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Seasonal changes of gonadotropin-releasing hormone in the Atlantic hagfish *Myxine glutinosa*

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Abstract

To investigate seasonal reproduction in *Myxine glutinosa*, we measured total brain gonadotropin-releasing hormone (GnRH) and determined gonadal stages of hagfish collected from the Gulf of Maine once a month for 12 months. Thirty hagfish from each of three different size classes of small (20–35 cm), medium (35–45 cm), and large (50–60+ cm) were sampled for brains and gonads. In the medium and large class hagfish there was an increase in GnRH concentrations during April and May that correlated with male and female gonadal maturity. Also in these size classes of female hagfish, there was a similar rise in GnRH in November and then again in January that preceded the highest incidence of large eggs (stage 7). The elevated GnRH may be influencing the onset of ovarian recrudescence which has been shown in other vertebrates. These data suggest an association of the concentration of brain GnRH with gonadal maturity and provide supportive evidence of a possible seasonal reproductive cycle in *M. glutinosa* shown in recent studies of [J. Exp. Zool. 301A (2004) 352], correlating steroid production with gonadal maturation.

Keywords: Gonadotropin-releasing hormone; GnRH; Hagfish; Agnathan; Steroid; Myxine glutinosa

1. Introduction

Hagfish and lampreys belong to the class of agnathans, and are the only known living representatives of the most ancient lineage of vertebrates. Since the introductory anatomical studies of the 19th century, hagfish have continued to be profound in their peculiarities in structure, life history, and in their position in vertebrate evolution. Hagfish belong to the family Myxinoidea, and are considered to be one of the most primitive of all vertebrates living or extinct. Hagfish are considered an important taxonomic group because modern representatives retain many structures and functions that are representative of the ancestral condition during the origin of vertebrates.

The regulatory hypothalamic neurohormone, gonadotropin-releasing hormone (GnRH), controls reproduction in all vertebrates through the hypothalamic–pituitary–gonadal axis. Members of the GnRH family that have been isolated are 10 amino acids in length, except for octopus which has 12 amino acids, and all have conserved regions at the NH_2-terminus (pGlu), Ser^4 and COOH-termini. To date the primary sequence for 24 forms of GnRH have been identified in invertebrates and vertebrates (Gorbman and Sower, 2003).

Most vertebrates have at least two forms of GnRH in the brain. In most cases, only one of the GnRHs controls the pituitary–gonadal axis. The second and third forms of GnRH are generally found in either the midbrain or olfactory septum and their function are not well understood. The earliest evolved vertebrate for which a

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functional role for GnRH has been established is the sea lamprey *Petromyzon marinus*. The two forms of GnRH, lamprey GnRH-I and lamprey GnRH-III, have been extensively demonstrated to modulate and control various reproductive processes in the sea lamprey via the hypothalamus–pituitary–gonadal axis (Sower, 2003). Matsuo et al. (1971) elucidated the first amino acid sequence of GnRH in porcine. In the early 1980s, four additional GnRHs were identified and sequenced in chicken (chicken GnRH-I and -II; King and Millar, 1982a,b; Miyamoto et al., 1982), salmon (Sherwood et al., 1983), and lamprey (Sherwood et al., 1986). During this period, attempts were made to determine if GnRH was present in hagfish. These early investigations trying to identify a GnRH-like molecule in the hagfish brain provided inconclusive results (Sower et al., 1995). Immunoreactive (ir) GnRH was reported in brains of the hagfish, *Heptatretus hexamtrema* (King and Millar, 1980) and *Eptatretus stouti* (Jackson, 1980) using radioimmunoassay and chromatographic techniques. However, others, using these same techniques, could not detect GnRH in the brain of the Pacific hagfish, *E. stouti* (Sherwood and Sower, 1985). In immunocytochemical studies, ir-GnRH was not detected in several species of hagfish brain by Nozaki and Kobayashi (1979), and Crim et al. (1979). Nozaki et al. (1984) hypothesized hagfish may contain an ir-GnRH that is not detected by current antisera or methods, or hagfish may lack GnRH.

