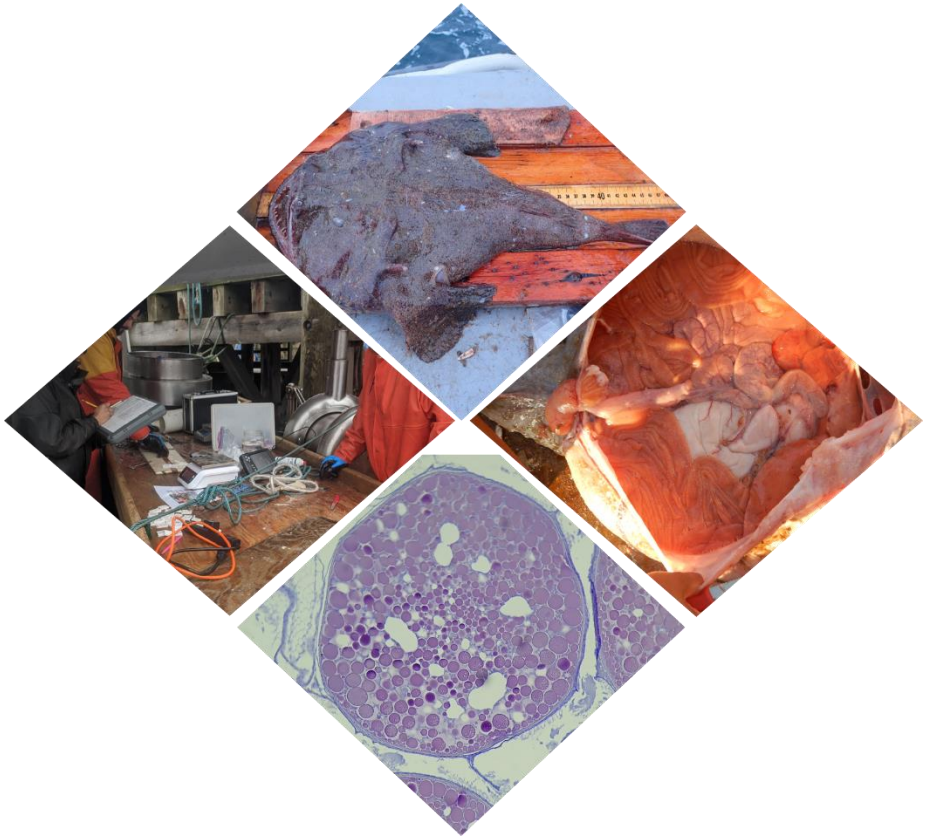
Exploring Non-lethal Techniques for Sex Determination and Evaluation of Maturity Stages of Southern New England Monkfish, *Lophius americanus*

Final Report

Prepared for the 2018
Monkfish Research Set-Aside Program
(Grant # NA18NMF4540331)
March 2022



Submitted By

Luisa Garcia and Farrell Davis
Coonamessett Farm Foundation, Inc

Coonamessett Farm Foundation, Inc
277 Hatchville Road
East Falmouth, MA 02536

508-356-3601 FAX
508-356-3603
contact@cfarm.org

www.cfarm.org

Table of Contents

INTRODUCTION.....3

OBJECTIVES.....4

METHODS.....4
Sampling design.....4

RESULTS.....8

DISCUSSION.....17

CONCLUSIONS AND FUTURE RECOMMENDATIONS.....19

REFERENCES.....19

APPENDIX A.....22
Introduction.....24
Protocol 1: Biometric Sampling.....25
Protocol 2: Ultrasound Technique.....26
Protocol 3: Blood Sampling.....29
Protocol 4: Endoscope Technique.....31
Protocol 5: Macroscopic Inspection.....34
Protocol 6: Histological Sampling.....35

List of Figures

Figure 1. Sampling locations of *L. americanus* collected by research and commercial trips between December 2018 and June 2020 in the SNE area. 5

Figure 2. Examples of the products from the non-lethal and lethal techniques used in all monkfish during this project. **a)** Male in developing phase, **b)** female in ripe phase and **c)** female in actively spawning phase. 7

Figure 3. Percentage of error in sex determination by **a)** non-lethal techniques and **b)** monkfish size. 9

Figure 4. Monthly distributions of **a)** females and **b)** males in the four phases of gonad maturity of monkfish collected in the SNE between December 2018 and June 2020 and monthly changes in the mean gonadosomatic index (GSI) and hepatosomatic index (HSI) for **a)** females and **b)** males. 10

Figure 5. Percentage of error in maturity stage evaluation by **a)** techniques and **b)** maturity stage. 10

Figure 6. Standard curve obtained in each one of the experimental trial **a)** February, **b)** May, **c)** September and **d)** October 16

List of Tables

Table 1. Monkfish collected during the length of the project by different sampling strategies..... 8

Table 2. Macroscopic description of four maturity stages in the reproductive cycle of female and male of monkfish collected in SNE between December 2018 and June 2020 (based on Colmenero et al. 2013 and Johnson and Grier 2017)..... 11

Table 3. Microscopic description of four maturity stages in the reproductive cycle of female of monkfish collected in SNE between December 2018 and June 2020 (based on Colmenero et al. 2013 and Johnson and Grier 2017). Bar = 50 μm..... 13

Table 4. Results for unextracted, undiluted fish plasma 16

EXECUTIVE SUMMARY

This report presents the results of the project entitled "Exploring non-lethal techniques for sex determination and evaluation of maturity stage of Southern New England monkfish, *Lophius americanus*" from the monkfish Research Set Aside (RSA) funding year 2018-2020. This project was executed from December 2018 to June 2020 in Southern New England and Georges Bank fishing grounds. The main project goals were:

1. Establish a method for sex and maturation stage determination in monkfish using an ultrasonic scanner.

2. Establish a method for sex and maturation stage determination in monkfish using a endoscope equipped with 6 light emitting diodes (LEDs).

3. Analyze plasma steroid hormone ratios in monkfish using ELISA techniques.

4. Calculate GSI values for male and female monkfish.

5. Use histological reference slides of gonads to verify assignment of sex and maturation stage by ultrasonic imaging, endoscopic imaging, ELISA, and gross examination, creating a reproductive profile for monkfish.

6. Identify spawning characteristics, such as multiple spawns, skip spawning, etc. as possible.

Monkfish were collected during commercial fishing trips, at the dockside, and research trips. Biometrics, ultrasound and endoscope inspections, blood sampling, macroscopic inspection, including the gonad sample collection, were completed on board for each monkfish. At the lab, the blood samples were used to perform the hormone analysis using two kits, Cayman estradiol and MyBiosource. All histological slides were analyzed for each individual.

A total of 193 monkfish, ranging in size from 33 to 82 cm, were analyzed using ultrasonography, endoscopy, macroscopic and microscopic inspection. Blood samples were taken from sixteen monkfish and a plasma steroid hormone analysis was done for five of these individuals. Comparisons of ultrasound and endoscopic sex determination showed that ultrasound inspection had a greater percentage of error (15%). Due to budget constraints it was necessary to find a low-cost solution which meant the crystal within the probe was of a lower quality and could not produce a detailed image. However, it is expected that this technique could have greater success if the appropriate equipment is purchased. Comparing macroscopic and endoscopic assignment of maturity stages revealed that these two techniques had a similar percentage of error (52% and 48%), indicating that human error was the cause for incorrect assignment. Hormone analysis did not provide the expected results due to a series of difficulties encountered during the project. Due to the inability to buy a -80°C freezer, the plasma samples were not stored properly; in addition, contaminants in the samples and limited range of detection in one of the kits utilized made this technique difficult to carry on during the entire project. For the successful implementation of this technique, it is necessary to have a well-equipped laboratory facility.

.

The Coonamessett Farm Foundation (CFF) has a long history managing RSA grants, and through those grants, has been able to provide a wealth of data that has been used to address a wide range of issues that impact the ecosystem mainly in Georges Bank (GB). However, this is the first time that CFF received a monkfish RSA. Unfortunately, the low demand for monkfish days at sea (DAS) amongst fishermen, challenged CFF's ability to sell DAS and ultimately secure funding for the project; in addition, the COVID-19 pandemic made this situation worse. As a result, this project was only able to sell 69 of the 295 DAS awarded, recuperating just 23% of the research costs needed to successfully complete this project. Despite this, detailed protocols of each technique are provided at the end of this report.

INTRODUCTION

Monkfish (*Lophius americanus*) are a fast-growing anglerfish species that are widely distributed throughout the Northwest Atlantic (Richards et al. 2008). The species supports an important commercial fishery in US waters with landings approaching 8,000 tons in 2020 (NOAA 2022). Despite of its importance to US fishing communities, estimates of key stock assessment parameters like recruitment are not clearly defined or understood for monkfish (NEFSC 2010). Growth patterns and recruitment are different in the northern and southern regions of the species US range (Johnson et al. 2008). However, there is no genetic difference between monkfish collected from North Carolina to Maine from depths up to 300 m has been demonstrated (Haring and Maguire 2008). Developing tools to better understand the reproductive biology of monkfish will help provide an explanation for observed regional differences in growth and recruitment as well as essential data for stock assessment models.

Tagging studies are often used to provide information about the relative movement and size of fish populations. Measuring tagged fish provides information about the population's age-structure but other key demographic data like sex and stage is often difficult to collect from tagged fish. Sexing and staging fish is often done by a postmortem examination of gonads which is why these data are not often collected from tagged animals before they are released. However, for researchers looking to relate movement patterns of tagged fish to changes in reproductive physiology a non-lethal method of sex and stage determination is required. This is especially important since post-mortem inspection of the gonads at recapture only provides one data point with regards to maturity stage, so if the goal is to evaluate animal movements relative to spawning activities, knowing the stage at release is key. Advances in technology over the last two decades have allowed non-lethal sex determination methods to be more accessible to fisheries researchers. Ultrasonography, endoscopy, and analysis of steroid concentrations are three methods that have been used to non-lethally identify the sex and stage of fish (Albers et al. 2013).

Since the early 1980s, ultrasonography has been used to determine the sex and maturational status of various freshwater and marine fish species by producing images of the size, shape, and location of reproductive organs (Martin et al. 1983, Reimers et al. 1987, Shields et al. 1993; Blythe et al. 1994, Arkush and Petervary 1998, Evans et al. 2004). Endoscopy, a method used in medical and veterinary settings to view internal organs via the insertion of a camera into the body cavity through a small incision has also been used to non-lethally sex and stage fish (Swenson et al. 2007, Hurvitz et al. 2007). Finally, circulating reproductive hormones (i.e. testosterone and 17β-estradiol) have been used to identify sex and reproductive status in sturgeon (Wheeler et al. 2016). Testosterone, an important androgen, contributes to the maturation of both males and females, whereas estradiol is not important for maturation of males, it is essential for oocyte production and maturation in females (Wheeler et al. 2016). By measuring testosterone and estradiol concentrations, the sex and stage of fish can be determined non-lethally by using the ratio of testosterone to estradiol (Wheeler et al 2016). Each of these techniques has its pros and cons.

