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Evaluation of an Automated 3-D Printed Sperm Cryopreservation Device for use with Eastern Oysters (*Crassostrea virginica*)

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Abstract

Many populations of Eastern oysters (*Crassostrea virginica*) in Louisiana are vulnerable to environmental changes. This has created a need for cryopreservation to preserve genetic diversity and offset declining availability of wild stocks for use in breeding. Eastern oysters have been subjected to over-harvesting and depletion of their main natural habitats. Oyster hatcheries and farms exist but can only do so much when it comes to preserving genetic diversity. They are limited not only in the availability of wild stocks to breed with, but also in maintaining these critical genetic lines as live animals. Sperm repositories would allow preservation of samples to ensure viability and longevity of the Eastern oyster genetic lines. Cryopreservation plays a critical role in this, but currently its accessibility to breeding programs is limited. Existing methods for cryopreservation can be expensive, and breeding programs do not have access to the necessary machines and devices. To address these issues, the goal of this research was to develop a 3-D printed Auto-Position Cooling Device (APCD) along with electronic components to automate freezing hardware for use with portable cryogenic shipping dewars. The objectives of this project were to: 1) develop a 3-D printed device that can accommodate French straws and has the capability to be integrated with a linear actuator; 2) develop a robust user interface (for input of user commands and data, and real-time monitoring) based on an LCD screen and navigation buttons; 3) construct a temperature measurement system based on sample and chamber thermocouples, and digital conversion and linearization modules; 4) develop and algorithm to control sample height using a microcontroller (Arduino) and a linear actuator to provide calibration procedures and freezing profiles based on recorded temperatures, and 5) perform cryopreservation of sperm from Eastern oysters to evaluate feasibility of freezing and recovering biological samples. A portable version was fabricated with a microcontroller (Arduino Uno), 3-dimenstional (3-D) printed components, Liquid Crystal Display (LCD), linear actuator, and a thermocouple. This device can control the freezing rate through a user-interface based on different heights within the dewar. Because of the fabrication methods and use of consumer-level electronics this device was inexpensive (~$200 total) and was easy to use with minimal training.

1 Introduction

Aquatic species are among the most threatened taxa on the planet. Conservation programs have not yet effectively utilized cryopreserved germplasm for repository development in fish and shellfish. Among these species, Eastern oysters (*Crassostrea virginica*) in Louisiana have great needs for preservation of genetic diversity through sperm cryopreservation because of declining availability of wild stocks for use in breeding programs (Yang et al. 2012). The development of sperm repositories for Eastern oysters is significantly limited by lack of standardized, inexpensive, reproducible technologies and quality management-based approaches to secure integration of accessions and associated data (Hagedorn et al. 2019). Programable freezers can provide a well-controlled temperature profile for cryopreservation procedures and are utilized for commercial and research applications that require high-throughput sample processing (Tiersch 2011). However, due to the high cost and complexity of these systems, they are generally not available for field applications. To address the high cost and complexity of these systems, the goal of this research is to design, develop and test a device that allows for user control of the freezing rates with 3-dimensional (3-D) printed and electrical components.

The objectives of this project were to: 1) develop a 3-D printed device that can accommodate French straws and has the capability to be integrated with a linear actuator; 2) develop a robust user interface (for input of user commands and data, and real-time monitoring) based on an LCD screen and navigation buttons; 3) construct a temperature measurement system based on sample and chamber thermocouples, and digital conversion and linearization modules; 4) develop and algorithm to control sample height using a microcontroller (Arduino) and a linear actuator to provide calibration procedures and freezing profiles based on recorded temperatures, and 5) perform cryopreservation of sperm from Eastern oysters to evaluate feasibility of freezing and recovering biological samples. A low-cost portable Auto-Position Cooling Device (APCD) was developed for sperm cryopreservation with standard nitrogen vapor shipping dewars. A linear actuator, along with an Arduino microcontroller that controlled vertical movement, allowed for programming of freezing rates chosen by the user, providing a cheaper alternative to commercially available freezers to ensure sample viability.

2 Materials and Methods

2.1 Design

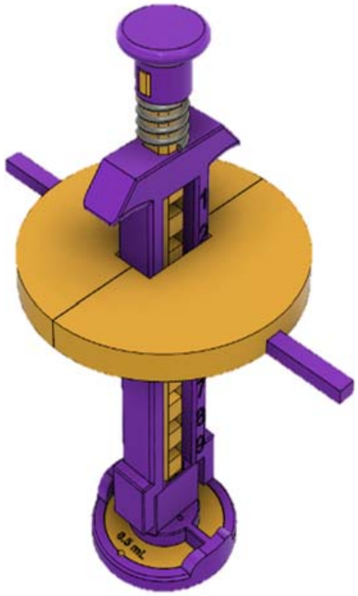


Figure 1: *Original manually operated Cajun Ejector.*

In our preliminary studies, a simple mechanical 3-D printed device ('Cajun Ejector') with low cost (<$5 material cost) was developed to generate standard cooling rates and curves (Figure 1) that were comparable to commercially available controlled rate freezers (which can cost $15,000-$50,000). The different cooling rates achieved by the Cajun Ejector were generated by temperature changes that occurred when samples were suspended at various heights surrounded by nitrogen vapor inside a standard cryogenic shipping dewar. Basically, the deeper the samples were held inside the dewar, the colder the surrounding environment, and the faster the cooling rate would be. After freezing was complete, a handle on top of the device was depressed to eject the straws into the bottom of the dewar for storage and transport, and allowing the device to be reloaded with additional straws for another freezing cycle.

This provided a portable freezer that can preserve and transport samples. It contains an inner wall of padding, or a 'core sock', that adsorbs 3.7 L of liquid nitrogen to keep samples frozen for days. The same dewar (Taylor-Wharton CXR100) used to develop the Cajun Ejector was used for this project (Figure 2).

