Louisiana Sea Grant Undergraduate Research Opportunity Program (UROP) Final Report

Elise Harmon (Biological Engineering)

Faculty Advisor: Todd Monroe, PhD

Project Co-advisor: Yue Liu, PhD

January 31, 2020

This report is formatted as a manuscript draft for submission to the peer-reviewed journal *Theriogenology*

Development of a Standardized Artificial Inseminator for Endangered Live-Bearing Fishes

Elise R. Harmon,[ab] Yue Liu,[ab] Grace H. Nyugen,[a] Val Browning,[a] Kallie Kilchrist, Terrence R. Tiersch,[b] , and [a] William Todd Monroe[a]

[a]Department of Biological and Agricultural Engineering, Louisiana State University, Baton Rouge, LA, USA
[b]Aquatic Germplasm and Genetic Resources Center, School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, La, USA

Corresponding author:

W. Todd Monroe, PhD

163 E. B. Doran Building, Department of Biological and Agricultural Engineering, Louisiana State University, Baton Rouge, LA 70803, USA
Email: tmonroe@lsu.edu

Abstract

Small-bodied live-bearing fish, also known as viviparous fish, are important to the overall diversity of fish species along with many areas of biological research. As endangered species, these fish are currently among the most at-risk groups in the world. It is of paramount importance to preserve the genomes of these endangered species in the case that their diversity is needed in the future. Sperm cryopreservation and captive breeding are two important methods to preserve genetic diversity of imperiled animals. To achieve these conservation approaches, artificial insemination is a core technology. The current method for artificial insemination of female viviparous fish is mouth-pipetting. This technique can be done by experienced researchers who are trained extensively, but the results can be unpredictable from an unexperienced user. This method lacks a standardized control of volume and pressure of the sperm sample injected into the female, because it is highly dependent on the air pressure exerted from mouths of individual operators. To address the low reliability issue of mouth-pipetting, the goal of this research is to design, develop, and test a device that controls volume and flow rate of sperm through mechanical and electrical components accurately and with low variation. A handheld device was fabricated with electronic components such as a linear actuator and an Arduino microcontroller that displace small volumes of liquids consistently. The device also contains mechanical components that facilitate fluid flow, including a capillary adapter, air displacement tube, metal rod, and O-ring. This system controls fluid flow through air displacement in a 3D-printed tube. The pressure produced by the fluid flow from the electronically controlled device showed less than 1% variation, which is significantly smaller than the variation seen from mouth-pipetting evaluation.

1 Introduction

Small-bodied live-bearing fish, such as Goodeidae and Poeciliidae, are among the most at-risk groups in the world (Huang et al., 2004a; Liu et al., 2019). Preservation of these fish is important, not only for the conservation of endangered species, but also for areas of biological research. For more than 70 years, platyfishes and swordtails of genus *Xiphophorus* have been used as research models to study genetic factors of carcinogenesis (Huang et al., 2004b). Interspecies hybrids between *Xiphophorus maculatus* and *Xiphophorus helleri* develop melanomas which can be investigated further (Water and Kazianis, 2001). These years of research with 25 species of has led to 60 pedigreed lines that are held in the *Xiphophorus* Genetic Stock Center (XGSC, xxx.Xiphophorus.org) (Liu et al., 2019). These lines are sometimes maintained as live animals or stored through sperm cryopreservation. Because of their status as endangered, it is important to preserve the genomes of *Xiphophorus* and other viviparous fish in case their diversity is needed in the future (Walter and Kazianis, 2001).

Live-bearing fish in research laboratories or the XGSC often have behavioral barriers to mating, so young are often produced through artificial insemination (Cuevas-Uribe et al., 2011; Yang et al., 2007). The current method for artificial insemination of female viviparous fish is called mouth pipetting. This method uses a tool that consists of a rubber tubing held in the experimenter's mouth at one end to control the flow of samples with breathing pressure, and a micropipette at the other end to deliver sperm into the female reproductive tract (Clark, 1950). This method is unpredictable, as it lacks a standardized volume and flow rate of the sperm sample because it relies on the user's exertion of air pressure which varies from user to user. This unstandardized method makes it almost impossible to accurately compare results due to user error. If a user does not exert enough air pressure, there may be no offspring produced, but if a user exerts too much air pressure, the fish may be harmed. Mouth pipetting also increases the risk of contamination to personnel.

The goal of this project was to develop a standardized inseminator device to assist artificial insemination of small-bodied live-bearing fish. The objectives of this project were to: 1) design and fabricate inseminator prototype devices, 2) develop standardized *in vitro* and *in vivo* methods to evaluate physical and biological performance of conventional and proposed inseminators, and 3) test physical and biological performance of the most promising prototypes *in vitro* and *in vivo*. This device would house a linear actuator that would drive fluid motion accurately. The entire device would be small enough to fit in the user's hand to ease the artificial insemination process.

2 Materials and Methods

2.1 Evaluation of Mouth-pipetting

To design a mechanism that standardizes the process of mouth pipetting, this process was closely examined; particularly the pressure generated through an operator's mouth pipetting technique. With a digital manometer (Omega Engineering, Newark, CT), the peak pressures generated by trained and untrained mouth-pipettors were measured (Figure 1). Each operator was asked to gather

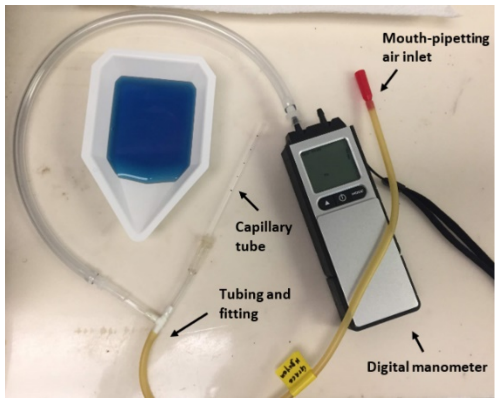

Figure 1. Measurement of pressure generated by mouth-pipetting.

3 µL, 5 µL, and 10 µL samples of water in the glass capillary and then exert the sample using a mouth-pipetting technique. The data from ten users each conducting ten repetitions was collected and analyzed to determine design parameters of the standardized device.

2.2 Evaluation and Calibration of Linear Actuator

The linear actuator chosen for this design is the Goteck GS-1502 Micro Servo because of its small size and its compatibility with Arduino. This linear actuator uses a small motor to rotate a screw which results in linear motion. The GS-1502 was introduced into a circuit with an Arduino microcontroller and two buttons to control its movement through coding (Figure 2A-B).