In these earlier investigations that produced negative results of ir-GnRH in the hagfish brain, there were a limited number of GnRH antisera available. By 1993, two isoforms of GnRH had been isolated and primary structures determined in another agnathan, the sea lamprey (Sower et al., 1993). The identification of the primary structures of GnRHs in lamprey and other vertebrates in the 1990s allowed new GnRH antisera to be produced that could be tested in hagfish. Sower et al. (1995), using the techniques of immunocytochemistry (ICC), HPLC, and radioimmunoassay (RIA) with a specific lamprey GnRH-III antisera localized a lamprey-III GnRH-like molecule in the hypothalamus, adenohypophysis, and the neurohypophysis of the Atlantic hagfish. Also reported in 1995, using six different antisera to GnRH (salmon PBL-49, lamprey 21–134, lamprey 1459, lamprey 1467, chicken-II, and mammalian), ir-salmon GnRH-like molecule was shown to be present in the preoptic cells, hypothalamic infundibular nucleus, hypophysial stalk, and distributed fibers of the hagfish brain (Braun et al., 1995). Based on these investigations, it is likely hagfish indeed have a GnRH or GnRH-like peptide in the brain, although, the primary amino acid structure of GnRH has not been identified in these ancient fish. The primary structure of the GnRH (-like) molecules needs to be determined to confirm the presence of specific GnRH(s) in the hagfish brain. The presence and location of a GnRH-like substance in the brain of the hagfish *Myxine glutinosa*, have led the authors to hypothesize that GnRH has a neuroendocrine function acting on the pituitary (Braun et al., 1995; Sower et al., 1995). However, to date no experimental evidence has been reported linking a GnRH or GnRH-like substances to the reproductive processes in hagfish.

Currently, little is known about reproduction in hagfish. Until recently, only one species *Eptatretus burgeri*, the Japanese hagfish was known to have an annual reproductive cycle (Tsuneki and Gorbman, 1977). In this cycle the hagfish migrates between deep and shallow water for spawning (Fernholm, 1974; Gorbman and Dickhoff, 1978; Kobayashi et al., 1972; Tsuneki et al., 1983). Hirose et al. (1975) demonstrated the presence of steroid hormone production in the mature ovary of the hagfish *E. stouti*. During the past three years, we have examined steroid concentrations from in vitro incubations of gonads, and correlated these concentrations with development and maturation of gonadal tissues in Atlantic hagfish captured monthly from the Atlantic Ocean (Powell et al., 2004). These data showed accompanying changes of gonadal estradiol and progesterone production corresponding to a seasonal reproductive cycle (Powell et al., 2004). To date, there have not been any studies that have investigated the relationship of a hypothalamic neuropeptide such as GnRH, in relation to a reproductive cycle in hagfish. Since GnRH is considered the "master control" of reproduction in all vertebrates (Gore, 2000) it is essential to gain an understanding of GnRH activity in relation to the reproductive cycle in hagfish. Therefore, the objective of this study was to determine the changes in the concentration of ir-GnRH during a one-year period in correlation with gonadal development to increase our understanding of reproduction in Atlantic hagfish.

2. Materials and methods

2.1. Hagfish collection

Atlantic hagfish, *M. glutinosa*, identified according to the characteristic and geographic locations as described by Bigelow and Schroeder (1948) were trapped monthly in the Atlantic ocean from November 2001, to October 2002, at Jefferies Ledge (42° 50.6′N, 70 10.079′W) in the Gulf of Maine using the University of New Hampshire Research Vessel Gulf Challenger. Hagfish were trapped using modified 200 L plastic barrels baited with salted herring and fitted with one-way cones. Traps were deployed for 45–60 min at a depth of 100–150 m. The collected hagfish were divided into three size classes small, 20–30 cm, medium 35–45 cm, and large 45+ cm. These classes were chosen based on length and weight data from previous studies in the Gulf of Maine (Sower, unpublished). It has been proposed that male hagfish

