Sexuality and Embryogenesis of the Atlantic Hagfish, *Myxine glutinosa*

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OCEAN TECHNOLOGY 797, APRIL, 2001

Faculty Advisor: Dr. Stacia A. Sower
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Department of Biochemistry and Molecular Biology
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Sea Grant
New Hampshire/Maine

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ABSTRACT

The objectives of these experiments were to determine gonadal development under controlled laboratory conditions and to stimulate gonadal development through injection of lamprey GnRH III to potentially obtain fertilized eggs. Atlantic hagfish *Myxine glutinosa* were obtained from the Gulf of Maine. For the first objective a total of thirty hagfish were held for five months at 4 °C in a recirculating saltwater tank at the University of New Hampshire Anadromous Fish and Invertebrate Research Laboratory (AFAIR Lab). Maintenance of these hagfish required daily temperature checks, and transfer of new seawater every two weeks from the Coastal Marine Laboratory. Hagfish were sampled once a month for five months from November through March for weight, length and to determine the stage of gonadal development through histological examination. In November, most of the hagfish were classified as undetermined, i.e., they had not undergone sexual differentiation. During the next few months, the majority of hagfish were female. For the second objective, six hagfish were injected in February with 48 μg of microencapsulated lamprey GnRH III in an attempt to stimulate gametogenesis. Hagfish were sampled after one month for comparison to a control non-injected group (n=6). Subsequent histological analysis showed that lamprey GnRH-III appeared to stimulate reproductive development in female hagfish compared to controls

INTRODUCTION

Background

Hagfish belong to a group of vertebrates called Agnathans, or jawless fish. Hagfish and lampreys are the only organisms belonging to this group. All other vertebrates are in the group of Gnathostomes, or jawed vertebrates. Hagfish belong to the family Myxinoidae which is the only surviving family of the class Pteraspidomorphi. This family is monophylenic and contains two genera with 55 species total.

Hagfish are considered to be the most primitive extant vertebrates and thought to have changed very little in the past 330 million years (Martini, 1998). Recent phylogenetic studies have shown that lampreys are more closely related to Gnathostomes than they are to hagfish. If this is true, the closest relative to hagfish would be fossilized fish.

The oldest fossils found of jawless fishes are from the Paleozoic era (Bardack, 1998). The fossilized hagfish and modern hagfish are alike in many ways. They both lack paired fins, have a rudimentary skeleton made of cartilage called a notochord, a rudimentary braincase, or cranium, and have several finger-like whiskers extending from around the mouth (Martini, 1998). The main difference between these fish is that the fossilized fish have relatively large eyes and extant hagfish only have residual eyes. It is believed that the water depth where the fossilized hagfish lived was relatively shallow (Bardack, 1998). Today hagfish are found at depths ranging from 30 to 920 m where there is significantly less light. Over time hagfish may have lost their eyes because they were no longer of necessity for them.

General Habitat

Hagfish are found in marine waters throughout the world with the exception of the Arctic and Antarctic seas (Martini, 1998). *Myxine glutinosa* are found off the northern Atlantic coast, *Eptatretus stouti* off the east Pacific coast and *Eptatretus burgeri* off the west Pacific coast (Patzner, 1998). They can survive at a variety of depths but are mostly found near the bottom (Martini, 1998). *M. Glutinosa* can be found at 30 m in the northern Gulf of Maine (Bigelow and Schroeder, 1948) and has been found at depths of 1100 m off the edges of the North American continental shelf (Bigelow and Schroeder, 1953). Temperature appears to be the primary factor that limits the habitat of hagfish. *M. glutinosa* prefer temperatures less than 15^0C and are usually held between 0-4^0C in captivity (Martini, 1998). Most species of hagfish remain in burrows most of the day and only emerge at night (Fernholm, 1974). *Myxine* tend to live in transitory burrows among soft and muddy sediment, whereas *Eptatretus* live individually in long-term burrows among rocks or hard substrates (Martini, 1998).

Mode of Prey Capture

Hagfish mostly scavenge for food, but have been found to feed on live prey (Martini, 1998). They have a unique feeding apparatus that protrudes from the mouth and consists of two dental plates, each supporting two curved rows of sharp, horny cusps (Fernholm, 1974). A fang is located above the dental plate and functions to hold the prey in place. The hagfish extends the feeding apparatus and presses the dental plates against the surface of the flesh. The apparatus is then drawn back into the mouth to consume the flesh (Martini, 1998). This feeding device is not strong enough to tear through the scales of fish or the skin of many animals. The hagfish may enter through openings of live prey, such as the mouth, gills or anus, and consumes it from the inside out.

Sexual Differentiation

Past studies on *E. stouti* have shown all post-hatching hagfish to be female with sexual differentiation as progynous (Sower and Gorbman, 1999). The sex ratio in *M. glutinosa* is strongly unequal with females being much more abundant (Nansen, 1887; Conel, 1931). Initially the gonads are paired organs, but the left part of the ovary atrophies during early development (Felix and Buhler, 1906). The gonads of both sexes are situated in the peritoneal cavity (Patzner, 1998). The peritoneum is the smooth, transparent serous membrane that lines the cavity containing the digestive organs. The gonads are suspended from the ventral side of the abdominal cavity, which extends the entire length of the body (Sower and Gorbman, 1999). The ovary comprises the entire two-thirds of the anterior end of the membrane and the testis comprise the remaining third at the posterior end (Sower and Gorbman, 1999). Some hagfish have been found to contain both the ovary and testis and are considered hermaphroditic, but whether these individuals are sexually functional is not known (Patzner and Adam, 1976). Some hagfish may not develop either ovaries or testes and are considered sexually sterile (Patzner and Adam, 1976). Female differentiation occurs in the anterior portion of the membrane with the development of vitellogenic eggs and the eventual production of mature eggs. Male differentiation is determined with the degeneration of the oocytes and the development of spermatic follicles in the posterior end of the membrane (Sower and Gorbman, 1999).

Oogenesis and the Egg

The ovary is attached to the intestine by a mesovarium throughout its total length (Patzner, 1998). Oogenesis in hagfish is very similar to that of humans except that there are no oviducts. This process can be described based on studies of *M. glutinosa* (Patzner, 1977) and *E. stouti* (Tsuneki and Gorbman, 1977). The oogonia coming from the germinal epithelium develop into oocytes with a diameter of approximately 1-2 mm. The small oocytes migrate up the membrane, although growth within the membrane may just give the appearance of this upward movement (Sower and Gorbman,

1998). This stage can be identified through a microscope because of the movement of the nucleus from the middle of the cell toward the animal pole (Patzner, 1974). When the eggs reach a certain size (1-2 mm), they enter a resting period known by Patzner (1974) as *ovum expectans.* They resume growth after a preceding generation of eggs has been ovulated. Only a limited number of eggs will complete maturation. While the production of oocytes continues, the eggs that have not been selected become atretic (Sower and Gorbman, 1999). Mature eggs are identified as having vacuoles of approximately 8 μm in diameter (Patzner, 1998). At the end of egg development, the follicular epithelium is very thick, especially at the poles. The extra, folded epithelial material form hooks at either end of the shell called anchor filaments (Sower and Gorbman, 1999). Ripe eggs break up their follicles and the peritoneal cover, and are released directly into the coelomic cavity (Patzner, 1998). They are then deposited from the abdominal cavity into the water through the cloaca (Sower and Gorbman, 1999).

Females of *M. glutinosa* produce 20-30 yolky, shelled eggs at a time (Martini, 1998). The size of the egg of most hagfish species, excluding the anchor filaments, is approximately 20 mm in length with a width of approximately 8 mm (Patzner, 1998). Anchor filaments are formed on all hagfish except *Eptatretus carlhubbi* (Patzner, 1998). They are surrounded by a gel-like matrix and contain a single micropylar opening located at the animal pole (Koch et al., 1991). A micropyle is a pore that all fish have to allow entrance of the sperm and to guarantee fertilization of eggs with a thick outer membrane.

Testis and Spermatogenesis

The testis is situated in the peritoneal cavity and is formed from the folding of the membrane in the posterior end (Sower and Gorbman, 1999). The testis is composed of lobules consisting of spermatic follicles of varying sizes (Patzner, 1998). A thin layer of connective tissue encapsulates each of the follicles and only one developmental stage of spermatogenesis can be found in each (Patzner, 1998). Only *E. burgeri* has a synchronous development within the testicular

follicles (Patzner, 1977). Studies on the spermatogenesis in *M. glutinosa* (Walvig, 1963) have allowed for identification of the stages. The spermatogonia can be identified as round cells containing a round nucleus (Patzner, 1998). The primary spermatocytes are surrounded by the *liquor folliculi,* which provides separation and allows them to be packed less densely than the spermatogonia. The secondary spermatozoa can be identified as having the chromosomes arranged closer together. The early spermatids are still surrounded by small amounts of the *liquor folliculi,* which enables them to move within the follicle. The cells are now spherical and approximately 13-14 μm in diameter. The nucleus is also spherical and moves from the center of the cell toward the anterior pole. The secondary spermatids can be identified as having a longitudinal shape with a longitudinal nucleus. They are also characterized by numerous microtubules parallel to the axis of the cell (Patzner, 1998). The sperm are deposited into the abdominal cavity and then released into the water through a large pore in the cloaca (Martini, 1998). Mature testis or mature, motile sperm have rarely been found in captive or wild hagfish (Patzner, 1982).

Spawning and Fertilization

Walvig (1963) studied the reproductive cycle of *Myxine glutinosa* and found no synchronized, seasonal pattern. Hagfish present no externally differentiated sexual features and the mode of fertilization is still unclear (Sower and Gorbman, 1999). Patzner (1998) suggested that sperm are released as a sperm mass mixed with mucus that may adhere to the surface of deposited eggs or be incorporated into the cloaca. No detectable difference in the anatomy of the cloaca has been found between males and females of *M. glutinosa* (Kosmath et.al, 1981). Females do not show any structures that would allow for this entrance of sperm into the abdominal cavity (Kosmath et.al, 1981), so a method other than internal fertilization is likely. Other studies suggest that external fertilization in the open ocean is unlikely because of the small amount of sperm produced in comparison to the size of the eggs (Sower and Gorbman, 1999). Different suggestions have been made on the mode of fertilization, such as the function of the slime or the function of the burrows in

keeping the sperm and eggs in close enough contact to allow fertilization (Sower and Gorbman, 1999; Patzner, 1998).

Reproductive Hormones

The importance of studying hagfish reproduction lies in the fact that the myxinoids (hagfish) represent one the oldest lineages of extant vertebrates, which evolved over 550 million years ago. Gonadotropin-releasing hormone (GnRH) is the major hypothalamic neurohormone involved in mediating reproductive activity in all vertebrates (Sower, 2000). GnRH is a peptide composed of ten amino acids and released from the hypothalamus from appropriate internal and external cues, and then travels to the anterior pituitary where it stimulates the release of gonadotropins. The gonadotropins, luteinizing hormone and follicle-stimulating hormone (GTH, LH, FSH) enter the bloodstream and act on the gonads to stimulate steroidogenesis and induce the maturation of eggs or sperm (Sower, 1998).

Twelve primary structures of GnRH have been determined in various vertebrate species (Sower, 2000). In all GnRH peptides, the length and the regions of this molecule (the NH2-terminal, pGlu1 and Ser 4, and the COOH-terminal) have been highly conserved over 500 million years of evolution (Sower, 1998). Featured in this group are nine vertebrate GnRHs. The GnRH structures of three ancient fish species are in an Agnathan, the sea lamprey, *Petromyzon marinus* (lamprey GnRH-I and –III); an elasmobranch, the spiny dogfish shark, *Squalus acanthias* (dogfish GnRH and chicken GnRH-II) and a holocephalan, the ratfish, *Hydrolagus colliei*, (chicken GnRH-II) (Sherwood *et al.*, 1993; Sower, 1993; Lovejoy *et al.*, 1991; Lovejoy *et al.*, 1992).