Figure 2: *Shipping dewar used for this project*

The original mechanical version of the Cajun Ejector required significant training and user management to ensure reliable results. Due to this, the APCD was conceived to combine the manually operated version of the Cajun Ejector with the necessary electrical components and microcontroller capabilities to increase reliability and consistency. The APCD allowed users to choose different cooling rates (based on sample heights) and could hold as many as 22 0.5-ml cryopreservation straws ('French Straws') (Figure 3).

Figure 3: *Bottom view of the device showing the positioning of five straws*

The device developed in this project was designed to be auto-calibrating and basically involved a redesign of the original Cajun Ejector concept to allow incorporation of the linear actuator and associated components (Figure 4). This involved incorporation of a variety of off-the shelf components and use of computer-aided design (CAD) software to re-engineer or create new parts that could accommodate the necessary activities and work within a cryogenic environment. As such this included multiple modifications to the parts that were 3-D printed using polylactic acid (PLA) thermoplastic filament.

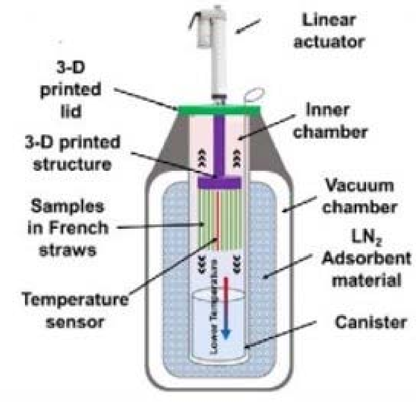


Figure 4: *Schematic view of the new design for the auto-calibrating Cajun Ejector*

2.3 Fabrication by 3-D printing

To support open availability of the designs, routinely available consumer-level software and 3-D printers were used. Designs of 3-D modeling were created using Inventor (Autodesk, CA, USA) CAD software. The 3-D renderings of prototypes were converted to stereolithography (STL) files in the software and imported to a free slicing software (Ultimaker Cura, V4.6, Ultimaker, Utrecht, Netherlands) to adjust the print settings (Table 1). The printing settings and 3-D models were converted to G-Code format and loaded onto an FDM-type 3-D printer (SOVOL, Sv01, SOVOL 3D®, Nova Silk Road Sarl, Paris, France).

Table 1: *3-D Printer settings used for part fabrication*

Setting parameters	In the present study
Printer name	Sovol SV01 3D Printer
Slicing software	Ultimaker Cura Version 4.6
Filament material	Polylactic acid
Filament diameter	1.75 mm
Hotend temperature	205 °C
Print speed	50 mm/s for infills and outer layers
Nozzle diameter	0.4 mm
Nominal layer height	0.2 mm
Retraction distance	5 mm
Retraction speed	50 mm/s
Print bed temperature	60 °C
Build surface material	Tempered Glass (300mm x 255mm)
Part cooling fan speed	100%
First layer printing speed	30 mm/s
Infill rate	60%
Infill pattern	Zig Zag
Perimeter layer number	See the Supplemental Table 2
Top layer number	2
Bottom layer number	3
Support usage	See the Supplemental Table 2
Build volume	11.2” L × 6.0” W × 6.1” H

3 Results and Discussion

3.1 Linear actuator Integration

The Cajun Ejector was integrated with a linear actuator in the APCD (Figure 5). The straw holding elements (inner and outer split rings) of the Cajun Ejector were maintained in the new device. A 3-D printed mount was created to fasten a linear actuator (Actuonix L16-140-35-6-R, Canada) on the device. A circular dewar collar (Supplemental Figure 1-2) that could be separated into two pieces in the middle was created to position the APCD on top of the opening of the shipping dewar and provide insulation for a stable freezing environment (Figure 6).

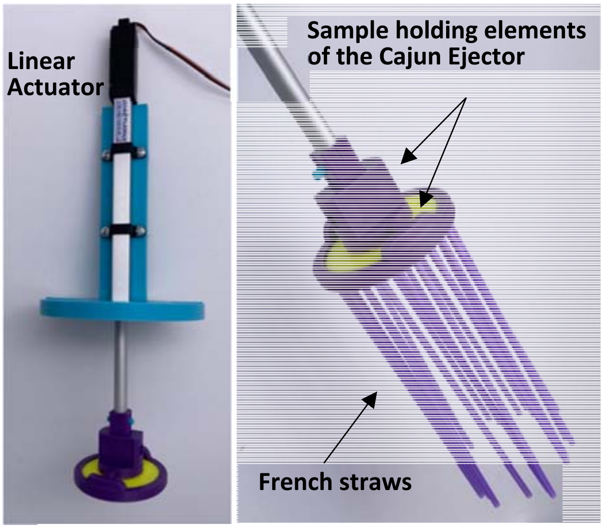


Figure 5: *Integration of a linear actuator (left) with the straw-holding elements of the Cajun Ejector*

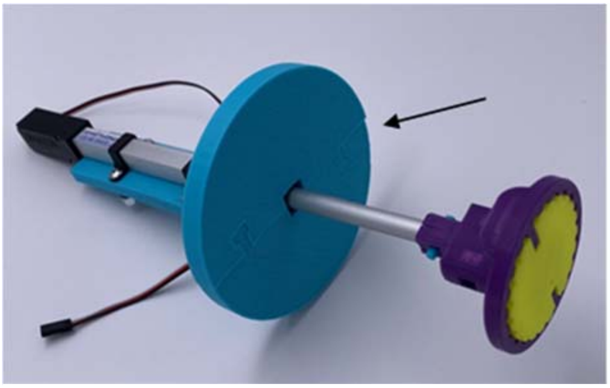


Figure 6: *Arrow indicates the 2-piece insulating collar with actuator shown on the left.*

