetic information (if available), and environmental descriptors (e.g., latitude, longitude, water pH, salinity). The Germplasm Resources Information Network (Animal-GRIN) (https://nrrc.ars.usda.gov/A-GRIN/database_collaboration_page_dev) provides an example of how comprehensive information systems can be developed for repositories and provide the information previously described and the various services needed by the germplasm repository and its user communities.

4.3. Genetic considerations of collection and utilization of germplasm repositories

The first consideration of repository collection planning is to determine the collection goals. This requires an assessment of two critical factors: 1) the quantity of germplasm needed to reconstitute a target population, and 2) the number of animals needed to ensure that the genetic diversity of the target population has been captured.

The quantity of germplasm needed for reconstitution entails computing the reproductive rate achieved with cryopreserved germplasm at animal numbers sufficient to establish a viable population. The Food and Agriculture Organization [107] has developed comprehensive guidelines for livestock and these approaches detailed can provide a starting point for aquatic species. For example, based on the FAO guidlines if the goal is to create a population (i.e., cattle) with a minimum effective population size (N_e: the roughly number of "breeders") of ~50 and the pregnancy rate is assumed to be 50%, it would require using 250 founder females and 1410 doses of semen. However, survival of reintroduced animals to the wild will probably be less than 100%, thus repeated reintroductions and therefore additional sperm collection will likely be needed to establish self-sustaining populations.

Genetic considerations need to be based upon the targeted N_e and the probability of maintaining rare alleles. The FAO (2012) has used an N_e of 50 as a base for its calculations, which theoretically results in an inbreeding level of 1% per generation. To estimate the probability of capturing rare alleles, the following formula can be used: $A = 1 - (1 - P)^{2n}$ can be applied, where: P is the allelic frequency, n is the number of males sampled, and A is the probability of capturing an allele of specified frequency [108,109]. Given this formula and sampling 50 males, there is 63% probability of capturing an allele with a frequency of 1%. Increasing the sample size to 200 males increases this probability to 98%. Therefore, repository managers need to determine the level of successfully capturing rare alleles that they believe will provide sufficient genetic security for the targeted species. For example, if there were two subpopulations of a species in relatively close geographic proximity, the number of males sampled from each location could be pooled if the population were judged to be sufficiently similar. However, as populations can become differentiated due to drift and natural selection, sampling would also need to take such differences into consideration.

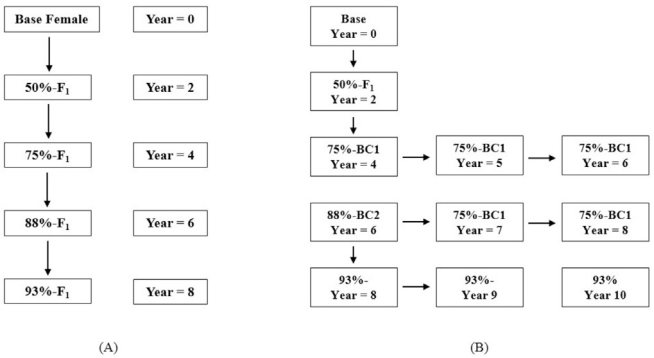

Fig. 6. Mating schemes for reconstitution of a breed or selected population using sperm in the repository with (A) a standard backcrossing plan and (B) an alternative plan [107,110]. Each box represents a new generation of animal in the backcrossing scheme and the increasing proportion of the genome from the population being reconstituted.

Repository samples can be used in various ways to either reconstitute a population or increase genetic diversity and thereby avoid or diminish genetic bottlenecks [110]. Increasing genetic variability can be accomplished by introducing alleles from the repository into the targeted population. This could be a one-time event or be repeated at several times. Modelling results demonstrate that repeated integration to the wild from multiple males in the repository is most effective for reducing inbreeding and increasing gene diversity [111,112]. Reconstitution of a population requires a more involved process spanning multiple generations. Typical reconstitution schemes often use repository germplasm in a backcrossing design over five mating cycles (Fig. 6). Starting with an unrelated population the goal is to increase the proportion of targeted genetic resources to 93% or higher. Therefore, each generation (e.g., F_1, or backcross, BC_1) will need to be mated with material (e.g. sperm) from the repository. Note that the time needed to reconstitute a population will vary based upon the generation interval.

4.4. Potential needs for conservation of threatened fishes

Germplasm repositories have been neglected in conservation programs of imperiled fishes. For example, in 86 current recovery plans of endangered or threatened fishes developed by the U.S. Fish & Wildlife Service, only two mention cryopreservation [113]. Such neglect of an important conservation tool can make restoration efforts ineffective and could waste resources, money, and time. For example, the plan for Pahrump poolfish (also Pahrump killifish, *Empetrichthys latos*) was one of the earliest recovery plans. It was published in 1980 to restore the Pahrump poolfish to non-endangered status by use of translocation and habitat protection projects. With these efforts, existing populations of Pahrump poolfish were maintained and three new populations were created via translocations as of 2016 [114]. However, comparison of nucleotide sequences for the mitochondrial genomes of two

individuals from each populations in 2017 revealed only three nucleotide point mutations across the entire 16,546 bp mtDNA genome, indicating high levels of inbreeding and loss of genetic diversity for the existing populations [114]. Unfortunately, populations with such low genetic diversity are unlikely to persist long term in the wild [93]. After almost 40 years of effort, the Pahrump poolfish is still listed as endangered today [114], and may go extinct in the future due to the low genetic diversity. If at the beginning, germplasm repositories had been incorporated into recovery plans, and were established from the original wild populations, genetic diversity of the existing population could have been improved, producing greater chances for protection and recovery, and fishes like the Pahrump poolfish could have better prospects to persist in the wild over the long term.

