Evolutionary studies on sex steroid hormone in the hagfish : plasma concentrations and biosynthetic enzymes

（ヌタウナギからみた性ステロイドホルモンの進化的研究：血中濃度と合成酵素）

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ABSTRACT

Reproduction in gnathostomes (jawed vertebrates) is controlled by a hierarchically organized endocrine system called the hypothalamic-pituitary-gonadal axis. In spite of the diverged patterns of reproductive strategies and behaviors, this endocrine network is remarkably conserved throughout gnathostomes. Gonadotropins (GTHs), in response to hypothalamic gonadotropin-releasing hormone, are secreted from the pituitary and stimulate the gonads inducing the synthesis and release of sex steroid hormones, which in turn elicit growth and maturation of the gonads. Hagfishes, which lack both jaws and vertebrae, are considered the most primitive vertebrate known, living or extinct. Accordingly, studies on hagfishes are indispensable for understanding the origin and evolution of the reproductive endocrine system in vertebrates. Recently, single GTH has been identified from the pituitary gland of brown hagfish, *Paramyxine atami*. It is suggested that the pituitary-gonadal system has been established during the early evolution of agnathans (jawless vertebrates). At present, little is known about gonadal sex hormones in hagfish. The present study was designed to explore sex steroid hormonal profiles and their biosynthetic enzymes in relation to gonadal developments in the brown hagfish.

Plasma concentrations of estradiol-17β, testosterone and progesterone were examined with respect to developmental conditions of gonads, sexual differences, and possible function of the atretic follicles in the brown hagfish, using a time-resolved fluoroimmunoassay. Plasma concentrations of these three hormones were low in juveniles of both sexes. In females, plasma estradiol-17β showed a significant correlation with ovarian development, with the highest concentrations in late vitellogenic adults. Plasma testosterone and progesterone also increased significantly in non-vitellogenic adult females; however, plasma testosterone showed no significant differences among adult females at different ovarian developments, while plasma progesterone was significantly lower in late vitellogenic adults than it was in non-vitellogenic adults. Vitellogenic females that possessed atretic follicles showed significantly lower concentrations of all three hormones than females that possessed only normal follicles. In males, no significant differences were found in plasma estradiol or

testosterone levels among groups of different developmental stages of the testis, while plasma progesterone showed an inverse relationship with testicular development. Thus, differences were found in plasma sex steroid hormone profiles between males and females.

Sex steroids are synthesized from cholesterol by a series of steroid synthetic enzymes such as cytochrome P450 enzymes (CYP11A, CYP17 and CYP19) and hydroxysteroid dehydrogenases (3β-HSD and 17β-HSD). Cytochrome P450 side-chain cleavage enzyme (CYP11A) which catalyzes the first step in the production of steroid hormones, was cloned by EST analysis of the brown hagfish testis. Phylogenetic analysis demonstrated that the hagfish CYP11A was positioned within the clade of vertebrate CYP11A, and was separated from CYP11B. By *in situ* hybridization, *CYP11A* mRNA signals were found in the theca cells of the ovarian follicles and Leydig cells and the tubule-boundary cells of the testis: these cells are established as steroid producing cells in the gonads of gnathostomes. The real-time PCR analysis was performed to examine the functional significance of hagfish CYP11A in relation to the gonadal developments. In females, the highest levels of *CYP11A* mRNA expression were found in the late vitellogenic adults, and clear positive correlation was noted in *CYP11A* mRNA expression levels in relation to ovarian development. In males, *CYP11A* mRNA expression levels were significantly higher in adults with medium and large GSIs than those in juveniles and adults with small GSIs. Thus, there was a clear discrepancy between males and females on the relationship between the transcriptional levels of *CYP11A* and plasma steroid levels. These results suggest a possibility that male hagfish uses other steroids than estradiol or testosterone as major androgens. From these results, it is suggested that CYP11A plays functional roles as a steroidogenic enzyme in the gonadal developments. Moreover, hagfish GTH stimulated the transcriptional levels of *CYP11A* in the cultured testis. This result further suggested that the steroidogenic activity of the hagfish testis is under the control of the pituitary GTH. Taking above-mentioned all results into consideration, it is suggested that vertebrates, during their early evolution, have established the pituitary-gonadal axis.

ABSTRACT IN JAPANESE

脊椎動物の多様な進化は、脳の神経情報を液性情報に変換・増幅して末梢の標的器官に送る情報伝達器官としての下垂体と、そこから分泌される腺下垂体ホルモンを介した視床下部–下垂体系を獲得したことで、もたらされたと考えられている。腺下垂体ホルモンの一つである生殖腺刺激ホルモン（Gonadotropin, GTH）は、顎を持つ脊椎動物（顎口類）において標的器官である生殖腺に作用し、様々な性ステロイドホルモンの合成・分泌を促す。それらのホルモンが生殖腺の発達や配偶子の形成・成熟、産卵行動などを誘起している。一方、脊椎動物の進化の最初期に出現した顎を持たない脊椎動物（無顎類）の遺存種であるヌタウナギ類は、化石種・現存種を含めて最も原始的な脊椎動物とされているが、その生態や生理学的機能などについてはほとんど調べられていない。従って、ヌタウナギ類の生殖内分泌機構を知ることは、この動物の生殖腺機能や、配偶子形成制御の理解だけでなく、脊椎動物の生殖内分泌機構の起源や、それがどのように進化し、多様性を生じたのかを考えるうえできわめて重要である。最近、ヌタウナギ類でも機能的な GTH が同定されたが、生殖腺から分泌されるホルモン分子についてはほとんど知られていない。本研究では、新潟県産クロヌタウナギ（*Paramyxine atami*）の性ステロイドホルモンの血中量を測定するとともに、ステロイド合成に関連する酵素群を探索し、得られた酵素の発現動態を生殖腺機能の調節と関連づけて理解することを目的とした。

まず、クロヌタウナギ尾部から採血を行った後、メスについては卵の長軸の長さ（卵径）を、オスは体重当たりの精巣重量の割合[GSI＝精巣重量(g)/体重(g)×100]を指標として、生殖腺の発達段階を区分した。そして時間分解蛍光免疫測定法（Time-Resolved-Fluoroimmunoassay、TR-FIA）によるクロヌタウナギ血液中のエストラジオール 17β（E2）、テストステロン（T）、プロゲステロン（P）の測定系を確立した。メスでは血中 E2 量と生殖腺の発達との間に相関がみられ、卵黄形成の進んだ群で最も高い値を示した。血中 T と P 量は卵黄形成の未熟な成体群で最も高い値を示し、P 量については卵黄発達に伴い減少した。このことから、E2 がヌタウナギのメスにおいて生殖腺の発達、特に卵黄形成に関連しており、T や P は中間産物として存在する可能性が考えられた。また、卵黄形成の途中で退化した退化卵胞をもつ

個体群では、3 ホルモンとも低い値を示したことから性ステロイドホルモンの合成が抑制されていると考えられた。一方、オスでは、生殖腺の発達と血中性ステロイドホルモン量の間に有意な相関は得られなかった。

E2 などの性ステロイドホルモンの合成には、シトクロム P450 酵素（CYP11A、CYP17、CYP19 など）やステロイド水酸基脱水素酵素（3β-HSD、17β-HSD など）といった様々な合成酵素が関わっている。コレステロール側鎖切断酵素(Cytochrome P450 side-chain cleavage enzyme = P450scc: CYP11A）は、コレステロールからプレグネノロンへの変換酵素であり、性ステロイドを含むステロイド生合成の最初の段階として重要な役割を果たしている。成熟した精巣から作成した cDNA ライブラリーの EST 解析により、ヌタウナギ *CYP11A* が同定され、その全長構造が明らかになった。また、*in situ* hybridization により、クロヌタウナギ生殖腺における *CYP11A* 発現が、脊椎動物における性ステロイド産生細胞として知られる精巣の間細胞（ライデッヒ細胞）と管状境界細胞、卵巣の莢膜細胞で観察された。生殖腺における *CYP11A* の遺伝子発現を調べると、メスでは卵黄形成、オスでは精子形成に伴い有意な発現量の上昇がみられたことから、CYP11A が性ステロイドホルモン合成酵素として働き、生殖腺の発達に関与していると考えられた。さらに、クロヌタウナギ GTH を加えて培養した精巣では、有意に *CYP11A* mRNA の発現量が上昇していたことから、GTH により発現が誘導されたと考えられた。

ヌタウナギ下垂体 GTH 量が生殖腺の発達段階と一致しており、ヌタウナギ GTH を加えて培養した精巣から性ホルモンが放出されたこと、さらに E2 と T の生体内投与により下垂体 GTH の合成や分泌が抑えられたことなどこれまでの成果を踏まえて考えると、本研究により、脊椎動物の最初期に下垂体（GTH）－生殖腺軸が確立し、その後のホルモンの機能分化に寄与したと考えられる。また、オスのヌタウナギでは E2 や T 以外のステロイドを主要ホルモンとしている可能性が示唆された。

CONTENTS

GENERAL INTRODUCTION

Reproduction in gnathostomes (jawed vertebrates) is controlled by a hierarchically organized endocrine system called the hypothalamic-pituitary-gonadal axis. In spite of the diverged patterns of reproductive strategies and behaviors, this endocrine network is remarkably conserved throughout gnathostomes. Gonadotropins (GTHs), in response to hypothalamic gonadotropin-releasing hormone, are secreted from the pituitary and stimulate the gonads inducing the synthesis and release of sex steroid hormones, which in turn elicit growth and maturation of the gonads. Estradiol-17β, for example, stimulates ovarian development and hepatic vitellogenesis, and regulates pituitary GTH activity by the feedback action to the hypothalamic-pituitary system.

Hagfish and lampreys are the only two extant representatives of the oldest superclass of vertebrates, Agnatha (jawless vertebrates). All hagfish including fossil species have inhabited only seawater (Bardack, 1998). Hagfishes are considered to be the most primitive vertebrates known, extant or extinct (Forey and Janvier, 1993). Extant hagfishes belong to the class Myxini, the order Myxiniformes, the family Myxinoidae, and most of them occur in deep or semi-deep and cool water in all oceans except the Polar Seas (Fernholm, 1998). Hagfishes are considered to be indispensable animals to understand origin and evolution of endocrine system in vertebrates. However, little is known about biology of hagfish. Our knowledge of endocrine regulation during reproduction in hagfish is also poorly understood (for reviews, see Gorbman, 1983; Nozaki, 2008, 2013). For example, it was thought that they did not have the same neuroendocrine control of reproduction as gnathostomes, since it was not clear whether the hagfish pituitary gland contained tropic hormones of any kind (Matty et al., 1976; Gorbman, 1983). Recently, single functional GTH was identified in the pituitary of the brown hagfish (*Paramyxine atami*), one of the Pacific hagfish (Uchida et al., 2010). Cellular and transcriptional activities of hagfish GTH were significantly correlated with the stages of gonadal developments. In addition, purified GTH induced release of gonadal sex steroids (estradiol and testosterone) *in vitro*. Furthermore, tonic administration of estradiol resulted in significant accumulation of immunoreactivities of GTH in the pituitary of juvenile brown

hagfish (Miki et al., 2006; Nozaki et al., 2013). These findings clearly suggested the presence of a GTH-gonad feedback system, similar to that of gnathostomes, in the hagfish.

Only a few studies exist regarding sex steroid hormonal profiles in relation to gonadal function in hagfish. In a previous study, Matty et al. (1976) reported that estradiol and testosterone were measurable in the plasma of *Eptatretus stouti* using radioimmunoassay (RIA); however, the observed levels of these steroids were near the lower limit of RIA sensitivity. Schützinger et al. (1987) found using a more sensitive RIA that plasma estradiol content increased in relation to the stages of ovarian development in female Atlantic hagfish, *Myxine glutinosa.* Schützinger et al. (1987) also showed that concentrations of plasma estrogen were low in males, juveniles and sterile animals. In another study, Yu et al. (1981) demonstrated that the synthesis of hepatic vitellogenin was inducible by estrogens, estradiol and estrone, in *Eptatretus stouti.* Based on these results, estrogenic control of ovarian development and hepatic vitellogenesis seem to have arisen early in vertebrate evolution.

The biosynthetic enzymes of sex steroids and their products have been well studied in gnathostomes. Typically, there are three cytochrome P450 enzymes (CYP) such as P450 side chain cleavage (CYP11A), P450c17 (CYP17), and P450 aromatase (CYP19) and two types of hydroxylated dehydrogenases (HSDs) such as 3β-HSD and 17β-HSD. It is also well established in gnathostomes that sex steroid hormones are produced in the cells comprising the growing follicles (theca cells and granulosa cells) of the ovary and in the interstitial cells (Leydig cells) of the testis. Little is known on the steroidogenic enzymes in the hagfish. As to the localization of steroidogenic cells, Tsuneki and Gorbman (1977a) reported cells with the ultrastructural features of Leydig cells among the spermatogenic follicles in the adult *Eptatretus stouti*. However, neither Fernholm (1972) in *Myxine glutinosa* nor Tsuneki and Gorbman (1977b) in *Eptatretus stouti* found cells showing the ultrastructural characteristics associated with steroidogenesis in the hagfish.

