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Immunohistochemical detection of gonadotropin-like material in the pituitary of brown hagfish (*Paramyxine atami*) correlated with their gonadal functions and effect of estrogen treatment

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Abstract

Since hagfish are members of the most primitive group of living vertebrates, studies on their reproduction are indispensable for understanding phylogenetic aspects of vertebrate reproductive system. Nevertheless, our knowledge of the reproductive physiology of the hagfish, especially of the pituitary–gonadal axis, is almost completely lacking. In the present study, the relationship between the amount of immunoreactive gonadotropin (GTH)-like material in the pituitary gland and gonadal conditions was examined in the brown hagfish, *Paramyxine atami*. First, pituitary sections were stained immunohistochemically with anti-ovine LHβ, and the degrees of the accumulation of GTH-like material were compared among three different groups of gonadal conditions; juveniles and adults with and without developing gonads. Immunoreactive GTH-like material was heavily accumulated in adults with developing gonads, whereas it was not or only weakly accumulated in juveniles or adults without developing gonads. Thus, there was a strong positive correlation between the amount of GTH-like material and gonadal conditions. Second, effect of estradiol benzoate on GTH-like material was examined using three groups of juvenile hagfish: initial control, sham control, and experimental animals. Experimental animals received estradiol benzoate resolved in sesame oil intraperitoneally every third day for 1 month, whereas sham control animals received the same doses of sesame oil. GTH-like material was heavily or moderately accumulated in most estrogen-treated animals, whereas it was not or weakly accumulated in initial or sham control animals. Thus, estrogen treatment in juvenile hagfish resulted in the large increase in the amount of GTH-like material. From these results, it is suggested the presence of not only GTH but also the hypophysial–gonadal feedback system in the hagfish.

Keywords: Hagfish; Agnathan; Pituitary gland; Gonadotropin; Reproduction

1. Introduction

Lampreys and hagfish are the only two extant representatives of the oldest class of vertebrates, Agnatha (jawless vertebrates). The agnathans probably arose as the first vertebrates about 530 million years ago (Forey and Janvier, 1993), immediately after the evolutionary explosion of multicellular organisms in the Cambrian period. Since hagfish are members of the most primitive group of living vertebrates, studies on their reproduction are indispensable for understanding phylogenetic aspects of vertebrate reproductive system. Nevertheless, our knowledge of the reproductive physiology of the hagfish, especially of the pituitary–gonadal axis, is almost completely lacking. The lack of information is mostly due to the fact that hagfish pituitary function is difficult to assess from cytological and physiological studies (Gobman, 1983).

The adenohypophysis of the hagfish consists of clusters of cells embedded in connective tissue below the

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neurohypophysis, and there is no clear cytological differentiation between the pars distalis and the pars intermedia (Ball and Baker, 1969; Holmes and Ball, 1974). Moreover, the hagfish adenohypophysis was composed primarily of chromophobic cells stained with classical tinctorial stain (Ball and Baker, 1969), and of agranular cells observed by electron microscopy (Fernholm, 1972; Tsuneki, 1976). Complete hypophysectomy in *Eptatretus stouti*, involving removal of both neurohypophysis and adenohypophysis, has not provided any clear evidence for pituitary gonadotropic activity (Matty et al., 1976). On the other hand, in *Eptatretus burgeri*, the only hagfish known to have a definite breeding season (Ichikawa et al., 2000; Nozaki et al., 2000), there have been some indications that gonadal development and spermatogenesis may be retarded after hypophysectomy (Patzner and Ichikawa, 1977). Thus, some species may have a functional pituitary gland associated with seasonal reproduction, although the adenohypophysis of hagfish is considered to be nonfunctional (Sower and Gorbman, 1999).