Two forms of GnRH have been isolated and identified from the lamprey. Both lamprey GnRH-I and -III have been shown to induce steroidogenesis and spermiation or ovulation in adult sea lampreys (Sower, 1998). The difference between the two structures of lamprey GnRH lies in three amino acids. It has also been found that lamprey GnRH-III is more closely related to the other GnRH structures than lamprey GnRH-I (Sower *et al.*, 1993). Lampreys are the most basal

vertebrates next to the hagfish and serve as a demonstrative model of the multiple roles of GnRH molecules acting as reproductive neurohormones. Both lamprey GnRH-I and –III have been shown to induce steroidogenesis and spermiation and/or ovulation in adult *Petromyzon* (Sower, 1989; Sower, 1990; Sower, 1997). Thus, previous data may suggest that the structure and function of the GnRHs in vertebrates may appear to be highly conserved throughout vertebrate evolution.

However, in hagfish there seems to be a lack of, or poor regulation of reproduction by hypothalamic-pituitary peptides (Sower and Gorbman, 1999). Inadequate experimental support has confounded the presence and possible function of sex steroid hormones and hypothalamic hormones such as GnRH. In fact, GnRH has not been isolated in hagfish. Although, in two studies using antibodies to lamprey GnRH-III from Dr. Sower's laboratory, as well as other GnRH antibodies in Dr. Northcutt's laboratory, immunoreactive GnRH was detected in the brain of the Atlantic hagfish and Pacific hagfish (Sower et al., 1995; Braun et al., 1995). These data suggest the presence of a lamprey GnRH-III-like molecule in the hagfish brain and that hagfish may have reproductive control mechanisms that are similar to other vertebrates. Thus, in our experiment to enable us to obtain a fertilized Atlantic hagfish embryo, we injected microencapsulated lamprey GnRH-III into the hagfish in order to stimulate gametogenesis.

Hagfisheries

Hagfish have attracted increased attention from the scientific community in the past decade, not only due to their phylogenetic position, but also for their biological importance in sustaining the ecology of the marine environment. In addition, the fishery for hagfish has increased in the North Pacific rim and the Western North Atlantic between 1991 and 1996, where catch went from zero to 50 million fish in just five years (Martini, 1998). The role these fish play in their ecological community is also a concern for fisheries management because little is being done to ensure that the increased demand and catch does not pose a threat to the population. Over-fishing in Asia has significantly decreased the population of the hagfish, while Korea continues to consume 5 million lbs of hagfish meat each year (Barnaby, 1995).

The fishing industry is based on the supply and demand of the fish and consumers respectively. Generally fisheries have been managed by the demand method. This entails fishing until there are so few animals left that fishing has to completely cease to help regain a sustainable population number. Fishing will then resume and the process is repeated. The North Atlantic hagfish, *M. glutinosa*, was considered a nuisance in the bi-catch until recently. The fishing industry has undergone major changes with decreases in many of the different species of fish. In particular the toll on the fish stocks of marketable meat such as the cod, has probably undergone the most fluctuating existence of all the fisheries in the northeast. In the fall of 1995 George's Bank was closed to commercial fisheries, leaving commercial fisherman in the area looking for viable, marketable fisheries. As the hagfish fishery collapsed overseas, the Asian market was looking for another source for their prized catch that was no longer abundant in their waters. New England had a promising outlook, commercial fisherman were looking non-traditional catch, the species *Myxine glutinosa* was a quality equivalent to the Asian species in both skin and meat, and sufficient packing centers were available from the ports of Gloucester, MA, Portsmouth, NH and Stonington, ME (Nardi, 1995). The market for hagfish had grown, and a new commercial focus was on these fish. Most fish are either frozen on the East coast and shipped, or processed and shipped to he Asian market, where tanning of the eel skin leather and meat processing is completed. The American market for hagfish meat is being tested in New York City and Los Angeles by K.H. Cho, a Korean businessman who set up an eel processing company, the New England Eel Processor, Inc. located in Gloucester, MA (Nardi, 1995).

The increase in demand for products from this newly established fishery is a cause for concern for management officers and biologists. The fact that hagfish reproduce so slowly, and tend to cluster in groups on the bottom numbering 50,000 or more presents a problem for the population if fished heavily. Their low reproductive rate gives them little time to recover from a hagfishery that may take tens of thousands of hagfish from an area that might be fished numerous times throughout a season or successive seasons. It is important to gain an understanding of the reproductive and community behavior of these animals in order to manage this relatively new

market in the northeast. This young industry is an ideal test of the ability of scientists and fisherman to created a sustainable fishery for year to come, and maintain exploitable stocks continuously. This is a very opportune time, and a very fortunate time, for the hagfish fishery and scientists to determine the age, growth and reproduction of these hagfish.

Summary—Goals and Objectives

In summary, our overall goal was to contribute to our knowledge on the reproductive processes in hagfish and to try and obtain a fertilized hagfish egg. The objectives of our experiments were to describe gonadal development under controlled laboratory conditions and stimulate gonadal development through injection of lamprey GnRH III to potentially obtain fertilized eggs. Atlantic hagfish *Myxine glutinosa* were obtained from the Gulf of Maine. For the first objective eight hagfish were held for five months at 4 °C in a recirculating saltwater tank at the University of New Hampshire Anadromous Fish and Invertebrate Research Laboratory (AFAIR Lab). Hagfish were sampled once a month for five months and the stages of gonadal development were determined through histological examination. For the second objective, six hagfish were injected with lamprey GnRH-III along with a non-injected control group. These hagfish were then examined for gonadal maturation five weeks after injection by histological examination.

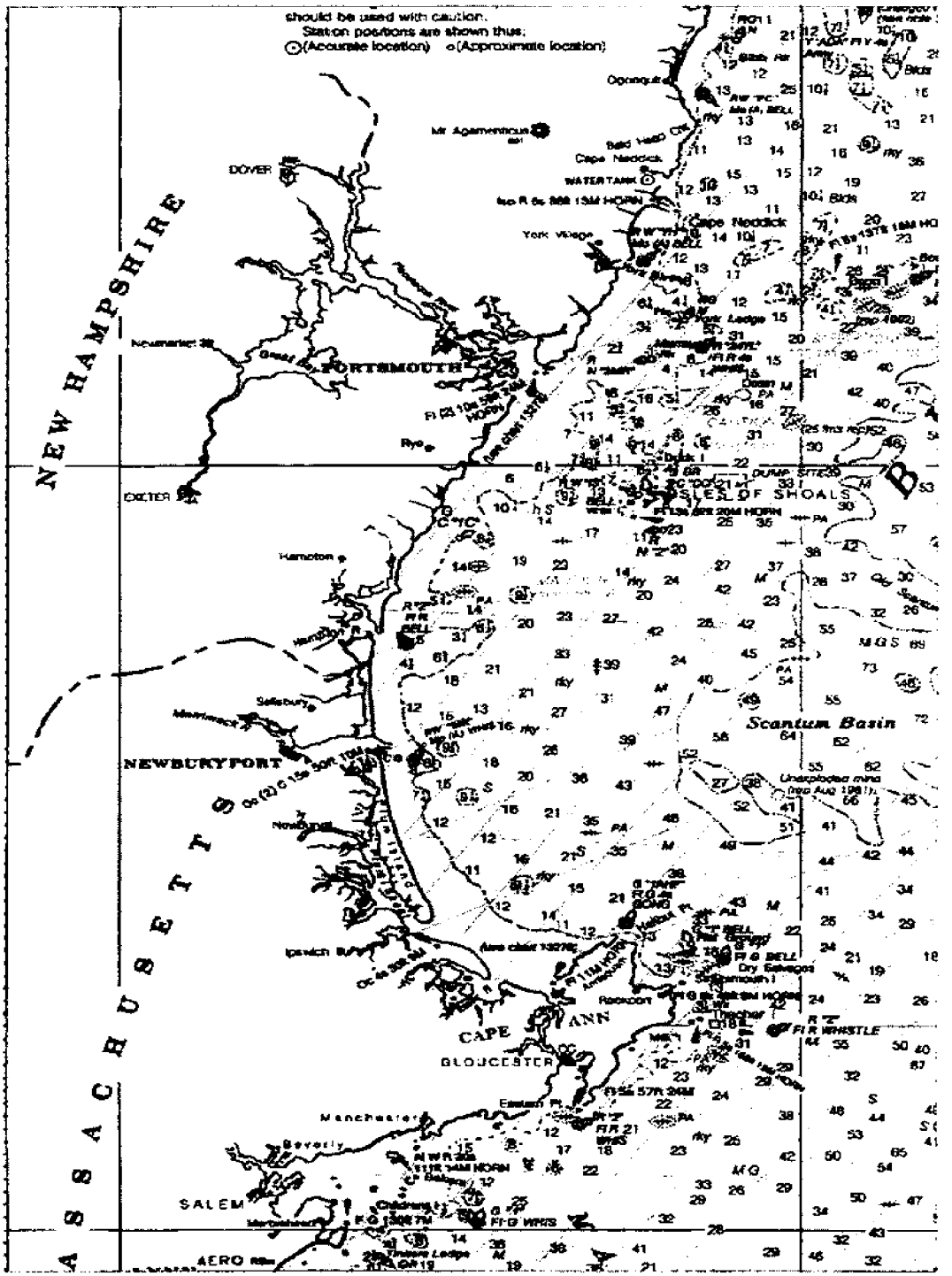
METHODS AND MATERIALS

The experiment to obtain fertilized eggs from the Atlantic hagfish, *Myxine glutinosa*, was divided into two objectives based on two separate collections of hagfish. The two objectives were 1) to determine gonadal development under controlled laboratory conditions and 2) to stimulate gonadal development through injection of lamprey GnRH III to try and obtain fertilized eggs.

Objective One: To determine, in part, reproductive development by histological examination.

On November 4, 2000, one hundred and fifty Atlantic hagfish were caught approximately 16 km off the Isle of Shoals at depths ranging from 84 to 107 meters (for coordinates see Fig. 1). Five modified pickle barrels (208-liter drums) containing sediment and bait were used for the collection. When the barrels were brought aboard, the newly caught hagfish were first removed and then weighed, measured, and divided according to sex by external palpitation. Upon returning to the laboratory, twenty-one hagfish were dissected for brains and pituitaries (for use in other studies) and gonads. Thirty-three hagfish were then kept first in a cold room at Rudman Hall and then maintained in a large aquarium at the Anadromous Fish and Invertebrate Research Facility (AFAIR) at UNH for monthly sampling.

Figure 1: Trap Coordinates on Map (See Appendix A for numerical coordintes).



Holding and Transfer

The hagfish for Objective 1 were first maintained in the cold room, Rudman G45, at UNH starting from November 4, 2000 and then transferred on December 18, 2000 to the AFAIR laboratory at UNH. The initial plan was to maintain the hagfish in the AFAIR laboratory in a large aquarium that was originally built for freshwater. This required modifications of the tank to hold seawater and to obtain a functional chiller. Chillers were obtained from the Coastal Marine Laboratory, however, these chillers were not operable and not seawater ready. Thus, these chillers had to be repaired and coils had to be obtained to change these chillers to be used in seawater. The costs of these chillers for repair and modification were approximately $3,000. Thus, the hagfish were held in the cold room adjacent to Rudman G45 from November 4, 2000 until December 18, 2000 until the seawater chillers were ready. The cold room was kept at a fairly constant temperature of 4° C, and the thirty-three fish were divided into three fifty-gallon buckets containing saltwater and about three inches of sand substrate. The installation of the chillers were completed on December 18, and the hagfish were then transferred to the AFAIR laboratory.