The present study aimed to elucidate the gonadal steroidogenic functions in the hagfish from the evolutionary point of view of reproductive endocrine system in vertebrates. In Chapter 1, plasma levels of sex steroid hormonal profiles were studied in relation to gonadal development and sexual difference in the brown hagfish using a time-resolved

fluoroimmunoassay (TR-FIA). In Chapter 2, EST analysis was performed to clone steroidogenic enzymes in the brown hagfish testis. As a result, CYP11A, which is the first and essential enzyme for steroidogenesis, was cloned. Thus, the aim of the present study was to clone and analyze the expression patterns of *CYP11A* mRNA in the gonads of the brown hagfish in correlation to the gonadal developments and following GTH administration. Also, *in situ* hybridization of *CYP11A* mRNA expression was studied to identify the steroidogenic cells in the hagfish gonads.

Chapter 1

Relationships between Plasma Concentrations of Sex Steroid Hormones and Gonadal Development in the Brown Hagfish, *Paramyxine atami*

INTRODUCTION

Of all vertebrate species, both extant and extinct, hagfishes are considered the most primitive (Forey and Janvier, 1993, 1994). Accordingly, studies on the hagfish reproduction are indispensable for understanding phylogenetic aspects of vertebrate reproduction; however, our knowledge of endocrine regulation during reproduction in hagfish is poorly understood (for reviews, see Gorbman, 1983; Nozaki, 2008). Complete hypophysectomy in the Pacific hagfish, *Eptatretus stouti,* did not provide any clear evidence for pituitary gonadotropic activity (Matty et al., 1976), while partial hypophysectomy in *E. burgeri*, the only hagfish known to have a definite breeding season (Ichikawa et al., 2000; Nozaki et al., 2000), resulted in retardation of gonadal development and spermatogenesis (Patzner and Ichikawa, 1977). Furthermore, only a few studies exist regarding sex steroid hormonal profiles in relation to gonadal function in hagfish. In a previous study, Matty et al. (1976) reported that estradiol and testosterone were measurable in the plasma of *E. stouti* using radioimmunoassay (RIA); however, the observed levels of these steroids were near the lower limit of RIA sensitivity. Schützinger et al. (1987) found using a more sensitive RIA that plasma estradiol content increased in relation to the stages of ovarian development in female Atlantic hagfish, *Myxine glutinosa*. Moreover, they found that plasma estrogen decreased appreciably after ovulation. Schützinger et al. (1987) also showed that concentrations of plasma estrogen were low in males, juveniles and sterile animals. In another study, Yu et al. (1981) demonstrated that the synthesis of hepatic vitellogenin was inducible by estrogens, estradiol and estrone, in *E. stouti*. Based on these results, estrogenic control of ovarian development and hepatic vitellogenesis seem to have arisen early in vertebrate evolution.

Follicular atresia is a common feature of hagfish ovaries (Gorbman, 1983). Recently, using *in vitro* cultured *M. glutinosa* ovaries supplemented with pregnenorone, Powell et al. (2006) showed that larger concentrations of progesterone were released into the media from atretic follicles compared to normal follicles. They hypothesized that hagfish possess functional corpora lutea-like structures that produce progesterone. Progesterone may play a role in the regulation of ovarian function in hagfish; however, the secretion of progesterone in

hagfish has only been studied in the gonads (Hirose et al., 1975; Gorbman and Dickhoff, 1978; Powell et al., 2004, 2006) and no previous studies have determined concentrations of the hormone in plasma. Furthermore, evidence regarding sexual differences in sex steroid hormonal profiles in hagfish plasma is lacking, since most studies that examined these profiles were performed in *M. glutinosa*, a species in which most individuals are either females or hermaphrodites (Powell et al., 2004).

Single functional gonadotropin (GTH) was recently identified in the pituitary of the brown hagfish, *Paramyxine atami* (Uchida et al., 2010). Cellular and transcriptional activities of hagfish GTH were significantly correlated with the stages of gonadal development. In addition, purified native GTH induced release of gonadal sex steroids (estradiol and testosterone) *in vitro*. Furthermore, tonic administration of estradiol resulted in significant accumulation of immunoreactive GTH-like material in the pituitary of juvenile brown hagfish (Miki et al., 2006). These findings clearly suggested the presence of a GTH-gonad feedback system in hagfish. The purpose of the present study was to examine sex steroid hormonal profiles in plasma in relation to gonadal development, sexual differences, and possible function of atretic follicles in *P. atami* using a time-resolved fluoroimmunoassay (TR-FIA).

MATERIALS AND METHODS

Animals

A total of approximately 300 brown hagfish, *P. atami*, including individuals of both sexes were obtained from fishermen at Oyashirazu Fishing Port, Niigata Prefecture, which faced the Sea of Japan, during the summer (July to September) of 2011 and 2012. Hagfish were trapped at depths of 100 to 130 m in the Sado Strait off the coast of Niigata Prefecture and were subsequently transported to the Sado Marine Biological Station, Niigata University, and kept in circulating seawater tanks without food at 12~15°C (Kabasawa and Ooka-Souda, 1991, 1994) prior to sacrifice. Tanks were covered by a black plastic sheet in order to prevent exposure of hagfish to light. Hagfish were maintained for one to several days before they were killed for sampling.

Blood Sampling and measurement of gonadal parameters

After being anesthetized using 2-phenoxy ethanol (Wako), individual hagfish were sampled for blood from a cut tail, and stored on ice. The blood was centrifuged at 3,500 rpm for 10 min at 4°C, and plasma was stored at -50°C until extraction. After blood collection, total length and body weight of each hagfish were measured. Animals were then killed by decapitation followed by removal of the gonad. In males, the testes were measured for weight, and a gonadosomatic index (GSI=testicular weight/body weight × 10^2) was calculated. In females, length of the long axis of the largest egg was measured. Atretic follicles were often observed in the ovary of early vitellogenic females (Fig. 1), but were relatively rare in late vitellogenic females. Empty follicles resulting from the discharge of eggs were not observed in the specimens examined in the present study, which suggested that collection sites did not encompass spawning locations. Since it is reported that the atretic follicles of *M. glutinosa* form the corpora lutea and produce progesterone (Powell et al., 2006), females that possessed atretic follicles in their ovaries were separated from females that did not have these follicles. Moreover, a previous study by Miki et al. (2006) reported that the majority of brown hagfish of both sexes that were less than 39 cm in total length were juveniles. Miki et al. (2006)

further reported that brown hagfish do not demonstrate a clear annual reproductive cycle, and that developmental conditions of ovaries varied among individuals, with some specimens containing non-vitellogenic eggs less than 2 mm in size and others containing almost mature eggs greater than 20 mm in size. Patzner and Adam (1981) studied hepatosomatic index (HSI) in *M. glutinosa*, and reported that females with eggs larger than 10 mm in diameter showed a statistically significant difference in HSI when compared to all other groups. Therefore, females in the present study were divided into 5 categories depending on total length and gonadal conditions, while males were divided into 4 categories depending on total length and GSI. Animals that could not be assigned to any of the categories were excluded from further analysis. Animals from which volume of collected plasma was less than 1 ml were also excluded from further analysis, since at least 1 ml of plasma was required from each animal in order to measure concentrations of the three sex steroids in duplicate manner.

Females:

1) Juveniles: total length < 39 cm and egg length ≤ 2 mm; 2) non-vitellogenic adults: total length ≥ 39 cm and egg length ≤ 2 mm; 3) early vitellogenic adults that possessed only normal follicles: total length ≥ 39 cm and 2 mm < egg length < 10 mm; 4) early vitellogenic adults that possessed both atretic follicles and normal follicles: total length ≥ 39 cm and 2 mm < normal egg length < 10 mm; and 5) late vitellogenic adults: total length ≥ 39 cm and egg length ≥ 10 mm.

Mean body length and body weight of each group of animals collected in 2011 were: 1) juveniles, 34.6 ± 0.7 cm and 66.6 ± 3.0 g (n=12); 2) non-vitellogenic adults, 42.1 ± 0.5 cm and 107.6 ± 4.1 g (n=16); 3) early vitellogenic adults that possessed only normal follicles, 44.6 ± 0.5 cm and 124.6 ± 5.4 g (n=11); 4) early vitellogenic adults that possessed atretic follicles, 45.4 ± 0.9 cm and 118.8 ± 9.2 g (n=17); and 5) late vitellogenic adults, 44.0 ± 0.8 cm and 133.5 ± 6.7 g (n=16), respectively. Animals collected in 2012 showed similar values for these parameters (data, not shown).

Males:

1) Juveniles: total length < 39 cm and GSI <0.1; 2) Adults with small GSI: total length ≥ 39 cm and GSI <0.1; 3) Adults with medium GSI: total length ≥ 39 cm and 0.1 ≤GSI < 0.2; 4)

adults with large GSI: total length ≥ 39 cm and GSI ≥ 0.2.

Mean body length and body weight of each group of animals collected in 2011 were: 1) juveniles, 35.1 ± 0.7 cm and 68.1 ± 4.2 g (n=12); 2) adults with small GSI, 43.4 ± 0.7 cm and 105.3 ± 4.8 g (n=17); 3) adults with medium GSI, 46.3 ± 1.0 cm and 118.7 ± 4.5 g (n=17); and 4) adults with large GSI, 45.2 ± 1.3 cm and 112.9 ± 10.0 g (n=8), respectively. Animals collected in 2012 showed similar values for these parameters (data, not shown).

Histological examination of testes

At the time of autopsy, testes of representative individuals (n=9~14) in each group were fixed in Bouin's solution for histological study as previously described (Nozaki et al., 2000). Hagfish testes are composed of lobules that consist of follicles filled with spermatogenetic cells. Mean percentages of follicles containing spermatogonia, spermatocytes, spermatids, or maturing sperm were calculated from a few randomly chosen sections. Follicles containing both spermatogonia and spermatocytes were included with follicles containing only spermatocytes. Follicles containing both spermatocytes and spermatids were included with follicles containing only spermatids. Any follicles that contained spermatozoa were treated as follicles containing spermatozoa irrespective of the stages of maturity of the remaining cells in the follicle.

Extraction of sex steroid hormones from plasma

Extraction of sex steroid hormones from hagfish plasma was achieved using methodology developed by Yamada et al., (1997, 2002). Steroid hormones were extracted from plasma (0.5 ml) with diethyl ether (2 ml, Wako). The extraction ingredient was dissolved in assay buffer (250 μl; 0.05M Tris buffer, 0.9% NaCl, 0.5% BSA, 0.05% NaN_3, 0.01% Tween 40, 20 μM DTPA/pH 7.75) and extracted plasma samples were frozen and stored at -50 °C prior to assay. In addition, pooled sera (500 μl) collected from several sexually mature hagfish of both sexes were also extracted in order to examine parallelism equilibrium using a standard curve. The extraction ingredient was dissolved in 100 μl assay buffer, and was then subjected to a 4-fold serial dilution. Samples of pooled sera were also frozen and stored at -50 °C prior to assay.

These procedures were repeated to measure three sex steroids in duplicate in samples collected in 2011 and to measure two sex steroids in triplicate in samples collected in 2012.

Time-resolved fluoroimmunoassay (TR-FIA)

TR-FIA procedures used for estradiol, testosterone and progesterone in the present study were the same as those used by Yamada et al. (1997, 2002). Fifty μl of a standard or sample and 150 μl of antiserum from the assay buffer were dispensed into the wells of 96-hole well plate. The plate was stored at room temperature for 4 h, and left overnight at 4 °C. After three washes, 200 μl of europium (Eu)-labeled-IgG (PerkinElmer) was added, and the plate was shaken for 1 h at room temperature. After another three washes, 100 μl of enhancement solution (PerkinElmer) was added, and the plate was shaken for 10 min at room temperature. Eu-fluorescence was measured with a time-resolved fluorometer. Each set of duplicate determinants of the three hormones were performed in February 2012 using samples collected in 2011, while each set of triplicate determinants of estradiol and testosterone were performed in February 2013 using samples collected in 2012. Concentrations of progesterone in samples collected in 2012 were not measured due to insufficient sample volume.