4.5. Development of germplasm repositories for Xiphophorus species

For live-bearing fishes, the most developed program manages the genetic resources for *Xiphophorus* species, sponsored by the National Institutes of Health (NIH), at the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University, San Marcos and the USDA-NAGP in collaboration with the AGGRC at the Louisiana State University Agricultural Center (LSUAC). The program addresses several critical factors to achieve comprehensive germplasm repositories, including initial research, establishment of collaboration, protocol development, sample collection, germplasm and genetic material banking, establishment of quality control and quality assurance, inventory development, and database usage and management. For example, a quality assurance program was established with three quality control checkpoints during the cryopreservation process: 1) immediately after the collected testis are crushed (fresh), 2) 20 min after the addition of the cryoprotectant (equilibration), and 3) immediately after thawing. At each checkpoint, spermatozoa concentration, motility, and membrane integrity were evaluated. Samples were frozen and held at the AGGRC before being shipped to the USDA-NAGP. The Animal-GRIN database was used jointly by the AGGRC and USDA-NAGP to ensure seamless integration of data collected. Each sample was assigned numbers for inventory, animal, and repository coding when entered into the Animal-GRIN database, and were used for inventory control. Sample identification information was also directly printed on French straws with text and barcoding. To date, more than 3000 0.25-mL straws, representing 23 of 26 *Xiphophorus* species and 47 of the 54 lines at the XGSC have been banked at the AGGRC and transferred into long-term storage with USDA-NAGP (our unpublished data).

5. Practical strategies

5.1. Incorporation of germplasm repositories into a comprehensive conservation program

Incorporation of conservation programs with germplasm repositories should include three basic phases: 1) research for development of appropriate technologies, 2) establishment of germplasm repositories, and 3) coordination with and integration of repositories into comprehensive conservation programs. Currently, most germplasm repository projects fail to progress beyond the research phase for a variety of reasons [115]. For example, sperm cryopreservation have been studied in several imperiled species, such as razorback sucker (*Xyrauchen texanus*) [116], Formosan landlocked salmon (*Oncorhynchus masou formosanus*) [117], Caspian brown trout (*Salmo trutta caspius*) [118], Colorado pikeminnow (*Ptychocheilus lucius*) [119], piracanjuba (*Brycon orbignyanus*) [120], gila trout (*Oncorhynchus gilae*) [121], wolffishes (*Anarhichas minor and A. lupus*) [122], and pallid sturgeon (*Scaphirhynchus albus*) [11]. Among these, little progress was made toward application of cryopreservation to establish of germplasm repositories except for pallid sturgeon, for which a repository has been initiated and offspring produced with thawed sperm has been released into the wild. However, to our knowledge few germplasm repositories of imperiled fishes have been applied to comprehensive conservation programs (the third phase). Storage of sperm in liquid nitrogen itself is not the final goal of germplasm repositories. The destination of this time travel is to fertilize eggs using thawed sperm to reintroduce valuable genetic resources into captive or wild populations.

Germplasm repositories should be integrated into a comprehensive recovery strategy which also includes other important conservation activities such as habitat restoration and captive breeding. In such a strategy, genetic banking is achieved by collection, cryopreservation, and genetic characterization of sperm from wild populations (or offspring of wild broodstock) (Fig. 7). Sperm can be collected by well-equipped central facilities (such as AGGRC), which have the capability for animal maintenance, sperm collection, quality control and quality assurance, protocol development, freezing and thawing, commercial-scale processing, genetic characterization, database management, and long-term storage. Another option is to use self-contained mobile facilities [123] to perform on-site cryopreservation within driving distance, which can avoid reduction or sperm quality caused by shipment of fish of sperm to central facilities. The frozen sperm and related database containing sample information are maintained and managed within germplasm repositories. The frozen sperm can be used for routine genetic enhancement for captive breeding, long-term backup, or to address specific needs identified by genetic analysis (e.g., inclusion of rare alleles). If genetic diversity of wild populations declines in the future, previously stored genetic resources can be used for artificial insemination. Offspring produced in hatcheries with thawed sperm can be used for captive breeding or in wild populations to enhance genetic diversity.

5.2. Process generalization

The development of new cryopreservation protocols can be costly and time consuming. For example, there are about 138 fish species in the United States listed as endangered or threatened by the USFWS, however, it would require prohibitive amounts of money, labor, and time to develop cryopreservation protocols *de novo* for each species. Generalization of the research process can be an efficient and reliable approach for future conservation programs. A generalized development process integrates multiple key research steps, including (but not limited to) refrigerated (non-frozen) storage, choice of extender, spermatozoa concentration, acute toxicity of cryoprotectant, equilibration time, choice of cooling container, cooling rate, and thawing rate [115,124]. This process can be repeated for individual species as initial research to establish cryopreservation protocols (Fig. 8), which can be refined for each species and used in production as primary applied protocol. However, when new protocols are needed for closely related species, it is perhaps not necessary to repeat the research pathway to yield an optimized protocol for each new species. In practice, a primary applied protocol can be chosen as a foundational protocol and be evaluated with new species, and decisions can be made based on factors such as goals, costs, schedules, and sample resources. If the foundational protocol is satisfactory, it can be adopted as a secondary applied protocol in production, whereas if it is unsatisfactory, the foundational protocol can be refined based on previous knowledge. After repeated evaluation of the foundational protocol,