Recently, Nozaki et al. (2005) observed three types of immunoreactive cells, gonadotropin (GTH)-like, proopiomelanocortin (POMC)-like, and growth hormone (GH)-like cells, in the adenohypophysis of both the Atlantic hagfish (*Myxine glutinosa*) and the Pacific hagfish (*E. burgeri*) by means of heterologous antisera. They suggested that GTH, POMC, and GH are ancient adenohypophysial hormones, which appeared at the very early period of vertebrate evolution. Furthermore, Nozaki et al. (2003) reported that the brown hagfish (*Paramyxine atami*), another Pacific hagfish, had a well-developing adenohypophysis, and about half of the total adenohypophysial cells were stained intensely with several antisera to LH-related GTH, such as anti-ovine LHβ and anti-human LHβ. Since GTH-like cells were so numerous in the adenohypophysis of *P. atami*, it is likely that GTH-like material is involved in the regulation of gonadal functions (Nozaki et al., 2003).

The present study was designed to examine the relationship between the pituitary gonadotropic activity and gonadal functions in *P. atami*. For this purpose, the following experiments were carried out. (1) Gonadal functions in relation to seasonality and total body length. (2) Correlation between the accumulation of GTH-like material in the pituitary gland and gonadal conditions. (3) Effect of estrogen treatment on the accumulation of GTH-like material in the pituitary gland.

2. Materials and methods

2.1. Animals

The brown hagfish (*P. atami*) has been commercially caught as food, which is sold in the local markets in Niigata Prefecture, Japan (Gorbman et al., 1990; Honma, 1998). The brown hagfish of both sexes were obtained from fishermen at Oyashirazu Fishing Port facing the Sea of Japan, Niigata Prefecture, Japan. They were collected with baited traps placed on the bottom and strung on the long line at depths of 100–160 m in Sado Strait, off the Niigata district (for details, see Gorbman et al., 1990). Animals were transported to the Sado Marine Biological Station, Niigata University, and were kept at 15 °C in circulating seawater tanks until sacrifice without food. In Experiments 1 and 2, they were killed within 2 days after collection, while in Experiment 3 they were kept for at most 40 days including both pre-experimental acclimation period and experimental period.

2.2. Experiment 1: Monthly changes in gonadal indexes

Paramyxine atami obtained monthly during the period from August 2001 to May 2002 were analyzed. The total length and body weight of all hagfish were measured under the anesthesia with 2-phenoxy-ethanol, and they were then sacrificed by decapitation. The abdominal body was opened and the sex was determined. In females, the length of the long axis of the largest egg was measured. In males, the testicular weight was measured and the gonadosomatic index (GSI = testicular weight/body weight $\times 10^5$) was calculated.

2.3. Experiment 2: Correlation between GTH-like material and gonadal conditions

Results in Experiment 1 showed that gonadal development occurred in both males and females larger than about 38 cm in total length (see Section 3). Accordingly, animals larger than 38 cm in total length were considered to be adults, whereas those smaller than 38 cm were considered to be juveniles. However, considerable individual variation in gonadal development was evident at any month of the year even in animals larger than 38 cm in total length, and thus there were two kinds of adults, those with and those without developing gonads (see Section 3). Thus, in Experiment 2, *P. atami* collected in September 2002 were used. Among them, only animals satisfying the following criteria were randomly selected. (1) Adults more than 40 cm in total length with testes of which GSI was greater than 200 or eggs of which length of long axis was greater than 10 mm. (2) Adults more than 40 cm in total length with their GSI less than 100 or with their egg length less than 4 mm. (3) Juveniles smaller than 36 cm in total length. Each group comprised of at least six animals.

Practically, all animals obtained in September 2002 ($n = 60$) were examined, and the total length and body weight were measured. After decapitation, the brains attaching pituitary gland were rapidly removed and immersed in Bouin–Hollande sublimate solution (Romeis, 1948) for about 24 h. The gonadal conditions were also examined as described in Experiment 1, and the testes were fixed in Bouin's solution for about 24 h. The fixed tissues were put in 70% ethanol and dehydrated through a series of solutions increasing concentrations of ethanol. Deposited mercuric chloride was removed by treatment with iodine–potassium iodine in 90% ethanol for 1–2 days. All tissues were embedded in Paraplast. Pituitaries and testes selected for Experiment 2 were cut at 6 μm in thickness (sagittally in case of pituitaries) and were mounted on gelatin-coated glass slides.

