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LOUISIANA SEA GRANT COLLEGE PROGRAM

PROJECT:
3-D Printing of Cryopreservation Devices for Standardization and Commercialization of Genetic Resources in Aquatic Species

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PROJECT OVERVIEW

The genetic resources of aquatic species are not adequately protected because of insufficient development of germplasm repositories. These efforts are impeded globally by a lack of standardized, inexpensive, reproducible technology, and a shortage of options to provide on-site freezing capability. The goal of this project was to use 3-dimensional (3-D) printing to develop a standardized freezing device that can be used with nitrogen vapor shipping dewars for on-site cryopreservation of germplasm from aquatic organisms, especially threatened and endangered species. The objectives were to: 1) design and prototype multiple variations of devices using CAD software and 3-D printing with PLA filament; 2) alpha test the most promising designs for efficiency and consistency; 3) improve and retest each design, and 4) conduct closed beta testing with individuals experienced with cryopreservation.

The final device comprised eight 3-D printed parts. All parts were designed using Fusion 360 software (Autodesk, Inc.). There were nine height slots to provide a range of cooling rates. It can hold twenty-two 0.25 or 0.5-mL French straws and the quick-release ring design allows for the straws to be easily ejected into the bottom of a shipping dewar. Print time was approximately 9 hr based on a print speed of 60 mm/s to produce all parts. Based on the average prices of PLA filament at US$25 per Kg, seven devices could be produced from one roll and would cost roughly US$3.50 per device.

In-house alpha testing was performed to characterize functionality, including the cooling rates that could be achieved with different sized French straws, and to further test the overall performance of the design. Part failures were identified, and changes were made to the designs and printing settings to strengthen these parts. The final design was able to produce typical cooling rates for 0.5-mL French straws as used in aquatic cryopreservation. The 0.25-mL French straws froze faster and produced a narrower range of freezing rates. Overall, such devices could be used for laboratory and field work including reliable preservation of material on the coast or riverbank for wild fishes. Broad areas of application include aquaculture (e.g., breeding programs), wild fisheries (e.g., securing genetic diversity), and biomedical research (e.g., backing up valuable research lines of zebrafish).

INTRODUCTION

Most efforts in aquatic cryopreservation are focused on research and developing “optimum” cryopreservation protocols. Months to years can be spent trying to find what is thought to be the best extender, cryoprotectant, and freezing rate. When work of this type is reported in peer-reviewed publications they often lack standardization, do not use widely available and inexpensive equipment, and are difficult to reproduce which hinders application of that protocol by other laboratories and users (Torres et al. 2016).

There are three broad categories of freezing devices used in aquatic cryopreservation: 1) computer-controlled freezers; 2) polystyrene (Styrofoam) box methods, and 3) shipping dewars. Computer-controlled freezers have the capacity to freeze hundreds of samples at a time with reliable and repeatable freezing rates. These freezers meet the needs for well-established laboratories participating in commercial activities, but most laboratories cannot afford these, with a cost of between US$15,000 and US$60,000. The use of a polystyrene box

with raft is one of the most commonly used devices in aquatic cryopreservation (Cabrita et al. 2001; Horváth et al. 2005; Liu et al. 2015). Samples are floated on the surface of liquid nitrogen and cooled in nitrogen vapor at different rates depending on the height about the surface. Although this is an inexpensive device, there is no standardization of features such as box or raft sizes. This produces challenges when laboratories try to replicate methods. Shipping dewars have been a practical choice for freezing in the field (Harvey et al. 1998; Wayman et al. 1997). Samples can be placed at different heights inside the shipping dewar to produce different cooling rates. But like the polystyrene box methods, there is not a standardized shipping dewar model to use or methods of suspending samples inside the shipping dewar.