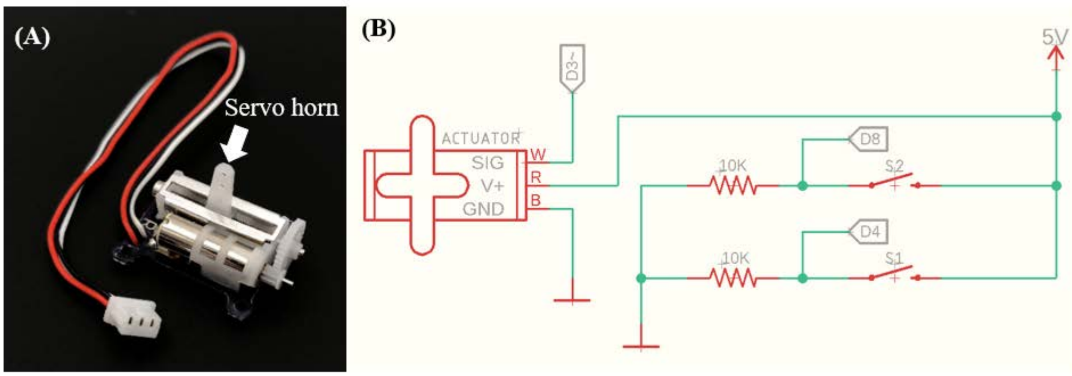

Figure 2. GS1502 Micro Linear Servo from Goteck. (A) The GS Micro Linear Servo with right-hand orientation and servo horn placement (B) Schematic diagram of the button-controlled circuit.

The linear actuator was programmed to move to a certain percentage of the maximum stroke with the push of a button. This distance corresponds to a certain length of travel, which is necessary information for predicting the volume gathered by the device. To gather a calibration curve to characterize the linear actuator's movement, the distance was measured with different coding inputs. Firstly, a measurement adaptor was fabricated with three-dimensional (3-D printing) using ae Anycubic Photon 3-D printer and associated Slicer software. The adaptor fitted over the servo horn to ensure the accuracy of measurement. This part had flat edges so that the measurements could be taken perpendicular to the surface (Figure 3).

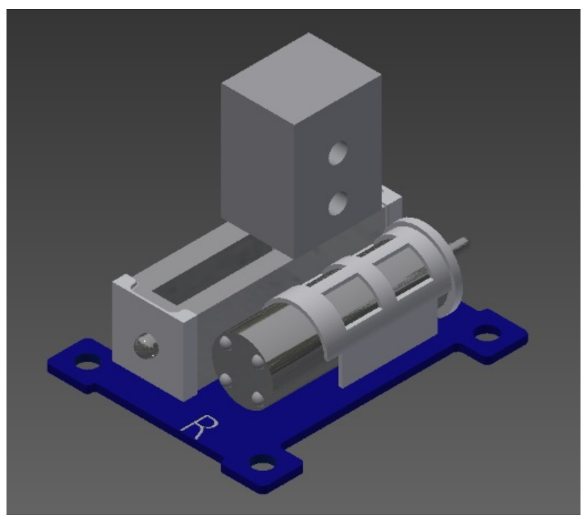

Figure 3. 3-D rendering of the GS-1502 servo with the 3D-printed measurement adaptor.

The actuator was programmed to move to 10% of its maximum distance when one button was pushed and then to 20% of its maximum stroke when the other button was pushed. The distance from the flat edge of the measurement adaptor to the surface underneath was measured after pressing each button (Figure 4). Because the actuator moved a net 10% of its maximum stroke, the difference between the locations of each percentage of maximum stroke describes the total movement. This data was taken to gain 15 repetitions for 10%, 20%, 30%, 40%, and so on, up to 80%.

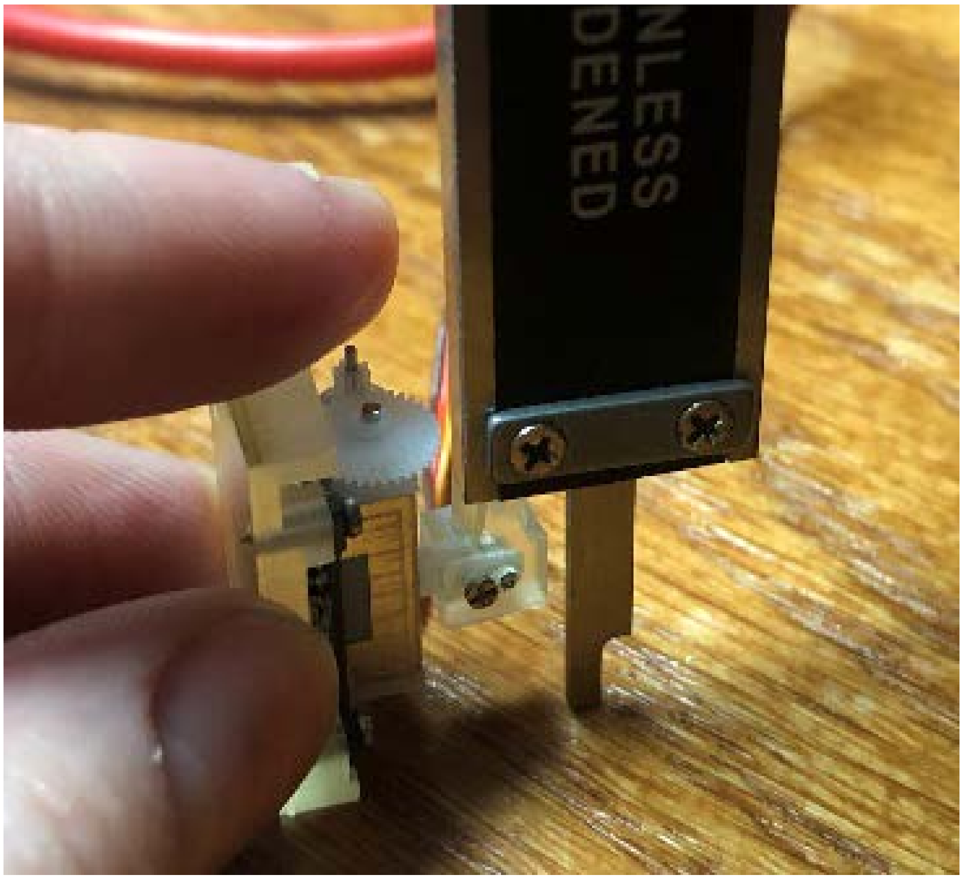

Figure 4. Demonstration of caliper measurement for determining total distance traveled.

2.3 Fabrication

All components for 3D printing were designed using Autodesk Inventor and printed with the Anycubic Photon Slicer. Once the designs were complete, the parts were saved as an STL file and transferred to a USB drive. The STL files were then prepared for the printer with the Anycubic Photon Slicer's computer software where its scaling, orientation, and print settings were manipulated to optimize the print (Figure 5). The part was saved as a photon file and transferred to a USB drive that was inserted into the printer. The desired file was selected and, after resin was poured into the vat, the printing started. Once the objects were finished printing, they were carefully removed from the platform and rinsed thoroughly with 99.5% isopropyl alcohol. This solvent removes any uncured resin to ensure that only the designed features appear. After thorough rinsing, the parts then cured further under a UV lamp for 5 minutes.