Immunohistochemical staining was performed for the pituitary by use of a Vectastain avidin–biotin peroxidase complex (ABC Elite) kit. Antiserum to ovine LHβ (dilution: 1/6000; lot No. AFP-697071P, source: NHPP) was applied as a primary antiserum in this study. The staining procedures have been described elsewhere (Nozaki et al., 1999). Specificity of the immunoreaction of the anti-ovine LHβ has been previously validated for localization of GTH-like cells in the *P. atami* pituitary (Nozaki et al., 2003). The testes were stained with Hematoxylin and Eosin.

2.4. Experiment 3: Intraperitoneal injection of estradiol benzoate

Only juvenile *P. atami* less than 36 cm in total length obtained in July 2004 were used. After acclimation for 10 days, animals were divided into three groups: (1) initial controls ($n = 7$), (2) experimental animals ($n = 13$), and (3) sham controls ($n = 11$). Initial control animals were sacrificed on Day 0.

Experimental and sham control animals were kept in separate seawater tanks at 15 °C without food during the experimental period. Estradiol benzoate (Sigma) was dissolved in sesame oil (Sigma) at concentration of

65 μg/0.1 ml and was injected intraperitoneally into experimental animals (0.1 ml) every third day for 1 month, whereas sham controls received only sesame oil. Thus, animals were received nine injections in total during the experimental period.

All experimental and sham control animals were killed by decapitation on the third day after the last injection. The total length, body weight, and gonadal conditions of all hagfish were measured under the anesthesia with 2-phenoxy-ethanol. After decapitation, the brains attaching pituitary gland were rapidly removed and immersed in Bouin–Hollande sublimate solution (Romeis, 1948) for about 24 h. The fixed tissues were dehydrated, treated with iodine–potassium iodide, and embedded in Paraplast as described in Experiment 2. Serial sagittal sections of 6 μm were mounted on glass slides. For immunohistochemical detection of GTH-like cells, the same procedures as that described in Experiment 2 were employed.

2.5. Statistics

All data obtained except for those of immunoreaction were expressed as the group means ± SEM. Differences between two means were evaluated with Student's t test or Cochran–Cox test. All statistical tests with $P < 0.05$ were considered significant. In addition, the degree of accumulation of immunoreactive GTH-like material in the adenohypophysis was estimated under a microscope, and was divided into the following five categories; heavily, moderately, weakly, faintly, and not accumulated (see Figs. 2 and 3 for representative examples). Moreover, the correlation coefficient (r) between total lengths and gonadal indexes was calculated in samples collected in each month.

3. Results

3.1. Monthly changes in gonadal indexes (Fig. 1)

A total number of 796 hagfish were analyzed. All hagfish were identified as either males ($n = 444$) or females ($n = 352$), and no hagfish were considered to be hermaphroditic.

3.1.1. Males

The mean total length collected throughout a year was 39.9 ± 0.3 cm (range: 27.0–57.5 cm). As shown in Fig. 1A, most males smaller than 36 cm in total length had small testis with GSI values less than 100, whereas several males larger than 39 cm in total length had well-developing testis with their GSI values more than 200. Thus, the hagfish collected included both juveniles and adults. When the mean GSI values were compared between 37 and 39 cm in total length, there was a significant difference between two (48.7 ± 10.1, $n = 20$ vs 123.3 ± 17.2, $n = 27$). On the other hand, the mean GSI value of 38 cm in total length (89.2 ± 11.5, $n = 28$) did not show a significant difference to either males of 37 or 39 cm in total length. Thus, males of