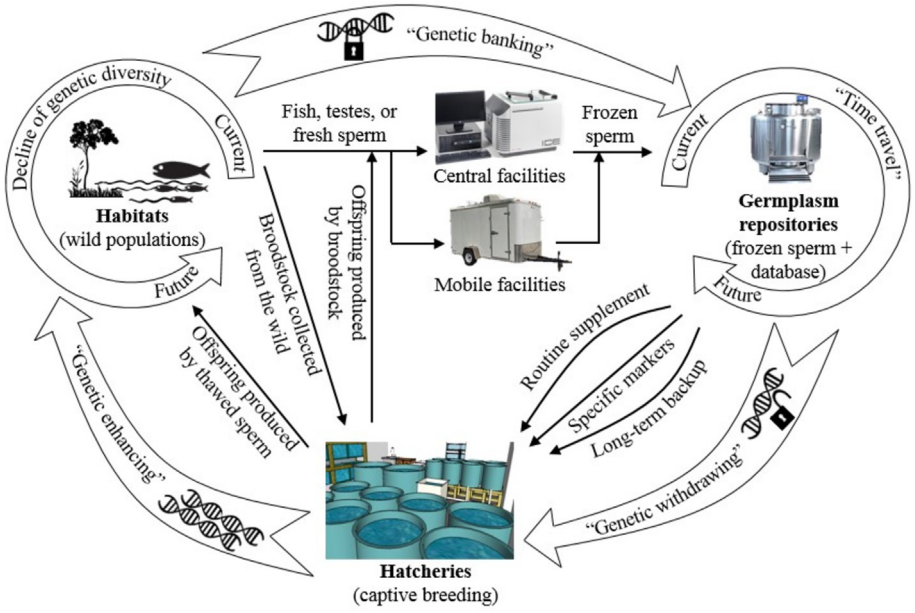

Fig. 7. A strategy to integrate germplasm repositories into a comprehensive recovery program. In the genetic banking process, sperm of wild populations (or offspring of wild broodstock) are collected, cryopreserved, stored, and genetically characterized. Fish, testes, or fresh sperm (diluted in buffer solutions) can be transported to well-equipped central facilities (e.g., AGGRC), followed by sample processing and freezing. If the distance is relatively close (within several hundred miles), on-site cryopreservation can be performed by use of mobile facilities, avoiding reduction of sperm quality caused by shipment. The frozen sperm and related database information are maintained within germplasm repositories, and can be used for routine genetic enhancement, long-term backup, or to address specific needs identified by genetic analysis. Should genetic diversity of wild populations decline in the future, previously stored genetic resources can be utilized by artificial insemination with thawed sperm. Offspring produced with thawed sperm can be used for breeding purposes or be incorporated into wild populations to enhance genetic diversity.

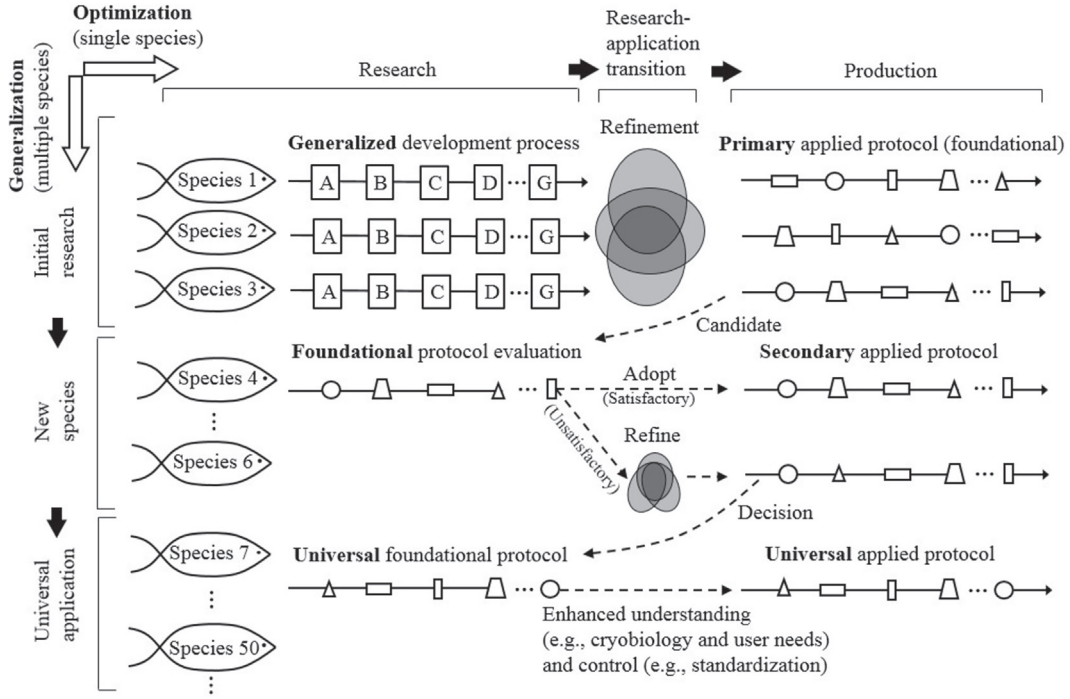

Fig. 8. A representative strategy of application of research to production by optimization or generalization. A generalized development process can be repeated for a single species (e.g., 3 species) as initial research to establish cryopreservation protocols. These protocols can be refined for each species and used in production as a primary applied protocol. However, when new protocols are needed for multiple (e.g., 50) closely related species, it is not necessary to repeat the research pathway. In practice, a primary applied protocol can be chosen as a foundational protocol to be adopted as a secondary applied protocol in production. After repeated evaluation a universal foundational protocol for multiple new species can be developed. Eventually, sufficient knowledge can lead to a future universal applied protocol in production.

the secondary applied protocol could perhaps be used as a universal foundational protocol for use with multiple other species. After repeated application of the universal foundational protocol to new species, sufficient knowledge could be gained to enhance basic understanding (e.g., cryobiology and user needs) and process control (e.g., standardization). Such enhanced understanding can

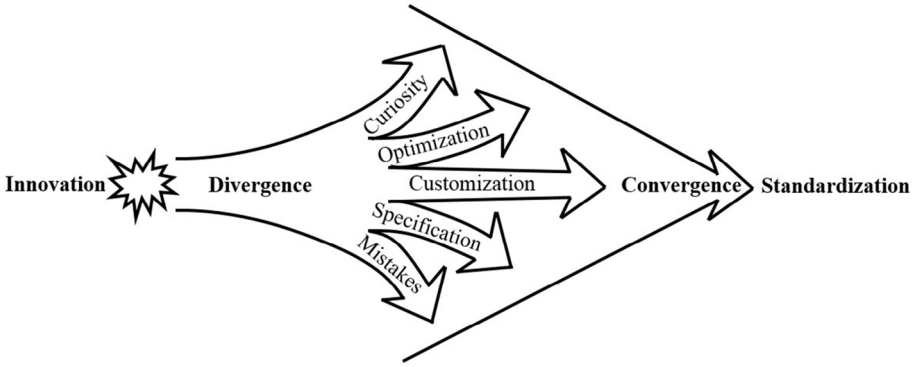

Fig. 9. A diagrammatic example showing how innovation can diverge with differential application and later be converged into standardization. An innovation is usually diverged into modified methods by individuals within a research community based on different applications. It is important to integrate and converge the modified methods into a standardized approach at the community level to enable direct comparison of research results and to foster technology application such as in germplasm repositories.

lead to a universal applied protocol for production purposes. The feasibility of this concept has been demonstrated by the establishment of germplasm repositories approaches for the XGSC *Xiphophorus* species at the AGGRC, in which protocol development for four species by generalization of the research process was integrated into a universal foundational protocol to cryopreserve more than 20 additional *Xiphophorus* species.

5.3. Standardization and quality control

Two important considerations in the transfer from research to establishment of germplasm repositories are standardization and quality management. Although protocols are developed and repeated, they can be interpreted or performed differently (or poorly) by different individuals without appropriate standardization and quality control [115]. Germplasm repositories represent a form of time travel. Samples cryopreserved today can be used tens or hundreds of years later by different users. Without standardization of protocols and quality assurance of operational processes, it can be problematic to assure production of offspring by use of thawed sperm.