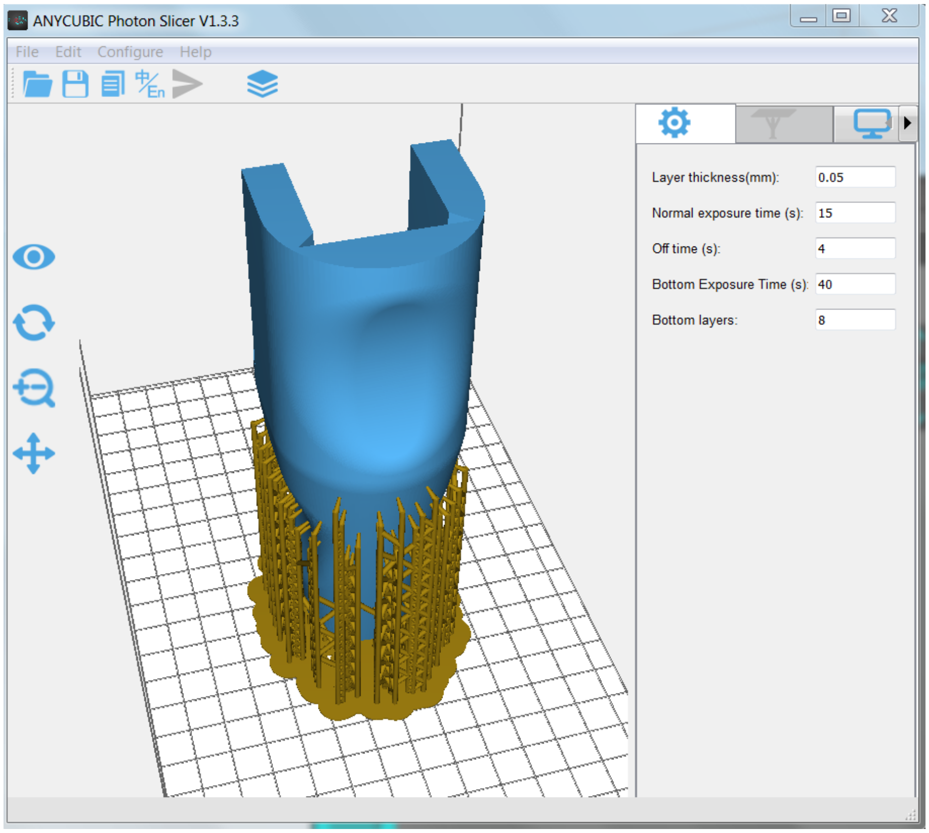

Figure 5. Print settings and preview of SLA-printed 3D model.

2.4 Design of Artificial Inseminator

The linear actuator acted as the piston of a micropipette, where a certain volume of air displacement inside the device drew fluid in and pushes fluid out. Further testing as to the device's capabilities would require design, fabrication, and assembly of parts to achieve this goal. A base that stabilized the linear actuator held the component in place while the servo horn was moving. An adapter piece fitted over the servo horn and connect to a metal rod to cause the air displacement. The air displacement took place inside a tube holding an O-ring to prevent air leakage from any gaps. The device should also accommodate for the usage of glass capillaries by including the silicone adapter tip commonly used in mouth-pipettes. A housing unit would be required to stabilize the individual components and aid in an accurate volume of air displacement.

2.4.1 Base piece

For the linear actuator base component, design considerations included the dimensions of the linear actuator, the 3D printer's resolution, and the overall size of the device. The GS-1502 actuator has four holes on each corner of the printer circuit board which could be used to fit small screws. The actuator also has an IC chip connected to the bottom of the component with a

1.5-mm depth. The overall handheld device should be as small as possible to provide functionality, yet large enough to be mechanically sound.

2.4.2 Metal Rod Adapter

The servo horn adapter piece was designed to fit over servo horn and hold a metal rod. The servo horn on the GS-1502 had three holes, each with a 1.3-mm diameter. The part was designed to fit over the 3-mm x 1.5-mm horn and hold a metal rod with a 1.4 mm diameter. Since the servo horn is not centered on the printed circuit board, the design would also have to shift the placement of the hole to be centered relative to the linear actuator. Several attempts to print this part were made to determine any necessary adjustments to the part.

2.4.3 Air Displacement Tube and O-ring Holder

The air displacement tube needs to accommodate the 1.4-mm metal rod, the silicone adapter tip, and the O-ring. It also needs to fit the silicone adapter tip so that a glass capillary could be used with the device. The tube would have to be long enough so that air displacement could occur. The inner diameter of the tube was designed to be greater than the 1.4-mm metal rod by about 100 microns because of the 3D printer's resolution. This would, theoretically, result in the inner diameter of the tube fitting the metal rod perfectly. One end of the tube also had to fit an O-ring with a 1-mm inner diameter, a 5.5-mm outer diameter, and a 2-mm thickness. It was determined that to keep the O-ring stable, another part would have to restain the O-ring in the tube. This part was the O-ring holder, which fit in the end of the tube and left a 2-mm gap for the thickness of the O-ring. This part would snap into the tube and hold the O-ring in place to prevent air leakage.

2.4.4 Case Unit

The case unit was designed in conjunction with all other components to design a small case that comfortably fit in the hand while maintaining functionality. The base piece would slide into the back of the case, so both parts would need triangular extrusions to fit snugly together. The air displacement tube was designed to have a larger outer diameter towards the tip of the case so that it would be stabilized in the case. The case unit would have a matching cutout. The other side would have a square extrusion which would fit the O-ring holder.

2.5 Evaluation of the Relationship of Linear Actuator Movement and Volume

With the assembled device, the volume of sample with a certain movement distance was measured to understand the relationship between volume obtained and the distance traveled by the actuator. The button-controlled circuit was used to move the actuator to a percentage of its maximum stroke, where one button moved the servo horn to 10% stroke and the other to 20%. When the servo horn finished moving to 20%, the capillary was placed in a dyed-blue sample of water. The other button was pushed, moving the actuator to 10% of its maximum stroke and drawing in a volume of water. To measure this volume, a picture was taken and opened in Fiji, an image processing software that facilitates scientific analysis. The length of the capillary from the end to the calibrated mark is 10 µL, so a line can be drawn from these two points to set the scale of the image (Figure 6). Once the scale is set, another line drawn the length of the blue liquid will have a length that is equal to the volume of water in the capillary. Because of the small volumes obtained in the capillary, this image analysis method was used to prevent the samples from evaporating and thus decreasing the accuracy of results. Volumes were gathered using 10%, 20%, 30%, and so on, up to 80% movement of the linear actuator, ten repetitions of each percentage.