As indicated above, this whole apparatus was designed to insert into a standard shipping dewar (Figure 7)

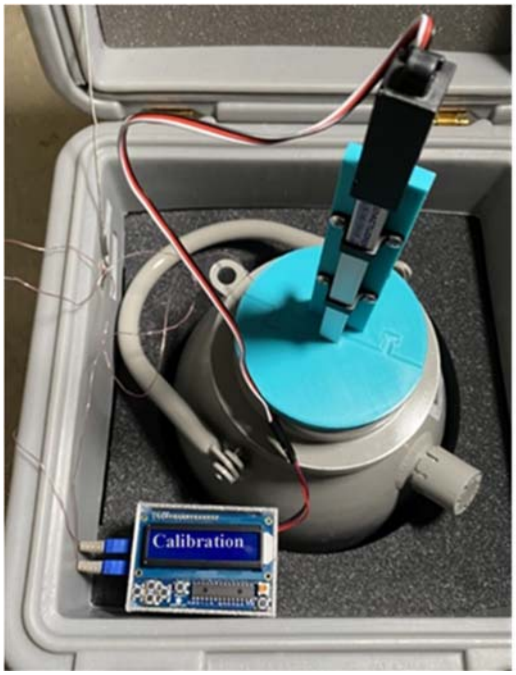


Figure 7: *The entire device situated on a dewar with prototype electronics in the lower left.*

An integration adaptor (Figure 8) was created to attach the linear actuator to the straw holder of the Cajun Ejector. This adaptor allowed the linear actuator to be attached to the straw holding components through a 3-D printed pin (Supplemental Figure 3) that inserted through a hole at the end of the actuator.

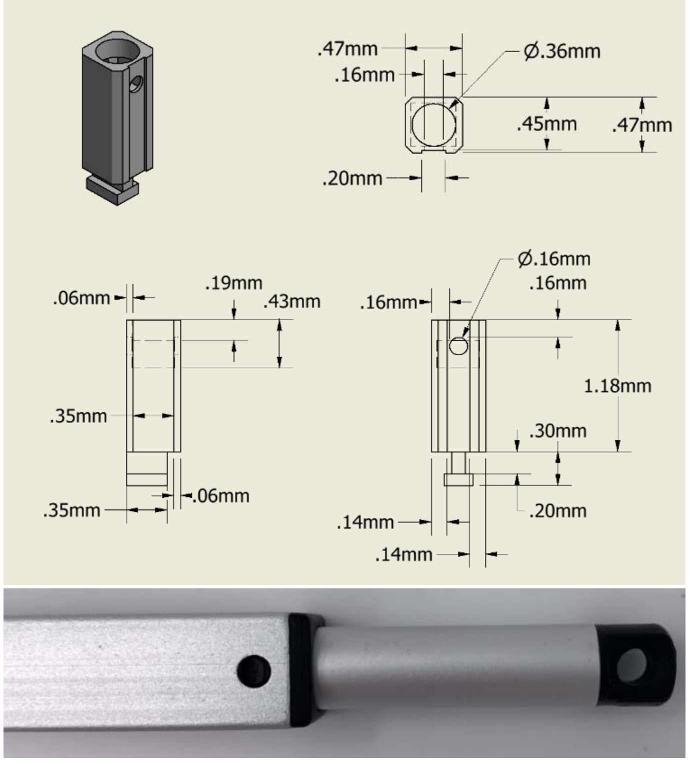


Figure 8: *An integration adaptor (upper) for fastening the linear actuator to the device through a hole and pin assembly (bottom) at the end*

3.2 User Interface

To create a user interface, we programmed the LCD (Figure 9) with the buttons to give the user a series of prompts to control the linear actuator, display temperatures and to calibrate the initial temperatures within the dewar. The user interface prompted the user to press the 'next' button to initiate temperature calibration, displaying the temperatures on the LCD and recorded in the Serial Monitor (Figure 9) of the Arduino programming software. Temperature data recorded by the Serial Monitor could be saved to the computer for further analysis. After calibration, temperatures were displayed on the LCD labeled (in °C) at 9 vertical positions. Three temperatures could be displayed at a time, and the subsequent (or previous) three temperatures could be displayed by pressing the 'Next' or 'Back' buttons. Once all the temperatures were displayed, the user could choose a desired height with appropriate temperature for sample cryopreservation. By pressing the 'Up' and 'Down' buttons, the device could be set at the demanded height by guidance of the height number displayed on the LCD. Once the demanded height was reached, the user could press 'Select' and linear actuator would automatically position samples to the chosen height.

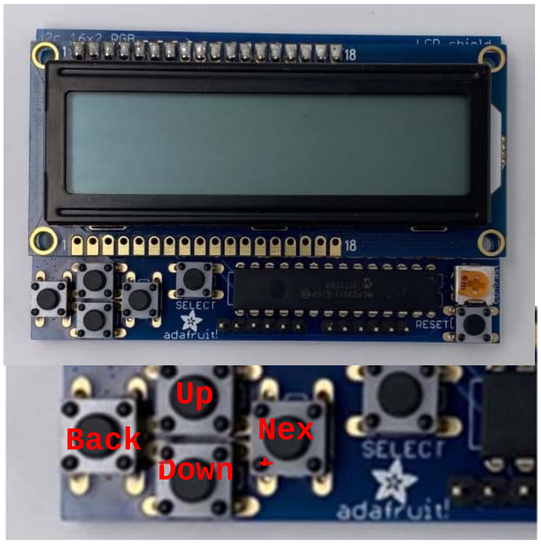
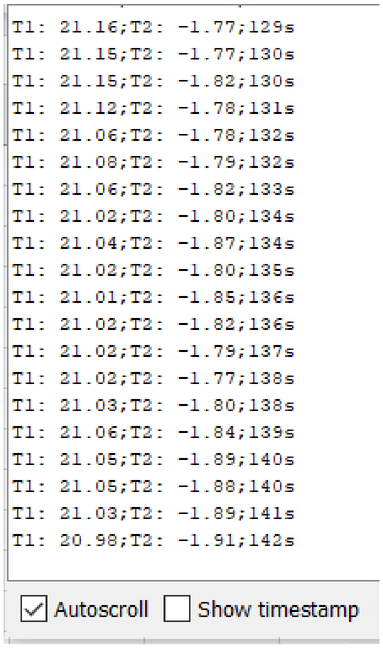