Aquarium Conditions, Maintenance and Monitoring

The aquarium in the fish hatchery measures approximately 472.4 X 105.4 X 38.1 cm and holds 1703.3 liters of water (See Figure 2). For the first part of the experiment, it was partitioned into four equal sections, each with the same conditions. Each partition was prepared with about three inches of sediment, two air stones, and black plastic covering. The covering ensured the hagfish were kept in constant darkness, only exposed to light for maintenance and monitoring. The chiller again kept the temperature fairly constant at 4° C.

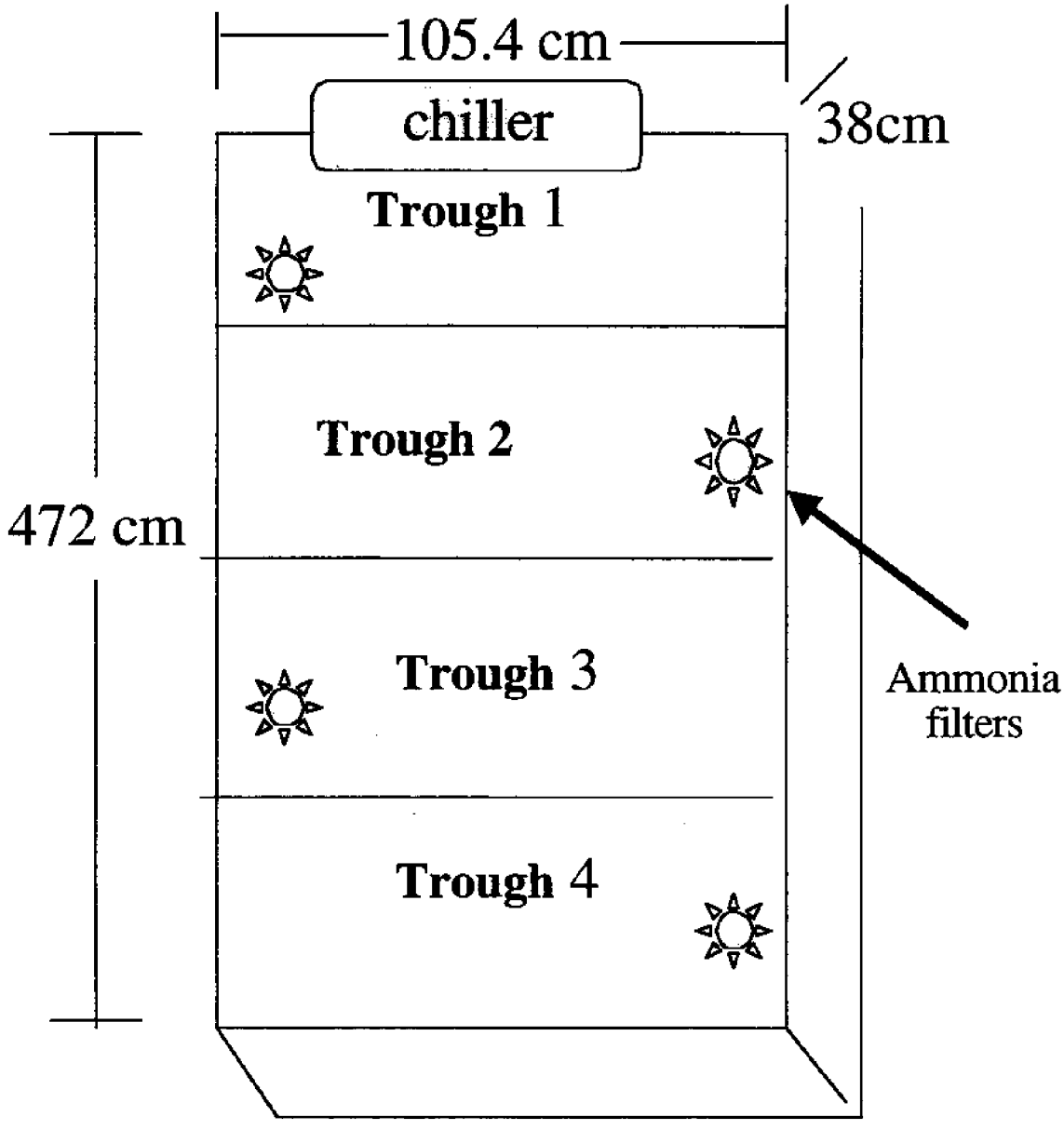
The holding tank required daily and monthly maintenance (See Table 1). The temperature was recorded daily and the tank was monitored for any changes, fatalities, and to check on the general condition of both the aquarium and the hagfish. Every two weeks the tank was emptied and filled with about 1510 liters of fresh seawater transported from the Coastal Marine

Table 1 This table is a summary of the aquarium (hagfish holding tank) maintenance. In February, a seawater filter was installed so that the seawater did not have to be changed until the end of the experiment.

Tank Maintenance

Sea Water Changed	Sediment Washed	Hatchery Cleaned
8-Dec	20-Jan	18-Dec
18-Dec		5-Jan
28-Dec		27-Jan
11-Jan		3-Feb
20-Jan		10-Feb
3-Feb		17-Feb
15-Feb		24-Feb
		3-Mar
		10-Mar
		17-Mar
		24-Mar
		28-Mar

Figure 2. Schematic Diagram of Aquarium—the holding tank for the hagfish.



Lab to keep the water clean and remove any accumulated slime and/or waste. This transfer of seawater generally required two vehicles, the Coastal Marine Laboratory truck and a van rented from Merchants, and generally three to five people. In addition, once a month the tank was completely emptied of both water and sediment. The sand was then transported back to the Coastal Marine Laboratory where it was thoroughly rinsed using seawater. At this time, the tank was scrubbed down and also thoroughly cleaned.

In order to reduce the major effort and time of the people and the financial cost of changing the seawater, an ammonia filtration system was designed in January by Dr. Mickie Powell and added to the aquarium. A modified 19 L bucket containing bio-balls was added to each of the four sections of the tank. Air stones were placed at the base of a PVC pipe adjacent to the bucket that pumped water to the top. A second air stone was located below the mesh bottom of the bucket to pull filtered water away. This system helped reduce accumulation of highly toxic ammonia to nitrite and then nitrate. After this system was installed, the seawater did not have to be transported.

Dissections and Gonadal Histology

The hagfish were sampled every month for length, weight and gonadal tissue in order to determine gonadal maturation. Dissections of hagfish occurred after being anesthetized with approximately 10% Finquel (Tricaine Methanesulfonate; MS222; Argent Chemical Laboratories). On the following dates, the number of hagfish were sampled on each of the following dates: 21 hagfish on November 4th, 2000, 11 hagfish on January 12th, 2001, 10 hagfish on February 9th, 2001, 7 hagfish on February 22nd, 2001, and 37 hagfish on March 28th, 2001. On February 9th, twelve hagfish were collected from ocean traps and transported to UNH to be used for Objective 2 for both lamprey GnRH-III injected (n=6), non-injected (n=6) . The length and weight of each hagfish sampled were recorded. The heads of the hagfish were then decapitated and gonadal tissues were dissected from the posterior end of each hagfish from all groups. The gonads that were collected were fixed in Bouins-Hollande sublimate solution (BHS) and then dehydrated by a

series of dilutions of ethanol for embedding in paraffin wax. Two designated group members performed tissue preparation, which included cutting gonadal tissue at 8 to 10 μm, placing them on slides and staining the gonads using hematoxylin and eosin. The gonads were then viewed under a microscope to determine the gonadal stage following the descriptions stated by Sower and Gorbman, 1999 and Patzner, 1998.

Objective Two: To stimulate hagfish reproduction by injecting hagfish with *Lamprey GnRH-III*.

Collection

On February 15, 2001, the traps were again deployed in the ocean for hagfish collection February 22, 2001. Thirty hagfish were caught, and were measured, weighed, and separated according to sex in a similar manner to the first collection. Ten of the hagfish were dissected for histological purposes while the remainder of the second collection was used for the second objective, to stimulate the production of fertilized eggs with lamprey GnRH-III.

Table 2 This table is a summary of the number of hagfish moved, dissected, or caught on certain dates for the two objectives.

Event	Dates	Remaining Number of Hagfish in Sections			
		#1	#2	#3	#4
33 Hagfish caught	**4-Nov**				
Moved to hatchery	18-Dec	9	8	8	8
3 Dissections	12-Jan	8	8	7	7
8 Dissections	19-Jan	7	5	5	5
2 Hagfish missing	19-Jan	5	5	5	5
10 Dissections	9-Feb	2	2	3	3
10 Moved to small tank	17-Feb	0	0	0	0
37 Hagfish caught	**21-Feb**				
37 Moved to hatchery	21-Feb	10	15	6	inject 6
37 Dissections	28-Mar	0	0	0	0

Experimental Design

Before beginning the second experiment, the remaining hagfish not yet dissected were moved to a smaller tank in the hatchery with the same conditions of the large tank. The new hagfish were then moved into the large tank with new seawater and clean sediment. The four partitions of the tank allowed for both experimental and control groups.

Immediately after returning from the collecting trip on Feb. 15, the hagfish were anesthetized with *MS222* and then weighed and measured. The six individuals of the experimental group were then injected with 48 micrograms of the lamprey GnRH-III in an implant (Syndell Inc.) using a Ral Gun (Syndell Inc.), and they were placed in the fourth trough of the tank. Six more fish without the injections were placed in the next trough as the control group. Fifteen extra fish from the trip were placed in the second trough while the ten previously caught hagfish were moved to the first trough.

The hagfish were then held in the large aquarium tank with daily monitoring from February 22, 2001 to March 28, 2001, or for a total time period of approximately five weeks. On March 28, 2002, the experiment was terminated and all remaining hagfish were decapitated and dissected. This included the control and experimental groups as well as the hagfish from the original collecting trip in November and the extra hagfish caught on the February trip. All groups were dissected for brain, pituitary, and gonad tissues. The gonadal tissues were sent to the diagnostic lab where they were processed in the same manner as for the first objective, and the slides examined for reproductive and development stages. The data collected was then compiled, analyzed, and statistical comparisons were made. Means and standard errors were determined for each group. The numbers were too low for accurate statistical analysis.

RESULTS

Objective One: Hagfish held under artificial conditions.

The sex of each hagfish was determined after histological examination and placed into one of four categories including male, female, male/female (hermaphroditic) and those of undetermined sex (have not undergone sexual differentiation). The percent in each category, with females comprising the majority, remained relatively constant from January through March (Fig.3). The greatest difference was noted between the months of November and January. In November, 71% of the hagfish were of undetermined sex (they had not yet undergone sexual differentiation) compared to January that had 45% females and none were undetermined sex.

The mean weight of both female and male hagfish showed a trend of decreasing weight in from January through March (Fig. 4). The mean weights of male/female (hermaphroditic fish that contained both ovarian and testicular tissue) and of undetermined sex remained relatively constant with an approximate mean of 60 g (Fig. 4). There was no change in the mean length of hagfish between November and March (Fig. 5).

Figure 3. Percent of hagfish that are males, females, hermaphroditic (male/female) or undetermined (hagfish that have not undergone sexual differentiation) that were sampled in Nov, Jan, Feb and March.