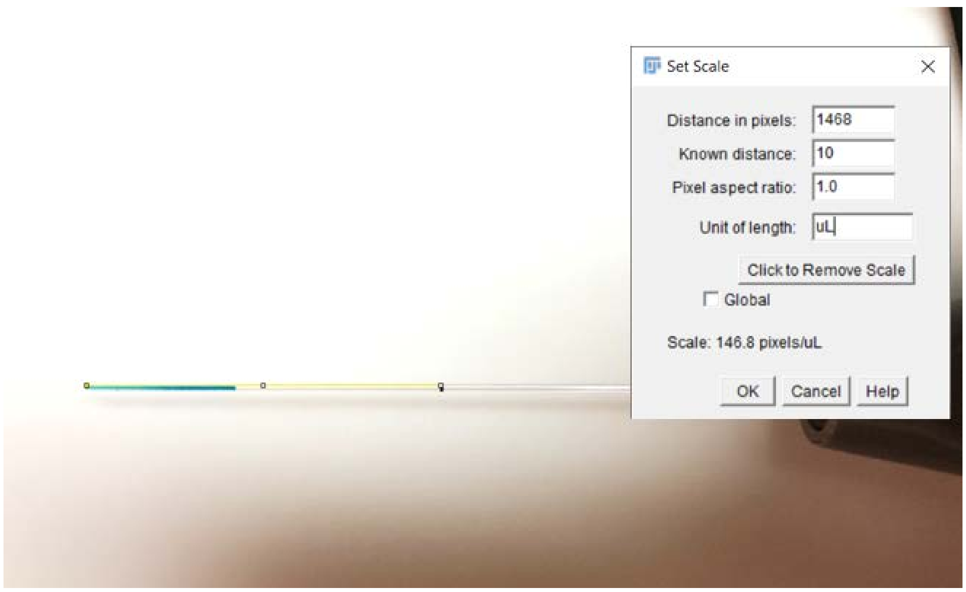

Figure 6. Process of setting the scale in Fiji software to measure the volume in the capillary.

2.6 Dynamic Pressure Generated by the Assembled Inseminator Device

To evaluate the dynamic pressures generated during linear actuator movement, a pressure sensor (Omega Engineering) was used in conjunction with the assembled inseminator device, an Arduino microcontroller, a computer, tubing junctions, and related electronic components (Figure 7). Firstly, the pressure sensor was calibrated using the digital manometer. The curve obtained by comparing the pressure readings from the digital manometer and the voltage readings obtained from the pressure sensor (Figure 8) can be used to calculate pressure readings based on the voltage output from the Omega Engineering pressure sensor. The calibrated sensor was then connected to the assembled inseminator device which, based on the calibration curve of linear actuator movement and volume obtained, moved to obtain a specific volume of air. The volume was then expelled by the actuator movement. Data points were gathered every millisecond during testing.

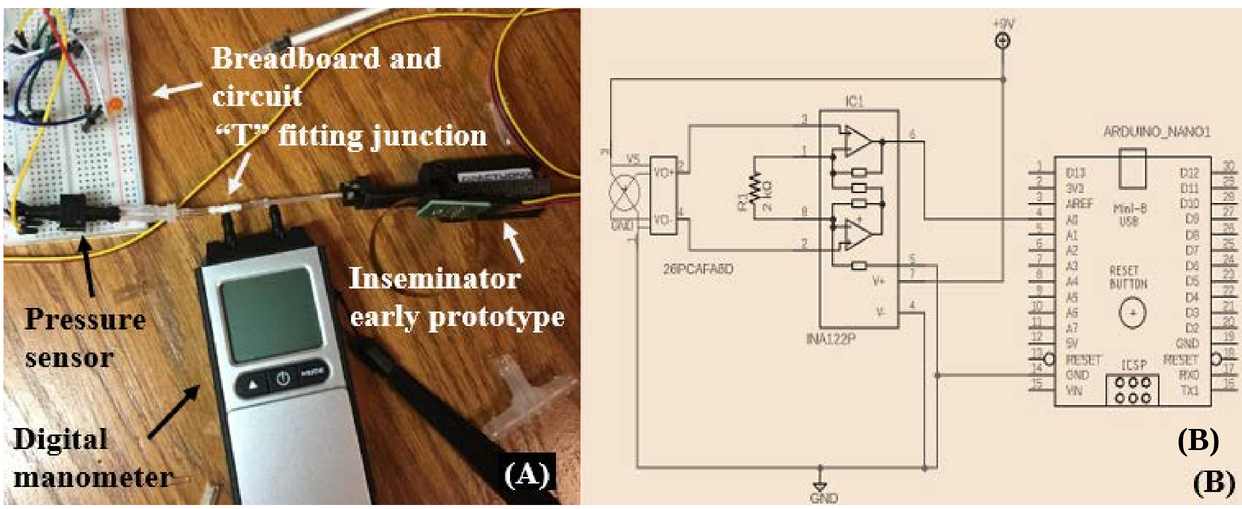

Figure 7. Measurement of air pressures for sample transfer by use of a pressure sensor (A) Omega Engineering pressure sensor and the digital manometer were connected by a "T" fitting junction to calibrate the pressure sensor. (B) The associated circuit for pressure analysis.

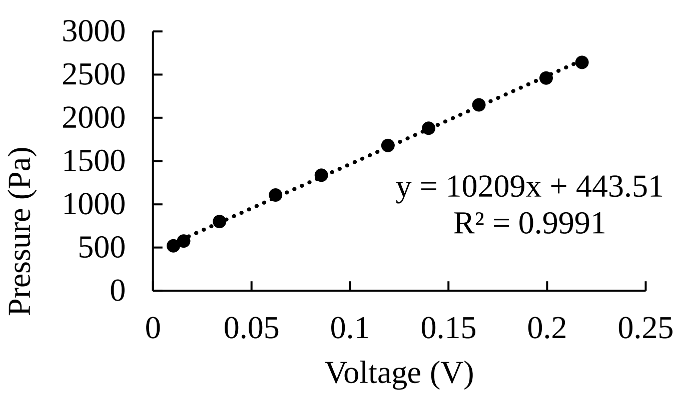

Figure 8. Calibration of the pressure sensor with a digital manometer.

3 Results and Discussion

3.1 Evaluation of Mouth-pipetting

The average pressure exerted during mouth pipetting techniques of trained and untrained individuals from each trial shows great variation among individuals in both groups (Figure 9). This variation is not only present from user to user, but there is also a great amount of variation among different trials by the same user. The results (Table 1) show the coefficient of variation of each individual operator's technique ranging from 18% to 48% for 3 µL samples, 20% to 49% for 5 µL samples, and 18% to 49% for 10 µL samples. Significant differences were found among 8 operators in dispensing pressures for 3 µL (P = 0.0009), 5 µL (P = 0.0002), and 10 µL dispensing (P = 0.0071).

A significant level of variation was apparent after the analysis of both experienced and unexperienced mouth pipettors, proving the need for a standardized insemination device.