Figure 9*: A RGB LCD Shield kit (upper) with buttons (bottom) to navigate user options in the user interface*

The LCD then displayed the temperatures of both thermocouples, and the time that has elapsed in seconds. To keep a continuous record of the temperature and time being recorded by the device, these values were displayed in the Serial Monitor as well (Figure 10). The LCD had a 16 x 2 digit display screen, so full data could not be constantly displayed. The Serial Monitor also provided a clear output from the thermocouples and time elapsed during freezing, which is critical in determining the freezing profiles of the samples.



```
T1: 21.16;T2: -1.77;129s
T1: 21.15;T2: -1.77;130s
T1: 21.15;T2: -1.82;130s
T1: 21.12;T2: -1.78;131s
T1: 21.06;T2: -1.78;132s
T1: 21.08;T2: -1.79;132s
T1: 21.06;T2: -1.82;133s
T1: 21.02;T2: -1.80;134s
T1: 21.04;T2: -1.87;134s
T1: 21.02;T2: -1.80;135s
T1: 21.01;T2: -1.85;136s
T1: 21.02;T2: -1.82;136s
T1: 21.02;T2: -1.79;137s
T1: 21.02;T2: -1.77;138s
T1: 21.03;T2: -1.80;138s
T1: 21.06;T2: -1.84;139s
T1: 21.05;T2: -1.89;140s
T1: 21.05;T2: -1.88;140s
T1: 21.03;T2: -1.89;141s
T1: 20.98;T2: -1.91;142s
```

***Figure 10:** example of the Serial Monitor*

3.3 Temperature measurement and signal conversion

A thermocouple interface (MAX31856, Maxim Integrated, CA, USA) (Figure 11) was used to convert temperature signals collected by thermocouples to digital signals. This was chosen based on its temperature range (-200 to 400°C), and compatibility with the Arduino Uno microcontroller and dual-channel T-Type thermocouples. When freezing samples, the cooling rate was determined by the time used to transition the samples from 4°C to -80°C, thus this thermocouple interface was within our desired operating range. The dual-channel thermocouple configuration was chosen for further development on the monitoring of environmental temperature outside shipping dewar and cryogenic temperatures inside the dewar.

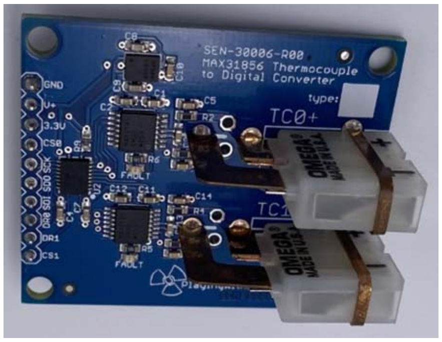


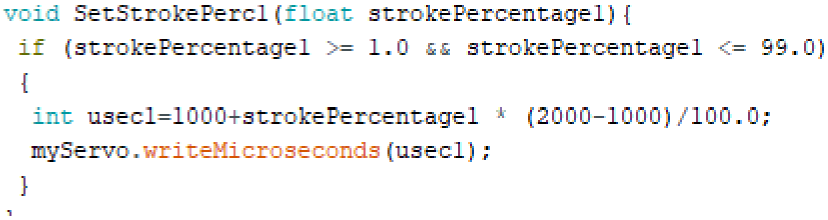
***Figure 11**: MAX31856*

3.4 Controlling system

As indicated above, a linear actuator (L16-140-35-6-R, Actunoix, Canada) was used to adjust sample heights inside the shipping dewar by vertical movement. This actuator was chosen because of its appropriate size, (140 mm stroke full distance), 150:1 gear ratio, and 6-V operating voltage. Because this voltage is too high to operate with an Arduino Uno microcontroller, an extra external 6-V power supply (Analog Devices, GT-41062-1806-T3, Massachusetts) (Figure 12) was used to power the linear actuator and its control was added to the Arduino unit.

***Figure 12:** Linear actuator next to external battery supply*

To calibrate the linear actuator, an equation was used to calculate the total distance a linear actuator extrudes or retracts (Figure 13). Total distance, or ‘strokePercentage1’ was the distance the linear actuator would extrude. To configure this into specific values that the linear actuator could execute, this number was converted using an equation that defined the maximum and minimum stroke of the linear actuator (1-99%), and the parameters were used to create our setup in the code, (or the void SetStrokePerc1).



```
void SetStrokePerc1(float strokePercentage1){
 if (strokePercentage1 >= 1.0 && strokePercentage1 <= 99.0)
 {
  int usec1=1000+strokePercentage1 * (2000-1000)/100.0;
  myServo.writeMicroseconds(usec1);
 }
```

Figure 13: *Linear actuator next to external battery supply*

Based on the maximum and minimum of the linear actuator movement, and the length of the straws to be attached on the end of the linear actuator, vertical heights 1-9 (10-90% extrusion length) were determined and programmed to generate height options. The ‘strokePercentage1’ was the distance in percent of extrusion entered by the user for the linear actuator.