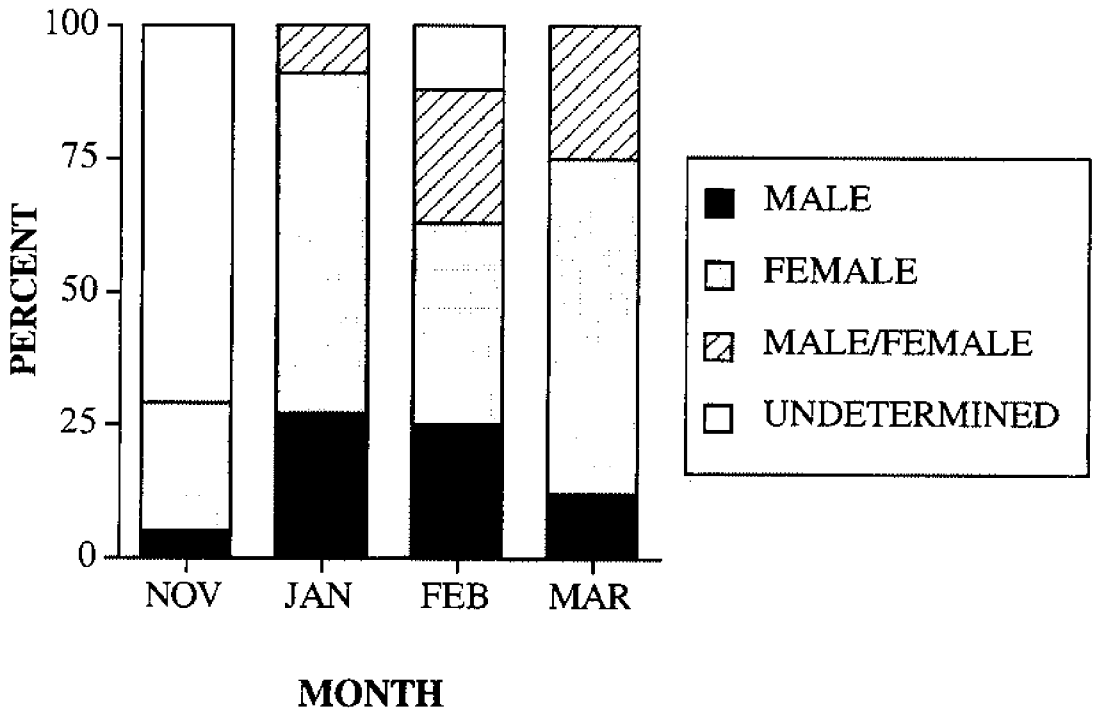


Figure 4. Mean weight and standard errors of hagfish that are males, females, hermaphroditic (male/female) or undetermined (hagfish that have not undergone sexual differentiation) that were sampled in Nov., Jan. Feb. and March.

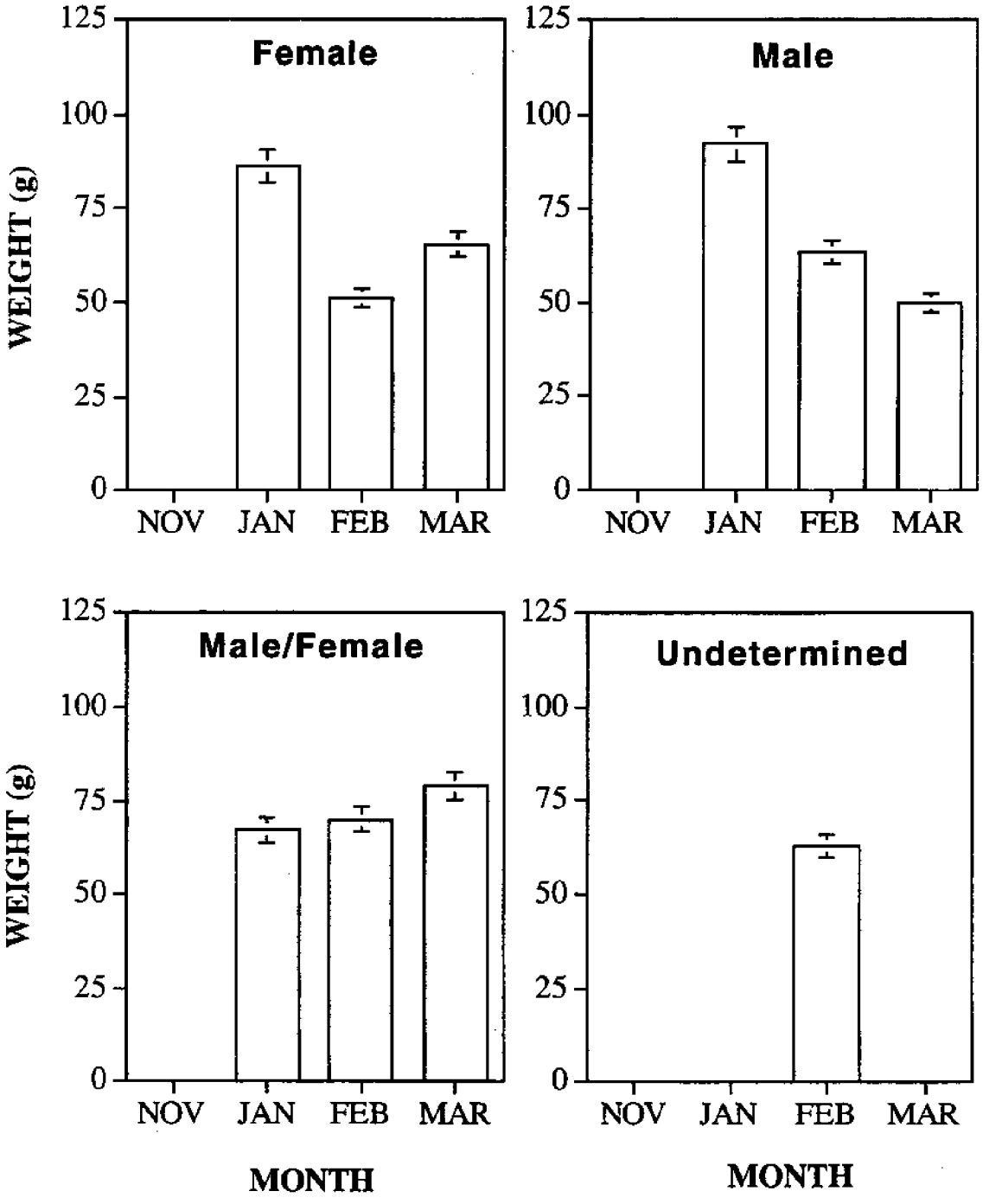
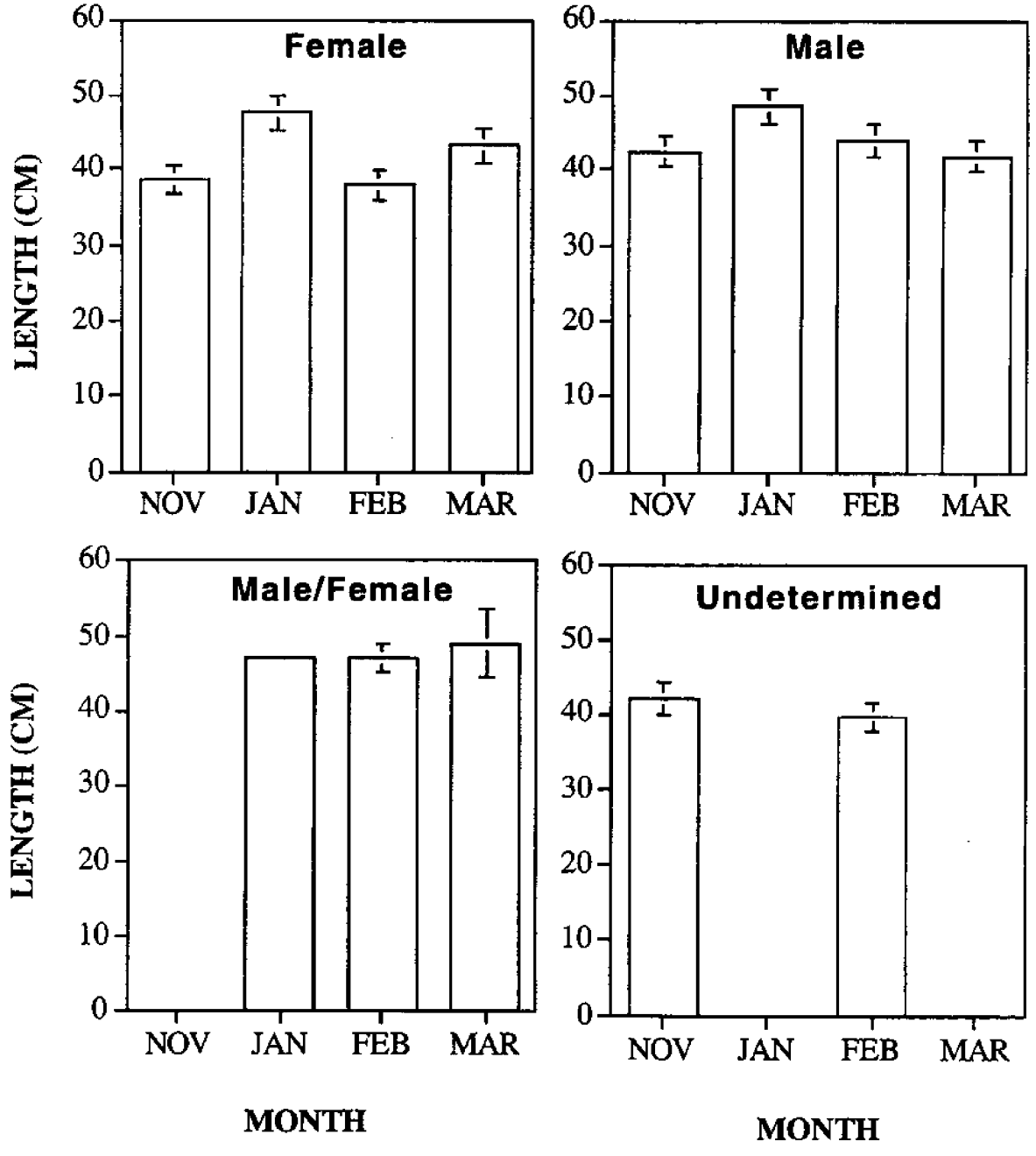


Figure 5. Mean length and standard errors of hagfish that are males, females, hermaphroditic (male/female) or undetermined (hagfish that have not undergone sexual differentiation) that were sampled in Nov, Jan, Feb and March.



Histological Analysis of Reproductive Development

A total of 33 hagfish were examined in this study. Reproductive development was determined by classifying oogenesis and spermatogenesis into distinct stages. Oogenesis was classified as containing either oogonia and/or early primary oocytes (F Stage 1), large primary oocytes (F Stage 2), yolk vesicle stage (F Stage 3) or large yolk granular stage (F stage 4) (See Fig. 6). Spermatogenesis was classified as containing either spermatogonia (M Stage 1) or primary spermatocytes (M stage 2) (See Fig. 7). No secondary spermatocytes or mature sperm were found in any hagfish. There were hagfish that were classified as undetermined, the gonads of these hagfish either had gonadal tissues that was undifferentiated or had gonadal tissue that had both testicular and ovarian tissue (Fig. 8). Three hagfish that were hermaphroditic that contained gonads with advanced stages of both testicular (M stage 2-primary spermatocytes) and ovarian (F stage 3- primary vesicles). This is an indication that these hagfish may be functional hermaphrodites. In November, we dissected 20 fish, with one in F stage 2, four in F stage 4 and 15 undetermined (Fig 9). The majority of hagfish between January and March were in F stage 1 with some females in later stages of development (Fig 9). Figure 9 shows a relatively equal distribution of stages in all remaining individuals.