Although it is a non-invasive technique, ultrasound requires expensive equipment to provide a detailed image for sexing and staging fish. Endoscopy is the least expensive technique but a small incision is required which puts the animal at risk for infection. However, if data loggers are being implanted into the fish body cavity the same incision can be used for sexing

and staging with an endoscope. Due to the invasiveness of measuring steroid concentrations, the procedure cannot be done non-lethally for fish less than 150 mm (Swenson et al. 2007). Despite its invasiveness, measuring steroid concentrations is considered the most precise way to non-lethally stage a fish. And, while each of these sampling methods has been used on other fish species, they have not been applied to monkfish. Therefore, we evaluated these three non-lethal techniques for determining the sex and stage of monkfish. The information provided by this project can be used to choose an appropriate non-lethal sex and stage determination technique to address knowledge gaps about monkfish reproduction and recruitment.

OBJECTIVES

The main goal of the project was to develop tools to improve monkfish stock assessment and ease the establishment of biological reference points throughout sex and maturity determination.

1. Establish a method for sex and maturation stage determination in monkfish using an ultrasonic scanner.

2. Establish a method for sex and maturation stage determination in monkfish using a endoscope equipped with 6 light emitting diodes (LEDs).

3. Analyze plasma steroid hormone ratios in monkfish using ELISA techniques.

4. Calculate GSI values for male and female monkfish.

5. Use histological reference slides of gonads to verify assignment of sex and maturation stage by ultrasonic imaging, endoscopic imaging, ELISA, and gross examination, creating a reproductive profile for monkfish.

6. Identify spawning characteristics, such as multiple spawns, skip spawning, etc. as possible.

METHODS

Sampling design

Prior to sample collection, CFF researchers were trained to collect blood samples in the field by Dr. Diana Papoulias, an expert in reproductive biology and hormone analysis. In addition, the researchers traveled to the Columbia Environmental Research Center (U.S. Geological Survey; USGS) to be trained by Dr. Mark Wildhabe, a researcher with extensive experience using ultrasound to determine sex and maturity stages of sturgeon. Starting December 2018, 193 monkfish were examined using the three non-lethal techniques for sex and maturity stage determination. Monkfish were collected from Southern New England (SNE) and Georges Bank (GB) fishing grounds (**Figure 1**). Sampling was conducted onboard, during commercial fishing trips, at the dock where catch was offloaded, and on specific research trips. Prior to using the three non-lethal techniques, each monkfish was weighed using a Marel 1100-series motion compensated scale and measured to the nearest centimeter. Once the non-lethal techniques were

used, each fish was then dissected for visual confirmation of the sex and maturity stages, and a piece of the gonad was collected for further laboratory analysis.

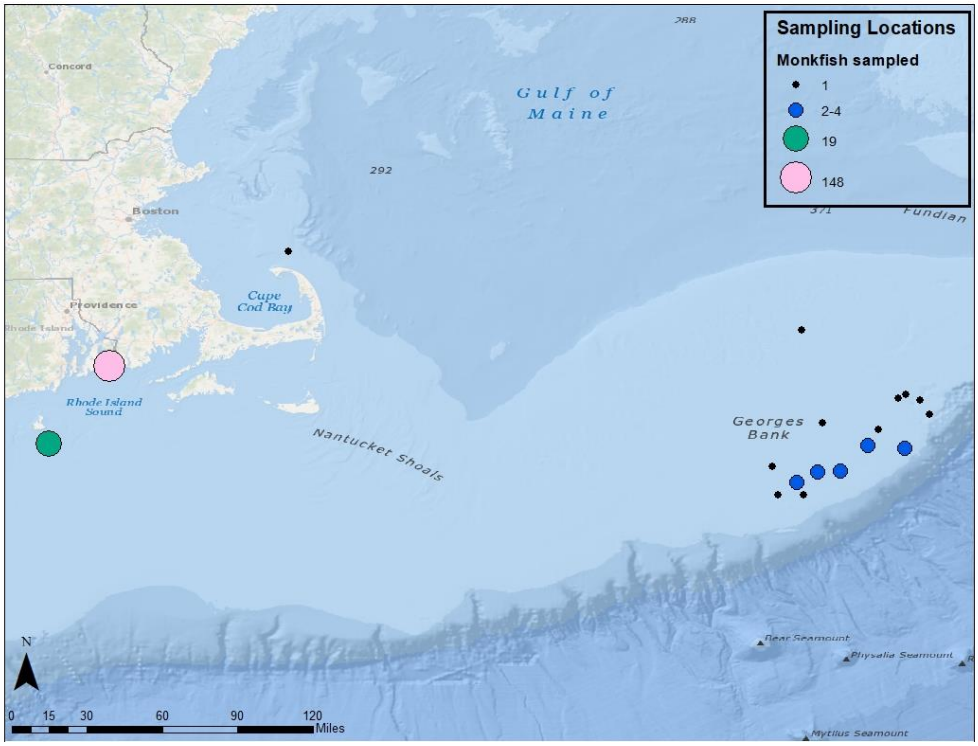


Figure 1. Sampling locations of *L. americanus* collected by research and commercial trips between December 2018 and June 2020 in the SNE area.

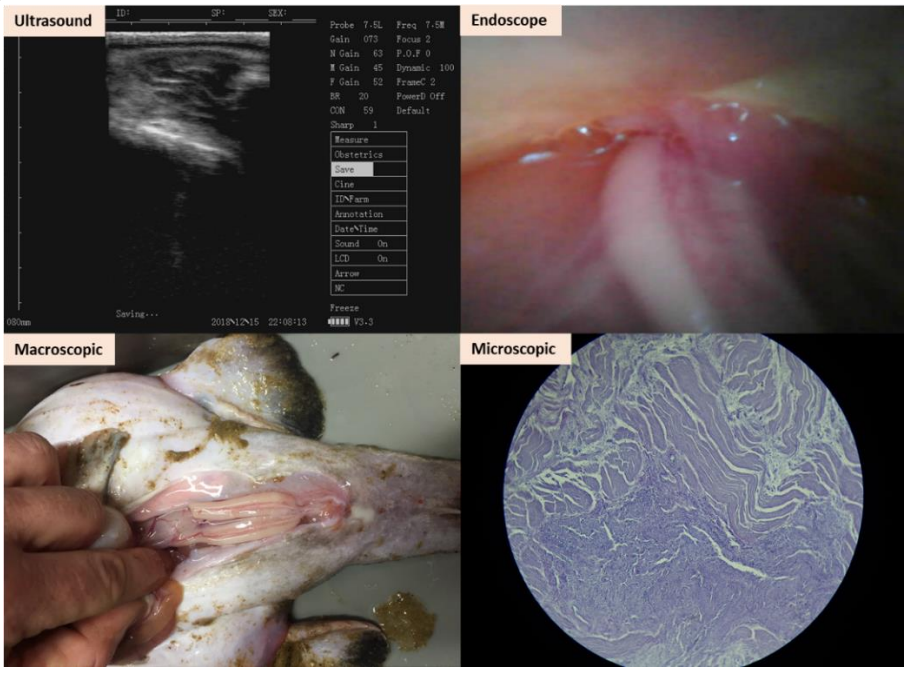
Ultrasound inspection: A portable ultrasound machine equipped with a 7.5-MHz linear probe was used to examine the monkfish gonads. The examination consisted of gently placing the ultrasound probe, with transmission gel, along each fish's abdomen and then moving the probe along the abdominal surface until the gonads were located. Pictures were taken when the gonads were located on the ultrasound screen; at least three pictures were stored by individual for later viewing (more details on the **Appendix A**: Field Collection Handbook).

Endoscopic inspection: A portable Depstech 2MP Wi-Fi unit was used to examine the monkfish gonads. A small incision was done with a scalpel in the center of the abdominal part of each monkfish. The endocospe, previously connected to a smartphone throughout Wi-Fi, was inserted into the slit in the abdominal wall to explore the body cavity and identify sex and maturity stage. Approximately three pictures were taken by individual (see **Appendix A)**.

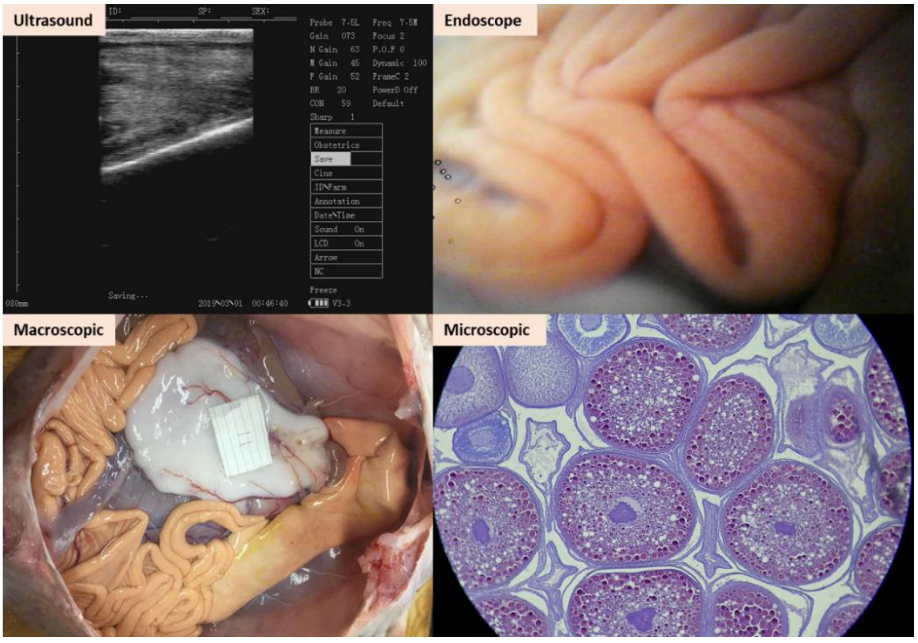
Blood sampling: For plasma hormone levels, a blood sample was taken from the caudal vein of each individual by use of a syringe with a hypodermic needle containing heparin to prevent coagulation. Blood samples were then separated by centrifugation for 10 minutes. The plasma was placed in microcryovials and they were stored at ~ -70°C in a liquid nitrogen container (see **Appendix A)**.