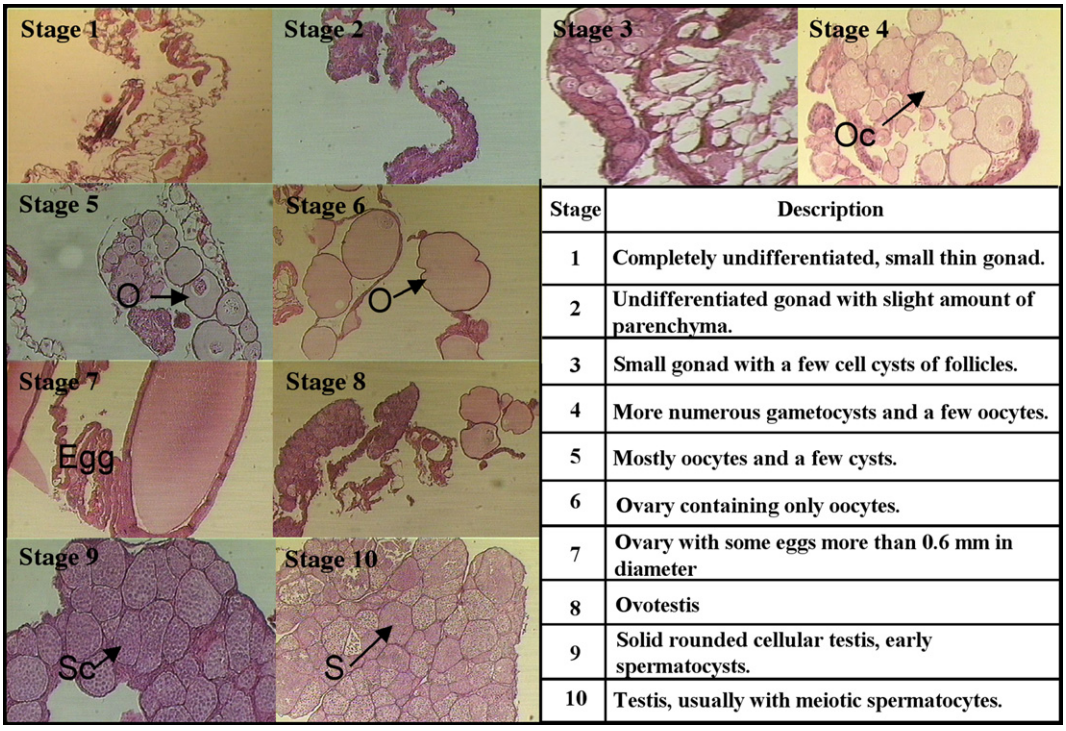
occur most frequently in the medium size class and females are the majority in the small and large size classes. Therefore, to increase the probability of including males and females in the sample population twenty hagfish from each size class were collected. Hagfish were maintained in 4 °C seawater and transported to the Anadramous Fish and Aquatic Invertebrate Research (AFAIR) Laboratory, University of New Hampshire, Durham, New Hampshire and held 24–48 h in chilled sea water (4 °C) tanks until dissected. Hagfish were anesthetized by immersion in a solution of 0.05 g/L tricaine-methylsulfonate (MS222), weighed, measured for length and decapitated. The brains were removed, frozen in liquid nitrogen, and stored at −80 °C until further use.

3. Histology of gonads

Previous examination of hagfish gonad histology had shown that the gonad of the hagfish has different developmental stages along its length to standardize the sampling. Gonad tissues for histology were sampled from three regions along the length of the gonad as described by Gorbman (1990). Gonad tissue was sampled from each hagfish at the 5th, 12th, and 44th muscle somite counting from the anterior wall of the cloaca, All tissue samples were prepared for histological examination by embedding in paraffin, followed by sectioning at 8 μm and staining with hematoxylin and eosin. They were evaluated for development as described by Gorbman (1990) (Fig. 1). Brains collected from hagfish caught from November 2001 to October 2002 were pooled by month, size class (small, medium, and large) and then further by similarity of posterior gonad development after histological examination of the gonad at the 5th muscle somite.

4. Extraction and radioimmunoassay of hagfish brains

Frozen brains from each monthly sampling time were aliquoted into six groups of three brains for each size class. The total weight for each group was recorded. The brains were kept on dry ice until extraction and radioimmunoassayed as previously described (Fahien and Sower, 1990; Sower et al., 1995; Stopa et al., 1988). Lamprey GnRH-III antiserum 3952 was used at an initial dilution of 1:16,000. The antiserum has a cross-reactivity of 123% with lamprey GnRH-I, 100% with lamprey GnRH-III, and less than 0.01% with mammalian, chicken-I and -II, and salmon GnRH forms by radioimmunoassay. Synthetic lamprey GnRH-I was iodinated using a modification of the chloramine T method as described by Stopa et al. (1988) and Carolsfeld et al. (2000). The lower limit of detection in this study was 9.8 pg/ml and the percent binding ranged between 30.1 and 48.4%.