3.5 Case Unit (‘Glitter Box’)

To make this device portable we created a case to contain the Arduino Uno, LCD, thermocouple, and linear actuator. This box contained all the wires and working pieces of the device, equipped with the stunning Cosmic Sparkle 3-D printed filament (coining the name ‘glitter box’) (Figure 14). This was developed to make the device neater and portable for applications in the field.

Figure 14: *Shows 'Glitter Box' alongside linear actuator.*

The wiring diagram (Figure 15) shows the MAX31856 (the dual-channel thermocouple), the linear actuator, RGB LCD Shield, and the Arduino Uno. The external power supply is connected to a port on the Arduino Uno (a port already built into the Arduino). Two extra wires were connected to the external power supply on the Arduino, one for the 6V power, and one for ground. This external power supply plugs into a wall outlet and connects to the port on the Arduino.

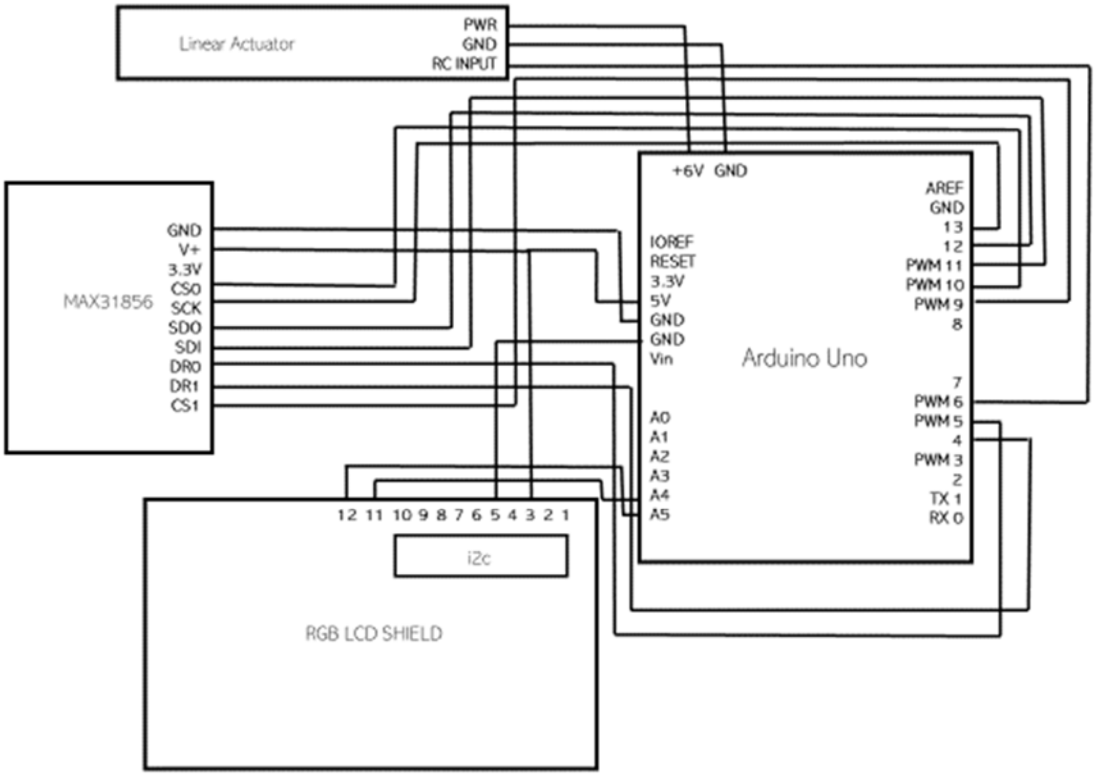


Figure 15: *Full wiring schematic (minus external power supply)*

For power distribution to the RGB LCD Shield and the MAX31856, the wire was split to enable one wire to go to the LCD and the thermocouple interface. An USB 2.0 male-to-male cable (not shown) connected to the Arduino Uno to a laptop to transfer digital data to the Serial Monitor on the Arduino programming software. The thermocouple was connected to the Arduino by 10 different ports, all enabling the Arduino to supply power and read temperatures from the thermocouples. The RGB LCD Shield was connected by 4 ports, 2 for power and ground, and the other two for input and output of the LCD to relay to the Arduino Uno.

3.6 Evaluation of the APCD

3.6.1 Calibration

To calibrate the APCD, temperatures at each of the 9 heights adjusted by the linear actuator were measured by a thermocouple in a fully charged dewar. To measure the temperature, the thermocouple was inserted through the thermocouple hole on the top of the APCD, and a 0.5-ml straw was cut in half to allow the thermocouple to read the temperature at the same height the samples would be (Figure 16). Because the thermocouple was inserted into the sample half-way through the straw, the same height is taken into consideration for obtaining the chamber temperature. The calibration average was calculated for all 3 trials (Figure 17).

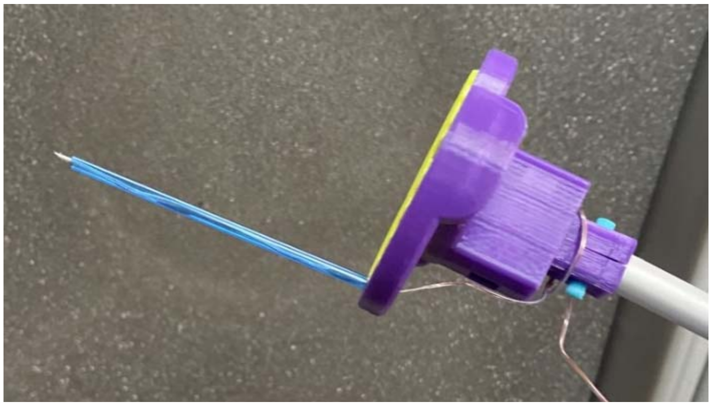


Figure 16: *Thermocouple inside of straw cut in half.*

This calibration allows for the user to consider the temperatures within the dewar before freezing, so they know the temperature at that height. This temperature curve can vary in a fully charged portable dewar, and as seen in Figure 20, there were many differences recorded at different points, but all followed a general curve throughout the calibration data recorded.