Currently, there are three pathways through which a germplasm repository can reliably acquire genetic material: 1) high-throughput processing at a specialized central facility; 2) on-site high-throughput processing with a mobile laboratory deployed from a specialized central facility, and 3) a newly emerging route, aggregate throughput (Childress et al. 2018; Torres and Tiersch 2018). Pathways 1 and 2 involve trained personnel and a well-equipped central facility but, in the United States there are only four facilities with central processing capabilities and a long-standing focus on aquatic cryopreservation: 1) United States Department of Agriculture (USDA),Agricultural Research Service (ARS), National Animal Germplasm Program (NAGP), in Fort Collins, Colorado; 2) United States Fish and Wildlife Service, Warm Springs Fish Technology Center, in Warm Springs, Georgia; 3) AGGRC at Louisiana State University Agricultural Center, in Baton Rouge, Louisiana, and 4) Zebrafish International Research Center at University of Oregon, in Eugene, Oregon. Aggregate throughput provides a pathway in which non-expert users can submit samples by use of standardized, inexpensive, and simple to use devices and protocols, but those standardized, inexpensive, and simple to use devices and protocols still need to be developed and accepted by the aquatic cryopreservation community.

Three-dimensional (3-D) printing technology has begun to be applied in aquatic cryopreservation to provide standardized, easy to use, and inexpensive freezing devices (Tiersch and Monroe 2016). A 3-D printed rack system for use inside a widely available polystyrene foam box was introduced to assist small-scale centralized laboratories in achieving standardized aggregate high-throughput (Hu et al. 2017). But there is still a need for a standardized approach for freezing samples in the field. As such, the goal of this project was to use 3-D printing to develop a standardized freezing device that can be used with nitrogen vapor shipping dewars for on-site cryopreservation of germplasm from aquatic organisms. The objectives were to: 1) design and prototype multiple variations of devices using CAD software and 3-D printing with PLA filament; 2) alpha test the most promising designs for efficiency and consistency; 3) improve and retest each design, and 4) conduct closed beta testing with individuals experienced with cryopreservation.

Through research and testing by four undergraduates a device was developed that was able to produce a range of freezing rates commonly used in aquatic cryopreservation for 0.5-mL French straws. Modification can be made in future designs to slow the cooling rate of 0.25-mL French straws and produce a broader range of cooling rates. Throughout this project these undergraduates learned how to: design objects using CAD software, assemble and operate 3-D printers, print and assemble device prototypes, and test cryopreservation parameters.

METHODS

Device Design: A list of device constraints was established to help the design process. The constraints were to: 1) fit within the neck and inner chamber of a standard shipping dewar; 2) reliably hold 0.25 and 0.5-mL French straws in a radial arrangement with minimal contact to avoid interference with freezing; 3) provide adjustable heights for a range of cooling rates commonly used in aquatic cryopreservation; 4) reliably release the straws directly inside the shipping dewar after freezing, and 5) require only basic 3-D printing skills with minimal experience on entry-level printers. Parts were designed using widely available CAD software (Fusion 360, Autodesk, San Rafael, CA).

Stereolithography (STL) files were imported into slicer software (Simplify3D, Cincinnati, OH) to control the print settings of each part. A FT-5 (Folger Technologies, Milford, NH) 3-D printer with an upgraded extruder (Titan, E3D, Oxford, UK) and hot end (V6, E3D, Oxford, UK) was used with widely available polylactic acid (PLA) filament (1.75 mm diameter) to fabricate the parts. Filament was extruded through a 0.4-mm brass nozzle at 195°C with a layer height of 0.25 mm, layer width of 0.48 mm, retraction distance of 0.6 mm, and retraction speed of 40 mm per sec onto a 60°C mirror build plate covered with a polyetherimide (PEI) (Gizmo Dorks, Temple City, CA) sheet. All parts were printed at 60 mm/s and had 3 printed top and bottom layers. To keep filament dry between use, spools were stored with desiccant (FG01K, DampRid, Memphis, TN) in a 63-L plastic bin (7105HFT-10-111-44, Hefty, Macedon, NY).