In recent years, reproducibility in science has received increasing attention [125,126]. The reproducibility of cryopreservation studies has been especially challenging, mainly due to a lack of comprehensive quality control strategies and standardized approaches [115,127]. For example, various procedures of motility evaluation reported from different sources make it difficult or impossible to directly compare research results. Indeed, basic quality evaluations such as spermatozoa concentration assessment (and adjustment) are rarely performed or reported within cryopreservation research community. The lack of standardized protocols, terminology, and reporting guidelines makes it difficult or impossible to reproduce outcomes [124].

These reproducibility and standardization problems are even more pronounced when working with small-bodied (<5 cm) fishes (such as goodeids and zebrafish) because of their minuscule sperm volumes. For example, less than 3 μL of sperm can be collected before dilution by stripping of a zebrafish male and less than 10 μL of sperm can be collected by dissection of the testis of male poeciliids and goodeids [62]. In addition, small sample volumes can affect the accuracy of analyses. For example, results of spermatozoa concentration measurements using 1 μL of sperm sample might be different from a measurement of same sample using 5 μL, because of sample evaporation, insufficient mixing, or pipetting inaccuracies that can lead to irreproducible results [127]. To address standardized quality evaluation problems caused by small body sizes, a preliminary study [127] was conducted using zebrafish as a model, indicating that concentration and spermatozoa motility could be confidently estimated using diluted volumes as small as 1 μL, whereas membrane integrity required a minimum of 2 μL (at 1×10^6 cells/mL). Based on these results, less than 5 μL of diluted sperm sample were used in measurement for quality evaluation.

Standardization is critical to reproducibility of research for sperm cryopreservation; however, it is often neglected. After a new method is developed, it usually diverges into modified methods for individuals within a research community based on different influences, such as customization, optimization, specification, curiosity, and errors (Fig. 9). However, such divergence can make it extremely difficult to compare results and replicate research. As such, it is important to eventually integrate and converge the modified methods into a standardized approach, which can be used by an entire community. Multiple standardized methods along a general research approach can result in a standardized research pathway or harmonization of approaches based on results [47].

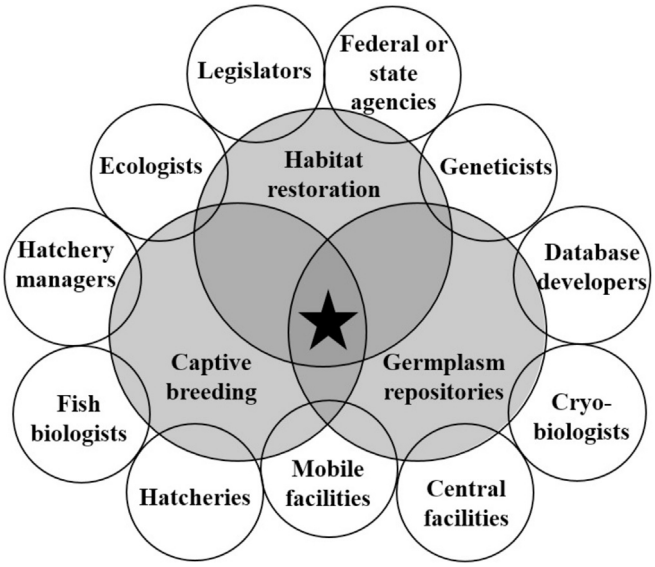

Fig. 10. An idealized comprehensive conservation or recovery program (indicated by the central star) is a combination of projects (grey circles), such as habitat restoration, captive breeding, and germplasm repositories. To achieve such programs, strong collaborations (white circles) are needed among people, agencies, and facilities with different specialized expertise and function.

5.4. Collaboration

Integration of germplasm repositories with conservation programs is an active process and requires collaboration among different sectors and people with different expertise. An ideal conservation or recovery program for imperiled species should include a comprehensive approach that combines major concerns such as habitat (by habitat restoration), population propagation and maintenance (by captive breeding or translocation), and preservation of genetic diversity (by germplasm repositories).

Lack of any of these can render a conservation or recovery program ineffective (Fig. 10). For example, ecologists and legislators are each needed for habitat restoration projects; reproductive biologists and hatchery managers are needed for captive breeding; cryobiologists and central facilities are needed for establishment of germplasm repositories, and conservation geneticists and management agencies are needed for comprehensive planning and implementation. Of course, all of these activities would require administrative support and dedicated funding. As we look at these needs it seems relevant to consider cross-training of students in the future address the development and integration of germplasm repositories for conservation. This could emerge as a new discipline in management of genetic resources. These efforts would also benefit from viewing the commercialization prospects including provision of services and markets for germplasm. These could be modeled on the decades-old, multi-billion dollar global industries that exist for livestock germplasm [4].

6. Conclusions

The development of germplasm repositories in the dairy improvement industry since the 1950's resembles a “S”-shaped curve, spanning technology development in the beginning, extensive global application during an expansion stage, and stable markets at a plateau stage. The application of germplasm repositories in aquatic species is currently in the technology development and the initial application stages, and has the potential to expand dramatically in the coming decades. Are aquatic user communities ready for the great demands of the future? More attention and efforts should be given to technologies and approaches for establishment of applied repositories instead of a conventional focus on research for protocol development. For research, tens to hundreds of samples are involved in protocol development. However, repository applications would involve thousands to hundreds of thousands of samples, and commercialized applications would involve millions of samples. Achievement of such large-scale application requires technologies and approaches to address practical aspects such as quality management, standardization, process generalization, high-throughput processing, and collaboration. In addition, community-based education efforts are needed to focus support from the public, researchers, resource managers, and administrative agencies, to realize the value of germplasm repositories in conservation programs.

Acknowledgments

This work was supported in part by funding from the National Institutes of Health, Office of Research Infrastructure Programs (R24-OD010441 and R24-OD011120), with additional support provided by the National Institute of Food and Agriculture, United States Department of Agriculture (Hatch project LAB94420), the USDA NAGP-AGGRC Cooperative Agreement (Award 58-3012-8-006), the Louisiana State University Research & Technology Foundation (AG-2018-LIFT-003), and the LSU-ACRES (Audubon Center for Research of Endangered Species) Collaborative Project (1617R0434). We thank W. Wayman for providing information on the USFWS. This manuscript was approved for publication by Louisiana State University Agricultural Center as number 2019-241-34001.