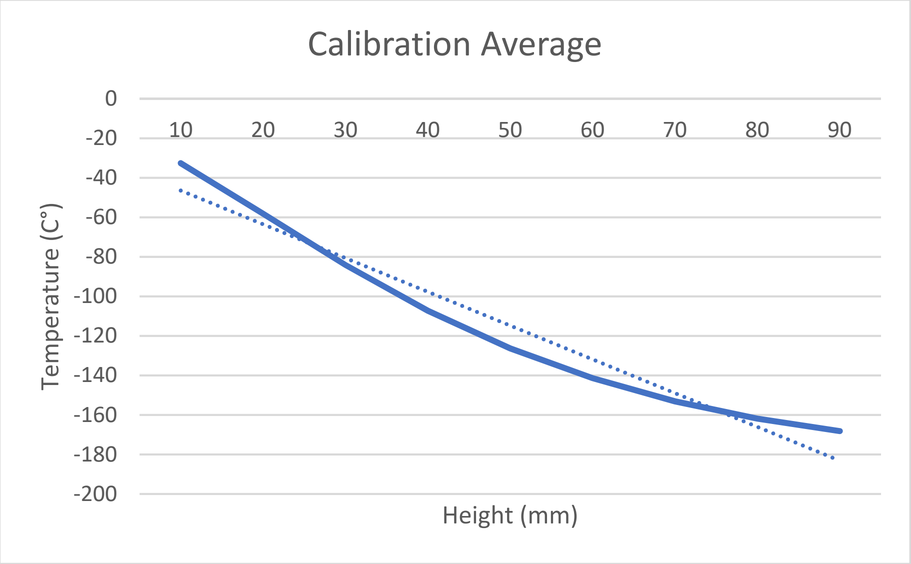


Figure 17: *Average Temperature at each height within the dewar before Trials 1-3.*

3.6.2 Cooling rate of samples with extender solution

The temperature of Hanks' Balanced Salt Solution (HBSS) 300 mOsmol/kg placed in 0.5-ml straws at 9 different heights were measured to mimic the actual solutions used when freezing sperm samples. To monitor the sample temperature, a thermocouple was inserted half-way through the filled straw and placed in the same slot every iteration (Figure 18). Time elapsed for cooling samples from 4C to -80C was recorded to calculate cooling rates. Measurements for each height was repeated 3 times.

Figure 18: *Orange straw showing the thermocouple inserted half-way into the sample.*

3.3.1 HBSS Results

As seen in Figure 19, the temperature curve profile within the dewar was measured before each trial of freezing samples.

To calculate the results, the amount of time it took for the sample to go from 4C to -80C and divided it by the total temperature change that occurred.

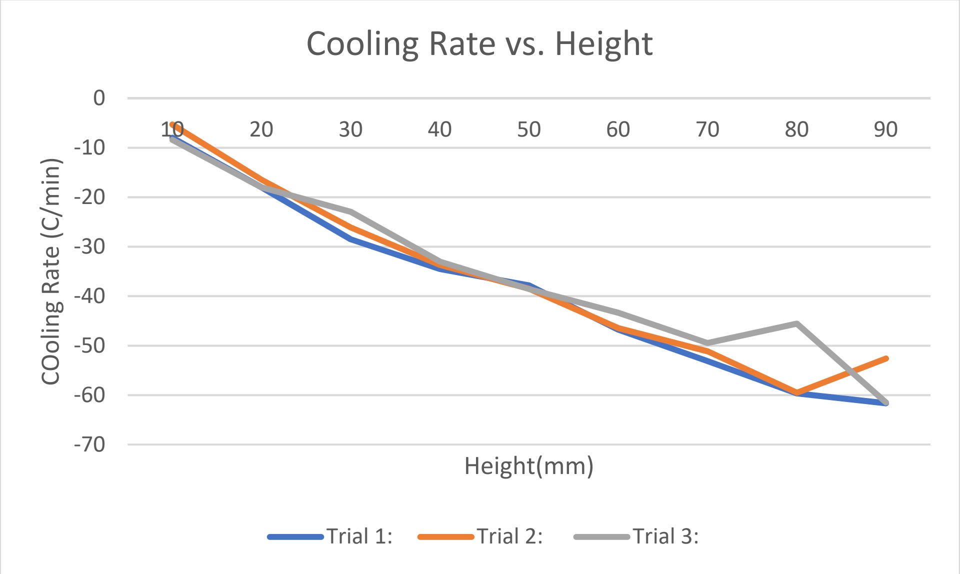


Figure 19: *HBSS 300 mOsmol/kg results. The freezing rate however increases at 80mm on Trial 3, this spike is likely caused by the thermocouple coming out half-way through freezing.*

The cooling rates of the sample followed the same general profile throughout the trials (Figure 19). The different freezing profiles varied at different points throughout the dewar and there was a consistent increase in the cooling rate. T temperature curve also decreased as the trials continued.

These data were all collected on the same day using the same dewar, so further evaluation is needed to understand the temperature curves within a variety of dewars. Many factors can affect this, depending on how much liquid nitrogen was put into the dewar, how long the dewar was charged before using, and how often the dewar cap was opened during freezing. Further comparison is also needed for the calibration, understanding this further with different conditions in the dewar can affect cooling rate that is measured while freezing.