Stage	Description
1	Completely undifferentiated, small thin gonad.
2	Undifferentiated gonad with slight amount of parenchyma.
3	Small gonad with a few cell cysts of follicles.
4	More numerous gametocysts and a few oocytes.
5	Mostly oocytes and a few cysts.
6	Ovary containing only oocytes.
7	Ovary with some eggs more than 0.6 mm in diameter
8	Ovotestis
9	Solid rounded cellular testis, early spermatocysts.
10	Testis, usually with meiotic spermatocytes.

Fig. 1. Hagfish gonadal development. The developmental stages, and description of the gonad in *Myxine glutinosa*. All stages are viewed at 40×. Oc, oocyst; O, oocytes; Sc, spermatocyst; and s, spermatocytes (modified from Gorbman, 1997).

5. Statistics

The resulting hormone concentrations were analyzed using one-way ANOVA followed by Fisher's PLSD test. The level of significance for differing groups was $p < 0.05$.

6. Results

6.1. Gonadal development

6.1.1. Small size class

Posterior gonadal stages 1, 2, 5, 6, 8, and 9 were identified in the gonads of the small class (25–35 cm) (Figs. 2 and 4). Hagfish with ovotestis (stage 8) and early spermatocytes were also identified in this size class. The majority of the gonads examined were stage 1 (34%) and stage 9 (33%). No ovaries with eggs were identified.

6.1.2. Medium size class

In the medium size class (35–45 cm) gonadal development was different from the small or large class, 59% of the animals were stage 9. There were no stages 2, 3, or 4 in this size class (Figs. 2 and 4).

6.1.3. Large size class

The large hagfish (45+ cm) were twice as likely to be female in the posterior gonad than the small or medium size class (Figs. 2 and 4). The large class hagfish were the only class to produce eggs longer than 10 mm in length. Thirty percent of the large size class were stage 1 and 27% were stage 9 in the posterior gonad.

6.2. GnRH

Gonadotropin-releasing hormone was detected in brains of all size classes of hagfish examined from November, 2001 to October, 2002. The greatest variability in GnRH concentration occurred in the small hagfish class (77 ± 5.5 mean $\pm$ SE to 1298 ± 147.9 pg/0.1 g brain). GnRH concentrations in the small hagfish during October and December were significantly higher ($p < 0.05$) compared to GnRH in hagfish in the remaining months (Fig. 3). Due to the low levels of GnRH and the number of individuals with undeveloped or undifferentiated gonads in small class hagfish, the rest of the paper will focus on the medium and large size classes of hagfish.

In May, 2002, there was a significant increase ($p < 0.05$) in GnRH concentrations in the medium class hagfish from 145 ± 6.8 pg/0.1 g brain to 935 ± 12.1 pg/0.1 g brain. During the following months until October, there was a gradual decline in GnRH concentrations from 935 ± 12.1 pg/0.1 g brain to 170 ± 13.1 pg/0.1 g brain (Fig. 3). The range of GnRH concentrations for this size class was from 136 ± 4.8 pg/0.1 g brain in February, 2002

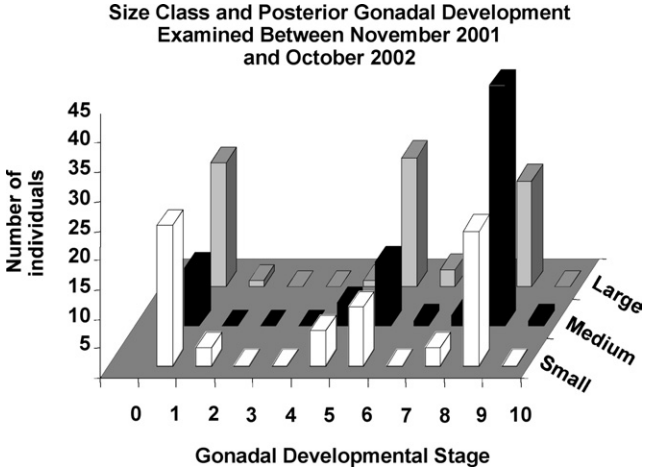


Fig. 2. Gonadal development. Comparison of size class, gonadal development, and the number of individuals at each developmental stage.