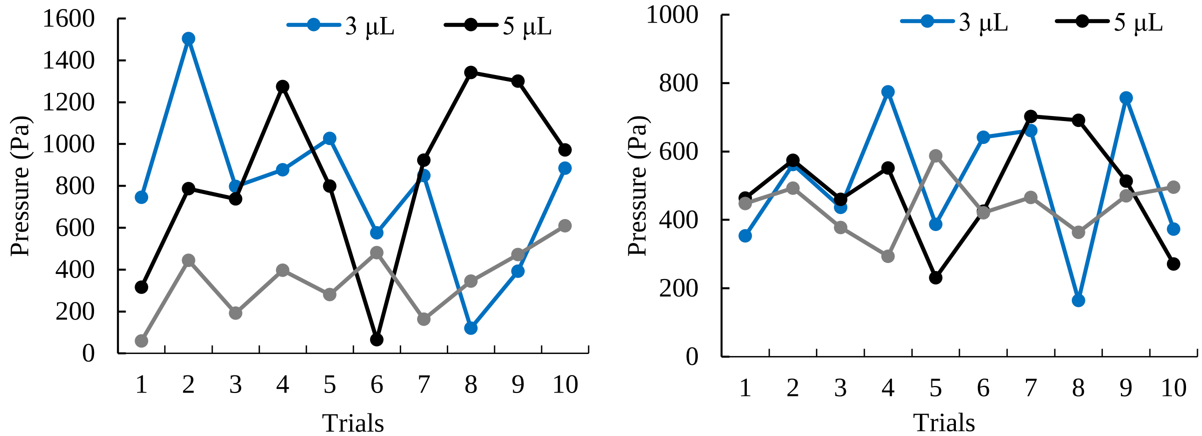

Figure 9. Average pressure exerted during mouth-pipetting among trained mouth pipettors (left) and untrained mouth pipettors (right).

Table 1. Average pressure exerted with each sample volume by different operators.

Operator No.	Pressure (Pa) (mean ± SD)			Coefficient of variation (%)		
	3 µl	5 µl	10 µl	3 µl	5 µl	10 µl
1	666 ± 120	7010 ± 175	420 ± 119	18	25	28
2	766 ± 182	666 ± 157	426 ± 89	24	24	21
3	931 ± 250	802 ± 182	507 ± 99	27	22	20
4	579 ± 214	675 ± 164	373 ± 67	37	24	18
5	716 ± 322	876 ± 266	458 ± 168	45	30	37
6	939 ± 238	1131 ± 454	591 ± 235	25	40	40
7	777 ± 371	852 ± 417	344 ± 170	48	49	49
8	511 ± 199	489 ± 156	442 ± 82	39	32	19

3.2 Evaluation and Calibration of Linear Actuator

3.2.1 Relationship between Linear Actuator Movement Distance and Coding Input

The relationship between the percentage input in the Arduino coding and the distance traveled by the actuator was determined to be linear (Figure 10). The results show that the actuator movement is consistent with each trial and the actuator moves 4.295 times the input percentage. Table 2 shows the average movement distance in millimeters with each percentage movement. The movement distance increases linearly with an increase in percentage input.

The linear actuator shows little variation in its movement when coded to move repeatedly. Because of this result, the linear actuator was chosen as the main electronic component to drive fluid motion.

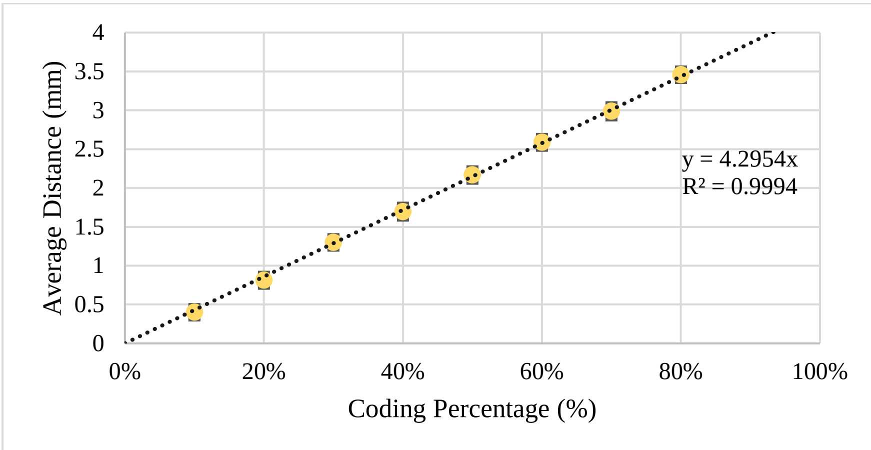

Figure 10. Relationship between percentage of maximum stroke input in Arduino code and movement distance of the actuator.

Table 2. Evaluation of linear actuator accuracy with movement percentage input.

Movement Percentage Input (%)	Average Movement Distance (mm)
10	0.401 ± 0.044
20	0.812 ± 0.059
30	1.299 ± 0.047
40	1.695 ± 0.082
50	2.167 ± 0.069
60	2.587 ± 0.055
70	2.987 ± 0.088
80	3.458 ± 0.047

3.3 Fabrication of Artificial Inseminator Device

The final design of the device includes both 3-D printed components to aid the assembly and function of the device, the linear actuator, and mechanical components. The 3-D printed components include the linear actuator base, metal rod adapter, O-ring holder, air displacement tube, and case unit which holds each piece in place. Mechanical components in this device are the screws, the metal rod, and the silicone adapter tip (Figure 11).

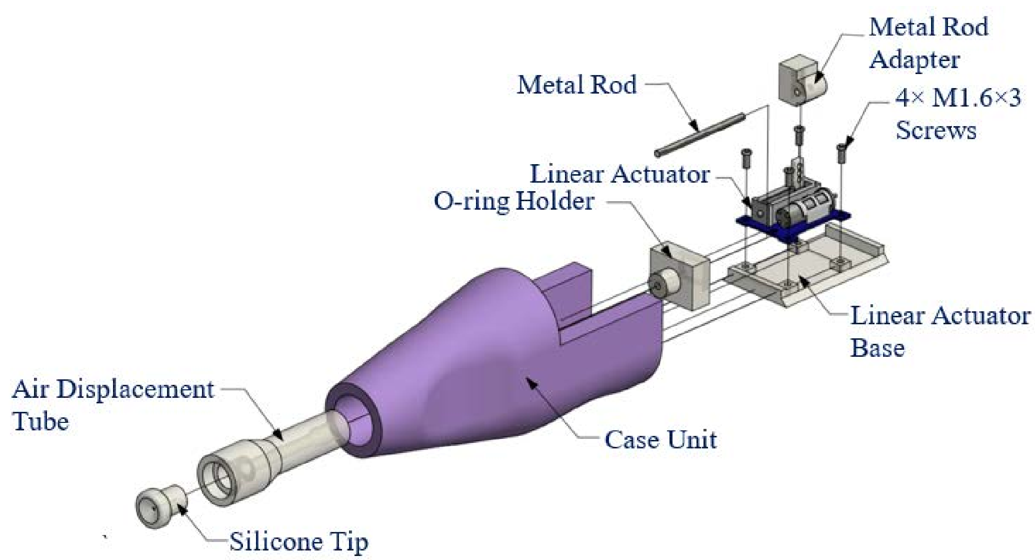

Figure 11. Assembled handheld device for artificial insemination.