References

[1] Fickel J, Wagener A, Ludwig A. Semen cryopreservation and the conservation of endangered species. Eur J Wildl Res 2007;53:81–9.
[2] Howard J, Lynch C, Santymire R, Marinari P, Wildt D. Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret. Anim Conserv 2015;19:102–11.
[3] Saint Jalme M, Lecoq R, Seigneurin F, Blesbois E, Plouzeau E. Cryopreservation of semen from endangered pheasants: the first step towards a cryobank for endangered avian species. Theriogenology 2003;59:875–88.
[4] Hu E, Yang H, Tiersch TR. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. Cryobiology 2011;62:74–82.
[5] Dong Q, Eudeline B, Huang C, Allen SK, Tiersch TR. Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. Cryobiology 2005;50:1–16.
[6] Yang H, Hu E, Buchanan JT, Tiersch TR. A strategy for sperm cryopreservation of Atlantic salmon, Salmo salar, for remote commercial-scale high-throughput processing. J World Aquac Soc 2017;49(1):96–112.
[7] Yang H, Carmichael C, Varga ZM, Tiersch TR. Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in zebrafish *Danio rerio*. Theriogenology 2007;68:128–36.
[8] Yang H, Tiersch T. Sperm motility initiation and duration in a euryhaline fish, medaka (*Oryzias latipes*). Theriogenology 2009;72:386–92.
[9] Yang H, Cuevas-Uribe R, Savage MG, Walter RB, Tiersch TR. Sperm cryopreservation in live-bearing *Xiphophorus* fishes: offspring production from *Xiphophorus variatus* and strategies for establishment of sperm repositories. Zebrafish 2012;9:126–34.
[10] Yang H, Hazlewood L, Heater SJ, Guerrero PA, Walter RB, Tiersch TR. Production of F_1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the swordtail *Xiphophorus helleri*. Biol Reprod 2007;76:401–6.
[11] Wayman WR, Looney GL, Holm RJ, Tiersch TR. Cryopreservation of sperm from endangered pallid sturgeon. N Am J Fish Manag 2008;28:740–4.
[12] Judycka S, Nynca J, Ciereszko A. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fish. Theriogenology 2019. https://doi.org/10.1016/j.theriogenology.2019.03.022. in press.
[13] Cuevas-Uribe R. A general approach for vitrification of fish sperm. Baton Rouge, Louisiana: Louisiana State University; 2011.
[14] Wourms JP. Viviparity: the maternal-fetal relationship in fishes. Am Zool 1981;21:473–515.
[15] Long JA, Trinajstic K, Johanson Z. Devonian arthrodire embryos and the origin of internal fertilization in vertebrates. Nature 2009;457:1124–7.
[16] Long JA, Trinajstic K, Young GC, Senden T. Live birth in the Devonian period. Nature 2008;453:650–2.
[17] Wake MH. Evolution of oviductal gestation in amphibians. J Exp Zool A Ecol Genet Physiol 1993;266:394–413.
[18] Guillette LJ. The evolution of viviparity in fishes, amphibians and reptiles: an endocrine approach. In: Norris DO, Jones RE, editors. Hormones and reproduction in fishes, amphibians, and reptiles. Boston, MA: Springer; 1987. p. 523–62.
[19] Blackburn DG. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. Am Zool 1992;32:313–21.
[20] Goodwin NB, Dulvy NK, Reynolds JD. Macroecology of live-bearing in fishes: latitudinal and depth range comparisons with egg-laying relatives. Oikos 2005;110:209–18.
[21] Thibault RE, Schultz RJ. Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). Evolution 1978;32:320–33.
[22] Meyer A, Lydeard C. The evolution of copulatory organs, internal fertilization, placentae and viviparity in killifishes (Cyprinodontiformes) inferred from a DNA phylogeny of the tyrosine kinase gene X-src. Proc R Soc Lond B Biol Sci 1993;254:153–62.
[23] Helmstetter AJ, Papadopulos AS, Igea J, Van Dooren TJ, Leroi AM, Savolainen V. Viviparity stimulates diversification in an order of fish. Nat Commun 2016;7:11271.
[24] Walter RB, Kazianis S. *Xiphophorus* interspecies hybrids as genetic models of induced neoplasia. ILAR J 2001;42:299–321.
[25] Shen Y, Chalopin D, Garcia T, Boswell M, Boswell W, Shiryev SA, et al. X. couchianus and X. hellerii genome models provide genomic variation insight among *Xiphophorus* species. BMC Genomics 2016;17:37.
[26] Boschetto C, Gasparini C, Pilastro A. Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). Behav Ecol Sociobiol 2011;65:813–21.
[27] Smith CC. Opposing effects of sperm viability and velocity on the outcome of sperm competition. Behav Ecol 2012;23(4):820–6. ars036.