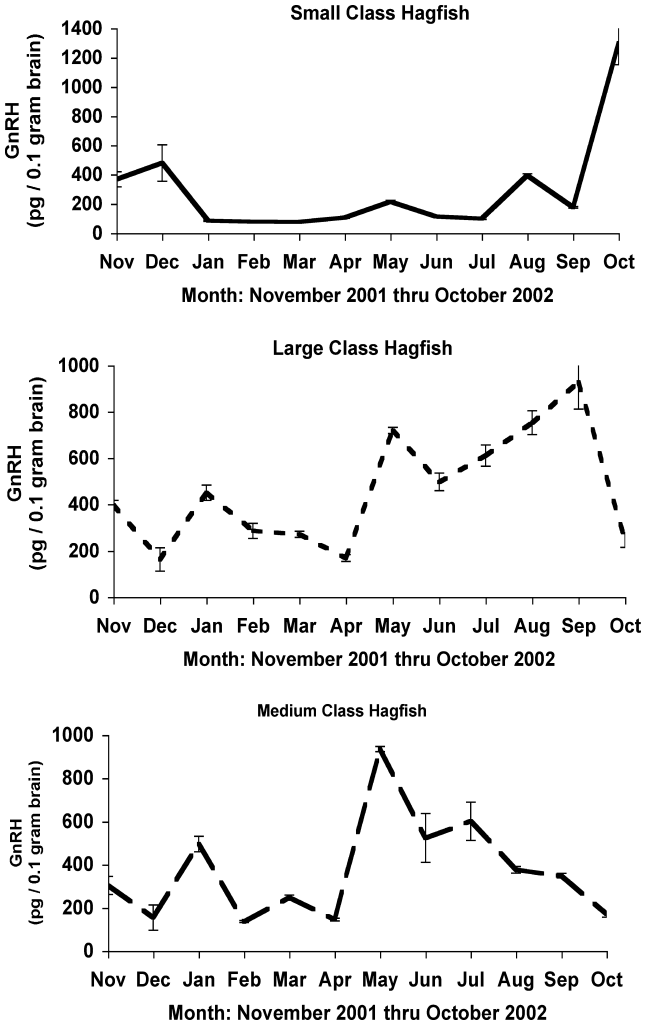


Fig. 3. Concentrations of GnRH. Concentrations of GnRH (pg/g brain) in the brains of Atlantic hagfish, *M. glutinosa* between November, 2001 and October, 2002.