3.3.1 Linear Actuator Base

The linear actuator base has triangular extrusions that slide into the case unit to hold it in place. There are four holes, each 1.4 mm in diameter, which match the dimensions of the actuator and fit 1.6 mm screws. Each hole is surrounded by a square extrusion with a 1.5-mm height to accommodate for the IC chip. Lastly, an 8.1×15-mm rectangular extrusion prevents the actuator's gears from losing torque due to friction (Figure 12).

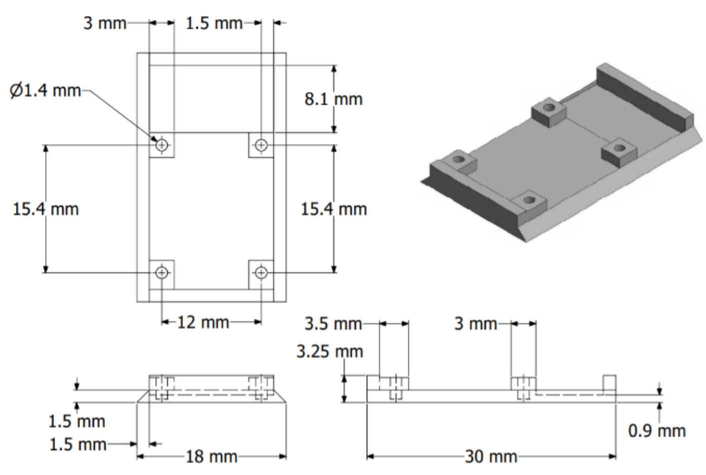

Figure 12. Linear actuator base dimensions for the handheld device design.

3.3.2 Metal Rod Adapter

The metal rod adapter has a rectangular extrusion in the middle of the part to fit the servo horn and can be secured by M1.4x2.6 screws. Positioned on the edge of the part is a hole to fit the 1.4-mm metal rod (Figure 13). This metal rod was secured in the part with steel bond epoxy in the hole. The metal rod is offset 2.25 mm from the servo adapter, a necessary adjustment that was made after several prototypes (Figure 14).

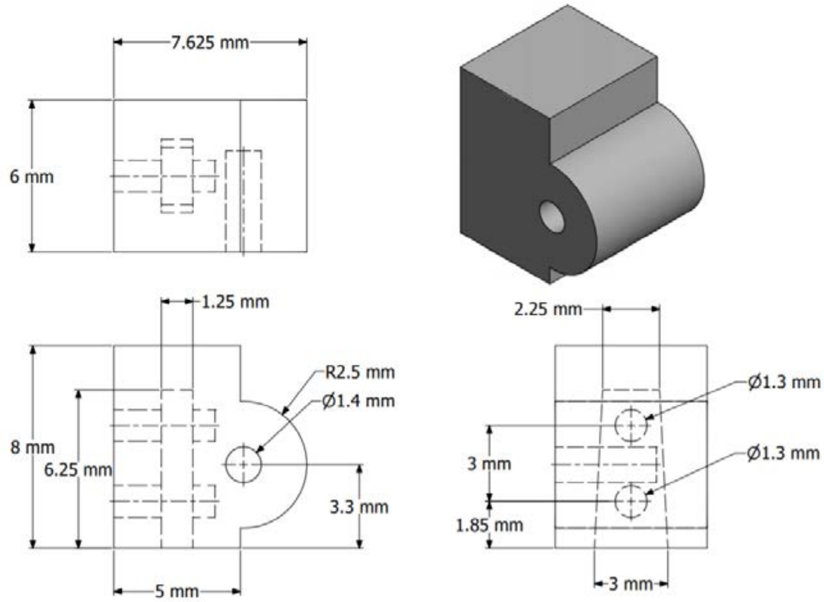

Figure 13. Technical drawing of the metal rod adapter fabricated for the insemination device.

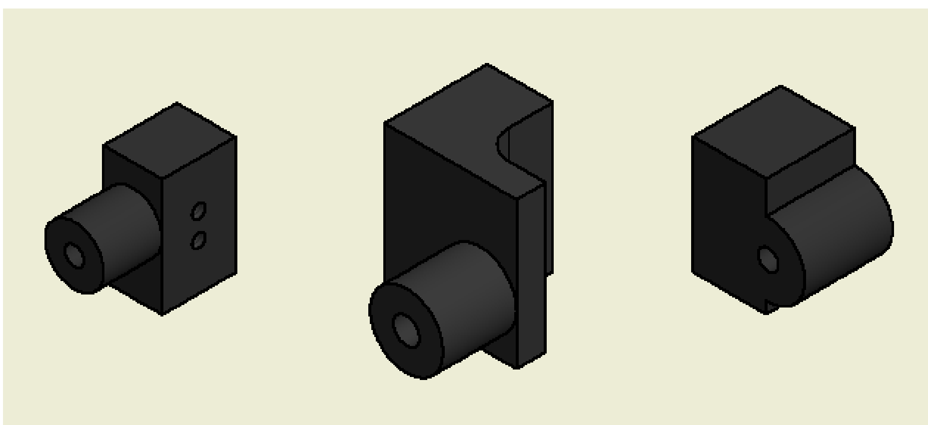

Figure 14. Evolution of the metal rod adapter, where the leftmost model is the first design and the rightmost model is the final design.

3.3.3 Air Displacement Tube and O-ring Holder

The air displacement tubing (Figure 15) had a maximum width of 11 mm and an inner diameter of 1.4 mm. The shape of the tube allowed for a matching extrusion to be made to the case unit so it could slide in easily for testing. The O-ring holder (Figure 16) would snap into the tube from the other end of the case to secure the O-ring in place and hold the tube together.

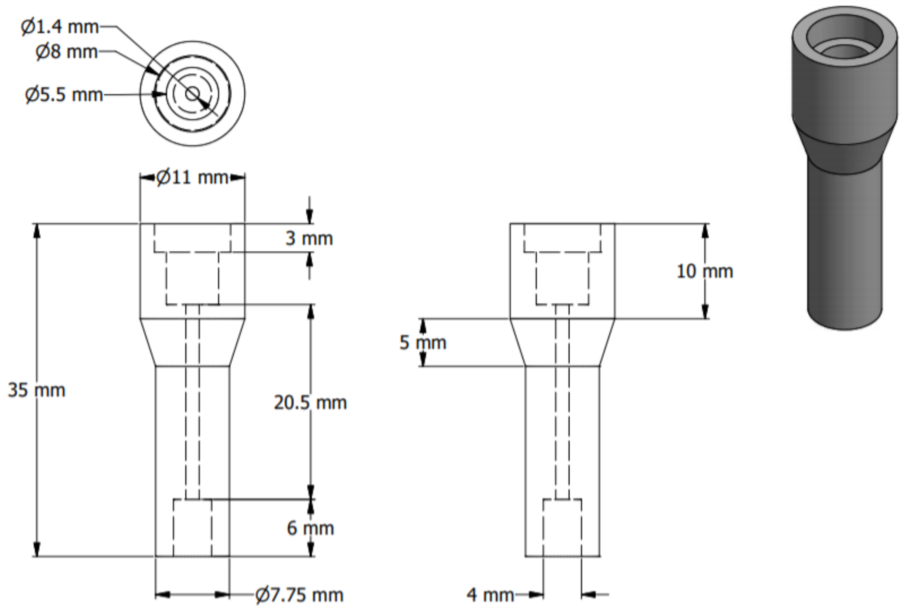

Figure 15. Technical drawings of the air displacement tubing for sample transfer.