Figure 6: Stages of oocyte development. **1.** Stage F1 – Oogonia. Nucleoli are apparent within the nuclear membrane. **2.** Stage F2 – Medium and large oocytes. Differentiated by developing follicular layers. **3.** Stage F3 – Yolk vesicle stage. Nucleus becomes irregular in outline; further development of follicular layers. **4.** Stage F4 – Yolk granular stage. Oil globules and yolk granules are present, and well developed thicker follicular layer

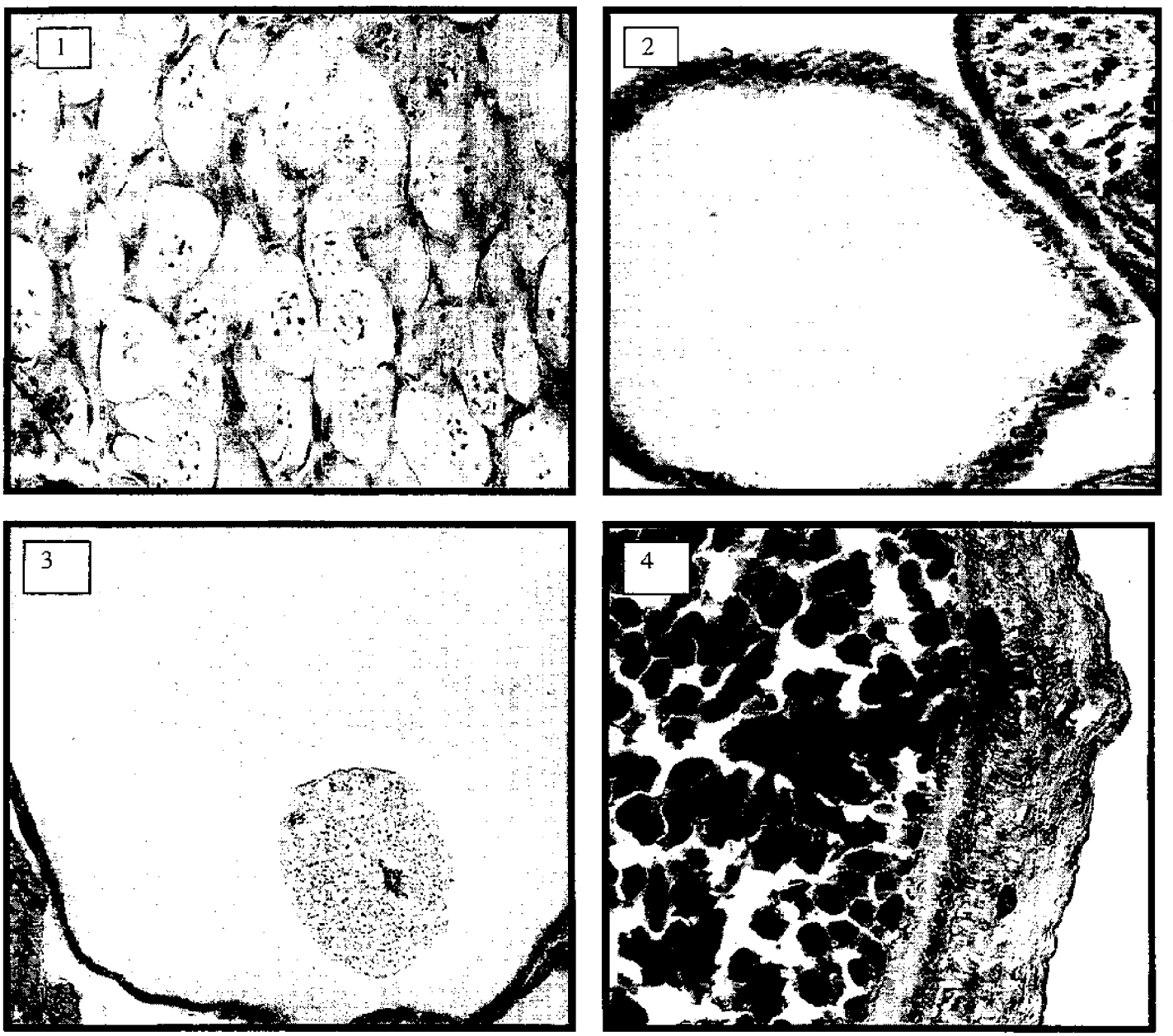
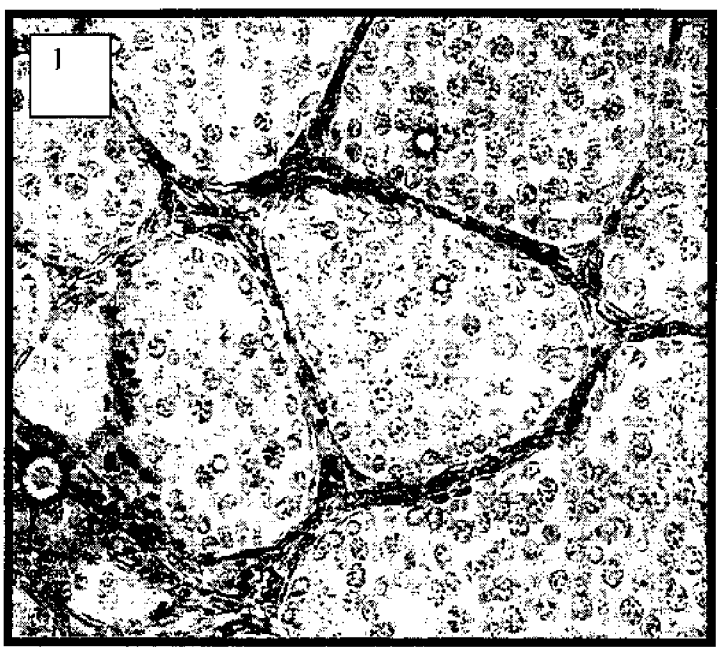


Figure 7: Stages of spermatogenesis. **1.** Stage M1 – Spermatogonia can be identified as round cells containing round nuclei. In each lobule, only one developmental stage of spermatogenesis can be found. **2.** Stage M2 – Primary spermatocytes are found within the *liquor folliculi*, which provides separation, and allow the spermatocytes to be packed less densely than spermatogonia.



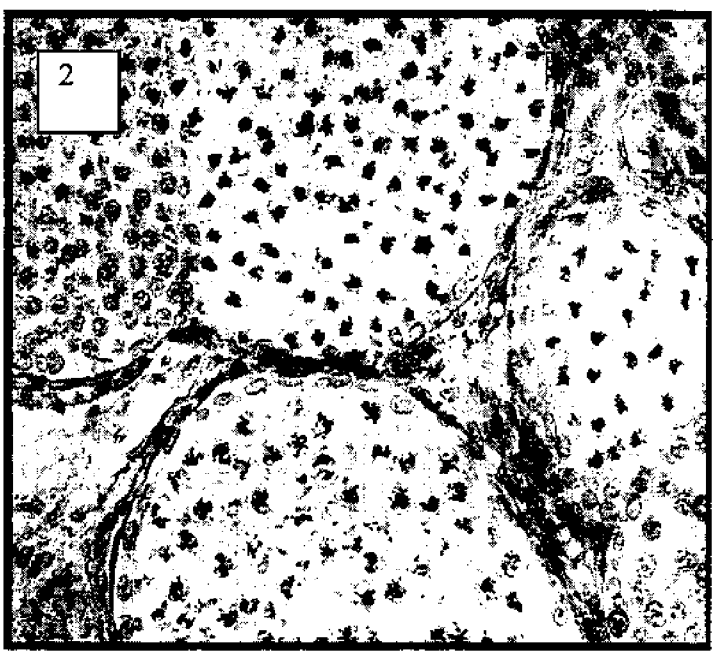


Figure 8: Several hermaphroditic hagfish were identified from this study (n=9). Hermaphroditic hagfish were defined as having both ovarian and testicular tissue. This figure shows a hermaphroditic hagfish that has both spermatogonia (Stage M1) and primary oocytes (Stage F2).

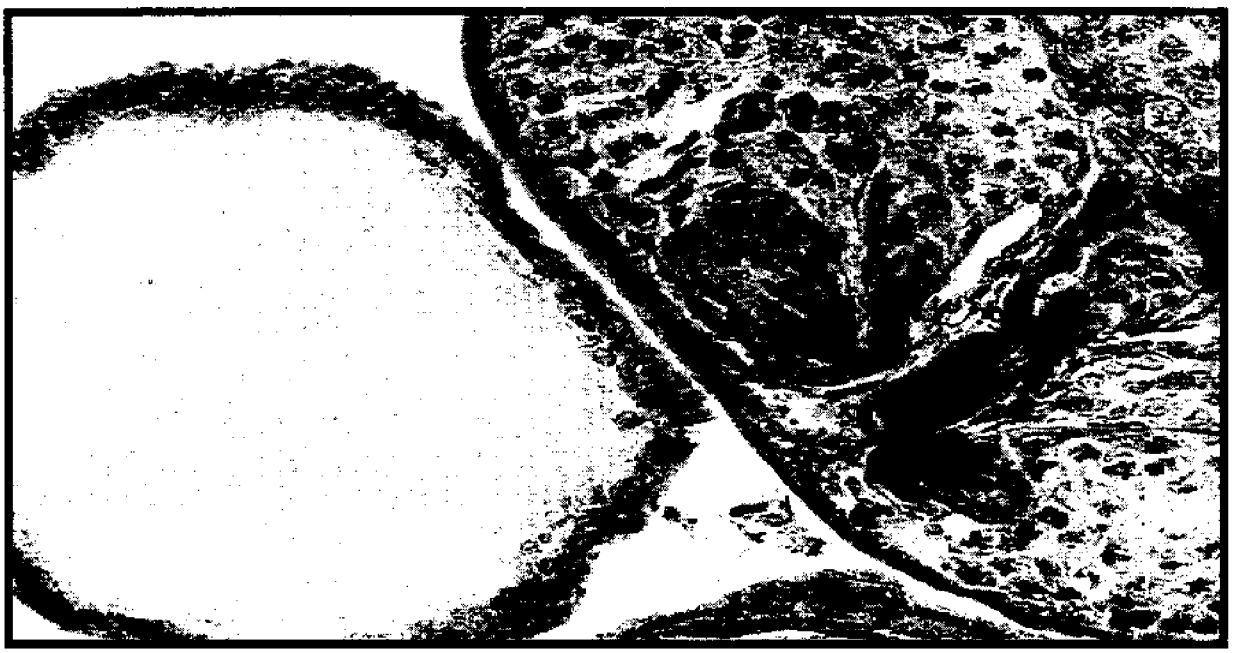
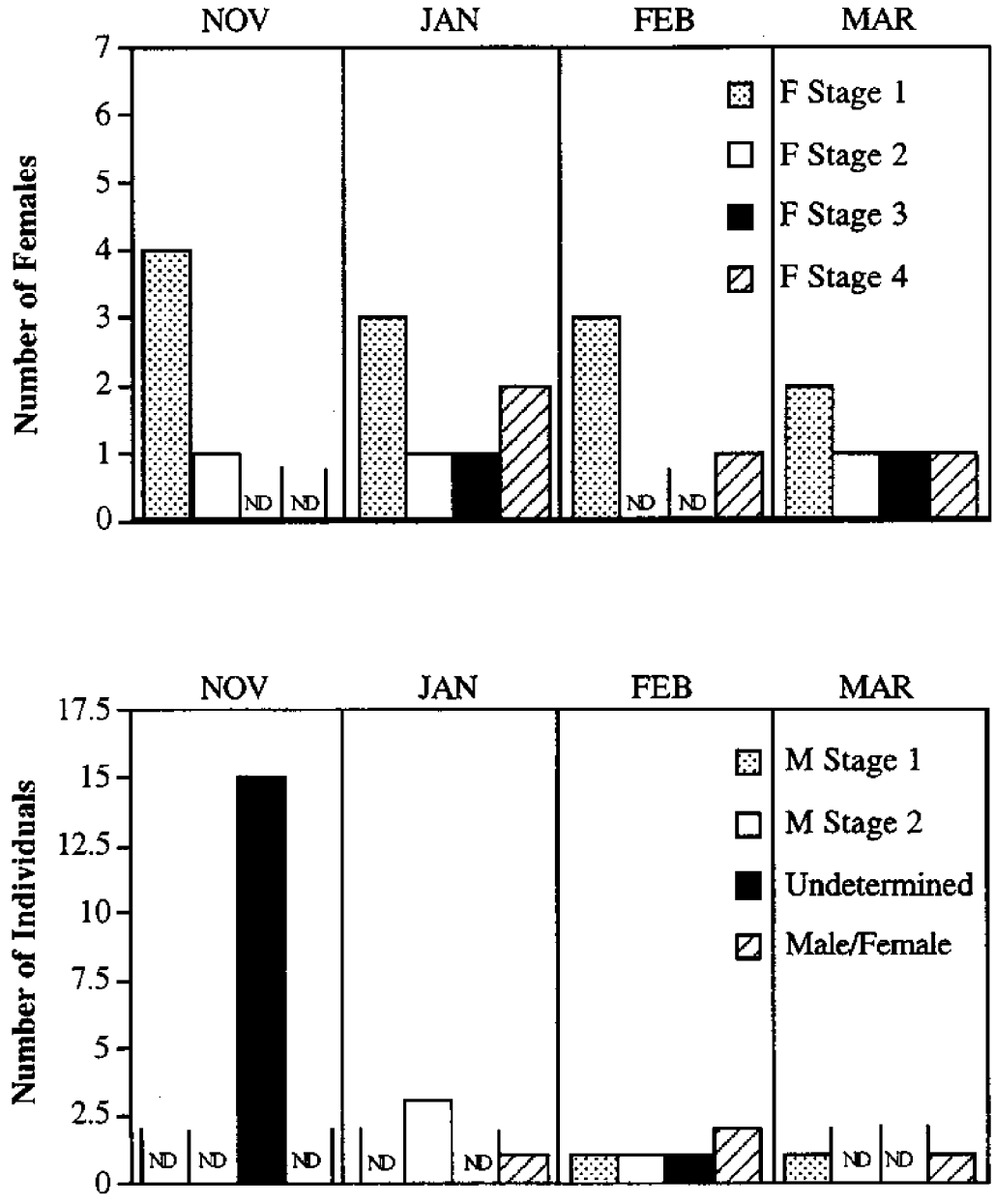