Macroscopic inspection: Each monkfish was dissected to confirm sex and stage previously evaluated with each of the non-lethal techniques. Each fish was photographed with their respective label. Egg veil/testes were carefully removed and weighed for future gonadosomatic index calculations. Using a scalpel, a piece of the gonad of each monkfish was stored in histology cassettes and preserved in Prefer fixative for further laboratory analysis. **Figure 2** show the products from each of the techniques mentioned above and explained in detail in **Appendix A**; also a reference histological slide picture of the example individual.

a)



b)



c)

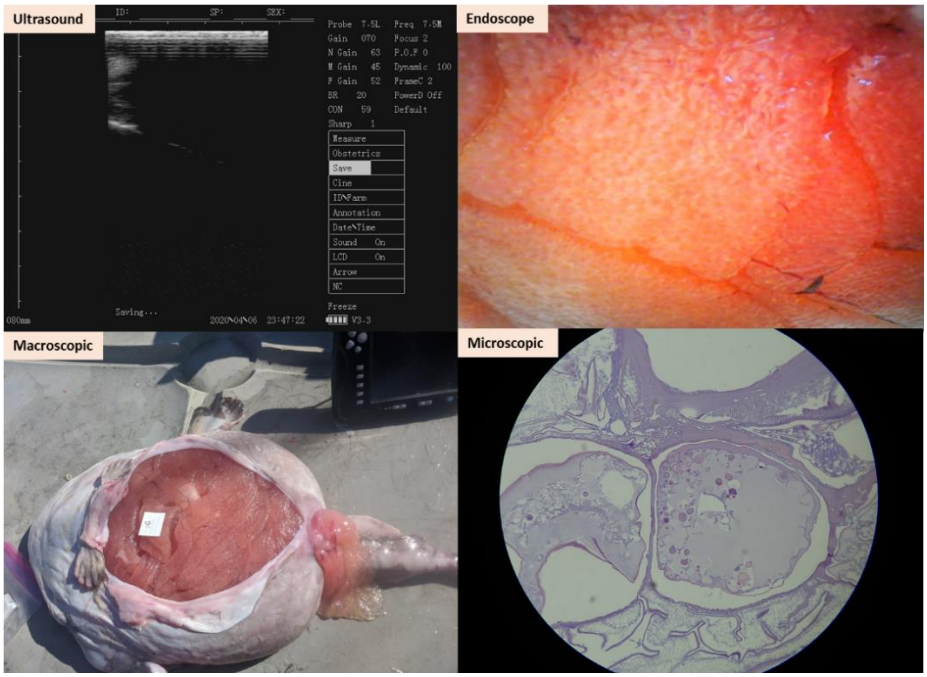


Figure 2. Examples of the products from the non-lethal and lethal techniques used in all monkfish during this project. **a)** Male in developing phase, **b)** female in ripe phase and **c)** female in actively spawning phase.

Laboratory analysis

Hormone analysis

For this analysis was used a Cayman estradiol kit and a MyBiosource kit. The first one was chosen because of the high-quality kits they produce and the company's reputation as a leading manufacturer. The MyBiosource kit was used because the first two trials with a Cayman kit did not give a strong signal response for the analysis. Manufacturer instructions provided with each kit were strictly followed (Cayman Chemical 2019**).**

Histological evaluation

All the gonad samples collected during the field work were sent to Horus Scientific to be processes into slides. At their lab, each sample was dehydrated, embedded in paraffin, sectioned at 5 mm, and stained with hematoxylin. Once at CFF, all histological slides were analyzed by light microscopy and assigned sex and maturity stages to confirm maturity stages assessed during macroscopic evaluation. Microscopic evaluation of the maturity stage was carried out by applying the criteria described in Colmenero et al. 2013 and Johnson and Grier 2017. Ovarian and testes stages were classified based on the stage of maturity of the most advance stage oocytes.

Data analysis

Ovaries were weighed, and the gonadosomatic index (GSI) was calculated using the following equation:

$$GSI = \frac{WG}{W}\ x\ 100$$

where WG is the gonad weight, and W the eviscerated body weight.

Percent error when using ultrasound and endoscopy was calculated by comparing the macroscopic sex determination to sex assigned using the non-lethal techniques. Error assigning maturity stage was calculated only for the endoscopic technique.

RESULTS

A total of 193 monkfish, ranging in size from 33 to 82 cm, were analyzed using ultrasonography, endoscopy, macroscopic and microscopic inspection. Blood samples were taken from sixteen monkfish and a plasma steroid hormone analysis was done for five of these individuals. Most samples were collected during dockside sampling in February, April, and June of 2020 (**Table 1**).

Table 1. Monkfish collected during the length of the project by different sampling strategies.

Year	Month	LA sweep trip	Monkfish dedicated trip	Seasonal survey	Dockside sampling	Total
2018	December	-	-	7	-	7
2019	January	-	-	3	-	3
	February	-	-	1	-	1
	March	1	-	4	-	5
	May	-	19		-	19
2020	February	-	-	10	21	31
	April	-	-	-	97	97
	June	-	-	-	30	30
Total		**1**	**19**	**25**	**148**	**193**

Sex determination

Of the monkfish examined, 80% were females ranging in size from 39 to 82 cm, while males had a much smaller size range (33-62 cm). Female and male monkfish can be differentiated by their reproductive organs. The ovarian structure in females consists of a flattened band with two distinct lobes that are folded up and connected to each other at their posterior end. The lobes form a single organ attached to the abdominal cavity of female

monkfish by a mesenteric tissue called the mesovarium (**Table 3**, Colmenero et al. 2013). In males, the testes are a pair of elongated and tubular structures located in the dorsal portion of the abdominal cavity, and they are bean-shaped in transverse section. The organization of the testes is lobular: the connective tissue extends from the testicular capsule to form lobules that have their blind ends on the surface of the gonads, converging ventrally towards the sperm duct (**Table 3**, Colmenero et al. 2013).

Results comparing the two non-lethal techniques: ultrasound, endoscopy, vs macroscopic sex assignment show that ultrasound inspection had greater percentage of error compared to endoscopic inspection (**Figure 3a**); although, the ultrasound inspection only had 15% error. A small portion (7.7%) of medium size monkfish were assigned to sex incorrectly; however, this size had greater percentage of error, for both non-lethal methods, than the other two sizes (**Figure 3b**).

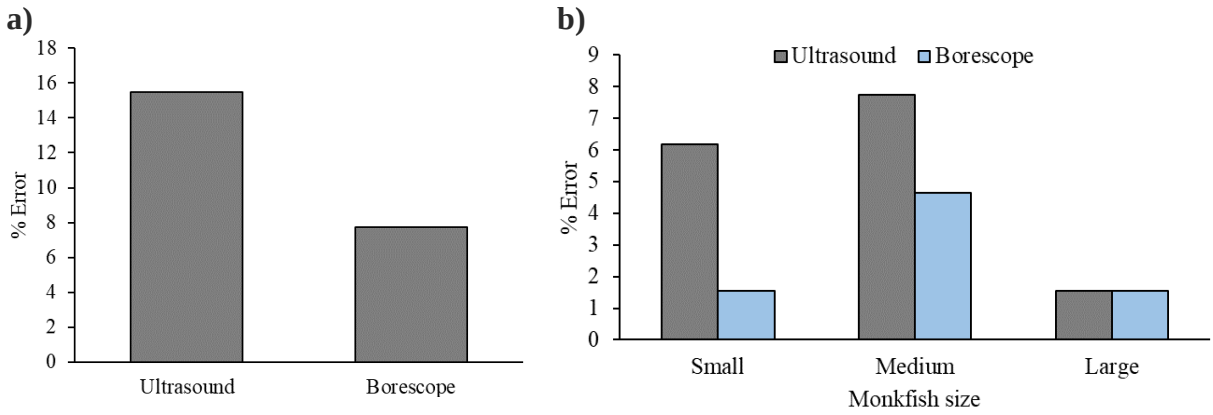


Figure 3. Percentage of error in sex determination by **a)** non-lethal techniques and **b)** monkfish size.

Maturity stages determination

Around half (54%) of the monkfish examined were mature. Monthly distribution of macroscopic classification of the maturity stages (**Figure 4**) revealed that the period of maximum occurrence of females in the ripe phase (III) was in February and April. The presence of females in the actively spawning stage (IV) was observed from February to April, with the highest percentage of spawning individuals seen in March. Females in the immature and developing stages (I and II, respectively) were found in February, April, May and June (**Figure 4a**). Based on the GSI values, there was one spawning period in spring (**Figure 4a**), which partly coincides with the results reported by Richards et al. 2008. Overall male samples were low during this project; mature males (IV) were observed in March, April and June (**Figure 4b**).

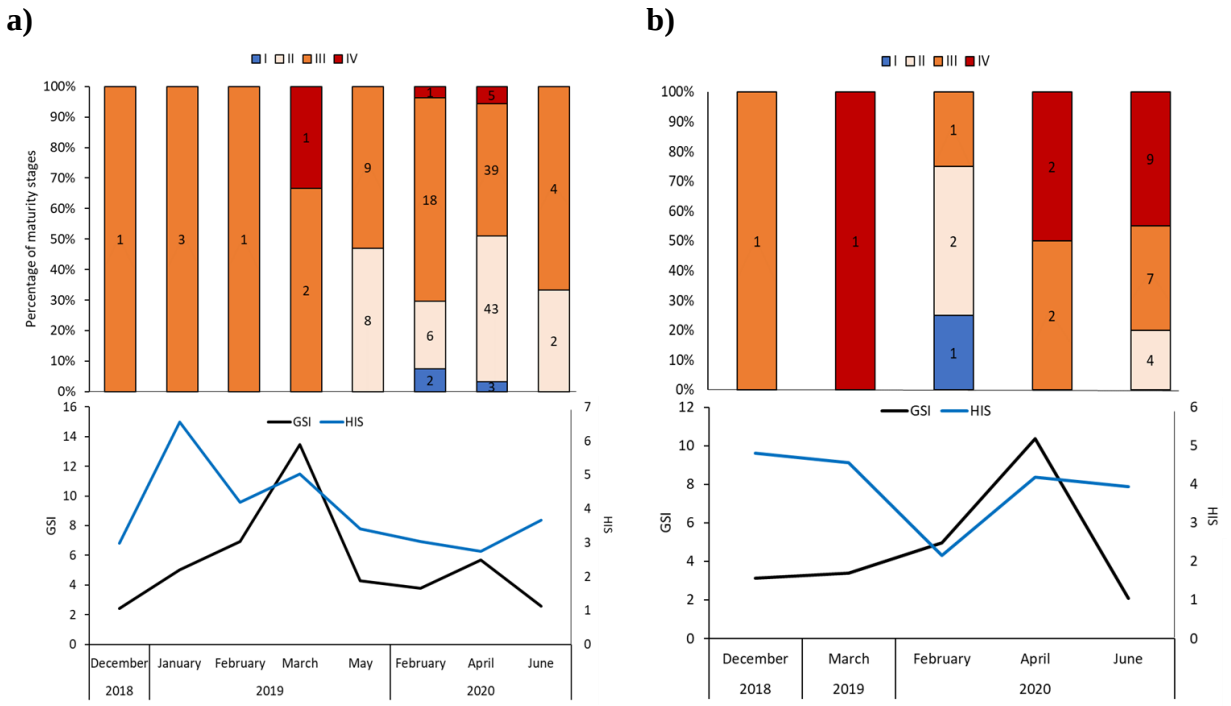


Figure 4. Monthly distributions of **a)** females and **b)** males in the four phases of gonad maturity of monkfish collected in the SNE between December 2018 and June 2020 and monthly changes in the mean gonadosomatic index (GSI) and hepatosomatic index (HSI) for **a)** females and **b)** males.