Primary antisera used in the present study were purchased from a commercial source (Cosmo-Bio, Tokyo, Japan): anti-estradiol (Catalog number: FKA236E), anti-testosterone (Catalog number: FKA102), and anti-progesterone (Catalog number: FKA302). Cross-reactivity of the antibodies used in the TR-FIA against chemically resembled steroids was as follows: for anti-estradiol antibody with testosterone (0.1%), estrone (5%), estriol (0.25%), and cortisol (less than 0.01%); for anti-testosterone antibody with estradiol, progesterone, 17α-hydroxyprogesterone, cortisol (less than 0.01%), 11-ketotestosterone (less than 1%), androstenedione (0.18%); for anti-progesterone antibody with pregnenolone (2%), 5α-pregnanedione (12.5%), 20α-hydroxyprogesterone (0.2%), 17α-hydroxyprogesterone (0.01%) (Yamada et al., 2002). TR-FIA of estradiol and testosterone in hagfish had been previously validated by parallel displacement curves obtained from dilutions of pooled plasma from multiple intact hagfish (Uchida et al., 2010). A parallel displacement curve was also obtained from the TR-FIA of progesterone with pooled hagfish plasma (Fig. 2). The limit of

detection of respective steroid hormone in our TR-FIA were: 1.2 pg/well (24 pg/ml) for estradiol, 0.3 pg/well (6.1 pg/ml) for testosterone, and 4.9 pg/well (98 pg/ml) for progesterone. The intra- and inter-assay coefficients of variation were 10.6% and 14.5%, 8.2% and 19.7%, and 12.8% and 13.4% for estradiol, testosterone, and progesterone, respectively.

Statistical Analysis

Data were expressed as group means ± SEM. Null hypothesis was tested using the Smirnoff test criterion for extreme values. Differences between two means were evaluated using either a Student t-test or a Cochran-Cox test. Statistical significance was determined at $P<0.05$.

RESULTS

Histological features of testes in relation to GSI

Testis of the hagfish consisted of a single thin membrane-like or thread-like organ that ran the length of the abdominal cavity and had several extensions (swellings) in the posterior region close to the anus, where the majority of spermatogenic follicles were distributed (Gorbman, 1983). Testes of juveniles were only 20~70 mg in weight, while those of adults were 50~600 mg in weight. In juvenile testes, almost all follicles were filled with spermatogonia (Figs. 3A, E). In adults with small GSI, most follicles displayed considerably increased volume compared to those of juveniles; however, the majority (84%) of them still contained spermatogonia and some follicles contained primary spermatocytes (16%) (Figs. 3B, F). Follicles containing neither spermatids nor maturing sperms were found in the testes of adults with small GSI. In adults with medium and large GSIs, follicles containing spermatogonia were still predominant (66% and 51% for adults with medium and large GSIs, respectively), while the occurrence of those containing spermatocytes increased considerably (27% and 34% for adults with medium and large GSIs, respectively) (Figs. 3C-D, G-H). Percentages of spermatids (5% and 10% for adults with medium and large GSIs, respectively) and maturing sperms (2% and 4% for adults with medium and large GSIs, respectively) were small in these two groups (Figs. 3C-D, G-H).

Plasma concentrations of steroid hormones in females

Concentrations of steroid hormones in plasma samples collected in 2011 and 2012 were measured independently. Samples from both years showed similar changes in plasma concentrations of estradiol and testosterone as a function of gonadal development in both females and males (Figs. 4, 5). In samples collected during both years, plasma concentrations of estradiol were low in juvenile females (Figs. 4E-1, E-2) and increased significantly in non-vitellogenic adults (Figs. 4E-1, E-2). Plasma estradiol concentrations further increased in early vitellogenic adults; however, concentrations observed in early vitellogenic adults were not significantly different from those observed in non-vitellogenic adults (Figs. 4E-1, E-2).

Plasma estradiol concentrations in early vitellogenic adults that possessed atretic follicles along with normal follicles were significantly lower than those in early vitellogenic adults that possessed only normal follicles (Figs. 4E-1, E-2). The highest concentrations of plasma estradiol were observed in late vitellogenic adults, where they were significantly greater than those observed in non-vitellogenic adults (Figs. 4E-1, E-2). Thus, a clear positive correlation was noted between plasma estradiol levels and ovarian development.

Plasma concentrations of testosterone were approximately ten times lower than concentrations of estradiol and progesterone (Figs. 4T-1, T-2). In samples collected in both 2011 and 2012, plasma testosterone concentrations in juveniles were low (Figs. 4T-1, T-2). The highest concentrations of plasma testosterone were found in non-vitellogenic adults samples from both 2011 and 2012; concentrations obtained from 2011 samples were significantly different from those observed in juveniles (Figs. 4T-1), while the difference was not significant in samples collected in 2012 (Fig. 4T-2). In both samples, no significant differences existed in plasma testosterone concentrations among the three adult groups examined (non-vitellogenic, early vitellogenic and late vitellogenic females) (Figs. 4T-1, T-2). Plasma testosterone concentrations in early vitellogenic adults that possessed atretic follicles along with normal follicles were significantly lower than those in females that possessed only normal follicles for samples collected in 2011 (Fig. 4T-1); however, the difference between these groups was not significant for samples collected in 2012 (Fig. 4T-2).

Plasma concentrations of progesterone were only measured in samples collected in 2011 (Fig. 4P-1), and were low in juveniles (Fig. 4P-1). The highest concentrations of plasma progesterone were noted in non-vitellogenic adults, and these concentrations were significantly different from those observed in juveniles (Fig. 4P-1). Plasma progesterone concentrations in early vitellogenic females that possessed only normal follicles were similar to those of non-vitellogenic adults; however, concentrations in early vitellogenic females that possessed atretic follicles as well as normal follicles were significantly lower than those that possessed only normal follicles (Fig. 4P-1). Plasma progesterone concentrations in late vitellogenic adults were significantly lower than they were in non-vitellogenic and early vitellogenic adults that possessed normal follicles (Fig. 4P-1). Thus, an inverse relationship

was found between plasma progesterone concentration and ovarian development in adult females.

Plasma concentrations of steroid hormones in males

In samples collected in both 2011 and 2012, plasma concentrations of estradiol observed in juvenile males were similar to those observed in juvenile females (Figs. 5E-1, E-2). In contrast with concentrations observed in females, no significant difference was found in plasma estradiol concentrations among juvenile males and adult males of the three different GSI groupings (small, medium and large) (Figs. 5E-1, E-2). In samples collected in both 2011 and 2012, plasma testosterone concentrations observed in juvenile males were similar to those observed in juvenile females (Figs. 5T-1, T-2). Concentrations of plasma testosterone were not significantly different among male groups, including juveniles (Figs. 5E-1, E-2). Plasma progesterone concentrations observed in juvenile males were similar to those observed in juvenile females and adult males with small and medium GSIs (Fig. 5P-1). Concentrations of plasma progesterone were significantly lower in adults with large GSI than they were in juveniles and adults with small GSI (Fig. 5P-1). Therefore, it was found that an inverse relationship existed between plasma progesterone concentrations and testicular development in males.

DISCUSSION

The present study revealed sex steroid hormonal profiles in plasma for both female and male brown hagfish. In females, plasma estradiol concentrations showed a significant positive correlation with ovarian development, while plasma testosterone and progesterone concentrations were highest in non-vitellogenic adults. In males, no relationships were observed between plasma estradiol or testosterone concentrations and testicular development, while plasma progesterone concentrations showed a significant inverse relationship with testicular development. Thus, there were clear differences in sex steroid hormonal profiles in plasma between females and males.

Using RIAs, biochemical techniques, or both, researchers have been able to detect low concentrations of estradiol, progesterone and/or testosterone in the circulating plasma (Matty et al., 1976; Weisbart et al., 1980; Schüzinger et al., 1987) and the gonads (Hirose et al., 1975; Gorbman and Dickhoff, 1978; Powell et al., 2004, 2006) of hagfish. Matty et al. (1976) did not find any correlation between plasma concentrations of estradiol and developmental conditions of the ovary of *E. stouti*, possibly due to the low sensitivity of the RIA method the used; however, Schützinger et al. (1987) used more sensitive RIA methods and found that a positive correlation between plasma estrogen concentration and egg size in female *M. glutinosa*. Powell et al. (2004) also reported using *in vitro* cultured ovaries that the number of females with large eggs increased following estradiol peaks in January in *M. glutinosa*. Data from the present study regarding plasma estradiol levels in female *P. atami* were consistent with the results of Schützinger et al. (1987) and Powell et al. (2004). Furthermore, the present study were successful using a TR-FIA in measuring plasma concentrations of estradiol, testosterone, and progesterone in all individuals from juveniles to adults in *P. atami*. In another study, Yu et al. (1981) demonstrated that synthesis of hepatic vitellogenin in *E. stouti* was induced by estrogens, estradiol and estrone. Thus, estrogenic control of ovarian development and hepatic vitellogenesis seems to be a mechanism that appeared early in the evolution of vertebrates.

In males, there was no clear relationship between plasma estradiol concentrations and

testicular development. However, none of the male groups examined in the present study contained sexually mature males with high incidence of spermatids or spermatozoa, which resulted in a lack of data regarding sexually active males. Therefore, it is still possible to consider that estradiol is involved in the regulation of male reproduction in hagfish. In support of this possibility, it is reported that purified native hagfish GTH induced secretion of estradiol and testosterone from cultured hagfish testes (Uchida et al., 2010). Moreover, intraperitoneal administration of estrogen in juvenile hagfish resulted in accumulation of immunoreactive GTH in the pituitary of both sexes (Miki et al., 2006). These results suggested the involvement of estradiol in the regulation of pituitary GTH functions through feedback action in both sexes of hagfish. Further studies are needed to clarify the function of estradiol in male hagfish.

Follicular atresia is a common feature in the hagfish ovaries (Gorbman, 1983), and it appears to be the method by which some 100 oocytes are reduced to approximately 20 that are grown and are ovulated (Dodd, 1986). Using in vitro organ cultured ovaries of *M. glutinosa* supplemented with pregnenorone, Powell et al. (2006) recently demonstrated that larger amounts of progesterone were released from atretic follicles (yellow bodies only) than from normal follicles. They hypothesized that hagfish possessed functional corpora lutea-like structures that produced progesterone; however, the present study showed that plasma levels of the three sex steroid hormones examined were significantly lower in adult females that possessed atretic follicles along with normal follicles than they were in females that possessed only normal follicles. These data clearly indicate reduced steroidogenic activity in females that possessed atretic follicles. Therefore, it is considered that progesterone production of atretic follicles is strongly suppressed under normal physiological conditions in hagfish. The present results in females having atretic follicles were unexpected for us, since normal follicles were still developing under reduced steroidogenic activity. It seems likely that normal follicles in those females will stop their development in course of time and are going to change to atretic follicles.

Previously, progesterone has been studied only in the ovaries in hagfish (Hirose et al., 1975; Gorbman and Dickhoff, 1978; Powell et al., 2004, 2006). In the present study, plasma

progesterone concentrations were the highest in non-vitellogenic adult females, and the lowest in late vitellogenic adult females. These changes in plasma progesterone concentrations were the opposite of those observed in the plasma estradiol concentrations of adult females. A similar inverse relationship was also observed in plasma progesterone concentrations of males, while no significant differences were observed in plasma estradiol concentrations among males of different GSI groups. Since plasma progesterone concentrations were the lowest in late vitellogenic females and early vitellogenic females that possessed atretic follicles, data from the present study do not support the idea proposed by Powell et al. (2006) that progesterone may be involved in retention of eggs and regulation of hepatic vitellogenin synthesis.

The difference on the results of atretic follicles between Powell et al (2004) and the present study might be originated by the species difference, since in *M. glutinosa* most individuals are females or hermaphrodites (Powell et al., 2004), whereas in *P. atami* all individuals are differentiated to males or females and the sex ratio is nearly 1:1 (Miki et al., 2006). Thus, it may be likely that the yellow bodies described in *M. grlutinosa* might be specific to that species. Alternatively, it is also possible that the progesterone released from the yellow bodies did not go into the plasma but acted locally at the gonadal level. Therefore, it remains unclear whether or not progesterone is involved in the regulation of hagfish reproduction.

In the present study, the highest concentrations of plasma testosterone were observed in non-vitellogenic adult females collected in 2011. In agreement with this result, Matty et al (1976) reported a significant negative correlation between body length and plasma testosterone concentrations in *E. stouti*. The function of testosterone in hagfish is not known, since physiological studies regarding testosterone have not been performed in hagfish. However, since purified hagfish GTH stimulated testosterone and estradiol release from organ cultured hagfish testes, testosterone may have some hormonal role. Alternatively, although hagfish gonads synthesize small amounts of testosterone, the hormone may not have a functional role in reproduction. The latter possibility is supported by the study of Thornton (2001), who has shown that nuclear steroid receptors arose at the time of or before vertebrate

radiation, with the exception of an androgen receptor that evolved only in gnathostomes.

In relation to our failure to correlate plasma concentrations of progesterone or testosterone to gonadal development, recent studies in the lamprey have emphasized the importance of non-classical steroids, such as androstenedione and 15α-hydroxylated sex steroids (15α-hydroxytestosterone and 15α-hydroxyprogesterone) in serving as functional androgens (Lowartz et al., 2003; Young et al., 2007; Bryan et al., 2007, 2008). Indeed, evidence demonstrating testosterone functionality in lampreys was scarce (see Young et al., 2004), while androstenedione was found in substantial amounts within the testicular tissue of lampreys, and plasma and tissue levels of the hormone increased significantly in prespermiating male sea lampreys after injection of GnRH (Bryan et al., 2007). In addition, prespermiating males implanted with androstenedione reached maturation significantly faster and exhibited larger secondary sex characteristics than placebo or non-implanted males. A receptor for androstenedione was recently described by Bryan et al. (2007). 15α-Hydroxylated steroids are also suggested to be involved in the regulation of lamprey reproduction (Bryan et al., 2008). Since hagfish gonads also produce substantial amounts of unusual androgens, such as 6β-hydroxy testosterone and 5α-androstane-3β, 7α, 17β-triol, as well as androstenedione (Hirose et al., 1975; Kime et al., 1980; Kime and Hews, 1980), some of these steroids may act as functional androgens. Further study is required in order to clarify the role of these steroids in hagfish.