Printed components were assembled, and the devices were tested under cryogenic temperatures to determine if the designs could reliably hold 0.25 and 0.5-mL French straws, reliably eject the straws, and to identify any structure weaknesses. Changes were made to the original CAD drawings and printing settings to correct any problems.

For use, shipping dewars were filled 3-4 times over several hours until no more liquid nitrogen was adsorbed and held full for at least 24 hr before use for testing. The remaining liquid nitrogen was poured out on the day of freezing. The nitrogen vapor levels inside the shipping dewar were allowed to stabilize with the dewar plug in place for a minimum of 5 mins. Filled containers were fully seated with the cotton end into the device and held at 4°C until the equilibration time was over. To start freezing, the dewar plug was removed and the device was slowly lowered into the shipping dewar. Once the device was at the desired height inside the dewar, a cross bar was used to hold the position and a two-piece cap was placed over the dewar neck opening to prevent nitrogen gas from escaping. After freezing, the samples were ejected into the bottom of the shipping dewar where they were held until the end of the day.

Alpha Testing: Comprehensive tests were performed using three type-T thermocouples (5SRTC-TT-T-30-36, Omega Engineering, Norwalk, CT) and a multichannel temperature data logger (UX120-014M, Onset Computer Corporation, Bourne, MA) to record the cooling curves for 0.25 and 0.5-mL French straws at 9 height increment inside a fully charged shipping dewar (CXR-100, Taylor-Wharton). French straws were filled with Hanks' balanced salt solution at an osmolality of 300 mOsmol/kg (HBSS300: 0.137 M NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3, and 5.55 mM glucose, pH 7.2) and loaded into the device. The device was always tested with the maximum amount of straws.

Thermocouples were inserted halfway into straws at specified positions around the holding ring and the device was carefully lowered into the shipping dewar. Thermocouple position inside the straw was examined at the end of the freeze. If the position had moved, the freezing run was discarded. The cooling rate was calculated as the temperature change over time from 4°C to -80°C. Each test was performed twice with thermocouples in the same position.

Engineering Performance: Each printed design was tested under cryogenic temperatures to evaluate the utility in holding the two sizes of French straws and the reliably of releasing them after each freezing trial. Extensive testing was performed to identify 3-D printing weaknesses which were corrected by redesign or changes in fabrication such as adjusting the infill (e.g., internal honeycomb) or wall thickness.

Beta Testing: An instruction manual was provided to beta testers to evaluate their ability to follow the directions for assembly, calibration, and freezing. This was preliminary testing, performed by the undergraduates, and will be expanded in future studies with larger groups including user communities from outside of LSU. This also helped us to edit the instruction manual for greatest comprehension. Information of this type will be used to guide design changes of the device for utility and ergonomics, and will identify problems encountered by people knowledgeable in this area, but new to the device.

RESULTS

Design: The overall height of the assembled device was 197 mm tall and 65 mm wide at its widest point (Figure 1).

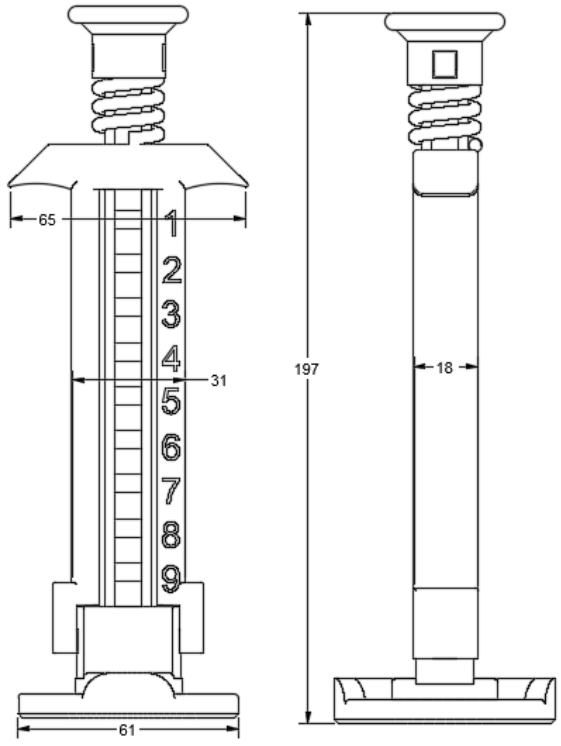

Figure 1. Schematic diagram (mm) of the front (left) and side (right) views of the device.