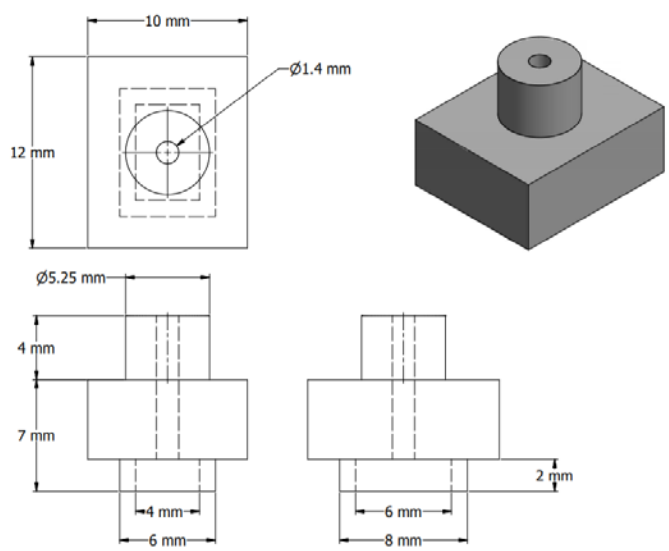

Figure 16. Technical drawings of the O-ring holder which stabilizes the O-ring for proper sealing and holds the air displacement tubing in place.

3.3.4 Case Unit

Several attempts were made to print the case unit which houses each individual component. The final case design held the components together while including some ergonomic features like finger grips (Figure 17). Once the air displacement tube was calibrated for the sample volume, it was combined with the case design (Figure 18).

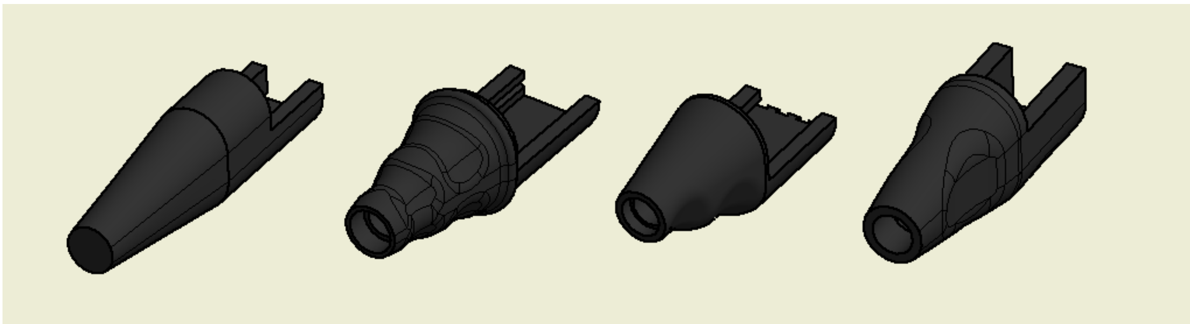

Figure 17. Multiple versions of the case unit, where the first functional version is the leftmost model and the final prototype is the rightmost.

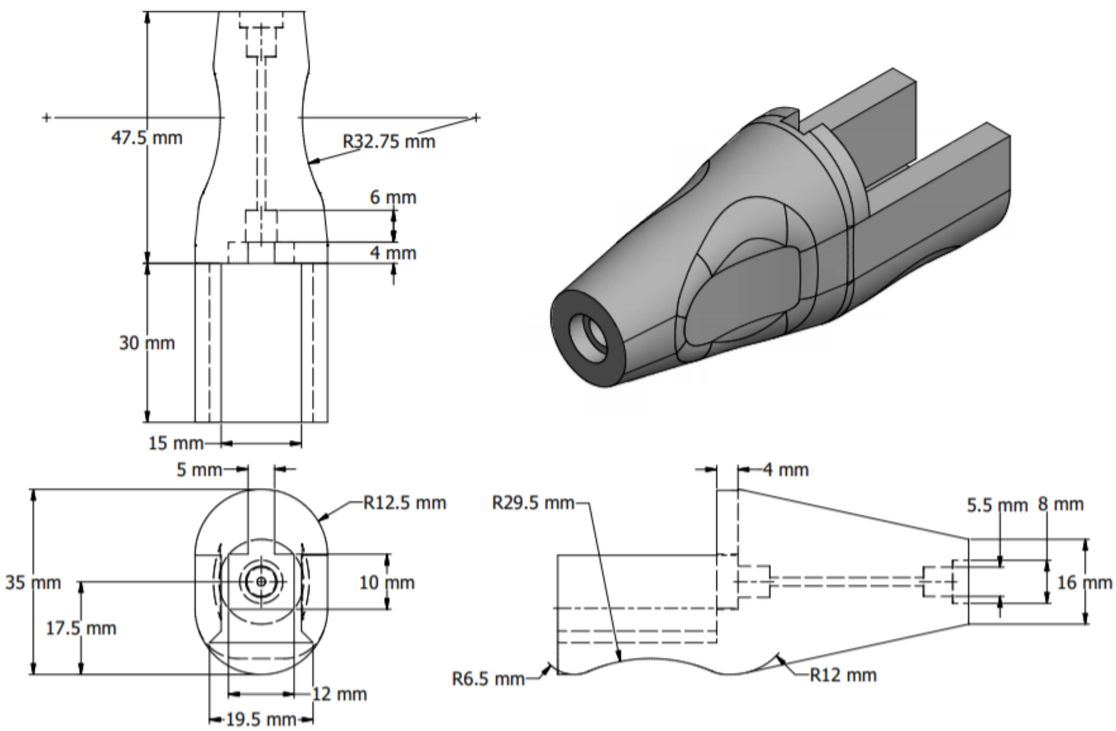

Figure 18. Technical drawings of the final case unit design. Initial case unit designs were combined with the air displacement tubing.

3.4 Linear Actuator Movement and Volume

A linear relationship between the linear actuator movement in percentage of maximum stroke and the volume obtained in the capillary was observed (Figure 19). This curve yielded an equation that estimates the volume of sample depending on the actuator's position. The movement of the linear actuator collected samples of water with low variation, less than 1%.