In conclusion, the present study revealed sex steroid hormonal profiles in plasma of both male and female brown hagfish, *P. atami*. Of the three sex steroids examined, only estradiol showed a significant correlation with ovarian development in females, while the two other sex steroid hormones did not show a significant correlation with testicular development.

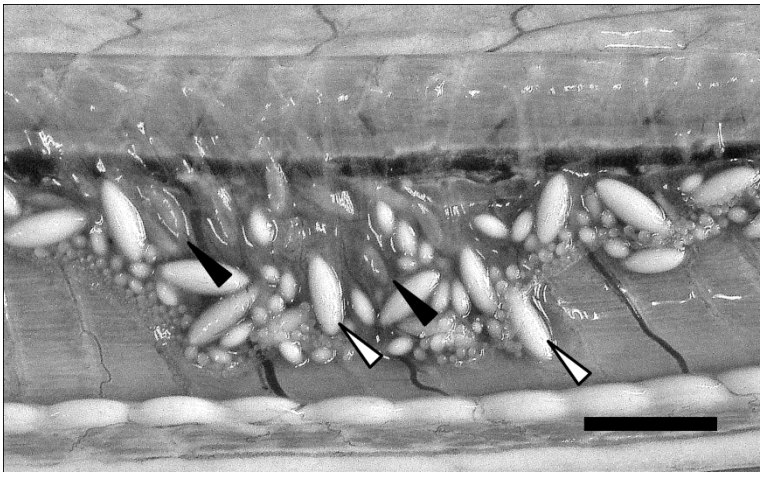

Fig. 1. Ovary containing atretic follicles. Black and white arrowheads indicate atretic (3 mm in length of long axis) and the normal follicles (6 mm in length of long axis), respectively. Scale bar: 10 mm.

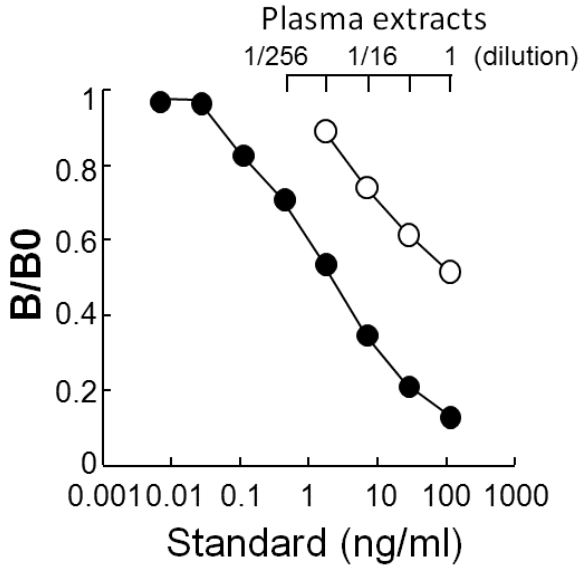

Fig. 2. Dose-response inhibition curves of standard (solid circles) and serially diluted (open circles) hagfish plasma in the progesterone TR-FIA. Each value represents the mean of triplicate determinations.

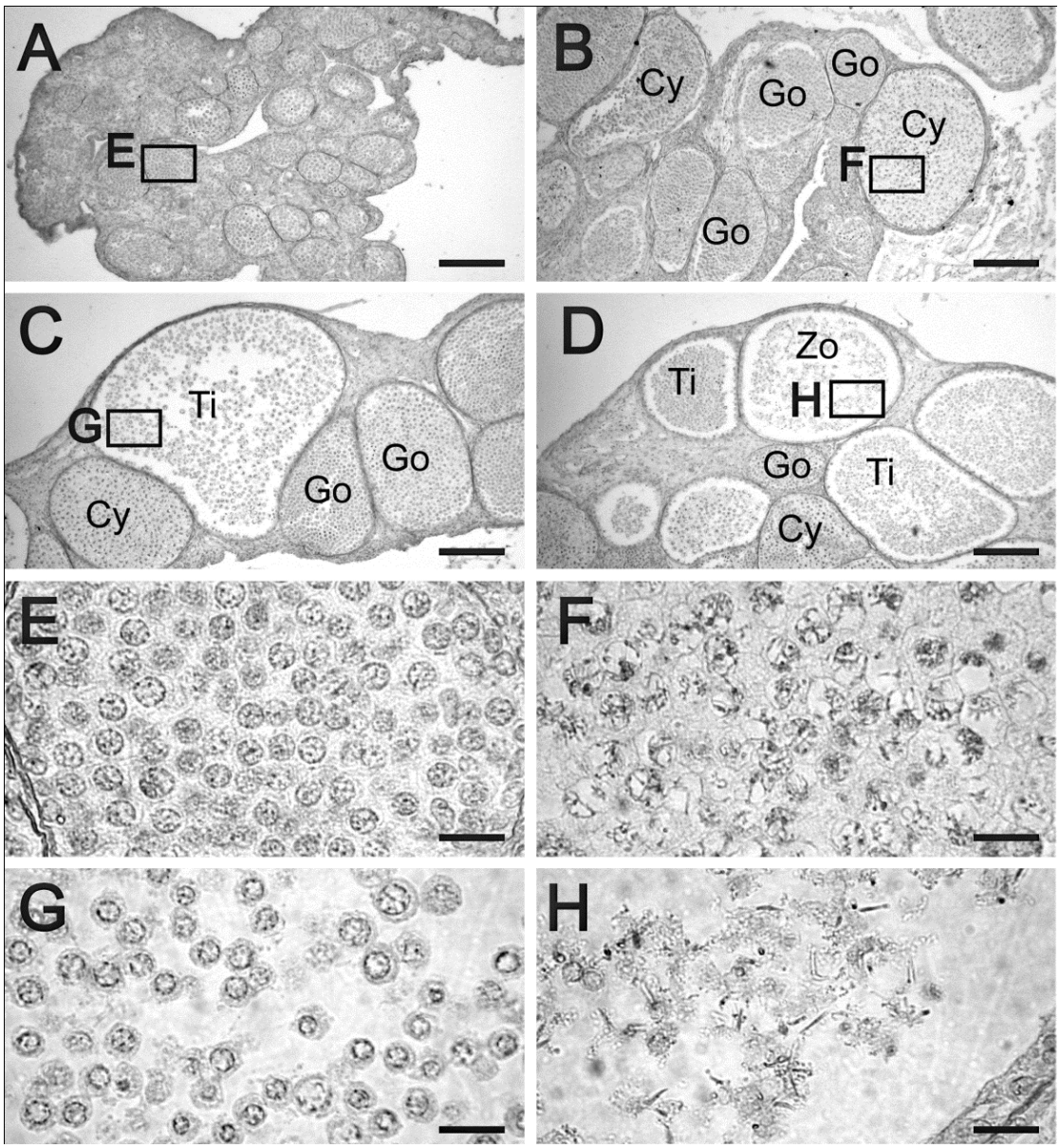

Fig. 3. Representative sections through testicular follicles of brown hagfish indicating spermatogenic stages in juveniles (A, GSI=0.027), adults with small GSI (B, GSI=0.068), adults with medium GSI (C, GSI=0.152), and adults with large GSI (D, GSI=0.292). Areas outlined by rectangles in a-d are enlarged and shown in e-h, respectively. Note follicles filled with spermatogonia (E), primary spermatocytes under cell division (F), spermatids (G), and spermatozoa (H), respectively. Cy, spermatocytes; Go, spermatogonia; Ti, spermatids; Zo, spermatozoa. Scale bars: A-D, 200 μm (x40); E-H, 20 μm (x400).

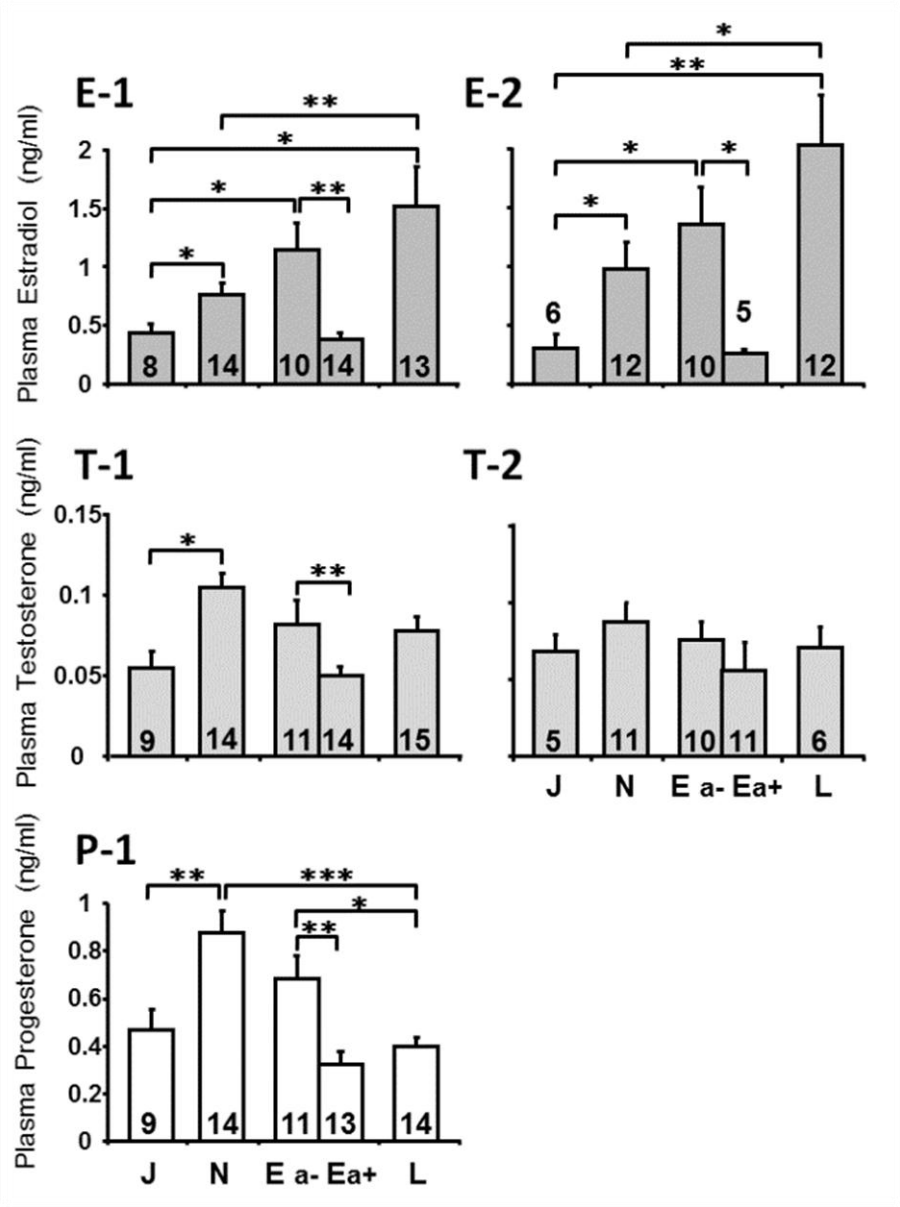

Fig. 4. Plasma concentrations of estradiol (E-1 and E-2), testosterone (T-1 and T-2), and progesterone (P-1 and P-2) in females sampled in 2011 (left column) and 2012 (right column). Bars indicate standard errors. Numbers in each column indicate number of animals examined. J, juveniles; N, non-vitellogenic adults; E a-, early vitellogenic adults possessing only normal follicles; E a+, early vitellogenic adults possessing atretic follicles as well as normal follicles; L, late vitellogenic adults. *P < 0.05; **P < 0.01; ***P < 0.001.