Comparison of the endoscope and macroscopic maturity stage inspections against microscopic analysis showed that endoscope inspection had the highest percentage of error (53%; **Figure 5a**); however, the difference was minimal between the two inspections (**Figure 5a**). Taking into account only macroscopic vs microscopic evaluation showed greater percentage of error in stages II (developing, 97%) and I (immature, 86%), and maturity stage III was correctly identified (91.8%) most often (**Figure 5b**). The macroscopic and microscopic characteristics of each maturity stage are listed in **Table 2** and **Table 3**.

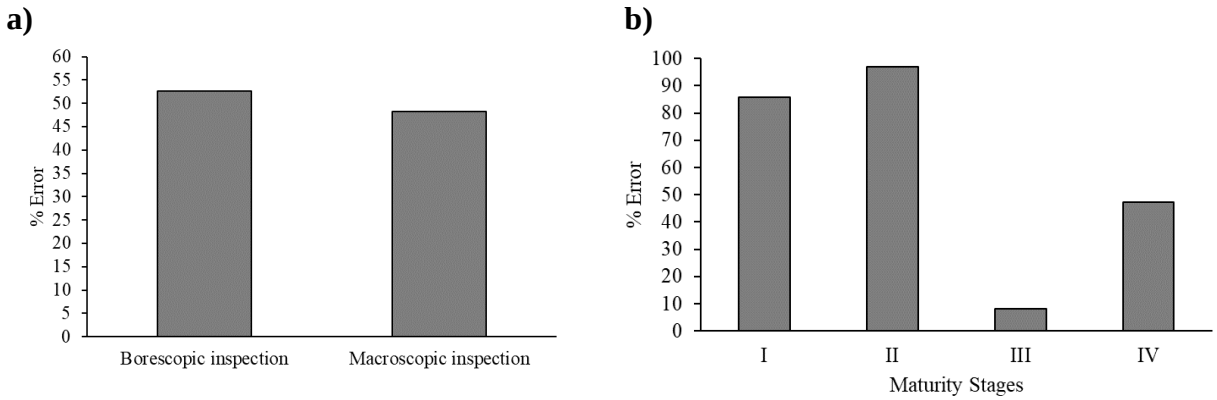
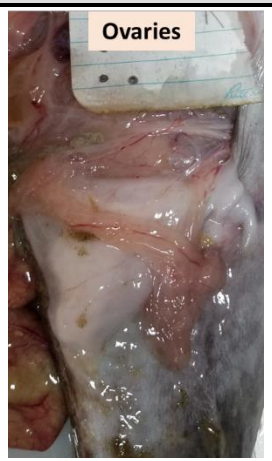
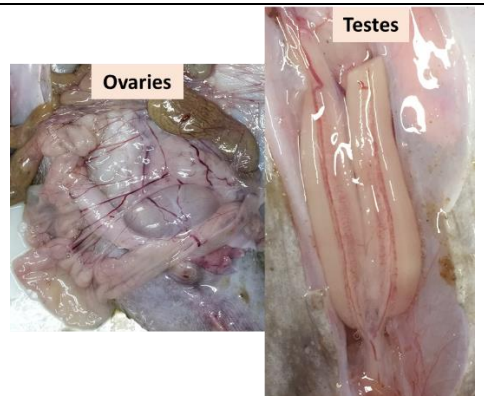


Figure 5. Percentage of error in maturity stage evaluation by **a)** techniques and **b)** maturity stage.

Table 2. Macroscopic description of four maturity stages in the reproductive cycle of female and male of monkfish collected in SNE between December 2018 and June 2020 (based on Colmenero et al. 2013 and Johnson and Grier 2017).

Maturity stage	Female	Male	Macroscopic picture
I – Immature	Ovaries are very narrow, thin, and ribbon-like. They are translucent and no oocyte clusters visible and minimal vascularization.	N/A	
II – Developing	Ovaries are small. Still no noticeable individual oocyte clusters. They become less translucent and vascularization is visible.	Testes are small with visible blood vessels around the seminal duct.	

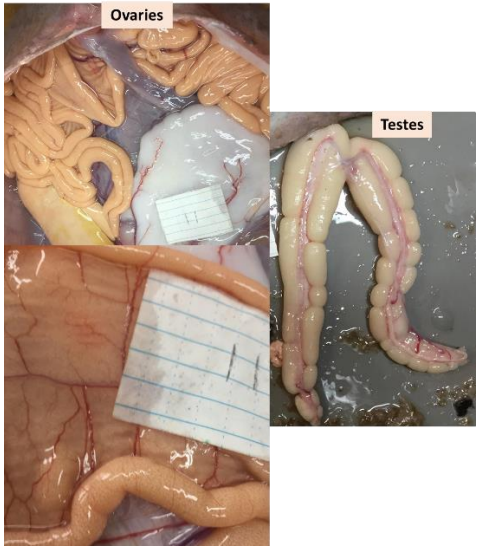
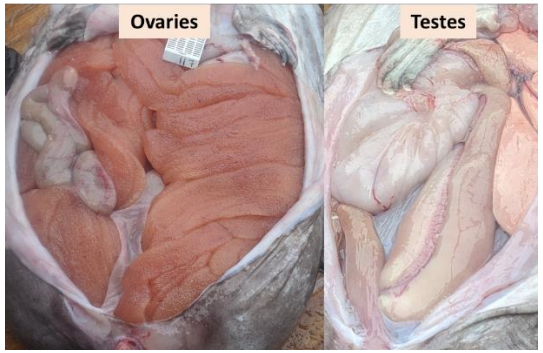
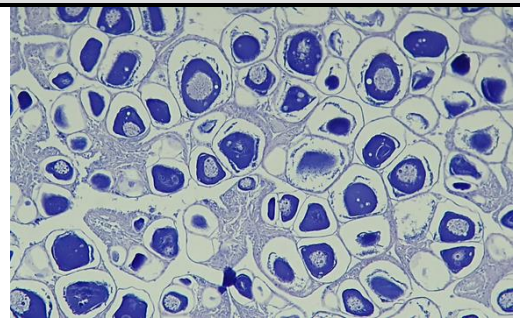
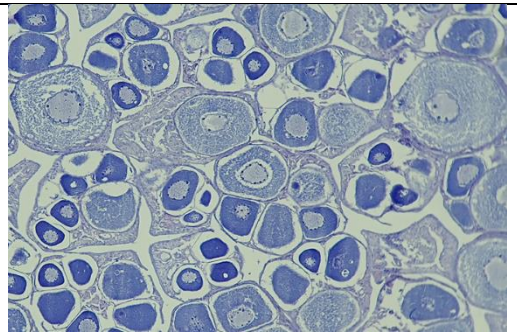
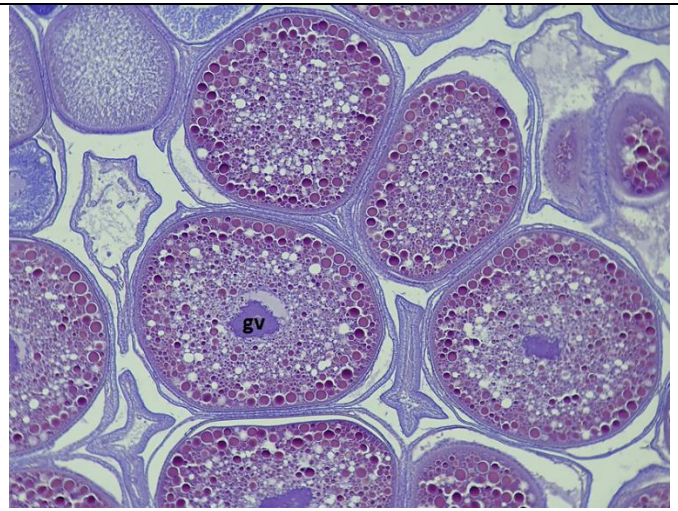
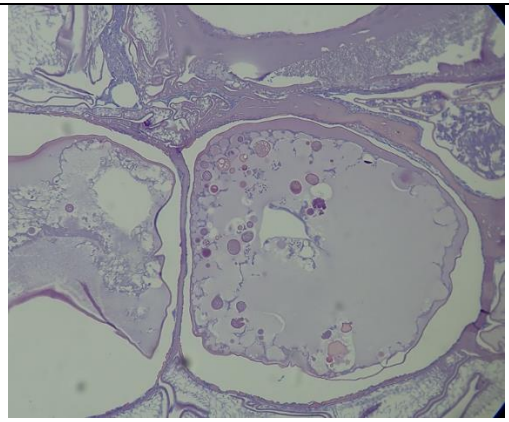
III – Ripe	Ovaries increase in width and length. They have a light orange color, and blood vessels are prominent. The edges of the ovaries start to curl, and they occupy a larger proportion of the body cavity.	Testes increase in length and width. They have a firm texture and cream color. Seminal duct is highly vascularized.	
IV – Actively spawning	Ovaries are extremely long and wide and occupy most of the body cavity. The color of the oocytes is orange, and they are visible macroscopically. Ovaries are characterized by the presence of large hyaline oocyte clusters enclosed in a transparent gelatinous matrix that is completely developed. High vascularization is present.	Testes are large and firm and have a creamy coloration. Large amounts of sperm produced when testes are dissected.	

Table 3. Microscopic description of four maturity stages in the reproductive cycle of female of monkfish collected in SNE between December 2018 and June 2020 (based on Colmenero et al. 2013 and Johnson and Grier 2017). Bar = 50 μm.

Maturity stage	Female	Microscopic picture
I – Immature	Only oogonia and primary growth oocytes are present.	
II – Developing	Secondary vitellogenic oocytes are present. The ooplasm has "expanded" containing a few oil droplets. Nuceloli may be around the germinal vesicle periphery or scattered.	