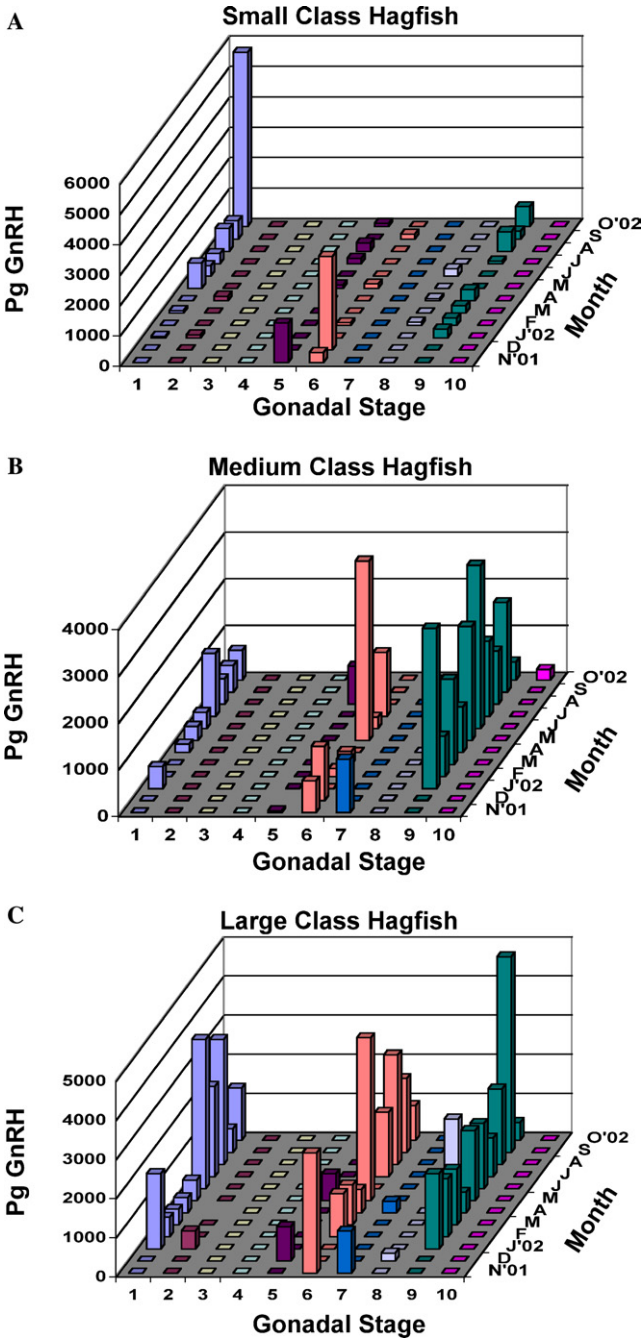


Fig. 4. Occurrence of developmental stage, time of year, and total GnRH. The occurrence of the posterior developmental stages, the time of year they occur, and the total GnRH (pg/g brain) for that stage/time period for three size classes of Atlantic hagfish small (A), medium (B), and large (C). Data were collected from November, 2001 to October, 2002.

to 935 ± 12.1 pg/0.1 g brain in May. The range of GnRH concentrations in the large hagfish was from 162 ± 50.8 pg/0.1 g brain in December, 2001, to 927 ± 116.6 pg/0.1 g brain in September, 2002 (Fig. 3). GnRH concentrations increased significantly ($p < 0.05$) in May, 2002 (719 ± 13.2 pg/0.1 g brain) through September, 2002 (927 ± 116.6 pg/0.1 g brain).

7. Discussion

In this study, brain concentrations of GnRH fluctuated significantly during one year, by size class, and stage of gonadal development in the Atlantic hagfish (*M. glutinosa*). In the medium and large class hagfish there was an increase in GnRH concentrations during April and May that correlated with male and female gonadal maturity. Also in these size classes of female hagfish, there was a similar rise in GnRH in November and then again in January that preceded the highest incidence of large eggs (stage 7). The elevated GnRH may be influencing the onset of ovarian recrudescence which has been shown in other vertebrates. These data suggest an association of the concentration of brain GnRH with gonadal maturity and provide supportive evidence of a possible seasonal reproductive cycle in *M. glutinosa* shown in recent studies of Powell et al. (2004), correlating steroid production with gonadal maturation.

Although the accompanying changes of GnRH and steroid concentrations during a one year period cannot be directly linked to a spawning event in the Atlantic hagfish, the gonadal development of the medium and large class hagfish correlated with reproductive hormones indicates a pattern of seasonality. In the medium and large class oscillations in GnRH are similar in time of year and stage of posterior gonadal development. In both size classes GnRH correlated with the highest occurrence of gonadal development stages one, six, and nine. Stage one was an undifferentiated gonad, stage six an ovary contained only oocytes, and stage nine consisted of solid rounded cellular testis with early spermatocytes. Similarly, as shown in an earlier study, gonadal steroids correlated with occurrence of gonadal stages one, six, and nine (Powell et al., 2004). In these studies, fluctuating changes of estradiol and progesterone production were associated with developing gonads in medium and large hagfish (Powell et al., 2004). From these data, we suggested hagfish may have an annual reproductive cycle. The current study examined brain GnRH from the same hagfish as our Powell et al., 2004 study. The peaks in estradiol and progesterone production accompanied noted increases in brain concentrations of GnRH in both the medium and large size classes of hagfish in January and May (Fig. 5). The increase in in vitro estradiol production was also correlated with an increase in the number of maturing eggs in female hagfish. However, whether hagfish spawn once or more a year will require much more extensive studies.