Figure 9. Number of identified females and males in the different reproductive stages from November through March.



Objective Two: Reproductive stimulation with GnRH injection.

A total of twelve hagfish from this study were examined for histological development following injection of GnRH (six hagfish) or non-injected (six hagfish) serving as controls. In Figure 10, as noted, GnRH-treated hagfish showed an increase in the number of females in F Stage 3 compared to the control group (one individual). A relatively equal distribution of stages was found in all remaining individuals of both groups (10). The numbers were extremely low so statistical analyses were unable to be used.

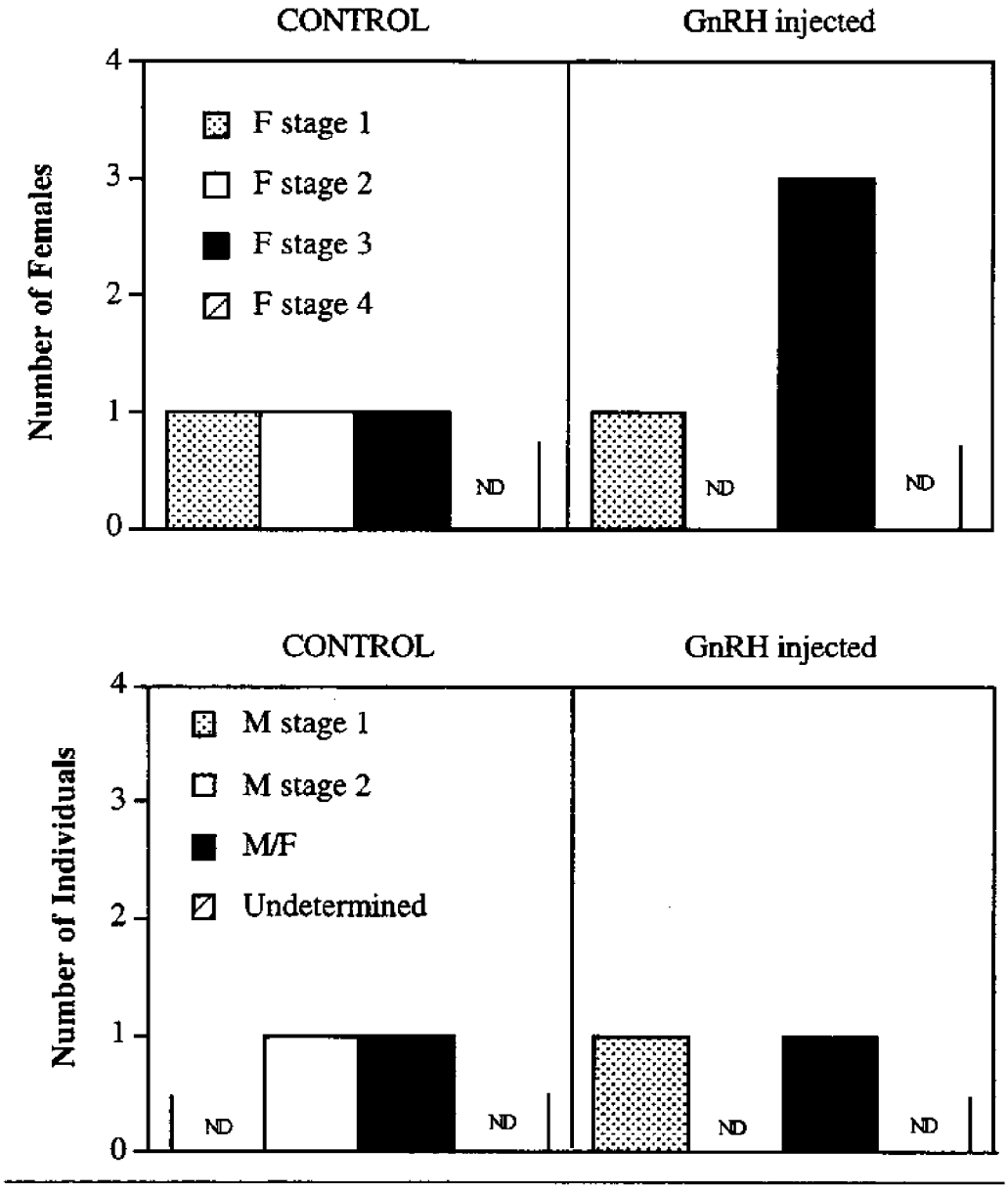


Figure 10. Reproductive stages of hagfish that were un-injected (controls) or injected with lamprey GnRH-III.

DISCUSSION

The reproductive development of hagfish has been a mystery to scientists for more than a century. The results of the histological examinations outlined in this study have contributed and helped to provide a clearer foundation on both the sex distribution and reproductive growth of the Atlantic hagfish, *Myxine glutinosa*. This study is also the first to indicate that this particular species of hagfish may act as functional hermaphrodites.

Previous studies on the sex ratio of *M. glutinosa* have shown an unequal distribution with females being much more abundant (Nansen, 1887; Conel, 1931). In our study, except in the first month of sampling, there was a greater percentage of females in January (70%), February (30%) and March (60%). The results of our study thus support the data from these previous studies. The timing of reproduction is unknown in this species of hagfish and it is unknown if they reproduce once a year or more than once a year. Our study showed a higher percentage of hagfish in November that were undetermined compared to hagfish from the January sample date. Undetermined status indicates that the fish have not undergone sexual differentiation, so the majority of animals collected in November were young and immature. Holding these hagfish for several months appeared to cause them to differentiate and mature with the greater percentage of nearly 50% being females. In addition, analysis of the reproductive development of the females over the five-month period showed a consistent decline in the number of females in Stage 1 of development to the more advanced stages of oogenesis from Stage 2 (primary oocytes) to Stage 4 (yolk granular stage). These data seemed to suggest either a time frame or season in which females reach sexual maturity, however, much more extensive studies need to be done to verify this statement.

The large number of hermaphrodites in this population is another interesting finding. As indicated by Gorbman (1997), adult hermaphroditism is rare, yet our results show that by February, an equal number of males and hermaphrodites could be found in this population. The functionality

of these male/female animals is questionable and Gorbman (1997) mentioned that they are most likely developing males in which the degeneration of the oocytes is delayed. If this were the case, we would expect to see an increase in the number of males in the population rather than a decrease as our results showed. Unlike previous studies cited by Gorbman (1997), the hermaphroditic hagfish had more mature stages of both ovarian and testicular tissue compared to what was previously reported. Thus, our data may be quite significant. However, further research with larger sample sizes will be necessary to determine the functionality and frequency of hermaphroditism in these species.

As opposed to the growth in reproductive development apparent in the females, a similar pattern was not found with the males. Neither, mature sperm or even secondary spermatocytes were found in any of the male hagfish that we dissected. In January, two males were found in Stage 2 (primary spermatocytes) of development and by March, only Stage 1 (spermatogonia) of development was found (Fig.11). Past studies on both captive and wild hagfish (Patzner, 1982), have found similar results, with the occurrence of mature testis or sperm rare. This indicates that the reproductive cycles of males and females do not follow a synchronized seasonal pattern. The reproductive cycle of *M. glutinosa*, as researched by Walvig in 1963, resulted in similar findings. If this is true, reproduction and the production of fertilized eggs in hagfish has become an even greater mystery, that is, unless these hagfish are truly functional hermaphrodites.

Our experiment with GnRH injection to stimulate gametogenesis resulted in limited yet exciting discoveries. Gonadotropin-releasing hormone is the major hypothalamic neurohormone involved in mediating reproductive activity in all known vertebrates (Sower, 2000). As explained earlier, GnRH has not been isolated in hagfish, yet the existence of reproductive control mechanisms similar to other vertebrates has been hypothesized (Sower et al., 1995; Braun et al.,1995). Our results showed an increase in the number of females in Stage 3 of development from one to three, in the control versus the GnRH injected, respectively. These are the first studies to show that GnRH may have an effect on reproduction in hagfish. Further experiments with a larger sample size on the effects of GnRH in stimulating gonadal development may hold profound

discoveries and enhance our understanding of the reproductive strategies of hagfish.

The majority of hagfish that were used in this study were captive and the effects of removal from their natural habitat are undoubtedly major. Temperature was monitored daily and the water was cleaned on a regular basis and replaced with fresh sea water to limit the build-up of toxins, yet other factors such as pressure were not incorporated, due to financial restraints. In order to make clear assumptions as to the reproductive strategies of hagfish, it is necessary to make comparisons between those held in captivity and those in the wild. Many factors, including weather and funding, limited this aspect of our research and we are unable to make suitable comparisons between our findings and those of hagfish caught from the ocean.

In addition, the sample size for our captive experiments was much smaller than originally intended and this impeded our ability to make statistically significant determinations from our results. The small sample sizes were also a result of the limited budget and presumably the weather conditions. Other factors, such as the effects of over-fishing also need to be taken into account when studying the sex ratio of the freshly caught hagfish. The use of the skin as leather and the limited knowledge that is known about hagfish adds to the problem of assuming our samples are representative of the population as a whole. It is a possibility that the reason we only caught juvenile hagfish in November is a product of over-fishing, rather than a result of the reproductive patterns of this particular species.

Extensions of this study and further investigations into the sex ratio, reproductive patterns and existence of reproductive control mechanisms are necessary to reveal the true nature of the hagfish. Contrary to the financial confines of this study, we have made some interesting findings and added to the present knowledge of the most primitive vertebrate known in existence.