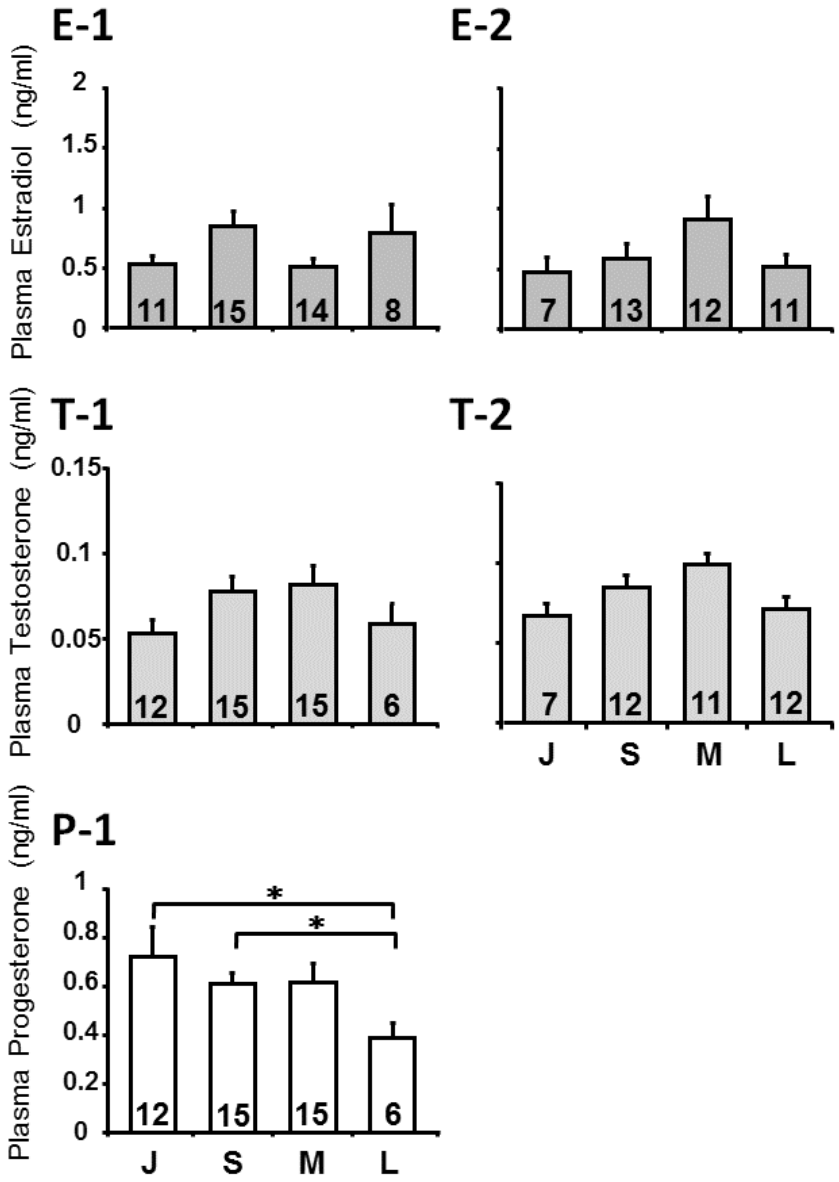

Fig. 5. Plasma concentrations of estradiol (E-1 and E-2), testosterone (T-1 and T-2), and progesterone (P-1 and P-2) in the males sampled in 2011 (left columns) and 2012 (right columns). Bars indicate standard errors. Number in each column indicates number of animals studied. J, juveniles; S, adults with small GSI; M, adults with medium GSI; L, adults with large GSI. *$P < 0.05$.

Chapter 2

Molecular Cloning of Cytochrome P450 side-Chain Cleavage Enzyme and its mRNA Changes during Gonadal Development of Brown Hagfish, *Paramyxine atami*

INTRODUCTION

Of all vertebrate species, both extant and extinct, hagfishes are considered to be the most primitive (Forey and Janvier, 1993, 1994). Accordingly, studies on the hagfish reproduction are indispensable for understanding phylogenetic aspects of vertebrate reproduction. However, our knowledge of endocrine regulation of reproductive processes is poorly understood in hagfish (for reviews, see Gorbman, 1983; Nozaki, 2008, 2013). For example, it was thought that they did not have the same neuroendocrine control of reproduction as gnathostomes (jawed vertebrates), since it was not clear whether the hagfish pituitary gland contained tropic hormones of any kind (Matty et al., 1976; Hardisty, 1979; Gorbman, 1983). Recently, single functional gonadotropin (GTH) was identified in the pituitary of the brown hagfish (*Paramyxine atami)*, one of the Pacific hagfish (Uchida et al., 2010). Cellular and transcriptional activities of both α- and β-subunits of hagfish GTH were significantly correlated with the stages of gonadal developments. In addition, purified native GTH induced release of gonadal sex steroids (estradiol and testosterone) *in vitro*. Furthermore, tonic administration of estradiol resulted in significant accumulation of immunoreactivities of both α- and β-subunits of GTH in the pituitary of juvenile brown hagfish (Miki et al., 2006; Nozaki et al., 2013). These findings clearly suggested the presence of a GTH-gonad feedback system, similar to that of gnathostomes, in the hagfish.

It is well established that the pituitary GTHs, luteinizing hormone (LH) and follicle-stimulation hormone (FSH), are the major hormones that act on the gonads to regulate steroidogenesis and gametogenesis in gnathostomes. Sex steroids in vertebrate gonads have crucial roles in reproductive phenomena including sex differentiation, gametogenesis, and gamete maturation. The biosynthetic enzymes of sex steroids and their products have been well studied in gnathostomes. Typically, there are three cytochrome P450 enzymes (CYP) such as P450 side chain cleavage (CYP11A), P450 17α-hydoroxylase (CYP17), and P450 aromatase (CYP19) and two types of hydroxylated dehydrogenases (HSDs) such as 3β-HSD and 17β-HSD. Among these enzymes, CYP11A is an enzyme that regulates the conversion from cholesterol to pregnenolone by its side chain cleavage activity, and it is the first and

essential enzyme of steroidogenesis. It is also well established in gnathostomes that sex steroid hormones are produced in the cells comprising the growing follicles (theca cells and granulosa cells) of the ovary and in the interstitial cells (Leydig cells) of the testis.

However, little information is available on the steroidogenesis or steroidogenic enzymes in the most primitive vertebrate, hagfish. Using radioimmunoassays (RIA), biochemical techniques, or both, researchers have been able to detect low concentrations of estradiol, progesterone and testosterone in the circulating plasma (Matty et al., 1976; Weisbart et al., 1980; Schüzinger et al., 1987; Nishiyama et al., 2013) or in the gonads (Hirose et al., 1975; Gorbman and Dickhoff, 1978; Powell et al., 2004, 2006) of the hagfish. Among those studies, Schützinger et al. (1987) reported that plasma estrogen content increased in relation to the stages of ovarian development in female Atlantic hagfish, *Myxine glutinosa*. Nishiyama et al. (2013) further observed that among estradiol-17β, testosterone and progesterone only plasma levels of estradiol-17β showed the significant correlation to the ovarian developments. In another study, Yu et al. (1981) demonstrated that the synthesis of hepatic vitellogenin was inducible by estrogens, estradiol and estrone, in *Eptatretus stouti*. Based on these results, estrogenic control of ovarian development and hepatic vitellogenesis seems to have arisen early in vertebrate evolution. As to the localization of steroidogenic cells, Tsuneki and Gorbman (1977a) reported cells with the ultrastructural features of Leydig cells (e.g., smooth ER, tubular cristae) among the spermatogenic follicles in the adult *Eptatretus stouti*. However, neither Fernholm (1972) in *Myxine glutinosa* nor Tsuneki and Gorbman (1977b) in *Eptatretus stouti* found cells showing the ultrastructural characteristics associated with steroidogenesis in the hagfish ovary.

The general objective of the present study was to elucidate all the processes of steroidogenesis and the involvement of steroidogenic enzymes in the hagfish gonads. Following EST analysis of the hagfish testis, *CYP11A*, which is encoding the first and essential enzyme for steroidogenesis, was cloned. Thus, the crucial objectives of the present study were to analyze the expression patterns of *CYP11A* mRNA in correlation to gonadal developments and following GTH administration. We also aimed to identify the steroidogenic cells in the hagfish gonads using *CYP11A* by *in situ* hybridization. Our results showed that the

expression patterns of *CYP11A* mRNA were very similar to those in gnathostomes. It is suggested that vertebrates, during their early evolution, have established the pituitary-gonadal reproductive system.

MATERIALS AND METHODS

Animals

A total of approximately 170 brown hagfish, *Paramyxine atami*, including individuals of both sexes were obtained from fishermen at Oyashirazu Fishing Port, Niigata Prefecture, during the summers (July to September) from 2008 to 2012. Hagfish were trapped at depths of 100 to 130 m in the Sado Strait off the coast of Niigata Prefecture and were subsequently transported to the Sado Marine Biological Station, Niigata University, and kept in circulating seawater tanks without food at 12~15°C (Kabasawa and Ooka-Souda, 1991, 1994) for one to several days before they were sacrificed for sampling. Tanks were covered by a black plastic sheet in order to prevent exposure of hagfish to light. The experimental protocol for the handling of the hagfish was reviewed and approved by the committees of Niigata University and were performed according to their Guide for the Care and Use of Animals.

Procedures of tissue preparations

Animals were anesthetized with 2-phenoxy ethanol (Wako), and the total body lengths and body weight were measured. They were killed by decapitation, the abdominal body was opened, and sex was determined. To estimate the developmental condition of female gonads, the length of the long axis of the largest egg (=follicle) was measured. In each female, several representative eggs of which egg yolk was removed by squeezing the egg were collected. To estimate the developmental condition of male gonads, testicular weight was measured, and a gonadosomatic index (GSI = testicular weight / body weight $\times 10^2$) was calculated.

For cloning of cDNA encoding hagfish sex steroidogenic enzymes and quantitative Real-Time PCR analysis, whole or a part of the gonadal tissue was snap frozen in liquid nitrogen, and were kept at -80°C until the extraction of total RNA. For *in situ* hybridization, egg follicles of late vitellogenic eggs (egg length: 16-17 mm) with normal development and testicular tissues of adult testes with large GSI (GSI: 0.28-0.42) were fixed with 4% paraformaldehyde at 4°C for overnight. The fixed tissues were dehydrated, and embedded in Paraplast; all procedures for tissue preparations of *in situ* hybridization were the same as those

described previously (Uchida et al., 2010). Serial sections (10 μm thick) were mounted on MAS-coated glass slides (Matsunami).

Classification of developmental conditions of gonads

Ovaries were divided into four categories depending on the total body length and the egg length. Testes were divided into four categories depending on the total body length and the GSI as described previously (Nishiyama et al., 2013). Gonads not fulfilling the following criteria were excluded from further analysis.

Ovary: 1) Juvenile ovary: total length < 39 cm and largest egg length ≤ 2 mm; 2) Adult ovary with non-vitellogenic eggs: total length ≥ 39 cm and largest egg length ≤ 2 mm; 3) Adult ovary with early vitellogenic eggs: total length ≥ 39 cm and 2 mm < largest egg length < 10 mm; and 4) Adult ovary with late vitellogenic eggs: total length ≥ 39 cm and largest egg length ≥ 10 mm.

Testis: 1) Juvenile testis: total length < 39 cm and GSI <0.1; 2) adult testis with small GSI: total length ≥ 39 cm and GSI <0.1; 3) adult testis with medium GSI: total length ≥ 39 cm and 0.1 ≤GSI < 0.2; 4) adult testis with large GSI: total length ≥ 39 cm and GSI ≥ 0.2.

Cloning of cDNA encoding hagfish sex steroidogenic enzymes

Total RNA was extracted from the testis of one sexually mature male (testicular weight, 726 mg; GSI=0.406; total length, 50.0 cm; body weight, 178.8 g) of *P. atami*, which was one of animals collected in 2008. The testicular tissue was extracted using a RNeasy kit (QIAGEN). Reverse transcription, randomly selection of cDNA clones, and preparation of recombinant plasmid DNA were delegated to the Hokkaido System Science Inc. (Hokkaido, Japan). A total of 5136 clones (96-hole well plate x 53.5) were used for the sequence analysis. The 5' sequence of each clone was determined using a capillary DNA sequencer (3730xl DNA Analyzer, Applied Biosystems) using a BigDye Terminator Cycle Sequencing Kit Ver. 3.1. (Applied Biosystems). The BLASTX Internet programs (http://blast.ddbj.nig.ac.jp/) from the Center for Information Biology and DNA Data Bank of Japan (Mishima, Japan) were used for homology searches for cDNA sequences. Genetyx (GENETYX CORPORATION) was

used for processing the sequence data and aligning the sequences. After EST analysis of 5136 clones, 10 genes were identified as the sequences related to gonadal functions. Among them, only one clone showed high sequence homology to chordate cytochrome P450 side-chain cleavage enzyme (CYP11A), which is the converting enzyme from cholesterol to pregnenolone. Among the remaining nine clones, one clone showed high sequence homology to steroidogenic acute regulatory protein (data, not shown).

Following the gene-specific primers for 3'and 5' sequence analysis were listed in Table 1 on the basis of the partial sequence of putative hagfish *CYP11A* that was provided by the EST analysis. Amplified cDNA fragments were extracted from 1.5% agarose gels using a QIAEX II Gel Extraction Kit (QIAGEN), and cloned into pCR II TOPO® Vector (Invitrogen) and transformed into One Shot TOP10 Chemically Competent cells (Invitrogen). More than three independent clones per gene were sequenced by using a 3130XL Genetic Analyzer (Applied Biosystems).