The Japanese hagfish, *Eptatretus burgeri*, is the only known species of hagfish that has a regular annual reproductive cycle and undergoes an annual migration (Ichikawa et al., 2000; Kobayashi et al., 1972; Nozaki et al., 2000). Based on histological examination of gonads from small, medium, and large size classes of Atlantic

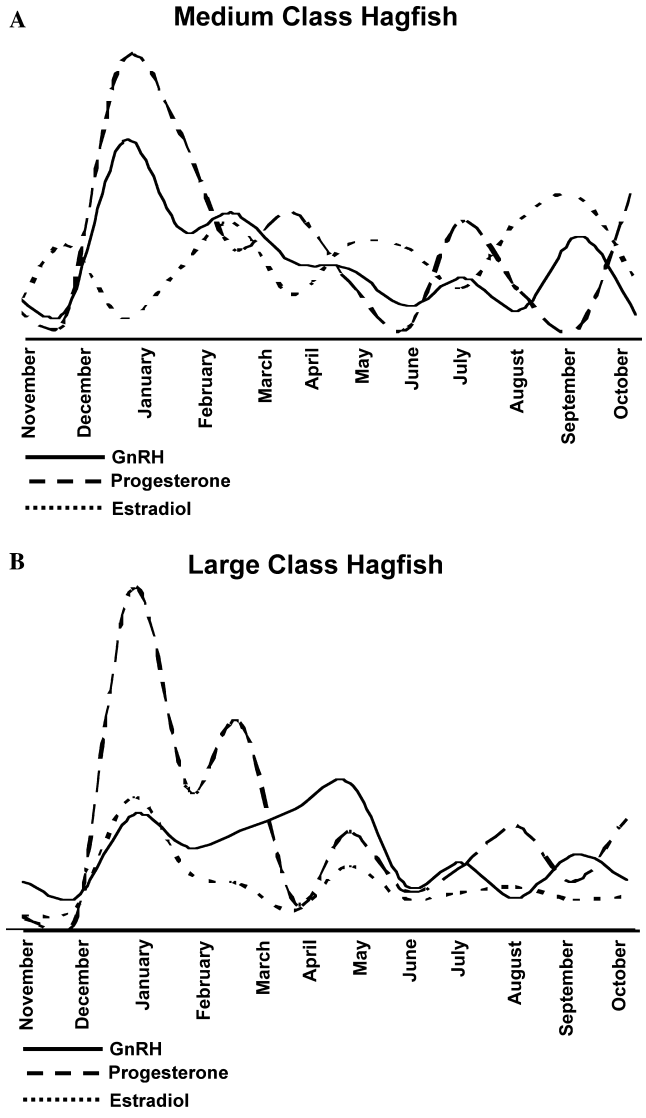


Fig. 5. Schematic of relative changes of seasonal reproductive hormones. Relative seasonal concentrations of estradiol and progesterone produced by the gonads of Atlantic hagfish compared to the seasonal concentrations of GnRH in the brain for (A) medium and (B) large hagfish.

hagfish, annual changes in gonadal development were seen (Powell et al., 2004). In the current study, a higher incident of the largest eggs (stage 7) occurred March through May in the large class size of hagfish correlating with increasing concentrations of brain GnRH. The majority of male hagfish occurred in the medium size class. Mature males were most abundant in the early spring occurred from March through May correlating with the highest peak of brain GnRH. Females with large developing eggs were observed in all months with the exception of December, January, and June. In earlier studies, it was recorded that mature and maturing *M. glutinosa* females were found during all seasons of the year (Bigelow and Schroeder, 1948). Our histological data and the measured seasonal changes of GnRH, estradiol and progesterone lead us to a different conclusion than Bigelow and Schroeder such that we hypothesize the Atlantic hagfish may spawn only once in a season possibly during the time period of April to May. During the 1940s and until the 1990s, there was not an active fishery for hagfish, thus there were no demands on the hagfish population off the coast of New England. A hagfishery started in the 1990s in New England. The landings for hagfish off the coast of Maine and Massachusetts have ranged from 1 to 12 million lbs each year during 1996–2002 (Gerry Gaipo, Fishery Information Section, DOC/NOAA/NMFS). It has been suggested that this demand for hagfish has greatly depleted the hagfish populations (Martini, 1998). Whether there is a true depletion of hagfish and if there has been a major impact on hagfish population and its reproductive patterns since the 1940s is unknown. Until more studies are done on reproduction in Atlantic hagfish, the patterns of reproductive cycles will not be fully established.