[28] Doadrio I, Domınguez O. Phylogenetic relationships within the fish family Goodeidae based on cytochrome b sequence data. Mol Phylogenetics Evol 2004;31:416–30.
[29] Blackburn DG. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. J Morphol 2015;276:961–90.
[30] Piller K, Kenway-Lynch C, Camak D, Domínguez-Domínguez O. Phylogeography and population structure of the imperiled Redtail Splitfin (Goodeidae: *Xenotoca eiseni*): implications for conservation. Copeia 2015;103:440–54.
[31] Webb SA, Graves JA, Macias-Garcia C, Magurran AE, Foighil DO, Ritchie MG. Molecular phylogeny of the livebearing Goodeidae (Cyprinodontiformes). Mol Phylogenetics Evol 2004;30:527–44.
[32] Sovrano VA, Bisazza A, Vallortigara G. Modularity as a fish (*Xenotoca eiseni*) views it: conjoining geometric and nongeometric information for spatial reorientation. J Exp Psychol Anim Behav Process 2003;29:199.
[33] Vega-López A, Martínez-Tabche L, Martínez MG. Toxic effects of waterborne polychlorinated biphenyls and sex differences in an endangered goodeid fish (Girardinichthys viviparus). Environ Int 2007;33:540–5.
[34] Chapman FA, Fitz-Coy SA, Thunberg EM, Adams CM. United States of America trade in ornamental fish. J World Aquac Soc 1997;28:1–10.
[35] Maceda-Veiga A, Domínguez-Domínguez O, Escribano-Alacid J, Lyons J. The aquarium hobby: can sinners become saints in freshwater fish conservation? Fish Fish 2016;17:860–74.
[36] Offill Y, Walton W. Comparative efficacy of the threespine stickleback (*Gasterosteus aculeatus*) and the mosquitofish (*Gambusia affinis*) for mosquito control. J Am Mosq Control Assoc 1999;15:380–90.
[37] Duncan JR, Lockwood JL. Extinction in a field of bullets: a search for causes in the decline of the world's freshwater fishes. Biol Conserv 2001;102:97–105.
[38] Goodwin NB, Dulvy NK, Reynolds JD. Life-history correlates of the evolution of live bearing in fishes. Phil Trans Biol Sci 2002;357:259.
[39] IUCN. The IUCN red list of threatened species (version 2017-3). The International Union for Conservation of Nature 2017. accessed February 2018, http://www.iucnredlist.org.
[40] Lyons J. Conservation status and population trends of Mexican Goodeids. Goodeid Working Group; 2011. accessed February 8, http://www.goodeidworkinggroup.com/conservation-status-and-population-trends-of-mexican-goodeids-2011.
[41] Domínguez-Domínguez O, Mercado-Silva N, Lyons J. Conservation status of Mexican goodeids: problems, perspectives, and solutions. In: Uribe MC, Grier HJ, editors. Viviparous fishes. Florida: New Life Publications; 2005. p. 515–24.
[42] Domínguez-Domínguez O, Mercado-Silva N, Lyons J, Grier HJ. The viviparous goodeid species. In: Uribe MC, Grier HJ, editors. Viviparous fishes. Florida: New Life Publications; 2005. p. 525–69.
[43] Domínguez-Domínguez O, Martínez-Meyer E, Zambrano L, De L, Pérez Ponce G. Using ecological-niche modeling as a conservation tool for freshwater species: live-bearing fishes in central Mexico. Conserv Biol 2006;20:1730–9.
[44] Lyons J, González-Hernández G, Soto-Galera E, Guzmán-Arroyo M. Decline of freshwater fishes and fisheries in selected drainages of west-central Mexico. Fisheries 1998;23:10–8.
[45] Tobler M, Riesch R, Garcíade León F, Schlupp I, Plath M. Two endemic and endangered fishes, Poecilia sulphuraria (Alvarez, 1948) and Gambusia eurystoma Miller, 1975 (Poeciliidae, Teleostei) as only survivors in a small sulphidic habitat. J Fish Biol 2008;72:523–33.
[46] G.W.G.. Objectives of the goodeid working group. Goodeid Working Group; 2011. accessed February 2018, http://www.goodeidworkinggroup.com/about-us.
[47] Tiersch T. Process pathways for cryopreservation research, application and commercialization. In: Tiersch TR, Green CC, editors. Cryopreservation in aquatic species. second ed. Baton Rouge, Louisiana: World Aquaculture Society; 2011. p. 646–71.
[48] Grier HJ. Cellular organization of the testis and spermatogenesis in fishes. Am Zool 1981;21:345–57.
[49] Grier HJ, Burns JR, Flores JA. Testis structure in three species of teleosts with tubular gonopodia. Copeia 1981:797–801.
[50] Yushin VV, Yoshida M, Spiridonov SE. Riders on the sperm: sperm dimorphism and spermatozeugmata in nematodes from the genus *Steinernema* (Rhabditida: Steinernematidae). Nematology 2007;9:61–75.
[51] Braidotti P, Ferraguti M. Two sperm types in the spermatozeugmata of *Tubifex tubifex* (Annelida, Oligochaeta). J Morphol 1982;171:123–36.
[52] Sahara K, Kawamura N. Roles of actin networks in peristaltic squeezing of sperm bundles in *Bombyx mori*. J Morphol 2004;259:1–6.
[53] Lynn JW. The ultrastructure of the sperm and motile spermatozeugmata released from the freshwater mussel *Anodonta grandis* (Mollusca, Bivalvia, Unionidae). Can J Zool 1994;72:1452–61.
[54] Piprek RP, Pecio A, Szymura JM. Modifications of the testis in response to sperm bundle formation in the Mediterranean painted frog *Discoglossus pictus* Otth, 1837 (Amphibia: Anura: discoglossidae). J Herpetol 2013;47:331–6.
[55] Jones CJ, Hamlett WC. Glycosylation of the male genital ducts and spermatozeugmata formation in the clearnose skate *Raja eglanteria*. Histochem J 2003;34:601–15.
[56] Wourms JP. Reproduction and development in chondrichthyan fishes. Am Zool 1977;17:379–410.