For comparison of the molecular structure of hagfish CYP11A with cephalochordate and jawed vertebrates, following GenBank Accession numbers were used: amphioxus CYP11A (GenBank: BAF61103.1), medaka CYP11A (GenBank:BAG49048.1), Japanese eel CYP11A (GenBank : AAV67332), and human CYP11A (GenBank:AAA52162.1).

Phylogenetic analysis

Phylogenetic analysis of CYP11A was performed including the closely related cytochrome P450 enzymes, 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). Amino acid sequences were aligned using the Sea View (Galtier et al., 1996) and phylogenetic relationships inferred using maximum parsimony the Mega version 6 (Tamura et al., 2013) and Neighbor-Joining method in ClustalX 2.0 (Larkin et al., 2007).

Quantitative Real-Time PCR Analysis

Animals obtained from 2009 to 2012 were used to quantify the mRNA levels of *CYP11A* depending on the developmental condition of their gonads. For quantifying the mRNA levels, a quantitative real-time PCR assay was performed. Primers specific to the

hagfish *CYP11A* and *β-actin* (Uchida et al., 2010; AB546742) were designed as listed in Table 1.

Total RNA from the gonads was extracted using a RNeasy kit (QIAGEN). cDNA were reverse transcribed using a PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer's protocols. Quantitative real time PCR for *CYP11A* mRNA was performed in 5 μl SYBR Premix EX Taq II (TaKaRa), 0.2 μl of each primer, 3.6 μl water, and 1 μl cDNA using an Thermal Cycler Dice® Real Time System TP800 (TaKaRa). The PCR thermal profile was 95 °C for 30 s, followed by 40 reaction cycles of 95 °C for 15 s and 60 °C 30 s each and dissociation. The levels of *β-actin* mRNA in each sample were also measured and used as an internal control.

Effect of native GTH on *CYP11A* mRNA expression levels

GTH fractions purified from hagfish pituitary were prepared as described in Uchida et al. (2010). Testes with large GSI more than 0.2 from eight hagfish, which were collected in 2008, were divided uniformly sized pieces (about 20 mg per piece). Each piece of testis was pre-incubated in 500 μl of the culture medium (see Uchida et al., 2010), and incubated then in the fresh medium with or without hagfish GTH (5 μg/ml) for 24 hrs at the same conditions. After incubation, total RNA was extracted from each of the incubated piece of testis by RNeasy kit (QIAGEN). Reverse transcription and Real-time PCR were performed using a One Step SYBR PrimeScript PLUS RT-PCR Kit (TaKaRa) according to the manufacturer's protocols. Real time PCR was performed in 12.5 μl 2×One Step SYBR RT-PCR Buffer, 1.5 μl EX Taq (TaKaRa), 1 μl each primer, 0.5 μl PrimeScript PLUS RTase Mix, 6.5 μl water, and 2 μl RNA. The PCR thermal profile was 42 °C for 5 min, 95 °C for 10 s, followed by 40 reaction cycles 95 °C for 5 s and 60 °C for 30 s each and dissociation. The levels of *β-actin* mRNA in each sample were used as an internal control.

***in Situ* Hybridization**

Specific primers (Table. 1) were used to amplify the PCR fragment of *CYP11A* (nt 525 to nt 1011; 486 bp) for generating labeled riboprobes. The PCR fragment was sub-cloned into

pCR II-TOPO® Vector (Invitrogen) and sequenced as described above. The sub-cloned fragment was used as a template for synthesis of digoxigenin (DIG)-labeled anti-sense and sense riboprobes with DIG RNA Labeling Kit (Roche).

Paraffin sections were made from both ovaries (n=3) and testes (n=3). For *in situ* hybridization, the sections were deparaffinized, hydrated, and treated with 10 mg/ml proteinase K (Roche) at room temperature for 5 min. The sections were subsequently hybridized with antisense DIG-labeled RNA probes at 63°C for 18 h. Hybridization signals were detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche) and 4-nitroblue tetrazolium chloride／5-bromo-4-chloro-3-indolyl-phosphate (NBT／BCIP) (Roche) as a chromogenic substrate. Negative control signals were stained with sense-strand riboprobes, in which we detected no equivalent signals to those detected in this study.

Statistical Analysis

Data were expressed as group means ± SEM. Null hypothesis was tested using the Smirnoff test criterion for extreme values. Differences between two means were evaluated using either a Student t-test or a Cochran-Cox test. Statistical significance was determined at $P<0.05$.

RESULTS

Cloning of cDNA encoding *CYP11A*

The cDNA encoding putative hagfish *CYP11A* was 1800 bp long with an Open Reading Frame (ORF) of 1491 bp (497 amino acids) (Fig. 1-A). Multiple alignment analysis (GENETYX CORPORATION) revealed that the amino acid residues observed in the hagfish CYP11A were highly conserved and well shared with those of chordate CYP11A, especially in the regions involved in the function of enzymatic catalysis, namely the substrate binding domain, adrenodoxin binding domain, and heme-binding domain (Fig. 1-B). However, 5' end of the ORF of the hagfish was approximately 60 bases shorter than those of gnathostomes and amphioxus (Chung et al., 1986; Ijiri et al., 2006; Mizuta and Kubokawa., 2007; Nakamoto et al., 2010). The mean values of sequence identity of those three domains between hagfish and three gnathostome species (medaka, Japanese eel and human) and between hagfish and amphioxus were: substrate binding domain (34.4% and 31.2%), adrenodoxin binding domain (61.1% and 44.4%), and heme-binding domain (70.0% and 50.0%), respectively.

Phylogenetic analysis

Phylogenetic analysis demonstrates that the putative hagfish CYP11A was positioned within the clade of vertebrate CYP11A, and was separated from CYP11B (Fig. 2). Hereafter, putative hagfish CYP11A is referred as to hagfish CYP11A. Hagfish and stingray CYP11A further segregated from teleost and bird/mammalian CYP11A clades within the clade of CYP11A. The amphioxus CYP11A was positioned as an outgroup of vertebrate CYP11 clade. Human CYP19 was used as an outgroup in the CYP11 tree. The tree was also constructed by the Maximum parsimony method, which showed the hagfish CYP11A in the same position as that of neighbor-Joining method (data not shown).

In situ Hybridization of hagfish *CYP11A* mRNA

In females, cells expressing *CYP11A* mRNA were found only in the outer layer of the egg follicle corresponding to the theca externa and theca interna of the theca layer (Tsuneki and

Gorbman, 1976) (Fig. 3-A, D). In males, *CYP11A* mRNA signals were found in two types of cells, one is typical Leydig cell (Fig. 3-B, E, Fig. 4), and the other is the boundary cell (tubule-boundary cells) surrounding the testicular follicle (Gorbman and Bern, 1962) (Fig. 3-C, F, Fig. 4).

Correlation between gonadal expression of *CYP11A* and gonadal development

In females, mRNA expression levels of *CYP11A* were very low in the non-vitellogenic eggs of both juveniles and adults (Fig. 5-A). They were significantly higher in early vitellogenic eggs than those in non-vitellogenic eggs (Fig. 5-A). The highest levels of *CYP11A* mRNA expression were found in the late vitellogenic eggs (Fig. 5-A). Thus, a clear positive correlation was noted in *CYP11A* mRNA levels in relation to egg development. Similarly, in males, mRNA levels of *CYP11A* were low in the testis of juveniles and adults with small GSI (Fig. 5-B). They were significantly higher in the testis of adults with medium GSI than those of juveniles and adults with small GSI, and the highest levels were found in the testis with large GSI (Fig. 5-B).

Effect on *CYP11A* mRNA expression by culture with hagfish GTH

CYP11A mRNA expression levels were significantly higher in the testis incubated with hagfish GTH (5 μg/ml) than those incubated without hagfish GTH (Fig. 6), indicating that gonadal *CYP11A* expression was induced by pituitary GTH in hagfish.

DISCUSSION

CYP11A is a crucial steroidogenic enzyme that catalyzes an initial step in the production of all classes of steroids. Therefore, expression of *CYP11A* in steroidogenic tissues is of fundamental importance for determining the levels of steroid hormone production and gonadal development. In the present study, cDNA encoding *CYP11A* was cloned from the testis of brown hagfish. Based on the EST analysis and PCR cloning, we have identified the gene structure of hagfish *CYP11A*. The deduced amino acid sequence of hagfish CYP11A shows high similarity to other animal forms especially in two functional domains, adrenodoxin binding domain and heme-binding domain. In the phylogenetic analysis, hagfish CYP11A forms a clade with the vertebrate CYP11A. *In situ* hybridization study showed that positive signals of *CYP11A* mRNA expression were observed in the typical steroid producing cells of the hagfish gonads. Transcriptional levels of hagfish *CYP11A* in the gonads showed clear positive correlation with the gonadal development in both sexes. Moreover, the steroidogenic activity of the hagfish testis is under the control by the pituitary GTH, similar to that of more advanced gnathostomes. Our current studies clearly demonstrated that hagfish have functional CYP11A molecule in their gonads.

In the present study, transcriptional levels of *CYP11A* increased in accordance with the developmental stages of gonads in both sexes of the brown hagfish. These results are consistent with those in the more advanced gnathostomes. For example, transcript for *CYP11A* in the gonads increased in correlation to gonadal development in both sexes of rainbow trout (Nakamura et al., 2005; Kusakabe et al., 2006) and female Japanese eel (Kazeto et al., 2006). In a previous study, we have shown that plasma concentrations of estradiol-17β increased in correlation with the gonadal development in female brown hagfish (Nishiyama et al., 2013). Moreover, Yu et al. (1981) demonstrated that synthesis of hepatic vitellogenin in *Eptatretus stouti* was induced by estrogens, estradiol and estrone. Thus, CYP11A is suggested to play a crucial role in the synthesis of estradiol-17β, which in turn acts on ovarian development and hepatic vitellogenesis in the female hagfish.

In male hagfish, although transcriptional levels of *CYP11A* increased in accordance with

the developmental stages of testis, no relationship was obtained between the testicular development and plasma levels of estradiol or testosterone (Nishiyama et al., 2013). Thus, there was a clear discrepancy between males and females on the relationship between the transcriptional levels of *CYP11A* and plasma steroid levels. These results suggest a possibility that male hagfish uses other steroids than estradiol or testosterone as major androgens. In support of this possibility, recent studies in the lamprey have emphasized the importance of non-classical steroids, such as androstenedione and 15α-hydroxylated sex steroids (15α-hydroxy- testosterone and 15α-hydroxyprogesterone) in serving as functional androgens (Lowartz et al., 2003; Young et al., 2007; Bryan et al., 2007, 2008). A receptor for androstenedione was recently described in lampreyby Bryan et al. (2007). 15α-Hydroxylated steroids are also suggested to be involved in the regulation of lamprey reproduction (Bryan et al., 2008). Since hagfish gonads also produce substantial amounts of unusual androgens, such as 6β-hydroxy testosterone and 5α-androstane-3β, 7α, 17β-triol, as well as androstenedione (Hirose et al., 1975; Kime et al., 1980; Kime and Hews, 1980), some of these steroids may act as functional androgens. Further study is required in order to clarify the role of these steroids in hagfish.

It is well established that sex steroid hormones are produced in the cells comprising the growing follicles (theca cells and granulosa cells) in the ovary of female gnathostomes. A two-cell type model has been proposed for the follicular steroidgenesis in the ovary (see Nagahama et al., 1994; Gore-Langton and Armstrong, 1998). In that model, steroid synthesis from cholesterol to androgen is performed in the theca cells, followed by the synthesis of estrogen from androgen in the granulosa cells. In the hagfish, although substantial amount of steroid hormones are produced in the ovary (see Nishiyama et al., 2013), previous electron microscopy did not find cells showing the characteristics associated with steroidogenesis in the ovary of *Myxine glutinosa* (Fernholm, 1972) or *Eptatretus stouti* (Tsuneki and Gorbman, 1977b). However, the present study clearly demonstrated the expression of *CYP11A* mRNA in the theca cells of the ovarian follicles of *Paramyxine atami*. The reason of the difference of the results was still unclear, but could be attributed to that we used the follicular tissues expressing the highest levels of *CYP11A*.

It is also well established that sex steroid hormones are produced in the interstitial cells (Leydig cells) of the testis in male gnathostomes. For example, steroidogenic enzymes such as CYP11A, CYP17, and 3β-HSD were reported in the Leydig cells of the rainbow trout testis (Kobayashi et al., 1998). However, in some teleost species, such as pike and char, urodele amphibians, and turtles, typical interstitial cells are absent, and a ring of circumtubular cells (tubule-boundary cells) are considered to be the site of the production of sex steroid hormones (Gorbman and Bern, 1962). In the lamprey, Leydig cells in the testis are also suggested, in a histological study, to be sex steroid hormone producing cells (Larsen, 1973, cited by Gorbman, 1983). In the hagfish, Tsuneki and Gorbman (1977a) described the ultrastructure of the testis of *Eptatretus stouti*: they found no apparently steroidogenic cells until a body length of about 40 cm is attained. At that time cells with the features of Leydig cells (e.g., smooth ER, tubular cristae) appeared among the spermatogenic follicles. In well agreement with Tsuneki and Gorbman (1977a), the current study showed that expression levels of *CYP11A* mRNA were very low in juveniles less than 39 cm in total length, but increased significantly in relation to testicular development. Our histological observations by *in situ* hybridization further revealed that *CYP11A* mRNA was expressed in both the Leydig cells and tubule-boundary cells of the developing testis. Thus, the Leydig cells and tubule-boundary cells are considered the steroid producing cells in the hagfish testis.