One of the complicating factors in assessing the role of the hypothalamus via GnRH in the control of reproduction in hagfish is that in our gonadal examination, about 40–50% of medium and large hagfish had gonads with distinct male testicular tissue in the posterior region and ovarian tissue in the anterior region (Powell et al., 2004). From this same study, 58% of all hagfish studied ($n = 1080$) from all size classes contained only female gonad tissue, 41% were hermaphrodites, and 0.05% were males with no ovarian tissue present. We propose that a certain percentage of Atlantic hagfish may be functional hermaphrodites since a small percent of the identified hermaphroditic adult hagfish were found with large oval eggs and mature sperm (Powell et al., 2004). In our current study, we did not consider the role of GnRH in the hermaphrodite hagfish, although it is highly likely there is more than one form of GnRH and if indeed hagfish can be functional hermaphrodites the control is mediated by the hypothalamus.

The question arises in hagfish on whether there are seasonal cues that may initiate or modulate activity since the Atlantic hagfish live at depths of 100 m or greater. The integration of environmental cues is a strong indicator of neuroendocrine control. If as we propose there is a seasonal reproductive cycle in hagfish, this cycle may be influenced by external cues from the environment. Changes in water temperature and food availability due to seasonal tide changes could influence the seasonality of the reproductive cycle of hagfish in the Gulf of Maine via the hypothalamus–pituitary axis.

There are two extant species of agnathans, hagfish and lampreys. Unlike hagfish, lampreys spawn once in their lifetime and then die after spawning thereby having a synchronous development of gonadal maturation and reproductive hormones corresponding to seasonal cues of photoperiod and temperature (Sower, 2003).

During metamorphosis when a lamprey develops from a filter-feeding larval lamprey to a young parasitic maturing adult, there is an increase of brain lamprey GnRH-I and -III that coincides with the acceleration of gonadal maturation (Youson and Sower, 1991). In adult lampreys, there are seasonal correlations between changes in brain GnRH and gametogenic and steroidogenic activity of the gonads in adult male and female sea lampreys (Bolduc and Sower, 1992; Fahien and Sower, 1990). These data along with other numerous studies in lampreys provide overwhelming evidence that similar to all other vertebrates that reproduction in lampreys is controlled by the hypothalamus (Sower, 1990; Sower, 1998; Sower and Kawauchi, 2001; Sower, 2003). In the current study, total ir-GnRH was measured. It is considered that even though the primary amino acid structures of GnRH have not been determined in hagfish, the measurements of total GnRH can be an indicator of reproductive activity. The ir-forms of GnRH (ir-lamprey GnRH-III and chicken GnRH-II) that we detected in earlier studies by immunocytochemistry had been shown in the hypothalamus-neurohypophysis of hagfish (Sower et al., 1995). The antiserum that we used in our radioimmunoassay in the current study only recognizes lamprey GnRH-I and -III, so it is likely that we are only measuring one type of GnRH, a lamprey GnRH-III like peptide. However, our antiserum could certainly recognize a novel form. Whether our assay system is measuring one or two GnRHs, the current study showed that relative changes of total GnRH in hagfish do correspond with reproductive gonadal stages.

In summary, the results of our study have shown brain concentrations of ir-GnRH fluctuating significantly during one year, by size class, and stage of gonadal development in the Atlantic hagfish. In vertebrates, the neuroendocrine axis plays a central role in the control of reproduction by integrating internal and external signals during key developmental states and the structure and functions of GnRH have been highly conserved throughout vertebrates (Gorbman and Sower, 2003). Similar to all other vertebrates, it is therefore likely that reproduction in hagfish has retained conserved aspects of the complex neuroendocrine axis in the coordination and integration of environmental cues and hormonal mechanisms.

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