The device was composed of eight 3-D printed parts: 1) dewar cap; 2) cross bar; 3) ejector cap; 4) ejector locking bar; 5) positioning rod; 6) upright support; 7) inner quick-release ring with thermocouple port, and 8) outer quick-release right with thermocouple port (Figure 2).

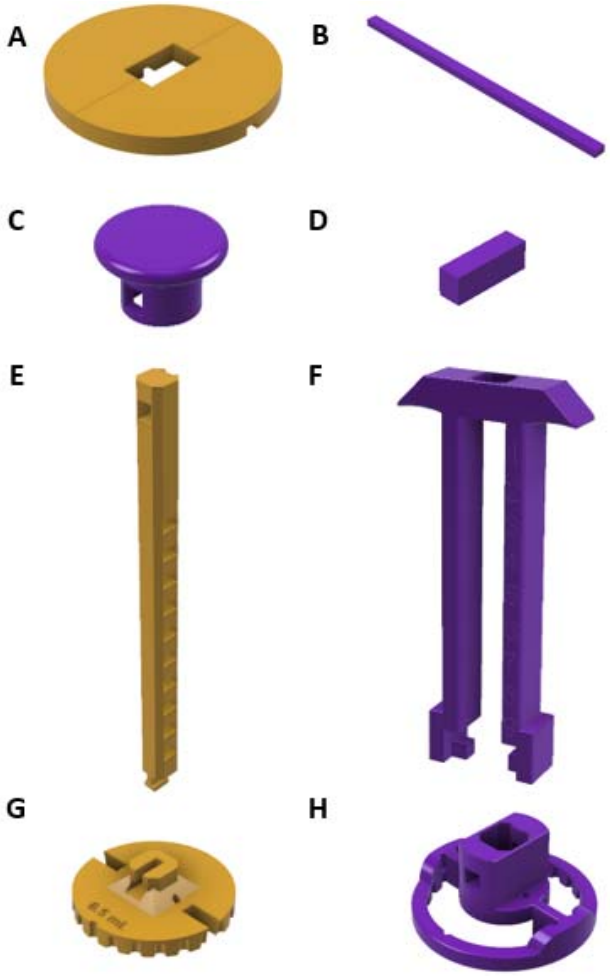

Figure 2. Individual components of the device: A) Dewar collar; B) Cross bar; C) Ejector cap; D) Ejector cap locking bar; E) Positioning rod; F) Upright support; G) Inner quick-release ring with thermocouple port; H) Outer quick-release ring with thermocouple port.

Each part went through multiple design changes with the most numerous changes occurring in the inner and outer quick-release rings (~45 design versions). There were nine height slots positioned 13.3 mm apart to provide a total working range of 109.4 mm within a standard dewar. The separate concentric quick-release ring design could hold twenty-two 0.25-ml or 0.5-ml French straws. The spring-loaded quick-release design allowed for the straws to be easily ejected into the bottom of a shipping dewar after freezing.

Each part varied in the percent infill used (i.e., use of an internal honeycomb structure rather than solid parts), number of wall perimeters (e.g., thickness), and the need for addition of removable support material to print sloping or overhanging features (Table 1). Print time to produce all final parts was approximately 9 hr based on a print speed of 60 mm/s. Most PLA

filament brands on the market cost about US$25 per 1kg roll. Based on this price, seven complete devices could be produced from one roll of PLA, and would cost roughly US$3.50 per device.

Table 1. The different infill percentages, wall perimeters, and need of support for each part.