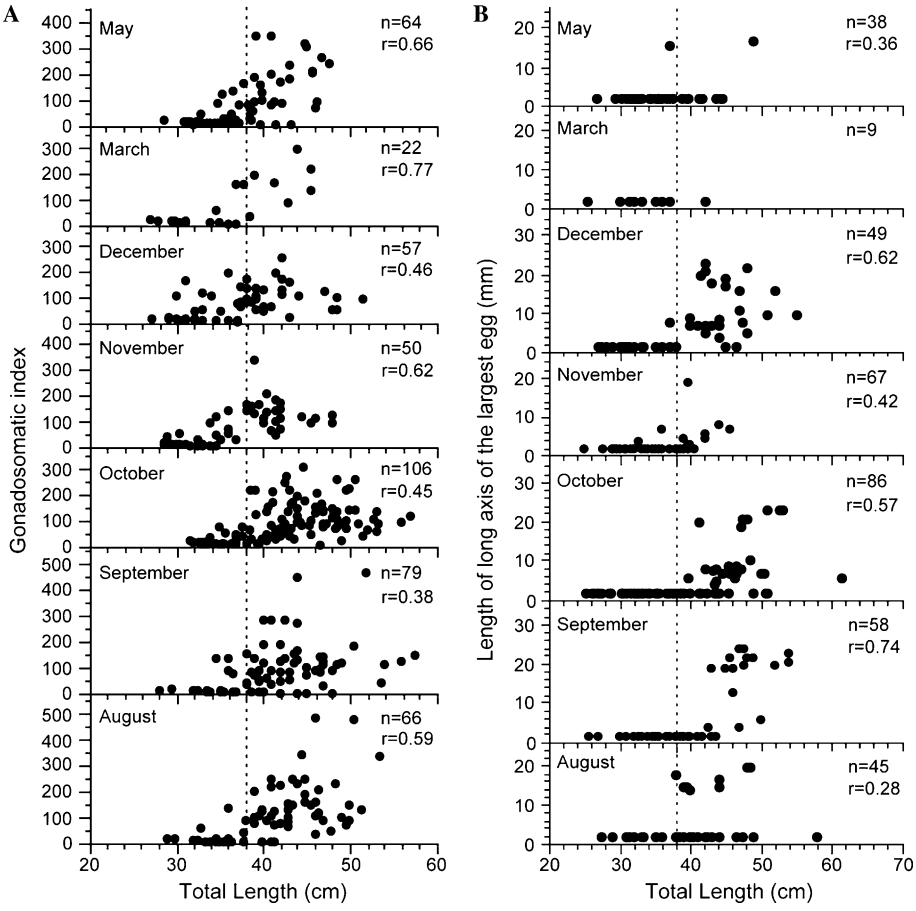

Fig. 1. Monthly distributions of gonadal indexes in the male (A) and female (B) hagfish. Animals obtained from August 2001 to May 2002 were analyzed. Gonadosomatic index (GSI = testicular weight/body weight × 10^5) was calculated for male gonadal index, whereas the length of the long axis of the largest egg was measured for female gonadal index. Vertical broken lines put on the 38 cm in total length of X-axis indicate the transition between juveniles and adults. r with value in each column indicates the correlation coefficient between total lengths and gonadal indexes. In both (A) and (B), note large individual variations in gonadal indexes at any months of the year. In (B), also note higher incidence of large eggs of more than 12 mm in August and September.

38 cm in the total length were transitional in gonadal development from juveniles to adults.

Monthly changes in mean GSI value were examined larger than 38 cm in total length (Fig. 1A). Since considerable individual variation in testicular development was evident in any month of the year, there were no significant differences in the mean GSI values among months. The mean GSI value throughout a year was 127.8 ± 5.1 ($n = 280$). Thus, males did not show a clear monthly change in testicular development.

3.1.2. Females

The mean total length was 37.8 ± 0.4 cm (range: 25.0–61.5 cm). As in the case of males, most females smaller than 38 cm in total length were juveniles, which had only previtellogenic oocytes (eggs) of 1–2 mm in length in the ovary (Fig. 1B). Adult females larger than 38 cm consisted of two groups, one with developing eggs of more than 5 mm (5–24 mm) in length and other with pre- or early vitellogenic eggs of less than 5 mm (mostly 1–2 mm) in length (Fig. 1B).