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APPENDICES

Appendix A: Trap Coordinates

Appendix B: Tentative Budget

Appendix C: Sexual Differentiation of Hagfish

Appendix D: Evolutionary Timeline

Appendix E: Project Photographs

Appendix F: Internal and External Anatomy of the Hagfish

APPENDIX A

Trap Coordinates

First Collection November 4, 2000

Five Traps Dropped

Trap 1	4251.65N	07021.45W	107m
Trap 2	4251.75N	07021.28W	84m
Trap3	4251.87N	07021.62W	84m
Trap 4	4251.87N	07121.62W	84m
Trap 5	4251.20N	07121.17W	84m

Second Collection Febuary 15, 2001

Five Traps Dropped

Trap 1	4252.742N	7023.048W	111m
Trap 2	4252.711N	7022.660W	88m
Trap 3	4252.089N	7021.981W	82m
Trap 4	4251.930N	7021.817W	84m
Trap 5	4251.851N	7021.601W	84m

Appendix B

Tentative Budget—OCEAN TECH COURSE

Supplies.....$55.46

Transportation.....$427.68

RV/Gulf Challenger.....$162.00

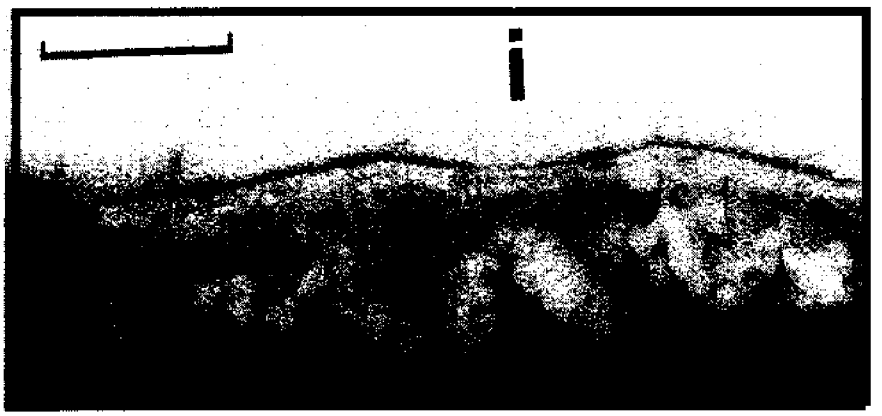
Histology.....$441.31

Total.....$1086.45

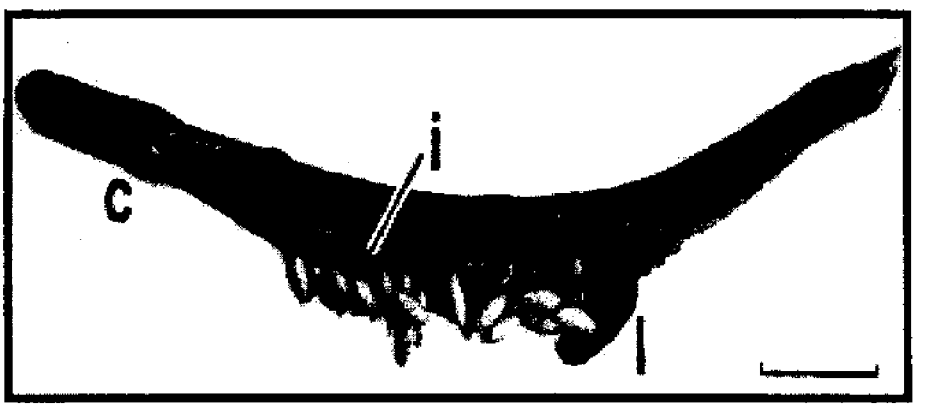
ADDITIONAL ITEMS—SOWER GRANTS-SEA GRANT AND OTHER FUNDS

Repair and Renovation of Chillers:	$3000
GnRH-III	$600
Injection Supplies	$180
Photocopying—color pages	$60.

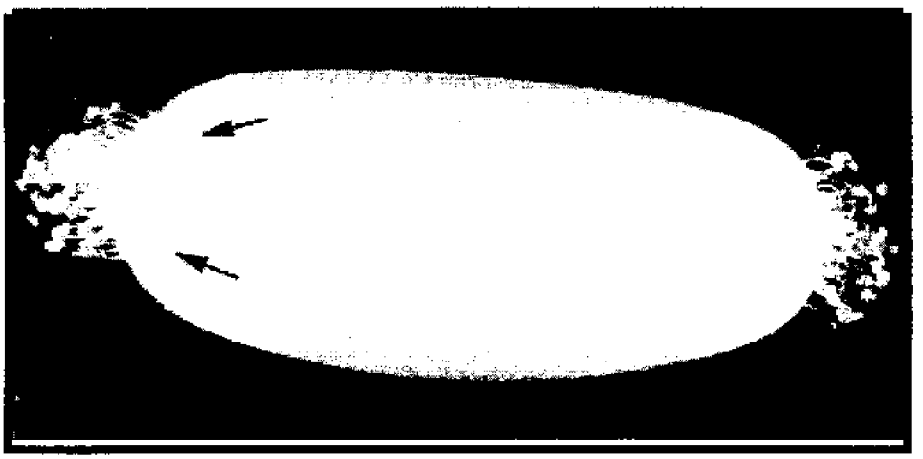
Appendix C



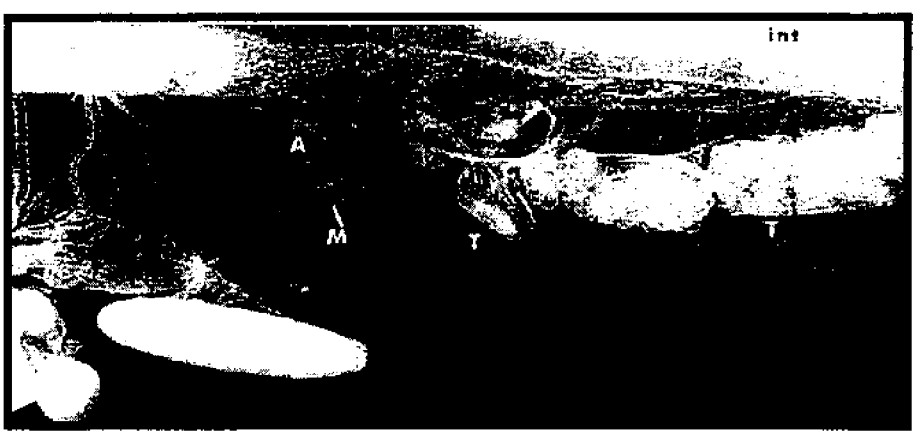
Appendix C1: Mature hagfish testes of *Paramyxine atami* (scale shows 10 mm). Testicular lobules are fixed to the intestine (I) by a mesenterium and are situated within the peritoneal cavity. From Patzner, R.A. 1982. Die Reproduktion der Myxinoiden. Ein Vergleich von *Myxine glutinosa* und *Eptatretus burgeri*. Zoolgischer Anzeiger (Jena), 208:132-44.



Appendix C2: Ovary of *Myxine glutinosa* with nearly mature eggs in the peritoneal cavity (scale shows 5 cm).
I = intestine, l = liver, c = cloaca. The ovary is attached to the intestine by a mesovarium throughout its total length. From Patzner, R.A. 1982. Die Reproduktion der Myxinoiden. Ein Vergleich von *Myxine glutinosa* und *Eptatretus burgeri*. Zoolgischer Anzeiger (Jena), 208:132-44.

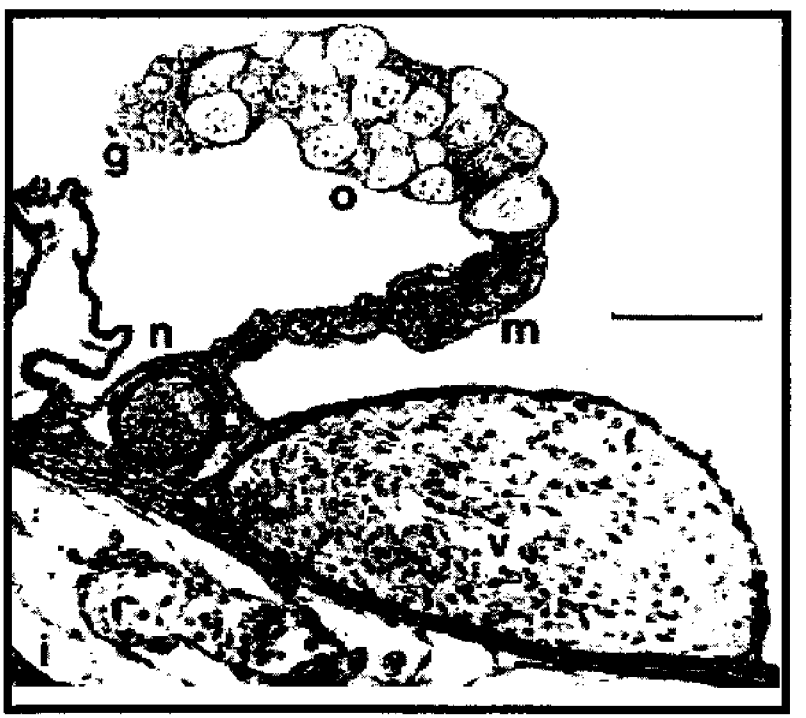


Appendix C3: A mature hagfish egg with a length of 18.5 mm, without anchor filaments. Arrows indicate the opercular ring where a young hagfish will hatch and break though the shell. From Patzner, R. 1978, Cyclical changes in the ovary of the hagfish Eptatretus burgeri (Cyclostomata). Acta Zool (Stockholm) 59:57-61.



Appendix C4: Gonad of an adult hermaphroditic hagfish, *Eptatretus stouti*. The testis (T) occupies the posterior end of the gonadal membrane. The ovary (O) contains a large vitellogenic egg. In this specimen large eggs are normal in shape but reduced in number. The mesovarium (M) contains an atretic egg (A) dorsal to the large egg. From Gorbman, A. 1997. Hagfish development. Zool. Sci. 14:375-390.

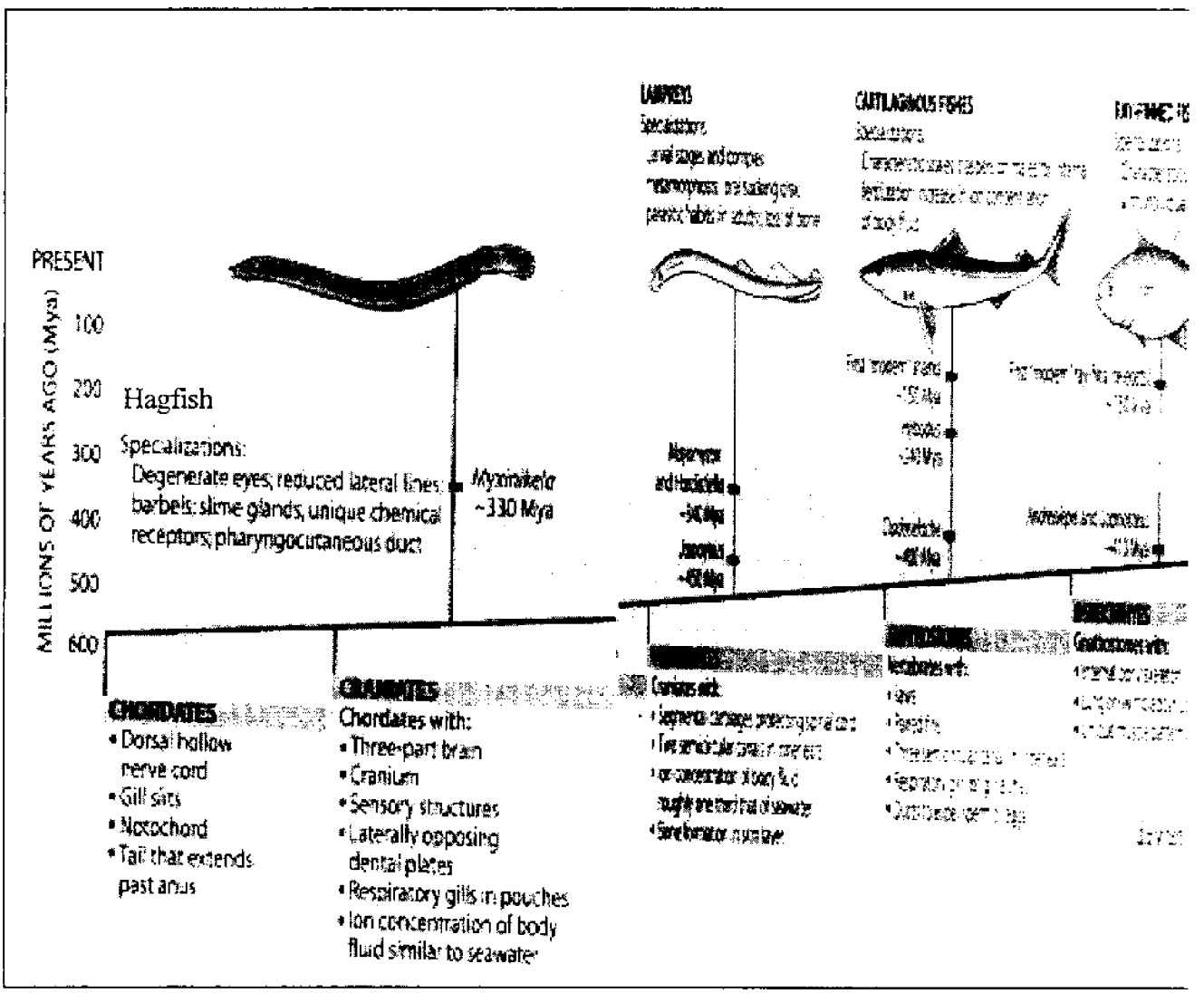
Appendix C5: Cross-section through a testis of *Eptatretus burgeri* (scale shows 250 um0. The testis is composed of follicular lobules of different sizes. In one lobule only one developmental stage of spermatogenesis is found. From Patzner, R.A. 1982. Die Reproduktion der Myxinoiden. Ein Vergleich von *Myxine glutinosa* und *Eptatretus burgeri*. Zoolgischer Anzeiger (Jena), 208:132-44.