III – Ripe	Primary growth, cortical alveolar, and primary and secondary vitellogenic oocytes are present. Yolk globules and unstained oil droplets are around of the germinal vesicle (gv). Nucleoli may be peripheral or scattered in the germinal vesicle. The germinal vesicle can be slightly displaced from a central position.	
IV – Actively spawning	Oocytes are in tertiary vitellogenesis, migratory nucleus and hydration. The germinal vesicle is located at the animal pole, and its nucleoli are scattered. The coalescence of yolk vesicles into a fluid yolk at the vegetal pole dominates the ooplasm. Oil globules are larger than previously observed due to their coalescence	

Plasma steroid hormone analysis

Plasma from five individuals was used to evaluate hormone kits (four females and one male). The Cayman estradiol kit was the first one tested because of the high-quality kits they produce and reputation of the company. The Cayman kit was tested on 2 occasions (February and May). During the first test the antibody did not recognize the monkfish estradiol protein, but in the second test the antibody did recognize the monkfish estradiol protein since the relative concentrations obtained were consistent with the expected concentrations based on sex and ripeness. However, neither trial gave a strong signal response with the recommended incubation time and at room temperature and neither gave good QAQC results indicating low confidence in having obtained a good estimate of the true concentrations due to interfering substances most likely. Cayman replaced their estradiol kit with an update that improved the detection range and made it possible to extract the hormone from the plasma using only methanol. Previous kits required more complicated extraction procedures than would be possible without proper laboratory facilities. Cayman sent a free kit for us to try in September 2019. The standard curve was not that good, only 4 points were useable (**Figure 6c**). Again, the absorption signal was low at the prescribed incubation time, increasing with additional time but still not acceptable. Of the QAQC tests, linearity did work well although parallelism did not.

Based on this result, other kits designed specifically for fish were researched (Cayman uses antibodies developed for humans). MyBiosource supplies such a kit and was tested in October, 2019. This kit gave us the best standard curve to date (**Figure 6d**); but note that this may in large part be due to having purchased a vortex and a multi-channel pipettor that reduced cross-contamination during the rinse step. We only tested the 100% fish sample as not dilutions are allowed with this kit. The kit was easier to use, but less forgiving. Unfortunately, the fish samples did not give good results. The male was higher than the females, but still low; in fact at about the concentration that would be expected. As with the Cayman kits, the signal was weak.

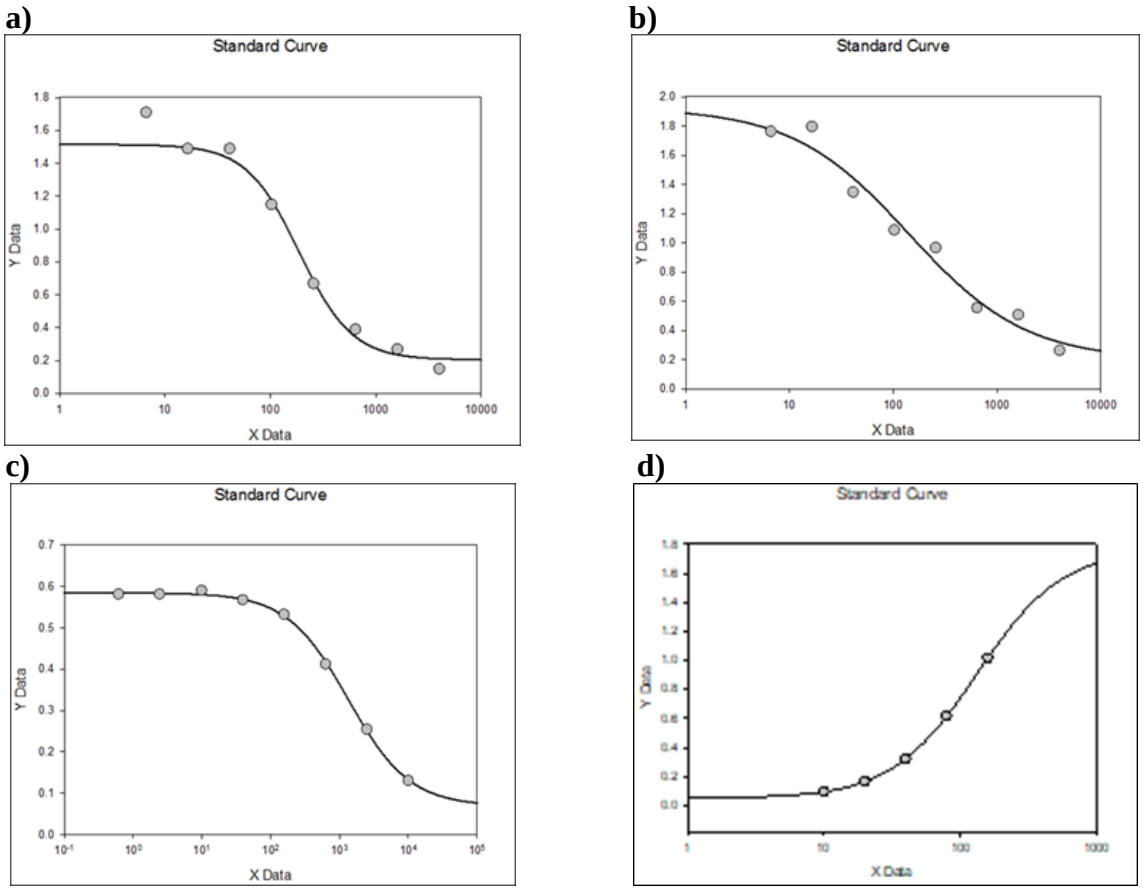


Figure 6. Standard curve obtained in each one of the experimental trial **a)** February, **b)** May, **c)** September and **d)** October

Table 4. Results for unextracted, undiluted fish plasma

Fish Sample	Sex	Stage	February [Cayman1] (ng/L)	May [Cayman1] (ng/L)	September [Cayman2] (ng/L)	October [MyBiosource] (ng/L)
1	Female	Ripe	223	361	304	
2	Female	Developing	175	231		
3	Male	Male	70	65	69	13
4	Female	Ripe and running IV			830	1
5	Female	Ripe				1

DISCUSSION

All 193 monkfish were analyzed using ultrasound to attempt to identify their sex. This technique had a high percentage of error (15 %) in identifying sex compared to endoscopic and macroscopic inspection of the gonads. One reason for the relatively high error rate may be the quality of the ultrasound equipment used for this project. To work within our limited budget, an ultrasound machine was purchased from an online wholesaler rather than from a medical supply store. While this machine had a 7.5-MHz linear probe the crystal was of poor quality and could not provide a complete and accurate image. However, during the ultrasound training, USGS researchers had a veterinary-grade ultrasound available that was able to capture very clear and precise images of the monkfish's organs. Thus, it is expected that with a greater investment in the equipment, the percentage of error of this technique will decrease significantly.

The endoscope was the most accurate non-lethal method for determining sex of monkfish, with an error rate of only 7%. However, for determining maturity status it had a similar error rate to the macroscopic inspection (52% and 48%) with the highest error rates for immature and developing individuals. This indicates that the incorrect assignment of maturity is likely due to the technician's ability to distinguish between immature and developing. In addition to properly training technicians to determine monkfish maturity stage, research is required to determine if endoscopic inspection of monkfish is truly non-lethal for all monkfish sizes. Similar to internal dataloggers, an incision is required to perform an endoscopic inspection, and surgical tagging does not significantly increase mortality to monkfish (Richards et al. 2011) indicating that endoscopy may not increase monkfish mortality. This added to its high accuracy makes this technique the best non-lethal technique for reproductive assessment purposes on monkfish.

Only five monkfish were used for the hormone analysis. One of the main difficulties with this technique was keeping the plasma samples frozen. Containers with liquid nitrogen were used to store samples short-term, but this approach does not keep samples frozen for an extended period of time. To successfully store plasma samples, a freezer capable of reaching -80 °C is required. Despite not having the ability to store samples long-term, we were able to use two hormone analysis kits for this project: the Cayman and MyBiosource kits. The Cayman Kits showed that the antibody was detecting fish estradiol but contaminants in the samples confounded the results. The MyBiosource kit was limited in its range of detection and that likely contributed to the odd result for the female fish analyzed with this kit. Both of these kits have the potential to be a non-lethal method for determining monkfish sex and stage; however, successfully developing this technique required purchasing additional equipment or contracting a laboratory. Neither of the options were feasible due to our limited ability to sell monkfish DAS.

The most significant factor that contributed to the outcome of this project was the method by which the research is funded. RSA programs generate funding through the sale of fishing effort (e.g. quota or days-at-sea) with no direct exchange of federal funding. When successful, RSA programs can provide sustained funding to research to address management objectives. However, to be successful the unit of fishing effort being exchanged through an RSA program needs to have an appropriate value to incentivize industry participation. The Monkfish RSA Program 'sets-aside' DAS to be sold by researchers to fund monkfish research. Unfortunately, we found that there was not a significant demand for monkfish DAS which challenged our

ability to sell DAS and ultimately secure funding for the project. Prior to the COVID-19 pandemic demand for monkfish DAS was relatively low. However, the pandemic disrupted the foreign markets that the monkfish fishery depends on which caused the average price for monkfish to remain low throughout2020 (NOAA 2021). The low average price likely deterred fishers from participating in the monkfish fishery, as there was less seasonal demand. As a result, this project was only able to sell 69 of the 295 DAS awarded, recuperating just 23% of the research costs needed to successfully complete this project. Under these circumstances, our ability to purchase the appropriate equipment was hindered and only 193 of the proposed 500 monkfish were sampled.

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Several non-lethal techniques are available to determine sex and maturity status of fish species; however, alternative approaches are either costly, or must be conducted in laboratory settings. In the case of the ultrasound, in order to obtain accurate results, a larger sum investment should be considered on equipment. The endoscope requires a lower cost investment and the accuracy is superior; however, this technique is mildly invasive requiring an incision. Furthermore, for the endoscopic technique it is necessary to evaluate the effects of this incision on the overall health of the fish. Blood plasma indicators can also be useful, but this approach requires well-structured laboratory facilities.

The overall idea of this project was to explore non-lethal techniques for sex and maturity stage determination that could be used in the field, and due to our budget constraints, it was necessary to find low-cost solutions. Although there was some indication that purchasing low-cost equipment negatively impacted our results, the most effective method was also the endoscope, which is easy to use in the field and has the lowest costs. Future research is needed to determine the lethality of this technique; however, there is indication that monkfish can recover from tagging surgeries (Richards et al. 2011).

REFERENCES

Albers, J.L., Wildhaber, M.L., and DeLonay, A.J. 2013. Gonadosomatic index and fecundity of lower Missouri and middle Mississippi river pallid sturgeon estimated using minimally invasive techniques. Journal of Applied Ichthyology. 29: 968–977.

Arkush, K.D., and N.A. Petervary. 1998. The use of ultrasonography to predict reproductive development in salmonid fish. International Association for Aquatic Animal Medicine. 29: 19-20.

Armstrong, M.P. Musick, J.A., and Colvocoresses, J.A. 1992. Age, growth, and reproduction of the goosefish *Lophius americanus* (Pisces:Lophiiformes). Fishery Bulletin, U.S. 90:217-230.

Blythe, B., Helfrich, L.A., Beal, W.E., Bosworth, B., and Libey, G.S. 1994. Determination of sex and maturational status of striped bass (*Morone saxatilis*) using ultrasonic imaging. Aquaculture. 125: 175-184.