Monthly changes in ovarian indexes were examined in females larger than 38 cm in total length (Fig. 1B). Females collected in August were clearly divided into two groups, one with developing eggs of more than 12 mm in length (16.8 ± 0.9 mm, $n = 8$) and other with previtellogenic eggs of about 2 mm in length ($n = 37$). A similar tendency was also observed in females collected in September. Moreover, the mean length of developing eggs (19.6 ± 1.3 mm, $n = 14$) in September was slightly, but not significantly, larger than that in August. On the other hand, females with developing eggs of 5–10 mm in length were predominantly observed in October, as well as a few females with large eggs of more than 20 mm. In December, most adult females had developing eggs of more than 5 mm in length, but the size varied among individuals from 5 to 24 mm. Data collected in November, March, and May were difficult to analyze due to scarcity of numbers of adult females. The mean lengths of developing eggs of larger than 5 mm collected in August and September were significantly larger than those in October and December. Thus, females exhibited monthly changes in ovarian indexes, although considerable individual variations were also evident as well as males. Finally, since females with maximal development of eggs were found in September and October, the spawning appeared to occur during these months.

3.2. Correlation between GTH-like material and gonadal conditions

The mean total lengths of respective groups were 32.6 ± 0.6 cm ($n = 7$) in the juveniles, 42.1 ± 0.5 cm ($n = 7$) in the adults without developing gonads, and 44.2 ± 1.1 cm ($n = 6$) in the adults with developing gonads. There was no significant difference in the total lengths between the two adult groups.

The group of juveniles consisted of one male and six females. The GSI value of that male was 40, and its testicular follicles were full of spermatogonia (data not shown). The ovary of all juvenile females consisted of previtellogenic oocytes that were 1 mm in length. The group of adults without developing gonads consisted of two males and five females. The GSI values of those males were 33 and 87, and their testicular follicles were predominated by spermatogonia with some follicles containing spermatocytes. All females contained only previtellogenic oocytes of 1–2 mm in length. The group of adults with developing gonads consisted of three males and three females. The mean GSI value was 285.7 ± 16.7, and their testicular follicles were predominated by spermatocytes and spermatids. Follicles full of spermatozoa were also observed in all males. All females had large developing eggs of 20.0 ± 0.1 mm in length. The number of large eggs per female was 30–35.

In the group of juveniles, GTH-like immunoreaction was observed in the adenohypophysis in only two of seven animals (Table 1). In those positive animals, a faintly accumulated immunoreactive material to anti-ovine LHβ was observed in a small number of adenohypophysial cells (Figs. 2A and D). In the group of adults without developing gonads, GTH-like immunoreaction was observed in the adenohypophysis in four of seven animals (Table 1). In those positive animals, a weakly or a faintly accumulated GTH-like material was observed in a considerable number of adenohypophysial cells (Figs. 2B and E). In the group of adults with developing gonads, a highly or a moderately accumulated GTH-like material was observed in about half number of whole adenohypophysial cells in all animals ($n = 6$) (Table 1 and Figs. 2C and F). Thus, the accumulation of GTH-like material was apparently different between two adult groups, whereas it was not apparently different between groups of the juveniles and the adults without developing gonads.

3.3. Effect of estradiol benzoate on GTH-like material

During the experimental period, three animals in the sham control group and two animals in the estrogen-treated group died. The total lengths of respective group at the time of sacrifice were 32.3 ± 0.7 cm ($n = 7$) in the initial controls, 32.5 ± 1.1 cm ($n = 8$) in the sham controls, and 31.4 ± 1.8 ($n = 11$) in the estrogen-treated group. There were no significant differences in the total lengths among the groups.

In the initial controls, GTH-like immunoreaction was observed in the adenohypophysis in five of seven animals (Table 2). In those positive animals, a faintly accumulated GTH-like material was observed in a few adenohypophysial cells (Figs. 3A and D). In the sham controls, GTH-like immunoreaction was observed in the adenohypophysis in seven of eight animals (Table 2). In those positive animals, a weakly or a faintly accumulated GTH-like material was observed in a considerable number of adenohypophysial cells (Figs. 3B and E). In the estrogen-treated group, GTH-like immunoreaction was observed in the adenohypophysis in all animals (Table 2). In most animals (seven of 11