Component	Infill (%)	Wall perimeters	Support needed?
Ejector cap	25	2	No
Cross bar	25	2	No
Dewar collar	25	2	No
Positioning rod	25	2	No
Upright support	25	3	Yes
Inner quick-release ring	50	3	Yes
Outer quick-release ring	75	3	Yes

Alpha Testing: The nine slots in the positioning rod provided sufficient variation in height inside the shipping dewar to produce the range of cooling rates typically used for fish sperm (i.e., from 4°C/min to 40°C/min). Slot 1 positioned the device at the lowest point inside the shipping dewar resulting in the fastest cooling rate, and slot 9 positioned the device at the highest point resulting in the slowest cooling rate. The device was able to produce a range of cooling rates from 4°C to 64°C/min for 0.25-mL French straws, and 6°C to 37°C/min for 0.5-mL French straws (Figure 3).

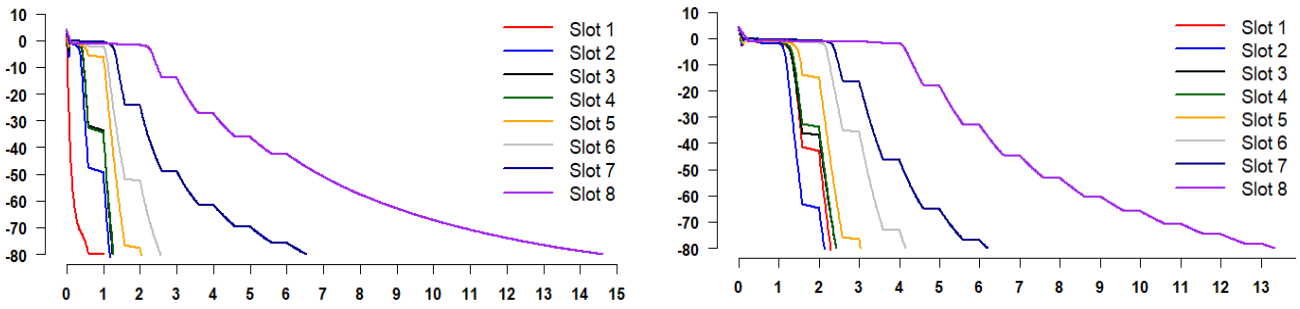

Figure 3. Average cooling curves for the different height increments for 0.25-mL (left) and 0.5-mL (right) French straws. In general, slot 8 provided the slowest cooing rate as the straws were positioned in the top of the shipping dewar, and slot 1 provided the fastest as the straws were positioned at the lowest point in the shipping dewar.

Engineering Performance: Early designs of the straw release ring relied upon small pegs to eject the straws from the ring to release them into the bottom of the shipping dewar (Figure 4). But, under cryogenic temperatures, the straws would not eject, and the small pegs would break easily. This led to the current split ring design which reliably releases straws every time. Further testing under cryogenic temperatures with the split ring design was performed to identify any 3-D printing weaknesses. Weaknesses were identified in the upright support and both quick-release rings (Figure 5). Multiple changes were made to the design files and the printing settings to strengthen these parts.

