BIOLOGY OF REPRODUCTION **65,** 1057–1066 (2001)

Involvement of Sex Steroid Hormones in the Early Stages of Spermatogenesis in Japanese Huchen (*Hucho perryi*)[1]

Mohamed A. Amer,[3] Takeshi Miura,[2,4] Chiemi Miura,[3] and Kohei Yamauchi[3]

Division of Marine Biosciences, Graduate School of Fisheries Science,[3] and Field Science Center for Northern Biosphere,[4] Hokkaido University, Hakodate 041-8611, Hokkaido, Japan

ABSTRACT

In higher vertebrates, considerable progress has been made in understanding the endocrine regulation of puberty; however, in teleosts, the regulatory mechanisms of spermatogenesis during the first annual cycle remain unclear. The present study was conducted to understand the regulatory mechanisms of spermatogenesis throughout the different stages of the first spermatogenic cycle and to check the ability of various steroids and hormones to induce in vitro spermatogonial proliferation in Japanese huchen (*Hucho perryi*). The results indicate that the serum level of 11-ketotestosterone (11-KT) was positively associated with germ cell type; the level first began to rise with the appearance of late-type B spermatogonia and continued to increase gradually throughout the active spermatogenic stages and spermiogenesis, reaching a peak value 2 wk before spawning, and then declined. During the spermatogenic stages, the serum concentration of 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) was undetectable. Only a small peak was detected with the appearance of spermatocytes and spermatids, and at the time of spawning, the level increased dramatically, reaching its maximum value with the onset of milt production. Despite the high variation in serum levels of 17β-estradiol (E_2) both between months and among the individuals, E_2 was found during the whole reproductive cycle. From these results, we concluded that 1) 11-KT is necessary for the initiation of spermatogenesis and sperm production, and it probably plays a role in spermiation, 2) 17α,20β-DP is essential for the final maturation stage, could play a significant role in the mitosis phase and meiosis process, and probably participates in the regulation of spawning behavior, and 3) estrogen is an indispensable male hormone that plays a physiological role in some aspects of testicular functions, especially during the mitotic phase. The three steroids were also able to induce DNA synthesis, spermatogonial renewal, and/or spermatogonial proliferation in vitro.

gametogenesis, puberty, spermatogenesis, steroid hormones, testis

INTRODUCTION

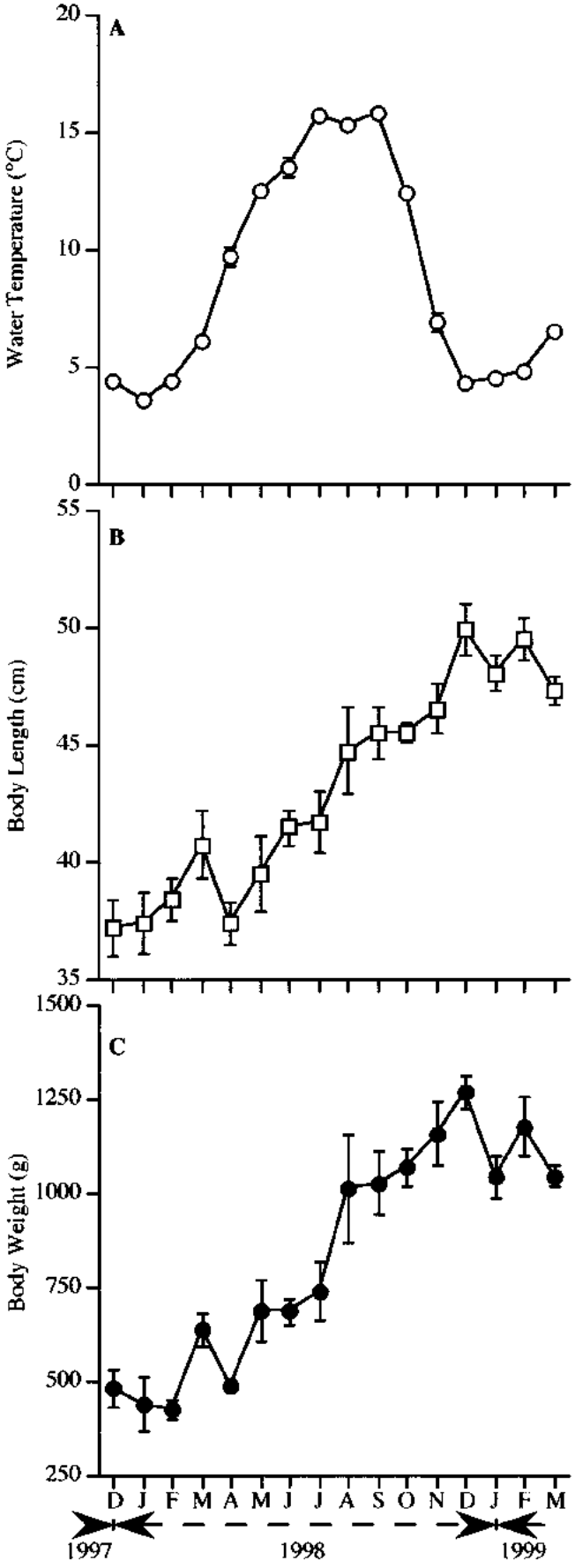
In vertebrates, puberty is the period during which a juvenile acquires the capacity to reproduce. It is characterized by an activation of the brain-pituitary gonadal axes. In higher vertebrates, considerable progress has been made in understanding the endocrine regulation of puberty, but in teleosts, the regulatory mechanisms underlying this activation are still poorly understood [1, 2]. Spermatogenesis is a continuum of cellular differentiation in which three principal phases can be discerned: spermatogonial renewal and proliferation, meiosis, and spermiogenesis [3, 4]. In vertebrates, including nonmammals, germ cell development and maturation appear to proceed in a similar fashion [5]. In most teleost species, including salmonids, the testis is of unrestricted spermatogonial-testis type, characterized by spermatogonia along the entire length of the testicular lobules, and the unit of spermatogenesis is the spermatocyst. Within each cyst, germ cell development occurs synchronously [6–10]. During the prepubertal period, spermatogonial stem cells and Sertoli cells occur as solid cords within the testicular lobule [5]. With the onset of spermatogenesis, spermatogonial stem cells divide to produce generations of spermatogonia, which enter the spermatogenic cycle. Spermatogonia type A divide several times to produce spermatogonia type B, which, in turn, develop into spermatocytes and then undergo meiosis to produce spermatids. The spermatids then proceed through a morphological metamorphosis during spermiogenesis to form spermatozoa. Spermiation results from the breakdown of Sertoli cells that form the spermatogenic cyst wall. In salmonids, the two major events of the testicular cycle (spermatogenesis and spermiation) are temporally separated by a stage of spermatozoal maturation during which the spermatozoa undergo physiological changes [6].