[57] Greven H, Brenner M. How to copulate without an intromittent organ: the external genital structures and mating behaviour of *Xenotoca eiseni* (Goodeidae). In: Uribe MC, Grier HJ, editors. Viviparous fishes II. Homestead, Florida: New Life Publications; 2010. p. 446–50.
[58] Greven H. Structural and behavioral traits associated with sperm transfer in Poeciliinae. In: Uribe MC, Grier HJ, editors. Viviparous fishes. Homestead, Florida: New Life Publications; 2005. p. 147–65.
[59] Grier H, Fitzsimons J, Linton J. Structure and ultrastructure of the testis and sperm formation in goodeid teleosts. J Morphol 1978;156:419–37.
[60] Kime D, Van Look K, McAllister B, Huyskens G, Rurangwa E, Ollevier F. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp Biochem Physiol C Toxicol Pharmacol 2001;130:425–33.
[61] Suquet M, Pouvreau S, Queau I, Boulais M, Le Grand J, Ratiskol D, et al. Biological characteristics of sperm in European flat oyster (Ostrea edulis). Aquat Living Resour 2018;31:20.
[62] Liu Y, Torres L, Tiersch TR. Quality evaluation of sperm from livebearing fishes: standardized assessment of sperm bundles (spermatozeugmata) from *Xenotoca eiseni* (Goodeidae). Theriogenology 2018;107:50–6.
[63] Dong Q, Huang C, Tiersch TR. Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. Cryobiology 2007;54:87–98.
[64] Liu Y, Torres L, Tiersch TR. Cryopreservation of sperm bundles (spermatozeugmata) from endangered livebearing goodeids. Cryobiology 2018;82:49–56.
[65] Cosson J, Groison AL, Suquet M, Fauvel C, Dreanno C, Billard R. Studying sperm motility in marine fish: an overview on the state of the art. J Appl Ichthyol 2008;24:460–86.
[66] Dzyuba V, Cosson J. Motility of fish spermatozoa: from external signaling to flagella response. Reprod Biol 2014;14:165–75.
[67] Cosson J. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J Fish Biol 2010;76:240–79.
[68] Alavi SMH, Cosson J. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol Int 2006;30:1–14.
[69] Alavi SMH, Cosson J, Bondarenko O, Linhart O. Sperm motility in fishes: (III) Diversity of regulatory signals from membrane to the axoneme. Theriogenology 2019 (in press).
[70] Krasznai Z, Márián T, Izumi H, Damjanovich S, Balkay L, Trón L, et al. Membrane hyperpolarization removes inactivation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. Proc Natl Acad Sci Unit States Am 2000;97:2052–7.
[71] Huang C, Dong Q, Walter RB, Tiersch TR. Initial studies on sperm cryopreservation of a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology 2004;62:179–94.
[72] Liu Y, Yang H, Torres L, Tiersch TR. Activation of free sperm and dissociation of sperm bundles (spermatozeugmata) of an endangered viviparous fish, *Xenotoca eiseni*. Comp Biochem Physiol, A: Mol Integr Physiol. 2018;218:35–45.
[73] Tanaka H, Oka Y. Chaotropic ions and multivalent ions activate sperm in the viviparous fish guppy *Poecilia reticulata*. Biochim Biophys Acta 2005;1724:173–80.
[74] Sun C, Huang C, Su X, Zhao X, Dong Q. Optimization of handling and refrigerated storage of guppy *Poecilia reticulata* sperm. J Fish Biol 2010;77:54–66.
[75] Morisawa M, Suzuki K. Osmolality and potassium ion: their roles in initiation of sperm motility in teleosts. Science 1980;210:1145–7.
[76] Pérez L, Vílchez MC, Gallego V, Morini M, Peñaranda DS, Asturiano JF. Role of calcium on the initiation of sperm motility in the European eel. Comp Biochem Physiol, A: Mol Integr Physiol. 2016;191:98–106.
[77] Loux SC, Crawford KR, Ing NH, González-Fernández L, Macías-García B, Love CC, et al. CatSper and the relationship of hyperactivated motility to intracellular calcium and pH kinetics in equine sperm. Biol Reprod 2013;89(123):1–15.
[78] Vines CA, Yoshida K, Griffin FJ, Pillai MC, Morisawa M, Yanagimachi R, et al. Motility initiation in herring sperm is regulated by reverse sodium-calcium exchange. Proc Natl Acad Sci Unit States Am 2002;99:2026–31.
[79] Brown M, Wittwer C. Flow cytometry: principles and clinical applications in hematology. Clin Chem 2000;46:1221–9.
[80] Liu Y, Cheng H, Tiersch TR. The role of alkalinization-induced Ca^{2+} influx in sperm motility activation of a viviparous fish Redtail Splitfin (*Xenotoca eiseni*). Biol Reprod 2018. https://doi.org/10.1093/biolre/ioy150.
[81] Huang C, Sun C, Su X, Zhao X, Miao M, Liu Y, et al. Sperm cryopreservation in guppies and black mollies — a generalized freezing protocol for livebearers in Poeciliidae. Cryobiology 2009;59:351–6.
[82] Potter H, Kramer CR. Ultrastructural observations on sperm storage in the ovary of the platyfish, Xiphophorus maculatus (Teleostei: Poeciliidae): the role of the duct epithelium. J Morphol 2000;245:110–29.
[83] Glogowski J, Kolman R, Szczepkowski M, Horváth Á, Urbányi B, Sieczyński P, et al. Fertilization rate of Siberian sturgeon (Acipenser baeri, Brandt) milt cryopreserved with methanol. Aquaculture 2002;211:367–73.
[84] Glogowski J, Kwasnik M, Piros B, Dabrowski K, Goryczko K, Dobosz S, et al. Characterization of rainbow trout milt collected with a catheter: semen parameters and cryopreservation success. Aquacult Res 2000;31:289–96.
[85] Yang H, Hazlewood L, Walter RB, Tiersch TR. Sperm cryopreservation of a live-bearing fish, *Xiphophorus couchianus*: male-to-male variation in post-