It is well established that pituitary GTHs, LH and FSH, are the major hormones that stimulate sex steroid hormones and gonadal activities in gnathostomes. Our previous study has revealed that hagfish GTH induces the release of sex steroids (estradiol-17β and testosterone) from the cultured testis in a dose-dependent manner (Uchida et al., 2010). In the present study, hagfish GTH stimulated the mRNA levels of *CYP11A* in the cultured testis. These results suggested that the steroidogenic activity of the hagfish testis is under the control of the pituitary GTH, similar to that of more advanced gnathostomes.

In conclusion, the present study revealed that expression of *CYP11A* in the gonads is of fundamental importance for determining the levels of steroid hormone production and gonadal development in the hagfish. Our data further showed that theca cells of the ovarian follicles and Leydig cells/tubule-boundary cells of the testis are steroidogenic cells of the

hagfish gonads.

Table 1. Sequence of primers

Primer name		Sequence (5’ → 3’)
For Sequencing		
SP1		GGGTATTGTACGGTGAGCGTATGGGC
SP2		GTGTACCCATGCTGTTCATCCCACCGG
SP3		ACACATGAATGCCTGGGACG
SP4		GGACCATGGGTCGACATCCTG
SP5		GCGGGACATACATGGTGAAGC
SP-R	reverse primer	GTAAGCCCATACGCTCACCG
SP-Rn	reverse primer	CAGACAGACTGCAGAGAGGG
For Real time PCR		
RT-F	CYP11A forward primer	GGCTGGAGGAGTAGACACGA
RT-R	CYP11A reverse primer	GCAAGGTTTCCCACGCTTT
AC-F	B-actin forward primer	TCAACCCCAAAGCCAACC
AC-R	B-actin reverse primer	CACCAGAGTCCATCACAATACCC
PCR subcloning for ISH		
SC-F1n	CYP11A forward primer	CCGTACCAGTGTACCCATGC
SC-R1n	CYP11A reverse primer	GGGTGACTGCCACAGGATGC

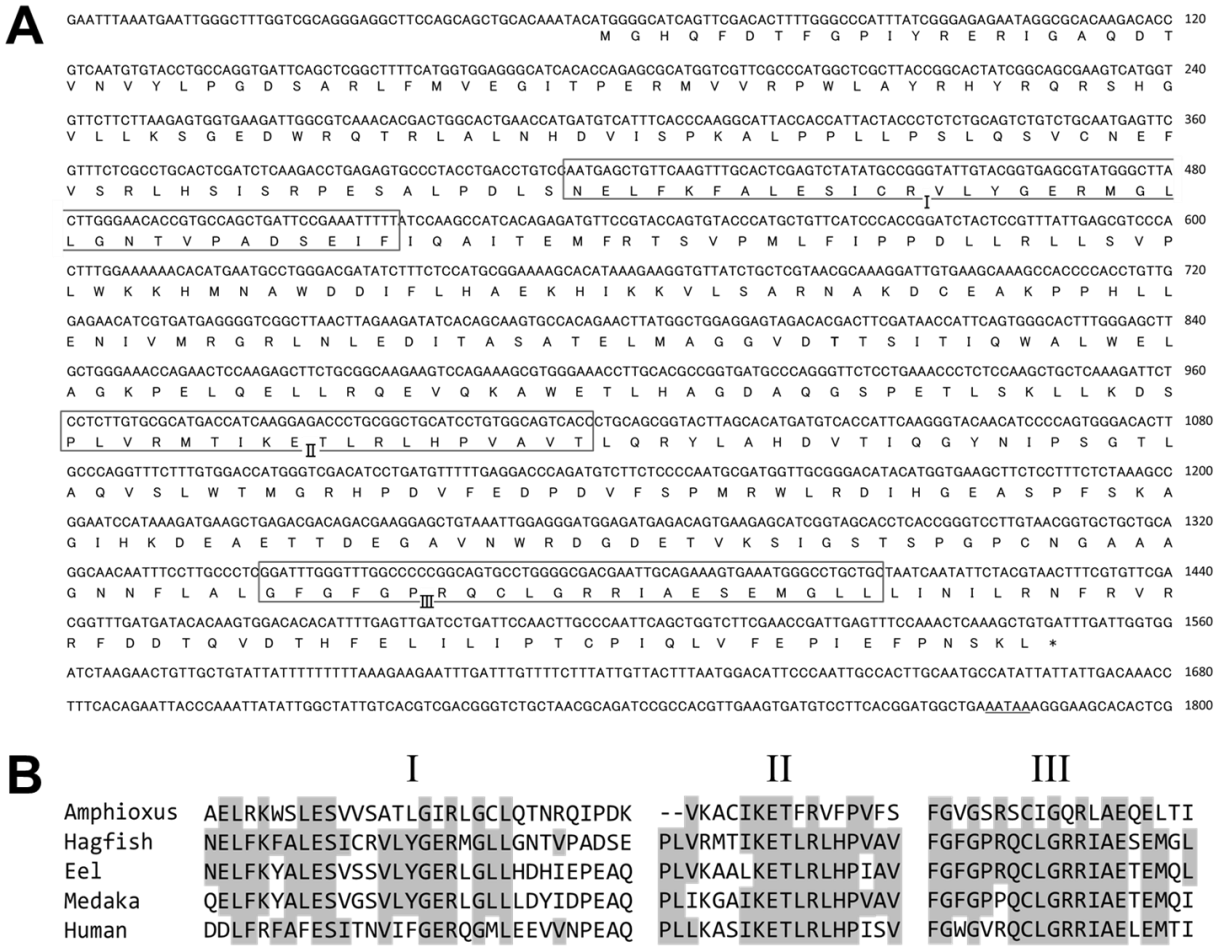

Fig. 1. A : Nucleotide and deduced amino acid sequences of hagfish CYP11A. Residues enclosed by the open rectangular boxes indicate the conserved regions as follows : I, substrate binding region ; II, adrenodoxin binding region ; III, heme ion binding site. B : Comparison of the binding domains in hagfish CYP11A with vertebrate homologs. The Roman numerals in B correspond to those in A. The sites in which the amino residue in the hagfish was shared in at least one vertebrate homolog are shaded. GenBank Accession numbers for the sequences are as follows: amphioxus CYP11A (GenBank: BAF61103.1), medaka CYP11A (GenBank:BAG49048.1), Japanese eel CYP11A (GenBank : AAV67332), and human CYP11A (GenBank:AAA52162.1).

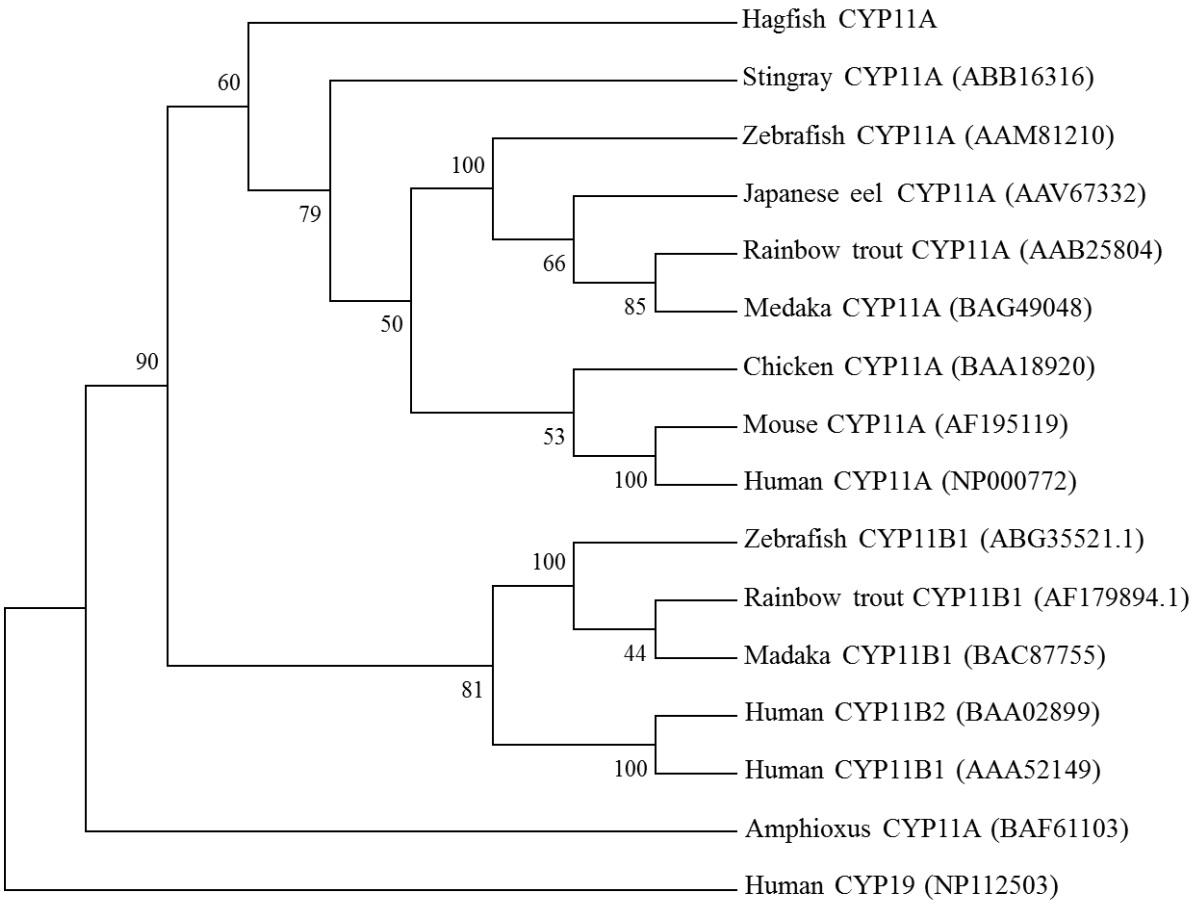

Fig. 2. Molecular phylogenetic tree of CYP11A including hagfish CYP11A constructed by Maximum parsimony method in Mega6 (Tamura et al., 2013) using deduced amino acid sequences of CYP11A, CYP11B1 and CYP11B2 proteins. Database accession numbers for each sequence are noted in parentheses. Numbers at the branch points indicate the percentage of 1000 bootstrap replicates supporting the division.