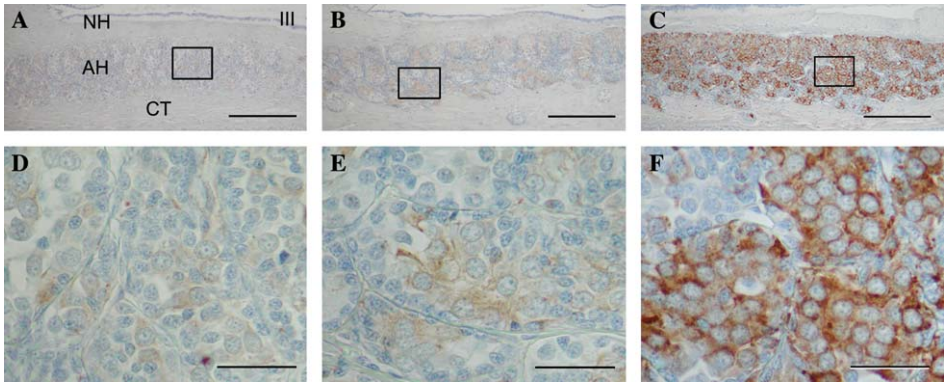

Fig. 2. Immunohistochemical detection of GTH-like cells in the adenohypophysis of brown hagfish. (A)–(C) Representative examples showing the degrees of the accumulation of GTH-like material of (A) juveniles, (B) adults without developing gonads and (C) adults with developing gonads, respectively. Each figure corresponds to the animal No. 1 indicated by symbol # in Table 1. The areas outlined by rectangles in (A)–(C) are enlarged and shown in (D)–(F), respectively. Note that the degree of accumulation of GTH-like material increases in accordance with gonadal development. AH, adenohypophysis; CT, connective tissue; NH, neurohypophysis; III, third ventricle. Scale bars: (A)–(C), 200 μm (42×); (D)–(F), 30 μm (330×).

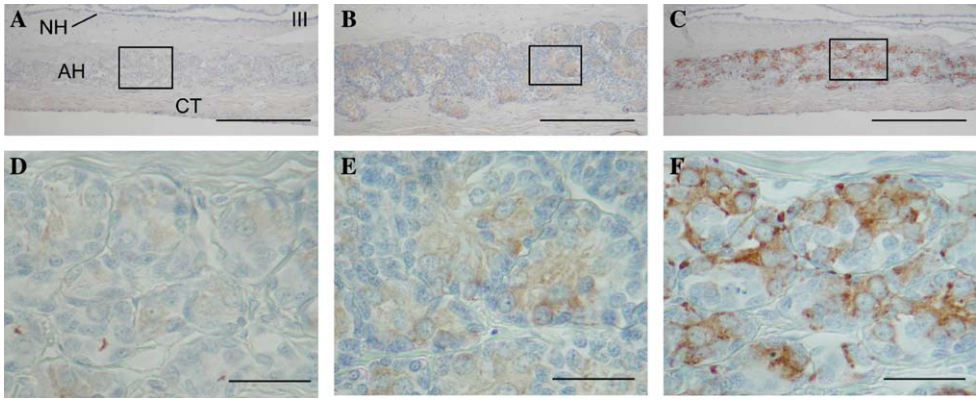

Fig. 3. Effect of estradiol benzoate on GTH-like cells in the adenohypophysis of juvenile brown hagfish. (A)–(C) Representative examples showing the degrees of the accumulation of GTH-like material of (A) initial controls, (B) sham controls, and (C) estrogen-treated animals. Each figure corresponds to the animal No. 3 indicated by symbol # in Table 2. The areas outlined by rectangles in (A)–(C) are enlarged and shown in (D)–(F), respectively. Note that treatment of estradiol benzoate in juvenile hagfish resulted in the large increase in the amount of GTH-like material in the adenohypophysis. AH, adenohypophysis; CT, connective tissue; NH, neurohypophysis; III, third ventricle. Scale bars: (A)–(C), 200 μm (60×); (D)–(F), 30 μm (335×).

animals), a high or a moderate accumulation of GTH-like material was observed in a considerable number of adenohypophysial cells, whereas a weak or a faint accumulation of GTH-like material was observed in the remaining animals (Table 2 and Figs. 3C and F). Thus, the accumulation of GTH-like material was apparently different between estrogen-treated and sham control groups. Apparent difference was not found between the initial and the sham control groups.