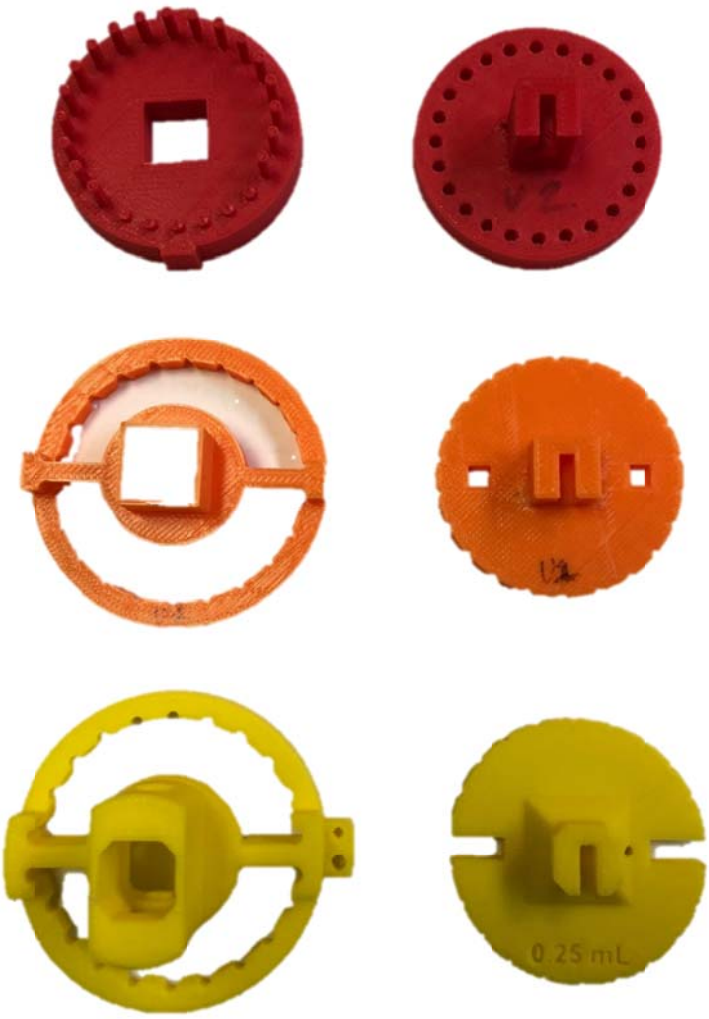

Figure 4. Evolution of the quick-release ring. 1: Our earliest versions relied on small pegs to eject the straws from the ring (top row). 2: An early version of the new split-ring design required two hands to eject the straws (middle row). 3: The final version of the split-ring design that integrated the upright support to allow the straws to be ejected with one hand (bottom row).