3.7 Freeing Live Samples

Once we completed the 3 trials with HBSS, we froze samples with zebrafish sperm samples. Due to COVID-19 and spawning season for Easter Oysters being so far off, we were not able to obtain oyster sperm samples to freeze. However, the results for the zebrafish sperm, for many reasons, was inconclusive.

We then chose a more reliable cell type to freeze: the single-cell microalgae (*Tetraselmis chuii*). These algae were chosen because of the availability in our laboratory, and because it is used as a food supply in oyster hatcheries. Twelve 0.5-ml straws were filled with algae 3.0 x 10^6 cell concentration, DMSO (dimethylsulfoxide) 10% solution and algae food solution. The DMSO served as a cryoprotectant for the cells. The algae food solution was included so the algae will have a food source to maintain them after thawing.

Table 2: Calibration Results. For HBSS the temperature average of each height is shown.

Height (mm)	HBSS (°C)	Algae (°C)
10	-32.5667	-30.92
20	-58.266	-57.3
30	-84.45	-84.43
40	-107.303	-110.26
50	-110.26	-131.28
60	-126.357	-147.04
70	-141.383	-158.58
80	-153.123	-166.54
90	-168.17	-171.8

To evaluate the capability of the APCD, six 0.5-ml straws with algae were frozen in a controlled rate freezer (Ice Cub 14M, Sy-Lab CryoBiology, Austria) and the 6 other samples were frozen with the APCD (Table 2). Algae has a freezing rate of -40°C/min and based on our preliminary results with the HBSS solution, the height closest to -40°C/min was the 50-mm extrusion. The Ice Cube was set to freeze at -40°C/min. Using the APCD, we froze the samples at 50 mm, and obtained a cooling rate of-49.8C/min. Although almost 10°C higher than anticipated, (this could be due to the dewar being slightly too cold). When comparing the calibration of the HBSS and the algae, the temperature at each height before freezing was -3°C colder than that of the HBSS at 50 mm, increasing the rate of freezing. Even so, none of the samples were viable after thawing, indicating that more work needs to be done.

5 Conclusions and Future Directions

5.1 Future Directions

Due to the lack of availability of Eastern oyster sperm, freezing with live material is still an objective for research, although it can be expected that producing the specific cooling rate that has been successful with other freezers will yield the same results with this device. Another objective to continue further development of this device is to make it portable with a battery pack. This will allow the user to work in the field and freeze samples anywhere with just the device and a laptop. Further understanding of the temperature profiles of the dewar is needed, in understanding how it is charged and how environmental conditions can affect the dewar. An additional objective to make the device more universal, including further development to work with 0.25-ml straws as well. For this specific design and temperature curve, we only evaluated the device with 0.5-ml straws.

5.2 Conclusions

Although we were not able to freeze live samples with Eastern oysters, the device was able to reliably produce a cooling rate profile within the dewar that is known to be effective with aquatic species (4^0C- 40^0C) and was a substantial improvement on the previous Cajun Ejector for freezing with a portable shipping dewar. Further improvement of this device could allow for preservation of oysters and other species. Oysters are critical to Louisiana’s food, culture, and seafood industry, and preserving them is one step to ensuring that when the next season rolls around, there are plenty of oysters to go around as well.

Acknowledgements

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Presentations supported by this funding:

Byrd, V., Shamkhalichenar, H., and Tiersch, T., Evaluation of an automated 3-D printed sperm cryopreservation device for use with Eastern Oysters (*Crassostrea virginica).* Annual conference of American Fisheries Association Louisiana Chapter. April 2020. Virtual Conference.