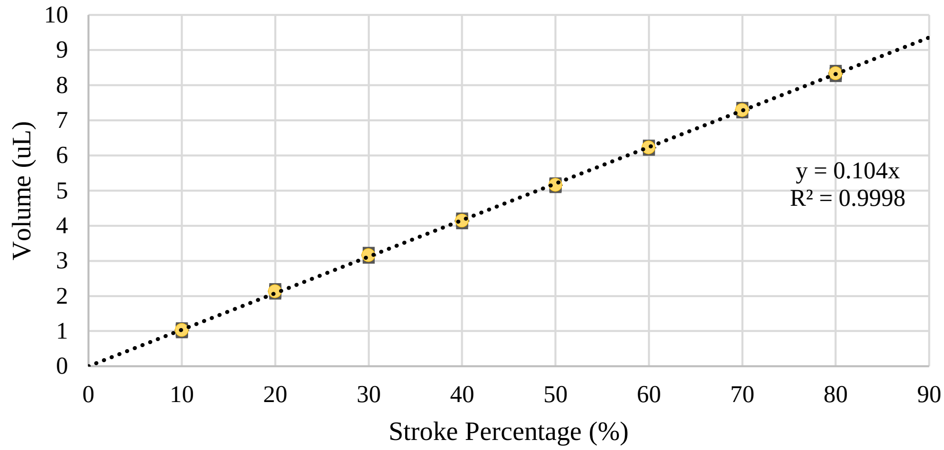

Figure 19. Calibration curve of the relationship between the volume of sample obtained in the capillary and the coding percentage of the maximum servo stroke.

3.5 Pressure Exerted by Linear Actuator Movement

The pressure generator by the inseminator prototype was tested with the calibrated Omega pressure sensor. The results (Figure 20) show that the prototype was able to deliver samples with consistent pressure and minimum variation. For example, for repetitive delivery of 3 µL of fluid, the dispensing pressures were constant at 483 ± 3 Pa with a coefficient of variation of 0.6%. Figure 20 shows the increase in pressure with higher speed of servo movement while there is a lower pressure with lesser speed.

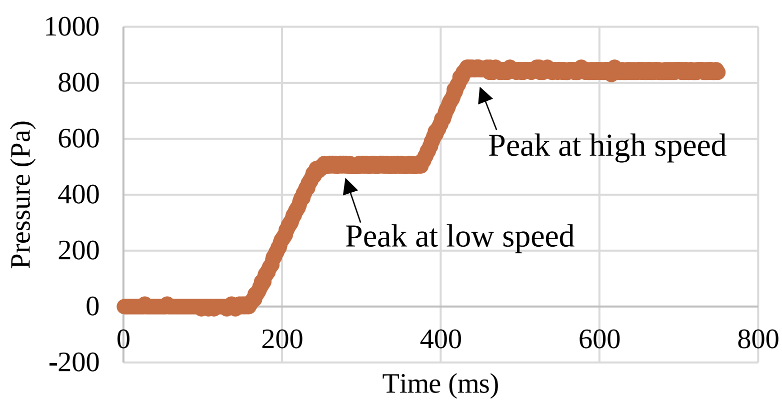

Figure 20. Dynamic pressure exerted by linear actuator movement

4.0 In-vivo insemination

The feasibility of insemination of female live-bearing fish was tested by dispensing of sperm extender solution Hank's Balanced Salt Solution (HBSS) at 300 mOsmol/kg into female fish of endangered species Redtail Splitfin (*Xenotoca eiseni*). Ten females were used of three different volumes (1, 3, and 5 µl). The body weight, standard body length, and mortality at one-week post sample transfer were evaluated. The results showed that sperm extenders were dispensed into the female reproductive tract with volumes tested in the device.

5. Conclusions and Future Directions

As endangered species, viviparous fish are currently among some of the most at-risk groups of species in the world. Artificial insemination is a core technology to achieve conservation of these fish. To supplant the current method for artificial insemination of female viviparous fish, which is mouth-pipetting, we developed a electromechanical device for dispensing small volumes for insemination. This device would enable untrained users to still achieve a standardized control of volume and pressure of the sperm sample injected into the female, because it is not reliant on the air pressure exerted from mouths of individual operators. A handheld device was fabricated with electronic components such as a linear actuator and an Arduino microcontroller that displace small volumes of liquids consistently. The volume and pressure produced by the linear actuator's movement was consistent with 1% variation, less than those measured in a mouth pipetting apparatus from untrained as well as trained individuals. In future work, the inner diameter of the air displacement tube will be optimized for the volume of sample transfer. Additions to the circuit, such as an LCD menu display, will allow for user customization and adapt to user preferences. Further testing *in vivo* is necessary to determine the effectiveness of the device. This technology will play an important role in research involving viviparous fishes.

Acknowledgements

This work was supported by the Louisiana Sea Grant Undergraduate Research Opportunity Program. We thank Perry, S. for valuable input and evaluation of device prototypes.

Publications and presentations supported by this funding:

During the 9 months of funding period, the publications and presentations supported by UROP include one two presentations and one manuscript in preparation:

Harmon, E., Nguyen, G., Liu, Y., Tiersch, R.T., and Monroe, W.T. Development of a standardized inseminator for endangered small-bodied live-bearing fishes. Annual conference of American Fisheries Association Louisiana Chapter. June 2019. Thibodaux, LA.

Harmon, E., Liu, Y., Nguyen, G., Browning, V., Kilchrist, K., Tiersch, T.T., and Monroe, W. T. Development of A Standardized Artificial Inseminator for Endangered Live-bearing Fishes. *In preparation*

Liu, Y., Tiersch, T. R., and Monroe, W. T. Development of a standardized artificial inseminator for endangered live-bearing fishes. World Aquaculture Society Annual Conference, March 2019, New Orleans, LA.

References

Clark, E. (1950). A method for artificial insemination in viviparous fishes. *Science*, *112*, 722-723.

Cuevas-Uribe, R., Yang, H., Daly, J., Savage, M.G., Walter, R.B., Tiersch, T.R., 2011. Production of F1 offspring with vitrified sperm from a live-bearing fish, the Green Swordtail *Xiphophorus hellerii*. Zebrafish 8, 167-179.

Huang, C., Dong, Q., Tiersch, T.R., 2004a. Sperm cryopreservation of a live-bearing fish, the platyfish *Xiphophorus couchianus*. Theriogenology 62, 971-989.

Huang, C., Dong, Q., Walter, R.B., Tiersch, T.R., 2004b. Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization. Cryobiology 48, 295-308.

Liu, Y., Blackburn, H., Taylor, S.S., Tiersch, T.R., 2019. Development of germplasm repositories to assist conservation of endangered fishes: Examples from small-bodied livebearing fishes. Theriogenology 135, 138-151.

Walter, R.B., Kazianis, S., 2001. *Xiphophorus* interspecies hybrids as genetic models of induced neoplasia. ILAR Journal 42, 299-321.

Yang, H., Hazlewood, L., Heater, S.J., Guerrero, P.A., Walter, R.B., Tiersch, T.R., 2007. Production of F_1 interspecies hybrid offspring with cryopreserved sperm from a live-bearing fish, the Swordtail *Xiphophorus helleri*. Biol. Reprod. 76, 401-406.