Cayman Chemical. 2019. Estradiol ELISA Kit Instructions. Available online: https://cdn.caymanchem.com/cdn/insert/501890.pdf

Colmenero A., V. Tuset, L. Recasens and P. Sanchez. 2013. Reproductive biology of Black Anglerfi sh (*Lophius budegassa*) in the northwestern Mediterranean Sea. Fish. Bull. 111:390–401 (2013). 12 p.

Evans, A.F., Fitzpatrick, M.S., and Siddens, L.K. 2004. Use of ultrasound imaging and steroid concentrations to identify maturational status in adult steelhead. North American Journal of Fish Management. 24: 967-978

Haring, P., and Maguire, J-J. 2008. The monkfish fishery and its management in the northeastern USA. – ICES Journal of Marine Science, 65: 1370–1379

Hurvitz A., K. Jackson, G. Degani, B. Levavi-Sivan. 2007. Use of endoscopy for gender and ovarian stage determinations in Russian sturgeon (Acipenser gueldenstaedtii) grown in aquaculture. Science Direct, Aquaculture 270 (2007) 158–166. 9 p.

Johnson, A.K., and H. Grier. 2017. Ovarian germinal epithelium, oocyte development and the secretory epithelium in monkfish (*Lophius americanus* Valenciennes). Journal of Morphology. 2018;1–10. 10 p.

Johnson, A. K., Richards, R. A., Cullen, D. W., and Sutherland, S. J. 2008. Growth, reproduction, and feeding of large monkfish, *Lophius americanus*. – ICES Journal of Marine Science, 65: 1306–1315

Matin, R.W., Myers, J., Sower, S.A., Phillips, D.J., and Mcauley, C. 1983. Ultrasonic imaging, a potential tool for sex determination of live fish. North American Journal of Fisheries Management. 3(3): 258-264.

Northeast Fisheries Science Center (NEFSC). 2010. 50th Northeast Regional Stock Assessment Workshop: Assessment Summary Report. Northeast Fisheries Science Center Reference Document 10-09.

NOAA. 2022. Commercial fisheries statistics. Available online: https://www.st.nmfs.noaa.gov/st1/commercial/landings/annual_landings.html

Reimers E., P. Landmark, T. Sorsdal, E. Bohmer, and T. Solum. 1987. Determination of salmonids' sex, maturation and size: an ultrasound and photocell approach. Aquaculture Magazine. November/December pp. 41-44.

Richards, R.A., Nitschke, P.C., and Sosebee, K.A. 2008. Population biology of monkfish *Lophius americanus*. ICES Journal of Marine Science. 65: 1291-1305.

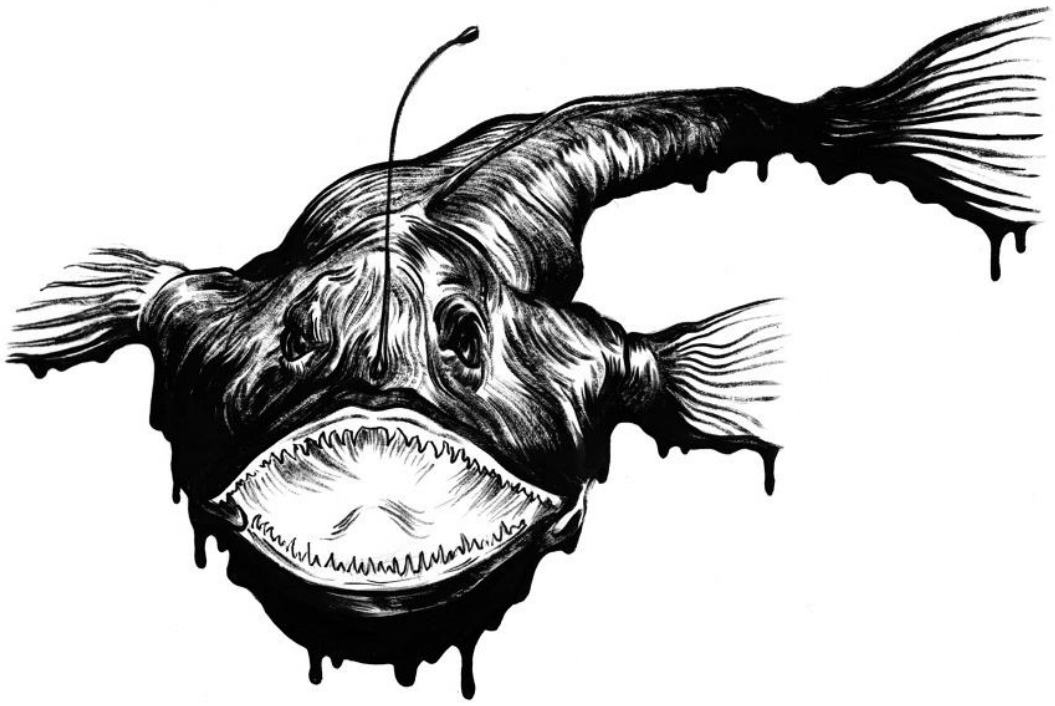
Shields, R.J., Davenport, J., Young, C., and Smith, P.L. 1993. Oocyte maturation and ovulation in the Atlantic halibut, *Hippoglossus hippoglossus* (L.), examined using ultrasonography. Aquaculture and Fisheries Management. 24: 181-186.

Swenson, E.A., Rosenberger, A.E., and Howell, P.J. 2007. Validation of endoscopy for determination of maturity in small salmonids and sex of mature individuals. Transactions of the American Fisheries Society. 136: 994-998.

Wheeler CR, Novak AJ, Wippelhauser GS, Sulikowski JA. 2016. Using circulating reproductive hormones for sex determination of Atlantic sturgeon (*Acipenser oxyrinchus* oxyrinchus) in the Saco River estuary, Maine. Conserv Physiol 4(1): cow059; doi:10.1093/conphys/cow059.

APPENDIX A

Exploring non-lethal techniques for sex determination and evaluation of maturity stage of Southern New England monkfish, *Lophius americanus*



Field Collection Handbook

Table of Contents

Introduction 24

Protocol 1: Biometric Sampling 25

Protocol 2: Ultrasound Technique 26

Protocol 3: Blood Sampling 29

Protocol 4: Endoscope Technique 31

Protocol 5: Macroscopic Inspection 34

Protocol 6: Histological Sampling 35

Introduction

Information on the sex ratios and maturity phases of monkfish is essential for managing of this species. Therefore, effective methods for determining sex and maturity stages in the field would greatly improve a fisheries manager's ability to collect vital population data. Non-invasive/non-lethal methods for sexing and staging fish have gained traction in recent years since they provide useful data without compromising the health of the animals. These protocols were developed during the Monkfish RSA project entitled "Exploring non-lethal techniques for sex determination and evaluation of maturity stage of Southern New England monkfish, *Lophius americanus*" and are presented here to ensure replicability of methods and quality of data. While all the non-lethal techniques mentioned in this document have been assessed in other fish species, this study is the first to apply these techniques to monkfish.

Protocol 1: Biometric Sampling

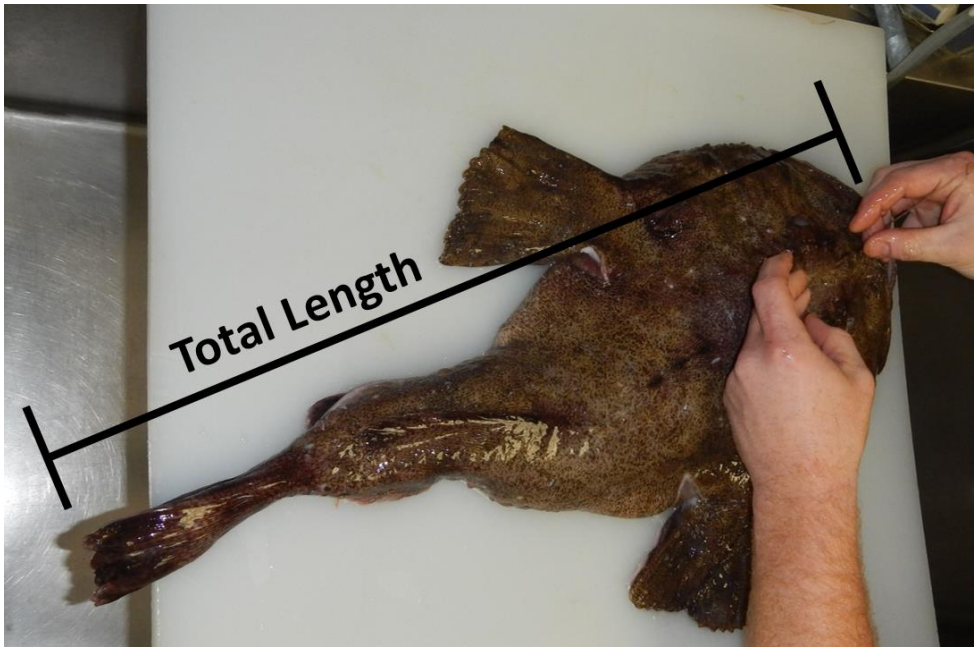
This section describes the initial data to be collected for each specimen.

Materials list

- Fish length board (in cm)
- 6 kg motion compensated waterproof scale
- Gloves (work gloves or deck gloves)
- Measuring tape

Procedures

- Total weight (kg)
    - CFF uses a 6 kg Marel wash-down scale with 1 gram resolution.
    - Monkfish can be placed directly onto scale or placed into a large bowl placed on the scale. Be sure to tare scale if a container is used.
- Total length (cm) – See photo below
    - Length state (relaxed or rigid) should be noted



Protocol 2: Ultrasound Technique

This section describes the process of ultrasound inspection as a non-lethal technique for sex determination on monkfish. This project used a portable waterproof veterinary ultrasound (PL-4018V).

Materials list

- Ultrasound with accessories in case. *Note*: *Make sure that the piezoelectric crystal of the transducer/probe has good efficiency in bandwidth, deep penetration, and high resolution.*
- Transmission gel
- Gloves
- Transducer/probe sleeves
- Small rubber bands
- Large stainless-steel bowl (if possible)

Procedures

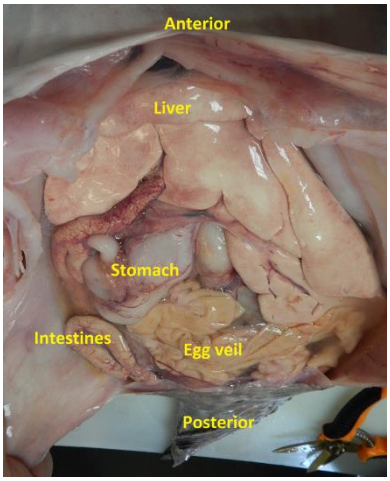
Setting up the ultrasound

To set up the ultrasound, make sure the battery is fully charged (consider the battery life of the machine) or plug the unit into a power source, if possible. Plug the probe into unit. Place transmission gel on the end of the probe, then insert the end of the probe into probe sleeve and secure it with two rubber bands.