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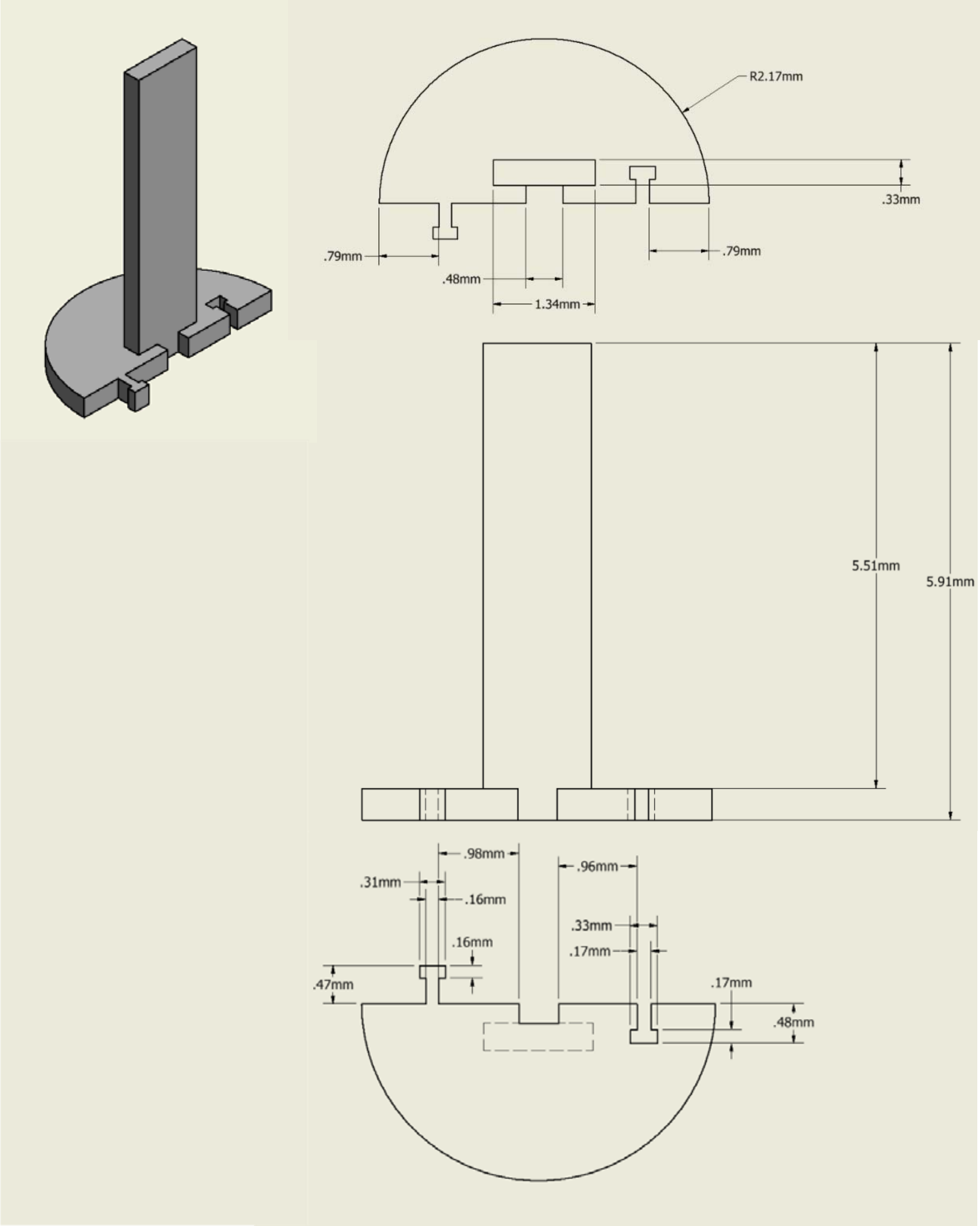
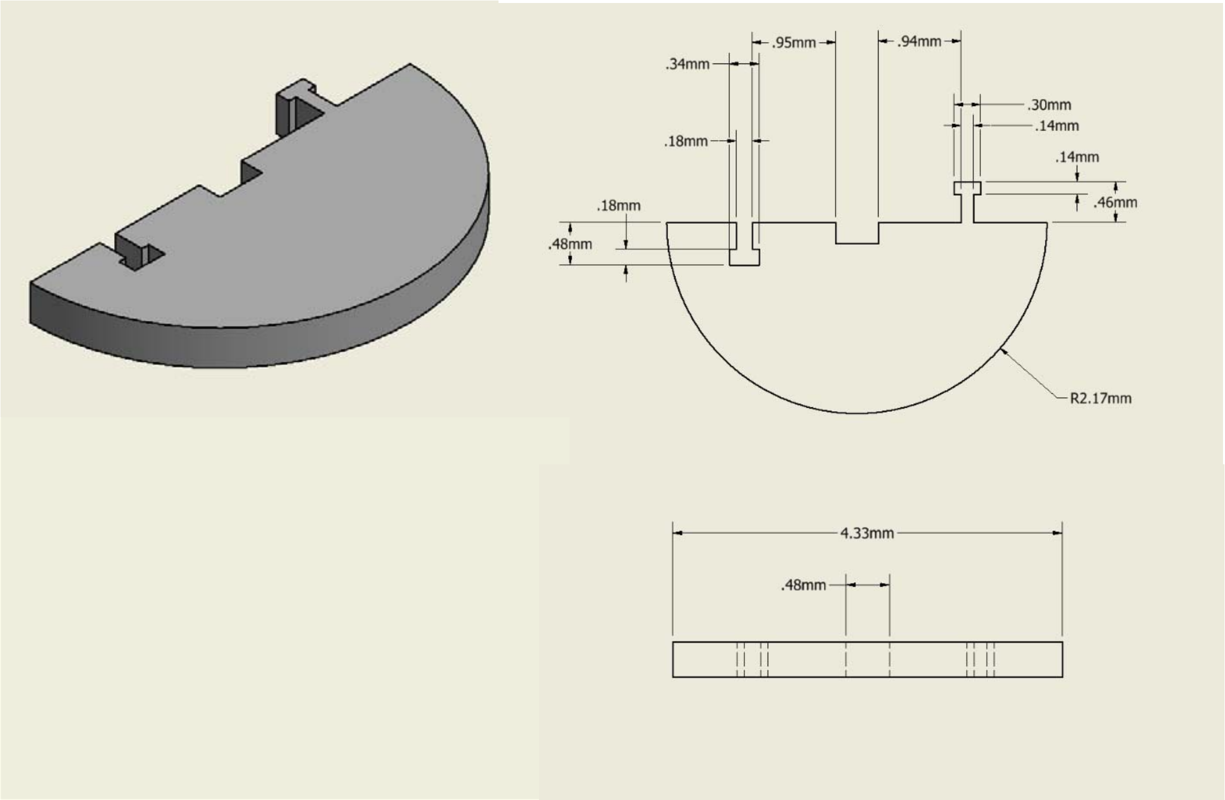
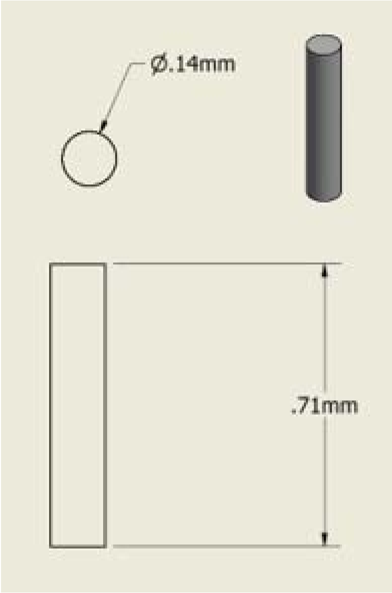
Supplemental Figures



Supplemental Figure 1: *Linear Actuator Holder 1*



Supplemental Figure 2*: Linear actuator holder 2*



Supplemental Figure 3: *Pin that secures linear actuator to adaptor.*