Table 1
Results on immunostaining on the amount of GTH-like material in the hagfish adenohypophysis

Animal No.	Juvenile	Adult	
		Without developing gonad	With developing gonad
1[a,#]	±[b]	+	+++
2	±	+	+++
3	−	+	+++
4	−	±	+++
5	−	−	+++
6	−	−	++
7	−	−	

[#]Results of Animal No. 1 are shown as representative examples of each group in Fig. 2.
[a] Data were represented individually.
[b] +++, Heavily accumulated; ++, moderately accumulated; +, weakly accumulated; ±, faintly accumulated; −, not accumulated.

4. Discussion

The present study clearly showed that there was a strong positive correlation between the amount of GTH-like material in the adenohypophysis and gonadal conditions of brown hagfish (*P. atami*). Namely, GTH-like material was heavily accumulated in adults with developing gonads, whereas it was not or only weakly accumulated in juveniles or adults without developing gonads. These results suggest that GTH-like material is involved in the regulation of gonadal functions in this animal. It is most likely that GTH-like material acts as a functional gonadotropin in the hagfish as well as in more advanced gnathostome vertebrates.

Table 2
Effect of intraperitoneal injection of estradiol benzoate (EB) on the amount of GTH-like material in the hagfish adenohypophysis

Animal No.	Control (initial)	Experimental	
		Control (sham)	EB treated
1[a]	±[b]	+	+++
2	±	+	+++
3[#]	±	+	++
4	±	±	++
5	±	±	++
6	−	±	++
7	−	±	++
8		−	+
9			+
10			+
11			±

[#]Results of Animal No. 3 are shown as representative examples of each group in Fig. 3.
[a] Data were represented individually.
[b] +++, Heavily accumulated; ++, moderately accumulated; +, weakly accumulated; ±, Faintly accumulated; −, Not accumulated.

Moreover, treatment of estradiol benzoate in juvenile hagfish resulted in the apparent increase in the amount of GTH-like material in the adenohypophysis. For this result, two possible explanations could be made: one is that estrogen treatment served to inhibit the secretion of GTH-like material from the adenohypophysis and hence resulted in the accumulation of the material in the adenohypophysis, and the other is that estrogen treatment served to stimulate the production of GTH-like material in the adenohypophysis. The former possibility, however, is not likely, since the adenohypophysis of juvenile hagfish produced apparently no or only a little amount of GTH-like material (see Section 3.2). Thus, even if estrogen treatment served to inhibit GTH release from the adenohypophysis, the accumulation of GTH-like material appeared to be very small. On the other hand, the latter possibility is supported by the following facts. Administration of sex steroid hormones, such as estrogen and aromatizable androgen, in juvenile teleosts resulted in a large increase in pituitary GTH contents (salmonids: Crim and Evancs, 1979; Crim et al., 1981; Gielen et al., 1982; Magri et al., 1985; eels: Dufour et al., 1983; Huang et al., 1997; Olivereau and Olivereau, 1979). This phenomenon in juvenile teleosts is explained by the operation of positive feedback system of steroid hormones to hypothalamo–hypophysial system in relation to the onset of puberty (Crim et al., 1981; Dufour et al., 1983). Thus, the present result further suggests the pituitary–gonadal feedback system in the regulation of gonadal functions in hagfish as well as in higher vertebrates.