Ultrasound inspection

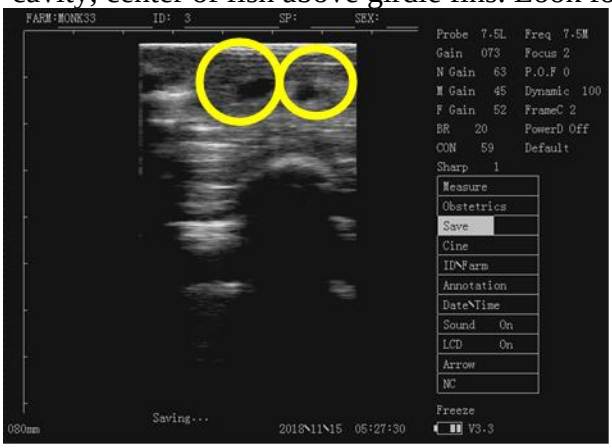
Position the monkfish ventral side up (on its back). Gently placing the ultrasound probe along each fish's abdomen and then moving the probe along the abdominal surface until the gonads are located. Take pictures when the gonads are located on the ultrasound screen, stored pictures by individual for later viewing. *Note: transmission is most effective when sleeved probe is submerged in water*.

Reference images

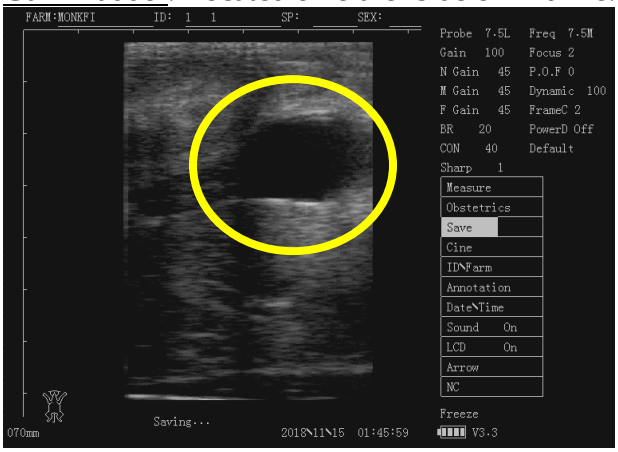


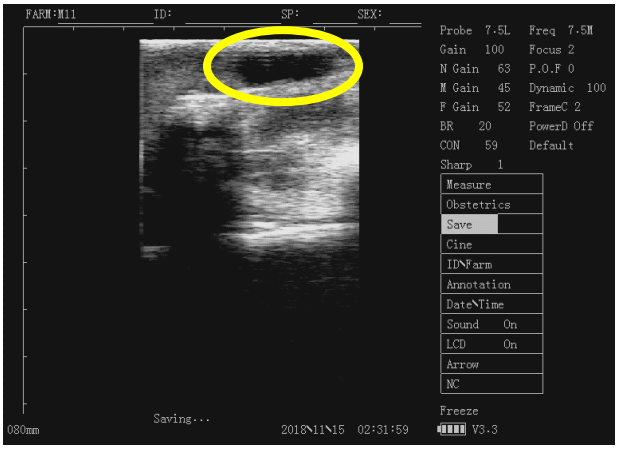
The following are reference images of some of the monkfish organs/structures examined through the ultrasound machine. Certainly, it is necessary to have clear where are the monkfish organs or structures located:

Heart: Located in hollow cavity, center of fish above girdle fins. Look for two valves

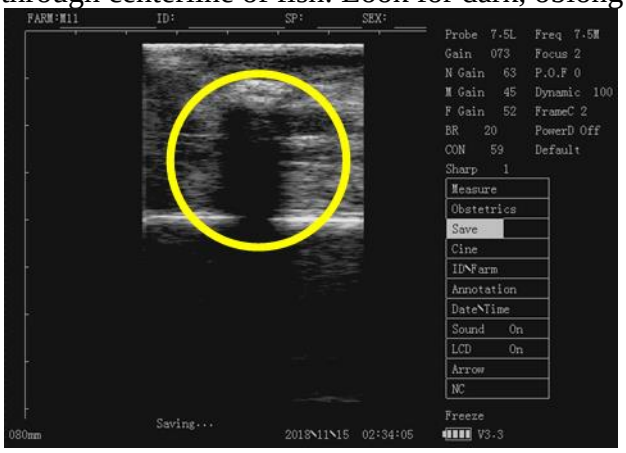


Gall Bladder: Located on either side of midline. Look for solid round black sac.

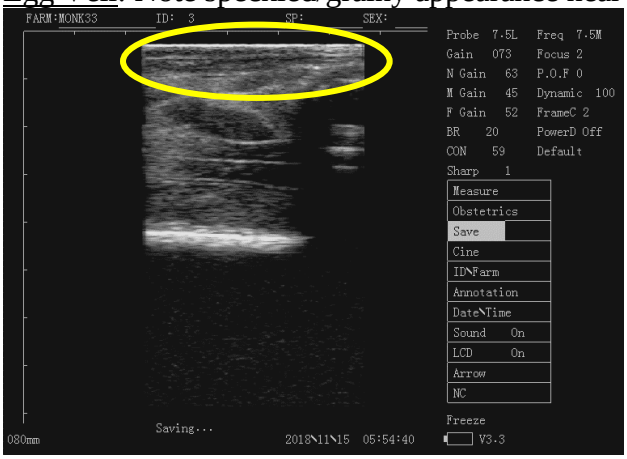


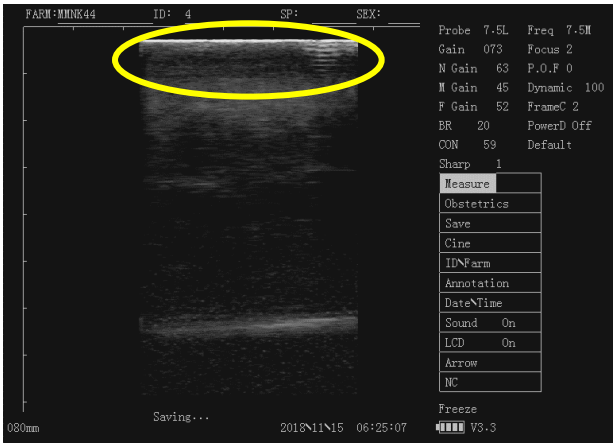


Spinal cord: Ubiquitous through centerline of fish. Look for dark, oblong void

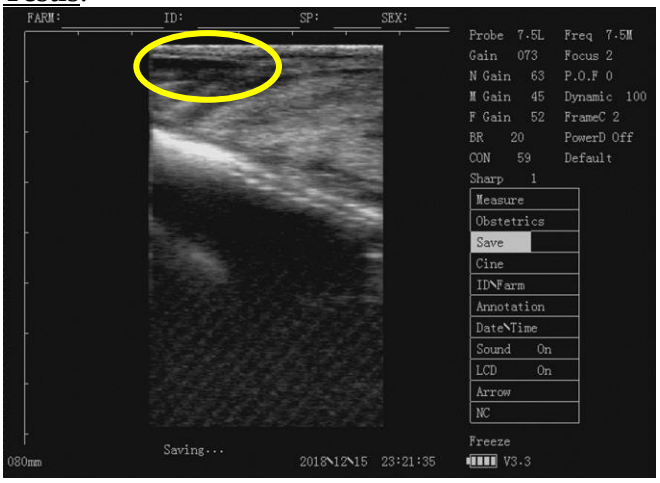


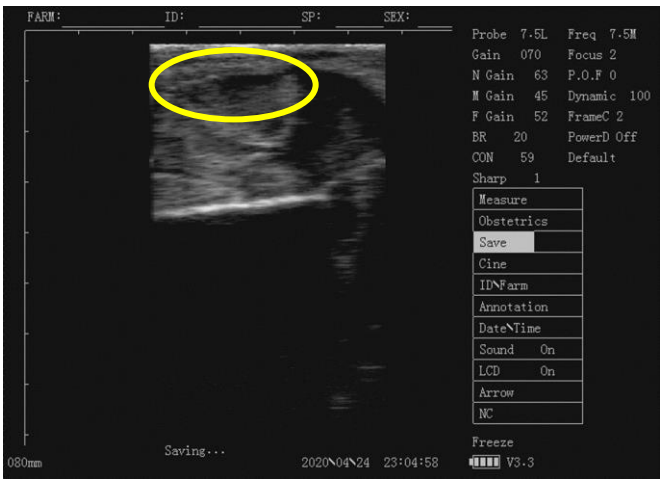
Egg Veil: Note speckled/grainy appearance near top of image





Testis:





Protocol 3: Blood Sampling

This section describes the process of drawing blood, centrifuging the blood sample, extracting plasma, and rapid freezing and storage of the plasma sample. These samples are being collected to test for hormone concentrations (estrogen, progesterone, testosterone, and vitellogenin).

Materials list

- Heparin 6 uits/µl (H-6279 Sigma Aldrich)
- 20G and 18G hypodermic needles
- 10 mL Luer-lock syringes
- Disposable transfer pipettes
- Micro-cryovials
- Labelled heparnized 6 mL BD vaccutainers (or sterilized centrifuge tubes)
- Lab gloves
- Small cooler full of chipped ice
- Styrofoam custom made sample holder insert for cooler
- Dewars container full of liquid nitrogen
- Sharps container (or Tupperware with lid)
- Cryocanes
- Panty hose
- Welding gloves
- Towel
- Centrifuge (3500 RPM)
- Three prong extension cord

Procedures

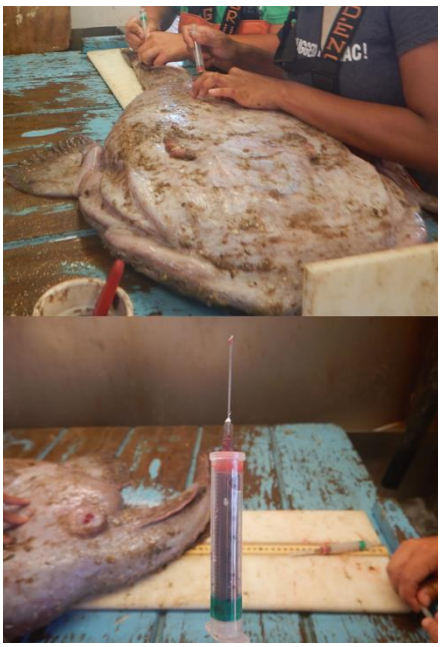
Setting up

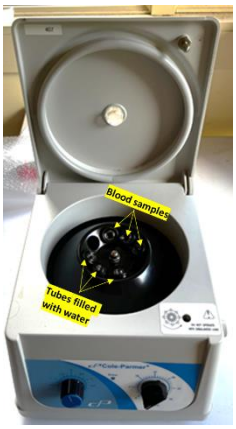
Fill a small cooler with chipped/crushed ice. Insert premade Styrofoam float*. This will be used for intermediate storage between fish samples, while collecting blood from other animals, and while transferring plasma into cryovials.