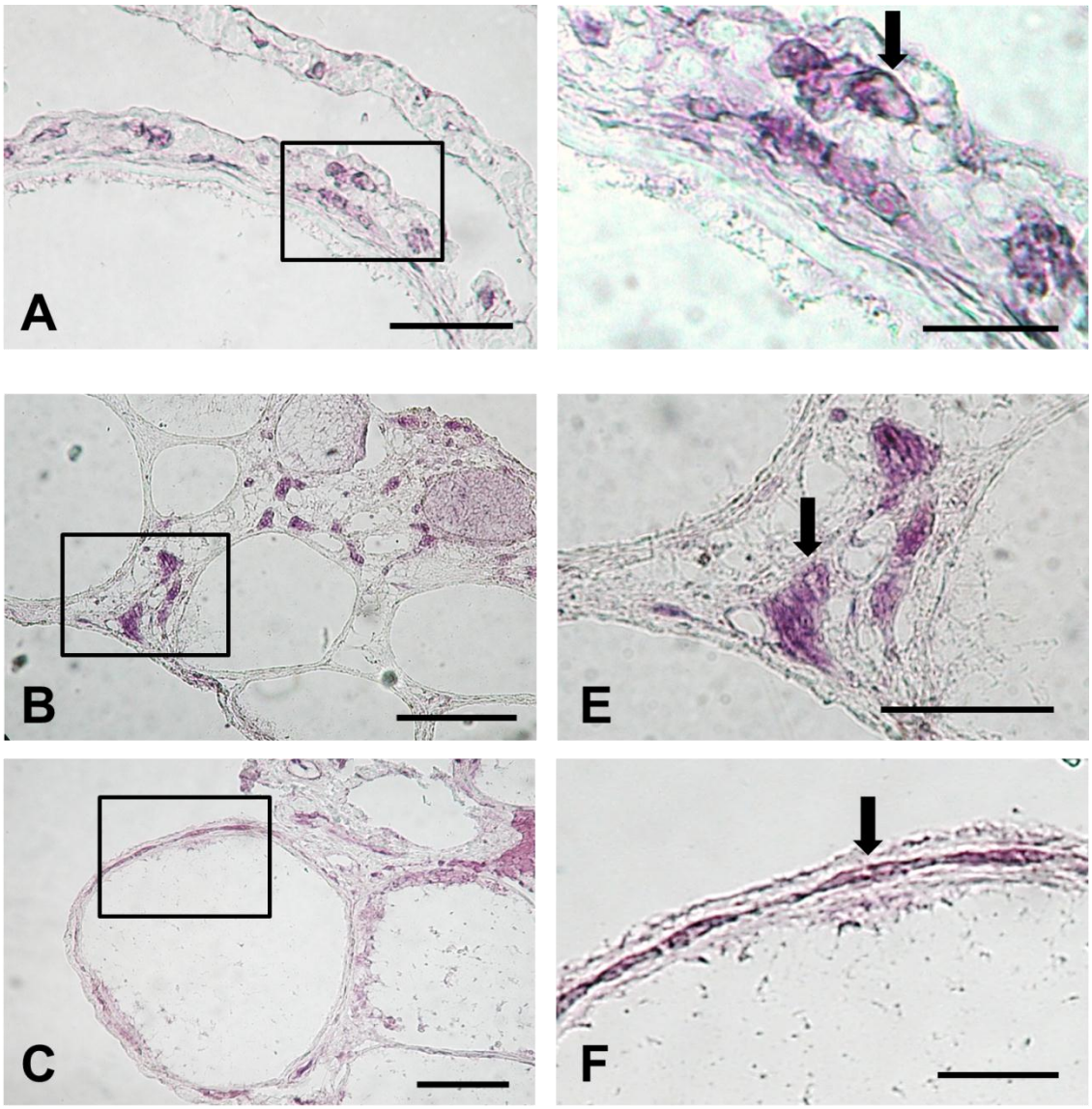

Fig. 3. Cellular localization of hagfish *CYP11A* in the gonads. A, *CYP11A* mRNA signals in the theca cells of late vitellogenic egg. Rectangular area in A is magnified in D. Arrow indicates cell of the theca externa expressing *CYP11A* mRNA. B and C, *CYP11A* mRNA signals in the Leydig cells (B) and tubule-boundary cells (C) in the testis with large GSI. Rectangular areas in B and C are magnified in E and F, respectively. Arrows indicate Leydig cells (E) and tubule-boundary cells (F) expressing *CYP11A* mRNA, respectively. Scale bars: A, C, E: 100 μm; B, D, F: 10 μm.

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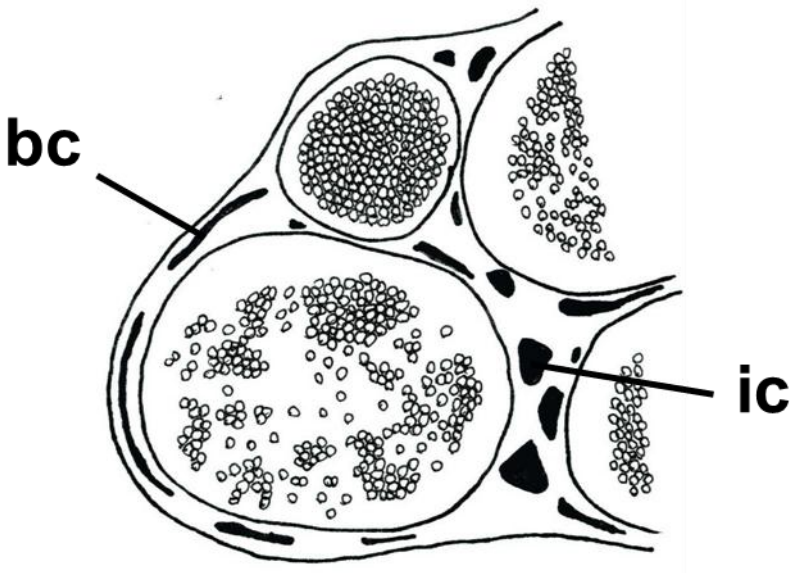

Fig. 4. Drawings of sections of testis treated by *in Situ* Hybridization *CYP11A* mRNA signals (black). ic: interstitial cell (Leydig cell), bc: boundary cell.

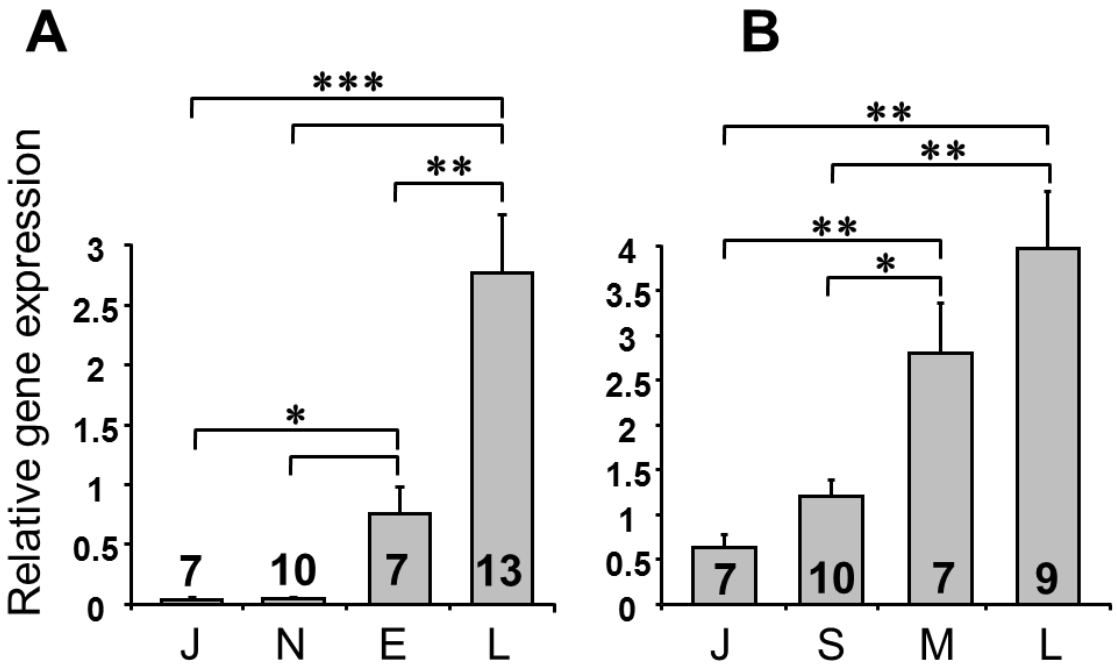

Fig. 5. Relative CYP11A gene expressions in the gonad of female (A) and male (B) hagfish. The *CYP11A* mRNA levels were normalized by *β-actin* mRNA levels. Relative values are expressed as mean ± SE. Number in each column indicates number of animals studied. (A): J, juvenile ovary; N, non-vitellogenic adult ovary; E, early vitellogenic adult ovary; L, late vitellogenic adult ovary. (B): J, juvenile testis; S, adult testis with small GSI; M, adult testis with medium GSI; L, adult testis with large GSI. *P < 0.05; **P < 0.01; ***P < 0.001.

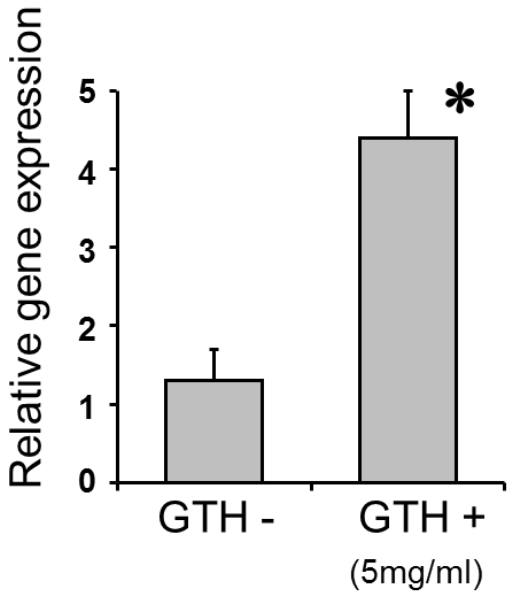

Fig. 6. *In Vitro* effects of pituitary GTH on relative CYP11A gene expressions in the testis. Testes with large GSI more than 200 were cultured with hagfish GTH (GTH+, 5 μg/ml) or without GTH (GTH-). The *CYP11A* mRNA levels were normalized by *β-actin* mRNA levels. Relative values are expressed as mean ± SE. *P < 0.05. Note that gonadal *CYP11A* expression was induced by pituitary GTH *in vitro*.

GENERAL DISCUSSION AND CONCLUSION

In Chapter 1, plasma concentrations of estradiol-17β, testosterone and progesterone were examined with respect to developmental conditions of gonads and sexual differences in the brown hagfish (*Paramyxine atami*). In Chapter 2, based on EST analysis of the testis, P450 side chain cleavage (CYP11A), which is the first and essential enzyme for steroidogenesis in chordates, was cloned. The deduced amino acid sequence of hagfish CYP11A shows high identity to other animal forms especially in two functional domains, adrenodoxin- binding domain and heme-binding domain. In the phylogenetic analysis, hagfish CYP11A forms a clade with the vertebrate CYP11A. Following the real-time PCR analysis, *CYP11A* mRNA expression levels were clearly correlated to the developmental stages of hagfish gonads in both sexes. By *in situ* hybridization, *CYP11A* mRNA signals were found in the theca cells of the ovarian follicles and Leydig cells and the tubule-boundary cells of the testis. These molecular and histological evidences indicate that CYP11A plays functional roles as a steroidogenic enzyme in the gonadal developments of the hagfish.

The hypothalamic-pituitary-gonadal (HPG) system is considered to be a seminal event that emerged prior to or during the differentiation of the ancestral agnathans. In gnathostomes, the hormones of the hypothalamus and pituitary have been extensively studied and shown to have well-defined roles in the control of reproduction. In the lamprey, although only α-subunit of the pituitary GTH has been identified (Sower et al., 2006), findings from many molecular, biochemical, physiological and morphological studies clearly indicate the presence of the HPG system (for review, see Sower et al., 2009). In contrast, endocrine regulation of reproduction in the hagfish is poorly understood, since it was not clear whether the hagfish pituitary gland contained tropic hormones of any kind (for reviews, see Gorbman, 1983; Nozaki, 2008). Recently, single functional GTH has been identified in the pituitary of the brown hagfish, *Paramyxine atami* (Uchida et al., 2010). The cellular and transcriptional activities of this hagfish GTH are significantly correlated with the developmental stages of the gonads. Additionally, purified native GTH induces the release of estradiol-17β and testosterone from the organ-cultured testis. In the present study, hagfish GTH stimulated the

transcriptional levels of *CYP11A* in the cultured testis. These results suggested that the steroidogenic activity of the hagfish testis is under the control of the pituitary GTH, similar to that of more advanced gnathostomes.

In the present study, in females, clear positive correlation was noted in *CYP11A* mRNA expression and plasma estradiol-17β levels in relation to ovarian development. Yu et al. (1981) demonstrated that synthesis of hepatic vitellogenin in *Eptatretus stouti* was induced by estrogens, estradiol and estrone. Thus, CYP11A is suggested to play a crucial role in the synthesis of estradiol-17β, which in turn acts on ovarian development and hepatic vitellogenesis in the female hagfish.

In contrast to females, in males, although transcriptional levels of *CYP11A* increased in accordance with the developmental stages of testis, no relationship was obtained between the testicular development and plasma levels of estradiol or testosterone. Thus, there was a clear discrepancy between males and females on the relationship between the transcriptional levels of *CYP11A* and plasma steroid levels. These results suggest a possibility that male hagfish uses other steroids than estradiol or testosterone as major androgens. In support of this possibility, recent studies in the lamprey have emphasized the importance of non-classical steroids, such as androstenedione and 15α-hydroxylated sex steroids (15α-hydroxy-testosterone and 15α-hydroxyprogesterone) in serving as functional androgens (Lowartz et al., 2003; Young et al., 2007; Bryan et al., 2007, 2008). Since hagfish gonads also produce substantial amounts of unusual androgens, such as 6β-hydroxy testosterone and 5α-androstane-3β, 7α, 17β-triol, as well as androstenedione (Hirose et al., 1975; Kime et al., 1980; Kime and Hews, 1980), some of these steroids may act as functional androgens. Further study is required in order to clarify the role of these steroids in hagfish.

Finally, the present study revealed sex steroid hormone profiles in the plasma in relation to the gonadal development in the brown hagfish. The present study further showed that expression of *CYP11A* in the gonads is of fundamental importance for determining the levels of steroid hormone production and gonadal development in the hagfish. In addition, the present study demonstrated that theca cells of the ovarian follicles and Leydig cells/tubule-boundary cells of the testis are steroidogenic cells of the hagfish gonads. Taking

all results into consideration, it is proposed that vertebrates, during their early evolution, have established the pituitary-gonadal reproductive system.

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