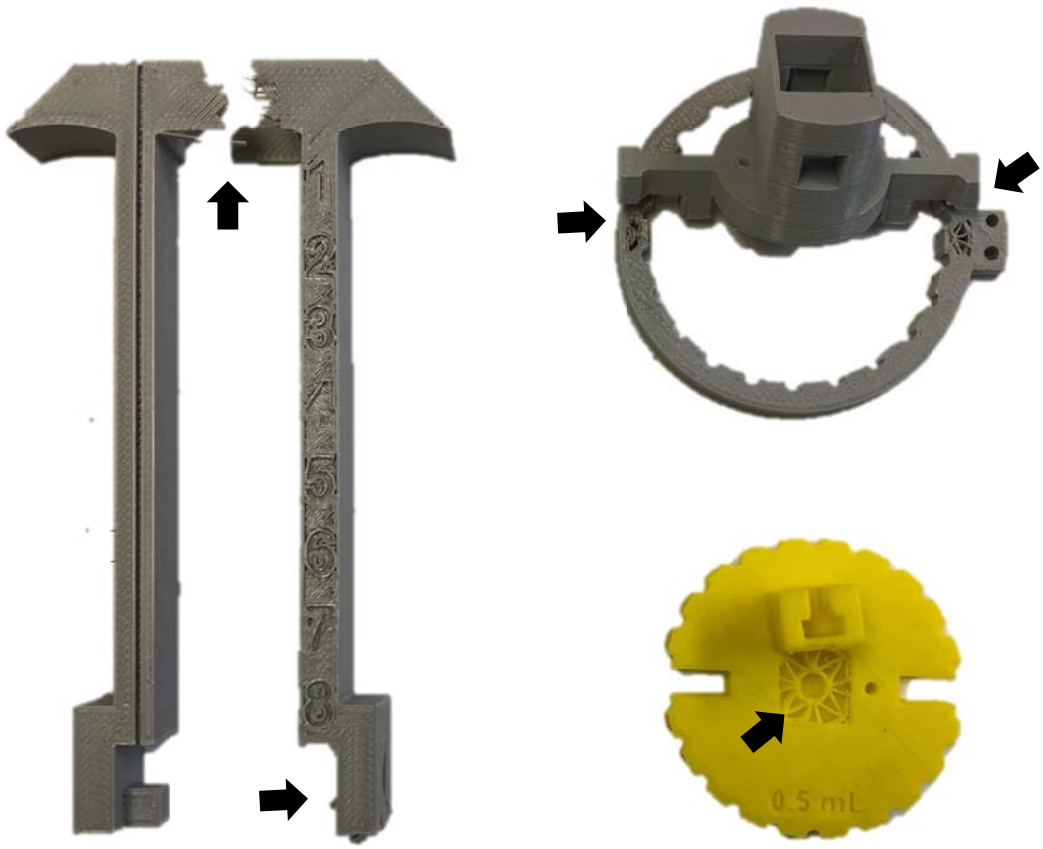

Figure 5. Examples of structural weaknesses in 3-D printing including those identified in the quick-release bar (left), outer quick-release ring (top right), and inner quick-release ring (bottom right). Multiple changes were made to the designs and the printing parameters to strengthen these parts. Arrows indicate the locations of structural failures. The infill (internal structure) is visible at the areas of structural failure.

Beta Testing: An instruction manual was created for the assembly and use of the device. On-site beta testing at the Audubon Aquarium of the Americans helped provide feedback on the design and use. Personnel from the Audubon Aquarium of the Americans also helped improve the manual. The testers assembled a device following the instruction manual to find areas that needed to be rewritten. A website training portal with training videos is currently being developed to offer greater utility for training. This portal will be organized as a Learning Management System will allow users to complete training sections online and access the 3-D printing files. A workshop focus group is scheduled to be held on March 8, 2019 at the World Aquaculture Society meeting in New Orleans to solicit feedback and find additional beta testers. The undergraduates will assist with this workshop and will gain valuable experience in seeing their designs move into the hands of users.

PROJECT SUMMARY

On-site cryopreservation of germplasm in nitrogen-vapor shipping dewars has been used for more than 35 years on islands, boats, and riverbanks. But, a standardized method of freezing inside shipping dewars has yet to be developed. Most studies only report that a shipping dewar was used to freeze the samples and fail to report important parameters such as how the samples were suspended inside the dewar, at what depth were they suspended, and the cooling rate (Fuller and Carmichael 2007; Nascimento et al. 2010). Other studies have used a customized polystyrene container or commercially available mammalian embryo freezer to suspend samples inside of shipping dewars. Our approach to address this lack of control and standardization was to utilize 3-D printing technology to produce inexpensive, standardized, and reproducible freezing devices.

During this project, the four undergraduates who did most of the research learned how to design objects using CAD software, and how to assemble, calibrate, and operate a 3-D printer. From there they learned about the cryopreservation process and were challenged to develop approaches to use the technology to address needs and problems. Multiple designs and versions were printed and tested, resulting in the current final version presented here. This device offers a standardized, inexpensive, and easy to use freezing device that can be used with nitrogen vapor shipping dewars for on-site cryopreservation of germplasm from aquatic species. The device has an approximate material cost of US$3.50 and can be printed on standard 3-D printers.

This project has been extremely beneficial for training of undergraduates and provided a great platform for them to perform independent research. We are strongly encouraged to continue the use of technologies such as 3-D printing to provide opportunities for undergraduate research experience, and we believe that this particular device will be useful in assisting development of germplasm banks to protect the genetic resources of aquatic species.

We will continue this work after the UROP project has ended and the project undergraduates and others will participate in future activities such as the workshop planned for March, 2019, at the annual meeting of the World Aquaculture Society. This workshop will allow us to collect the kind of systematic beta testing data (with large sample sizes) that will be necessary to produce a manuscript for publication.

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