The goal is to obtain four 1cc plasma samples per individual. Therefore, the goal is to draw as much blood as possible. Select an 18- or 20-gauge (depending on fish size; use 18 for larger and 20 for smaller fish) hypodermic needle and attach to a Luer-lock 10cc syringe. To prevent clotting, draw 1 cc heparin solution** into syringe; swirl the liquid inside the syringe to coat the walls then return remaining liquid to heparin bottle.

Sampling

Insert hypodermic needle syringe into the artery that runs along the spinal cord, about ½" to 1" posterior of the urogenital opening. To get blood to flow, you may need to adjust the needle by pulling it in or out, adjusting the angle, or spinning the needle. Rubbing the heart, located in a cavity in the center of the chest, may help the fish pump blood. Draw as much blood as possible, cap the needle, remove the needle from the syringe, then transfer the blood to a labelled centrifuge tube. Tip the centrifuge tube to a 45° angle and allow blood to run down the side of the tube to the bottom. If necessary, use more than one tube. Bend the used needle with pliers and place in sharps container. Break used syringe and place in sharps container.





Fill another tube(s) with water to the same level as the blood tube(s), so the centrifuge will be balanced, then place the tubes in opposite positions in the centrifuge. Once the centrifuge is full (all tube spaces are filled), spin down for ten minutes at 3500 RPMs. Use a transfer pipette to extract the plasma and distribute 1cc into each of four microcryovials. If you don't have 4cc, fill as many cryovials as you can. Label the vials with the specimen ID number, cover the label and the lid with clear tape, insert vials into cryo-cane, and place canes in Dewars liquid nitrogen container. It is important to check the level of liquid nitrogen in the container often, as this container is used to storage and rapid freezing.

**To make a new float*: cut a Styrofoam or a thick piece of foam board so that it will just fit inside of the cooler. Cut a hole in the foam just big enough for the heparin bottle to fit inside. Then, drill holes the size of microcryovials and centrifuge tubes.

***To make heparin solution:* Mix 4.2 mL of saline solution (PBS pH 7.2) with 250 mg of heparin sodium salt (from porcine), to obtain *6 units/microliter solution.* If using other products, dilute to 6 units/microliter.

Protocol 4: Endoscope Technique

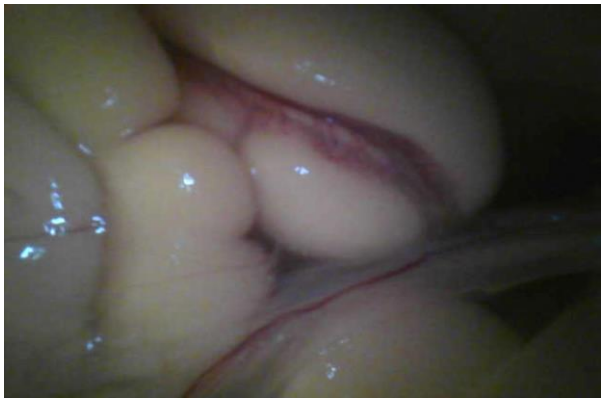
This section describes the process of endoscope evaluation, including incision method, exploration method, and visual assessment of sex and reproductive state.

Materials list

- Scalpel
- Endoscope/Borescope with Wi-Fi/Bluetooth
- A smart phone or Bluetooth enabled device with Depstech application downloaded

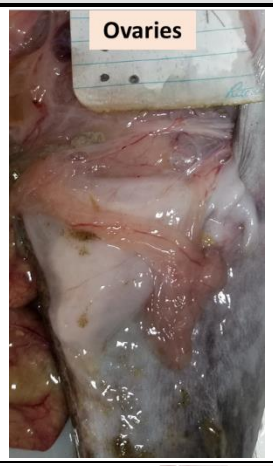
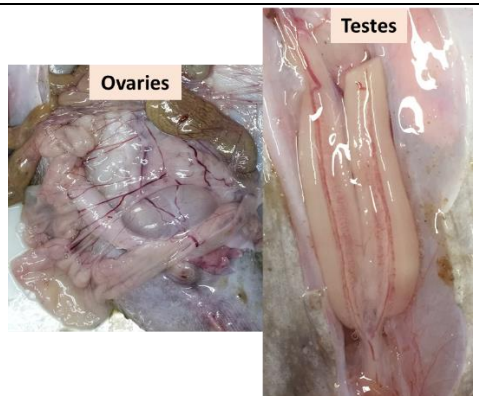
Procedure

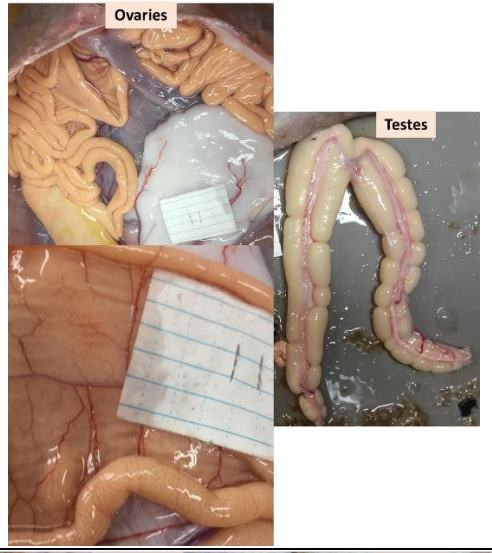
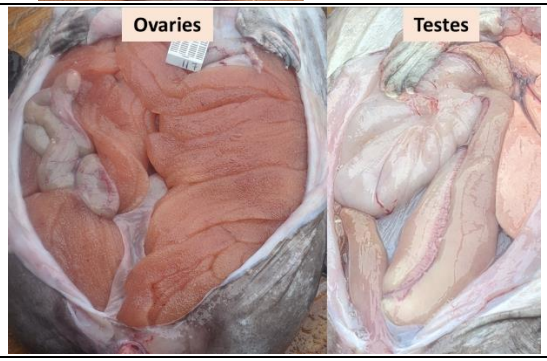
Make a small incision in the center of the abdominal wall by pinching the skin about halfway between the urogenital opening and the girdle fins and slicing an approximately ½ cm wide slit with a scalpel. Insert the endoscope into the slit in the abdominal wall to explore the body cavity. Starting just posterior to the girdle fins, identify the liver. Sweep the boroscope down along the fish's right side examining the internal organs. Locate the gonad, identify the sex (testes or egg veil), then characterize the maturity state in accordance with the following table.



The endoscope used in this project was a Depstech 2MP Wi-Fi unit that can be connected to the CFF tablet or any smart phone via a unique Wi-Fi connection (similar to GoPro). IMPORTANT – the program application must be installed in the viewing device for use.

Table 1. Macroscopic description of four maturity stages in the reproductive cycle of female and male of monkfish collected in SNE between December 2018 and June 2020 (based on Colmenero et al. 2013 and Johnson and Grier 2017).

Maturity stage	Female	Male	Macroscopic picture
I – Immature	Ovaries are very narrow, thin, and ribbon-like. They are translucent and no oocyte clusters visible and minimal vascularization.	Testes white to tan, similar in shape as mature testes but very small, medial groove less distinct. No males at this phase were identified during this project.	
II – Developing	Ovaries are small. Still no noticeable individual oocyte clusters. They become less translucent and vascularization is visible.	Testes are small with visible blood vessels around the seminal duct.	

III – Ripe	Ovaries increase in width and length. They have a light orange color, and blood vessels are prominent. The edges of the ovaries start to curl, and they occupy a larger proportion of the body cavity.	Testes increase in length and width. They have a firm texture and cream color. Seminal duct is highly vascularized.	
IV – Actively spawning	Ovaries are extremely long and wide and occupy most of the body cavity. The color of the oocytes is orange, and they are visible macroscopically. Ovaries are characterized by the presence of large hyaline oocyte clusters enclosed in a transparent gelatinous matrix that is completely developed. High vascularization is present.	Testes are large and firm and have a creamy coloration. Large amounts of sperm produced when testes are dissected.	
V – Spent	Ovaries gray, extremely flaccid, appear almost empty, atretic ova appear as black or white dots, moderately vascular.	Testes grayish-tan, edges appear translucent, extremely flaccid, small amount of milt sometimes present when dissected.	No individuals at this phase were identified during this project.

Protocol 5: Macroscopic Inspection

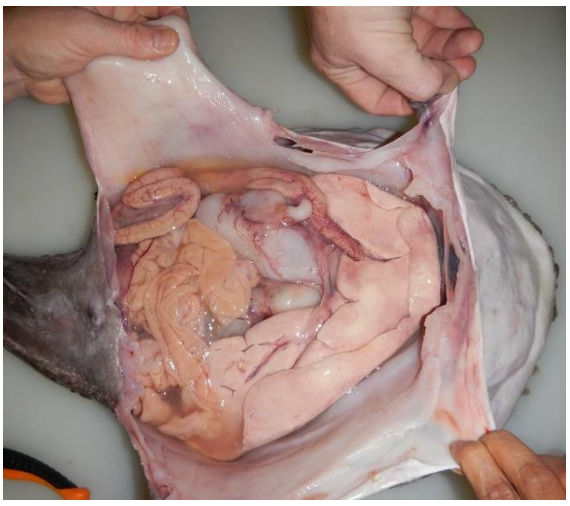
This section describes the process of macroscopic evaluation of monkfish for this project. This will include weighing of the liver, visual examination of the gonads, and notes on general condition of the specimen.

Materials list

- Dissection kit (scalpel, forceps, kitchen shears, small scissor)
- Gloves
- Camera
- Specimen ID label for all photos

Procedure

Expand the boroscope incision upward toward the heart, along the centerline of the underbelly, and then down to the urogenital opening. At either end of the incision, make perpendicular cuts, allowing a full view of the internal organs (see photo below).



Create a label with Specimen ID number for all photographs. Photograph internal organs with label in view. Photograph remove and weigh (g) the liver for hepatosomatic index calculation.

Photograph the gonads. Visually assess gonads, based on descriptions from **Table 1**. Remove and weight (g) the gonads.

Protocol 6: Histological Sampling

This section describes the process of removing the gonad and preserving a sample for histological processing.

Materials list

- Large jar full of Prefer fixative
- Ample supply of histology cassettes
- Scalpel
- Pencil to label cassettes

Procedure

Following macroscopic inspection, carefully and completely remove the testes or egg veil. Use a scalpel or small scissor to separate the gonad from the body cavity by cutting the thin mucoid layer that connects them. Weight the gonad (g) for gonadosomatic index calculation. Using a scalpel, collect a tissue sample from the middle of the gonad and place in a labelled (Specimen ID) histology cassette. Place the cassette into the jar of Prefer fixative or other fixative of your preference like formalin. For the purposes of this project, the samples were sent to a specialized laboratory to obtain the histology slides.