Appendix C6: Cross-section through the ovary of a 15 cm long Myxine Glutinosa (scale shows 0.1 mm). Germinal epithelium (g) can be distinguished from the group of oocytes (O). The ovary is attached to the intestine (i) by a mesovarium (m). A nerve (n) and the vena suprintestinalis (v) run along the intestine. From Patzner, R.A. 1974. Die fruhen Stadien der Oogenese bei Myxine glutinosa L. (Cyclostomata). Licht- und elektronenmikroskopische Untersuchungen. Norwegian Journal of Zoology, 22:81-93.

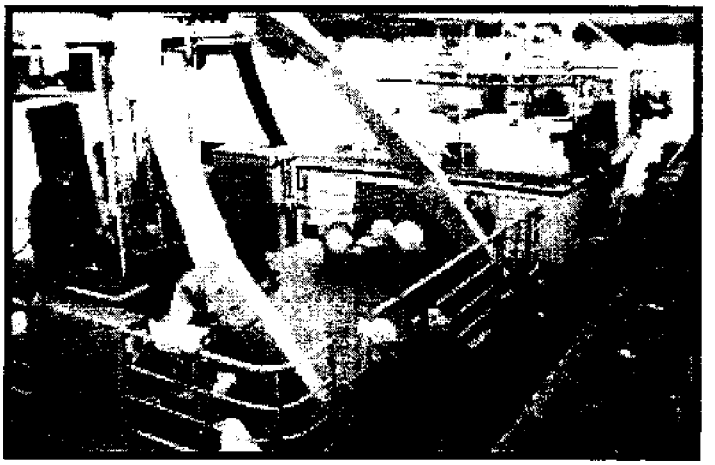
Appendix D

Appendix D: A representation of the evolutionary lineage from hagfish to modern fish. The hagfish is the most ancient of these groups. The fossilized remains of *Myxinikela* are over 330 million years old. Jana Brenning and William C. Ober drawing from martini, 1998. Secrets of the Slime. Scientific American 10: 70-75.



Appendix E

Appendix E1: Loading hagfish traps onto the Gulf Challenger.



Appendix E2: The Gulf Challenger loaded up and ready to go.

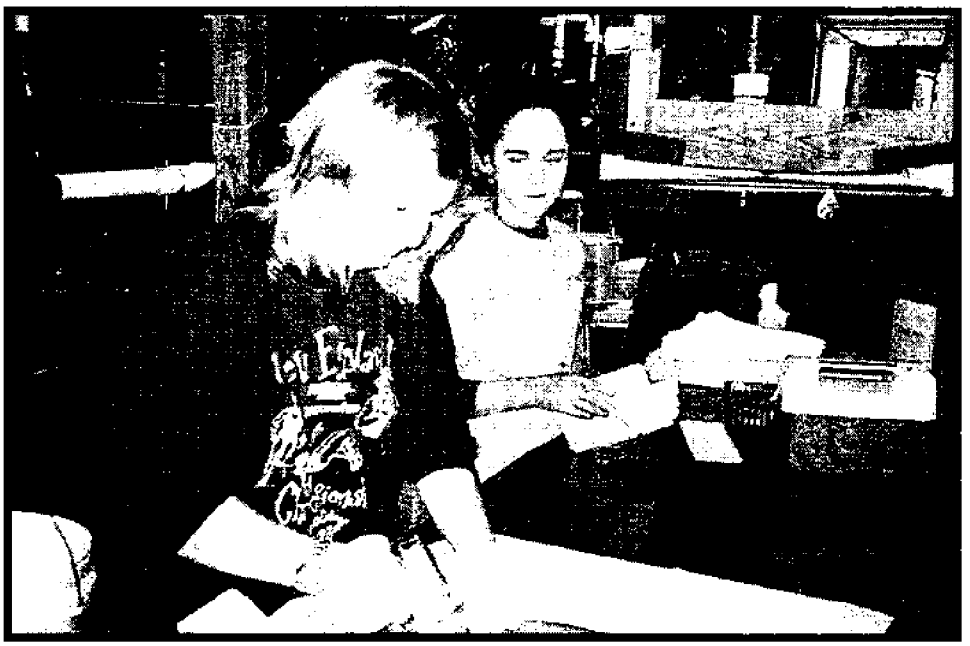
Appendix E3: Baiting the hagfish traps.

Appendix E4: Hagfish caught in traps dropped in the Gulf of Maine, 10 miles off the Isles of Shoals.

Appendix E5: Two hagfish in captivity.

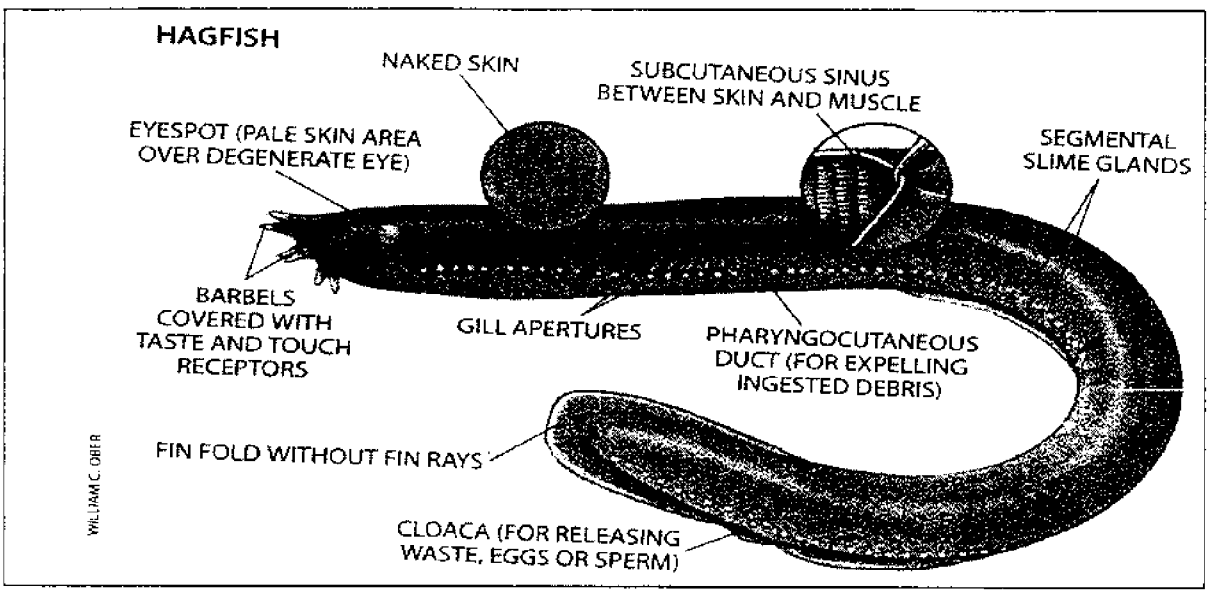
Appendix E6: Measuring a hagfish on the boat.

Appendix E7: Lamprey GnRH-III injections, at the fish hatchery (AFAIR Lab).

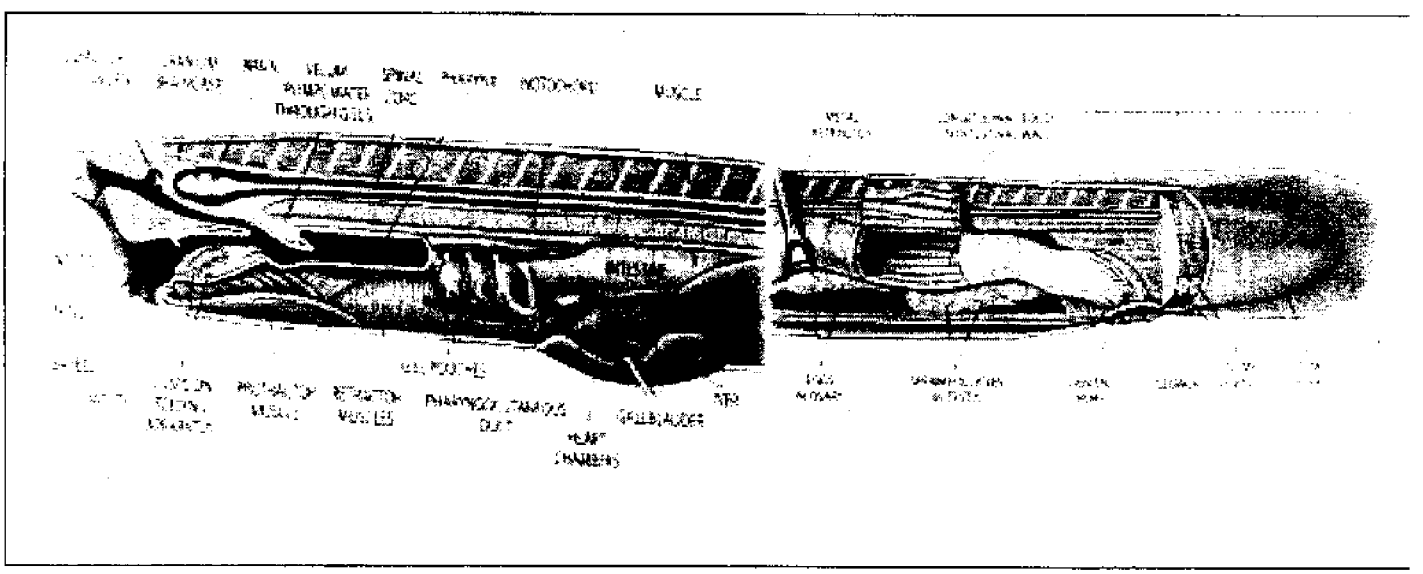


Appendix E8: Dissected mature female hagfish showing large eggs.

Appendix F



Appendix F1: External anatomy of a Pacific hagfish illustrates the unique characteristics of the Myxine genus. From Martini, F. 1998. Secrets of the Sline. Scientific American 10: 70-75.



Appendix F2: Internal anatomy of a Pacific hagfish. From Martini, F. 1998. Secrets of the Slime. Scientific American 10: 70-75.