Because of negative or equivocal data on the hagfish adenohypophysial hormones, many researchers have questioned whether there is a functional hypothalamo–hypophysial–target organ system in hagfish (Gobman, 1983; Hardisty, 1979). Surgical hypophysectomy in *E. stouti* has not provided any clear evidence for pituitary gonadotropic activity (Matty et al., 1976). In that study, gametogenesis appeared to be unaffected by hypophysectomy, suggesting that the hagfish gonad was independent of hypophysial gonadotropic control. On the other hand, in *E. burgeri*, the only hagfish known to have a definite breeding season (Ichikawa et al., 2000; Nozaki et al., 2000), there have been some indications that gonadal development and spermatogenesis may be retarded after hypophysectomy (Patzner and Ichikawa, 1977). The present study clearly suggests the involvement of the pituitary gland in the regulation of gonadal functions in *P. atami*, and thus contrasted to that of Matty et al. (1976). There are at least two possible, but not mutually exclusive, explanations for the difference of results on hypophysectomy between *E. stouti* and *E. burgeri*. First, the process of spermatogenesis and ovogenesis is relatively autonomous one, and is not completely arrested by hypophysectomy. In both *E. stouti* and *E. burgeri*, spermatogenesis still proceeded, although at a slower pace in *E. burgeri*, after hypophysectomy. In support of this idea, hypophysectomy experiments have been conducted extensively in lampreys, in which gonadal growth still continued after hypophysectomy (Evennett and Dodd, 1963; Larsen, 1973; also see Gobman, 1983). In intact conditions, gonadal growth is regulated by the hypothalamo–hypophysial feedback system in both lampreys and hagfish, and thus gonadal growth does not proceed autonomously.

Second, functional condition of the adenohypophysis is different among hagfish species, from minimally to fully functional. In support of this idea, Nozaki et al. (2003) reported that there was a remarkable difference in the thickness (= volume) of the adenohypophysis among hagfish species: *P. atami* had a well-developing adenohypophysis, which consisted of many clusters of cells, embedded densely in the connective tissue. On the other hand, *M. glutinosa* had a poorly developing adenohypophysis, which consisted of small numbers of clusters of cells, embedded sparsely in the connective tissue. The adenohypophysis of *E. burgeri* was the intermediate in thickness between above-mentioned two species. In all these three species, GTH-like cells were demonstrable in the adenohypophysis (Nozaki et al., 2003, 2005), but number of GTH-like cells per histological sections was also different among species. GTH-like cells were most numerous in *P. atami*, whereas GTH-like cells were a few in *M. glutinosa* (Nozaki et al., 2003). Indeed, as shown in the present study, about half of the total adenohypophysial cells were GTH-like cells in sexually maturing *P. atami*. Thus, it seems most likely that the functional condition of the adenohypophysis is different among hagfish species, from minimally to fully functional. *M. glutinosa* and *P. atami* may be the former and the latter examples, respectively.

Hagfishes mostly inhabit a deep or a semi-deep marine environment that is relatively free of circadian or even seasonal changes. The brown hagfish, *P. atami*, is one of those species, which lives in water of more than 100 m in depth. The present study revealed monthly changes in ovarian development in adult females, whereas monthly changes in

testicular development were not clearly observed. In agreement with the present findings, Powell et al. (2004) and Kavanaugh et al. (2005) recently reported the seasonal reproductive cycle in the Atlantic hagfish, *M. glutinosa*. They were trapped at a depth of 100–150 m, which was similar depth to our material. Thus, hagfish inhabiting a semi-deep marine environment also exhibit seasonal reproductive cycles, even though considerable individual variation in gonadal development is evident.

Another striking feature on the ecology of *P. atami* was that there might be two kinds of adults, those with and those without developing gonads, in the same population at any month of the year. Similar observations have also been reported in *E. burgeri* (Nozaki et al., 2000). Although the reason is not clear, several possible explanations could be considered as follows. (1) It may reflect the difference in nutritional conditions among individuals in relation to food availability. (2) They are repetitively cyclic breeders without clear seasonal limits to their cycles. (3) The interval of their reproductive cycle may be more than 1 year. (4) It may also be possible that they have only one cycle per life time, since it has not been proven that hagfishes are repetitively cyclic breeders.

Finally, the present study has provided first convincing evidence to indicate not only the presence of GTH in the adenohypophysis but also the presence of the hypophysial–gonadal feedback system in the hagfish. Indeed, quite recently cDNAs of both α- and β-subunits of GTH have been cloned from the pituitary gland of *P. atami* (Nozaki et al., unpublished). Since GTH-like cells were predominant cell type in the hagfish pituitary gland, the control and integration of reproductive processes is probably the oldest and original functions of the vertebrate pituitary gland.

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