thaw motility and production of F 1 hybrid offspring. Comp Biochem Physiol C Toxicol Pharmacol 2009;149:233–9.
[86] Yang H, Tiersch TR. Current status of sperm cryopreservation in biomedical research fish models: zebrafish, medaka, and *Xiphophorus*. Comp Biochem Physiol C Toxicol Pharmacol 2009;149:224–32.
[87] Cuevas-Uribe R, Yang H, Daly J, Savage MG, Walter RB, Tiersch TR. Production of F1 offspring with vitrified sperm from a live-bearing fish, the Green Swordtail *Xiphophorus hellerii*. Zebrafish 2011;8:167–79.
[88] Yang H, Savage MG, Hazlewood L, Walter RB, Tiersch TR. Offspring production with cryopreserved sperm from a live-bearing fish *Xiphophorus maculatus* and implications for female fecundity. Comp Biochem Physiol C Toxicol Pharmacol 2012;155:55–63.
[89] Linhart O, Rodina M, Cosson J. Cryopreservation of sperm in common carp *Cyprinus carpio*: sperm motility and hatching success of embryos. Cryobiology 2000;41:241–50.
[90] Liu Y, Grier HJ, Tiersch TR. Production of live young with cryopreserved sperm from the endangered livebearing fish Redtail Splitfin (*Xenotoca eiseni*, Rutter, 1896). Anim Reprod Sci 2018;196:77–90.
[91] Bond N, Lake P. Local habitat restoration in streams: constraints on the effectiveness of restoration for stream biota. Ecol Manag Restor 2003;4:193–8.
[92] Katz SL, Barnas K, Hicks R, Cowen J, Jenkinson R. Freshwater habitat restoration actions in the Pacific Northwest: a decade's investment in habitat improvement. Restor Ecol 2007;15:494–505.
[93] Williams SE, Hoffman EA. Minimizing genetic adaptation in captive breeding programs: a review. Biol Conserv 2009;142:2388–400.
[94] Snyder NF, Derrickson SR, Beissinger SR, Wiley JW, Smith TB, Toone WD, et al. Limitations of captive breeding in endangered species recovery. Conserv Biol 1996;10:338–48.
[95] Bailey N, Garcia CM, Ritchie M. Beyond the point of no return? A comparison of genetic diversity in captive and wild populations of two nearly extinct species of Goodeid fish reveals that one is inbred in the wild. Heredity 2007;98:360.
[96] Witzenberger KA, Hochkirch A. Ex situ conservation genetics: a review of molecular studies on the genetic consequences of captive breeding programmes for endangered animal species. Biodivers Conserv 2011;20:1843–61.
[97] Germano JM, Bishop PJ. Suitability of amphibians and reptiles for translocation. Conserv Biol 2009;23:7–15.
[98] Storfer A. Gene flow and endangered species translocations: a topic revisited. Biol Conserv 1999;87:173–80.
[99] Wolf CM, Garland Jr T, Griffith B. Predictors of avian and mammalian translocation success: reanalysis with phylogenetically independent contrasts. Biol Conserv 1998;86:243–55.
[100] Frankham R, Ballou JD, Eldridge MD, Lacy RC, Ralls K, Dudash MR, et al. Predicting the probability of outbreeding depression. Conserv Biol 2011;25:465–75.
[101] Szell AZ, Bierbaum RC, Hazelrigg WB, Chetkowski RJ. Live births from frozen human semen stored for 40 years. J Assist Reprod Genet 2013;30:743.
[102] Leibo S, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species. Theriogenology 2002;57:303–26.
[103] Bart A. New approaches in cryopreservation of fish embryos. In: Tiersch TR, Green CC, editors. Cryopreservation in aquatic species. second ed. Baton Rouge, Louisiana: World Aquaculture Society; 2011. p. 503–12.
[104] Gollin D, Evenson R. Valuing animal genetic resources: lessons from plant genetic resources. Ecol Econ 2003;45:353–63.
[105] Silversides F, Purdy P, Blackburn H. Comparative costs of programmes to conserve chicken genetic variation based on maintaining living populations or storing cryopreserved material. Br Poult Sci 2012;53:599–607.
[106] Blackburn H, Krehbiel B, Ericsson S, Wilson C, Caetano A, Paiva S. A fine structure genetic analysis evaluating ecoregional adaptability of a *Bos taurus* breed (Hereford). PLoS One 2017;12. e0176474.
[107] FAO. Cryopreservation of animal genetic resources. FAO Animal Production and Health Guidlines 2012;12. Rome.
[108] Smith C. Genetic aspects of conservation in farm livestock. Livest Prod Sci 1984;11:37–48.
[109] Blackburn H. Development of national animal genetic resource programs. Reprod Fertil Dev 2003;16:27–32.
[110] Blackburn H. Biobanking genetic material for Agricultural animal species. Annual review of animal biosciences 2018;6:69–82.
[111] Johnston L, Lacy R. Genome resource banking for species conservation: selection of sperm donors. Cryobiology 1995;32:68–77.
[112] Harnal V, Wildt D, Bird D, Monfort S, Ballou J. Computer simulations to determine the efficacy of different genome resource banking strategies for maintaining genetic diversity. Cryobiology 2002;44:122–31.
[113] USFWS. Listed FWS/Joint FWS and NMFS species and populations with recovery plans. The United States Fish and Wildlife Service; 2018. accessed February 2018, https://ecos.fws.gov/ecp0/pub/speciesRecovery.jsp?sort=1.
[114] Jimenez M, Goodchild SC, Stockwell CA, Lema SC. Characterization and phylogenetic analysis of complete mitochondrial genomes for two desert cyprinodontoid fishes, *Empetrichthys latos* and *Crenichthys baileyi*. Gene 2017;626:163–72.
[115] Torres L, Tiersch TR. Addressing reproducibility in cryopreservation, and considerations necessary for commercialization and community development in support of genetic resources of aquatic species. J World Aquac Soc 2018;49:644–63.
[116] Tiersch TR, Figiel Jr CR, Wayman WR, Williamson JH, Carmichael GJ, Gorman OT. Cryopreservation of sperm of the endangered razorback sucker. Trans Am Fish Soc 1998;127:95–104.
[117] Gwo J-C, Ohta H, Okuzawa K, Wu H-C. Cryopreservation of sperm from the endangered Formosan landlocked salmon (*Oncorhynchus masou formosanus*). Theriogenology 1999;51:569–82.
[118] Sarvi K, Niksirat H, Amiri BM, Mirtorabi S, Rafiee G, Bakhtiyari M. Cryopreservation of semen from the endangered Caspian brown trout (*Salmo trutta caspius*). Aquaculture 2006;256:564–9.
[119] Tiersch TR, Figiel CR, Wayman WR, Williamson JH, Gorman OT, Carmichael GJ. Cryopreservation of sperm from the endangered Colorado pikeminnow. N Am J Aquacult 2004;66:8–14.
[120] Maria A, Viveiros A, Freitas R, Oliveira A. Extenders and cryoprotectants for cooling and freezing of piracanjuba (*Brycon orbignyanus*) semen, an endangered Brazilian teleost fish. Aquaculture 2006;260:298–306.
[121] Fuller SA, Carmichael GJ. Cryopreservation of sperm of the endangered Gila trout, *Oncorhynchus gilae*. J Appl Aquac 2007;19:71–9.
[122] Le Francois N, Lamarre S, Tveiten H, Blier P, Bailey J. Sperm cryoconservation in *Anarhichas sp*., endangered cold-water aquaculture species with internal fertilization. Aquacult Int 2008;16:273–9.
[123] Childress WM, Caffey RH, Tiersch TR. Design and cost analysis of a self-contained mobile laboratory for commercial-scale Aquatic species cryopreservation. J World Aquac Soc 2018;49(5):805–26.
[124] Torres L, Hu E, Tiersch TR. Cryopreservation in fish: current status and pathways to quality assurance and quality control in repository development. Reprod Fertil Dev 2016;28:1105–15.
[125] Begley C, Ellis L. Drug development: raise standards for preclinical cancer research. Nature 2012;483:531–3.
[126] Hagedorn M, Varga Z, Walter RB, Tiersch TR. Workshop report: cryopreservation of aquatic biomedical models. Cryobiology 2018. https://doi.org/10.1016/j.cryobiol.2018.10.264.
[127] Torres L, Liu Y, Guitreau A, Yang H, Tiersch TR. Challenges in development of sperm repositories for biomedical fishes: quality control in small-bodied species. Zebrafish 2017;14:552–60.