In teleosts, as in other vertebrates, spermatogenesis is essentially regulated by pituitary gonadotropin and testicular factors, including androgens. Gonadotropin stimulates androgen production by Leydig cells and, eventually, controls spermatogenesis and spermiation [6, 11–13]. Among the androgenic steroids, 11-ketotestosterone (11-KT) is the major androgen in most teleost species [14], especially during the later stages of spermatogenesis [15–20]. The progestin 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) could play a direct or indirect role in the mechanism of spermiation of male salmonids via regulation of the ionic composition of the seminal fluid and the acquisition of sperm motility [21, 22]. A number of studies have mentioned that a distinct shift from androgen to 17α,20β-DP appears to occur in the testes of several teleost fish around the onset of spermiation. These results suggest that, in several species of salmonids and in the Japanese eel (*Anguilla japonica*), 17α,20β-DP is essential for the final testicular or sperm maturation [18, 19, 22–29]. Estrogens, derived either from local aromatization of androgens or produced by the testis, can exert feedback effects on the neuroendocrine components of the male reproductive axis, but recent evidence suggests that estrogen may also exert paracrine actions within the testis itself [30]. Recently, it has been reported that implantation of 17β-estradiol (E_2) promotes spermatogonial renewal in Japanese eel [31] and induces a full, qualitatively normal spermatogenesis in hy-

[1]Supported by a doctoral scholarship from the Mission Department of the Ministry of Education, Egypt, and in part by grants-in-aid from the Ministry of Agriculture, Forestry and Fisheries of Japan (BDP-01-1V-2-1), and from The Japan Society for the Promotion of Science.
[2]Correspondence. FAX: 81 138 40 5545;
e-mail: takeshi@fish.hokudai.ac.jp

Received: 26 March 2001.
First decision: 16 April 2001.
Accepted: 21 May 2001.

ISSN: 0006-3363. http://www.biolreprod.org



pogonadal mice [32], suggesting that estrogen is an indispensable male hormone. Thus, in fish, differentiation of spermatogonia into spermatozoa requires several steroid hormones. The role of androgen and progestin has been elucidated, in large part, through the correlation of seasonal changes in serum hormone levels with the different stages of the annual testicular cycle in a variety of fish species. However, it is not clear whether the progression of germinal cells into different stages of spermatogenesis is regulated by changes in these hormonal signals; in other words, the specific role played by a specific hormone of these steroids throughout the different stages of spermatogenesis remains unclear. Previously, our in vivo and in vitro studies on the role of these steroids in Japanese eel (an excellent system for analyzing the mechanisms controlling spermatogenesis due to the presence of only type A and early type B spermatogonia) showed that 1) 11-KT is necessary for the initiation of spermatogenesis [33], 2) E_2 promotes stem cell renewal [31], and 3) 17α,20β-DP is essential for the acquisition of sperm motility and milt production [24].

Although salmonids have been the subject of more published papers than any other group of teleosts, one of their subgroups, the huchen (*Hucho perryi*), has not been well studied. In Japan, huchen is the largest salmonid. It is also a late-maturing species, so the quantity of available sperm from a mature male is very limited [34]. Recently, huchen has been considered as a new species for aquaculture; therefore, a source of gametes is necessary for propagating and preserving the species. To our knowledge, no information exists concerning its reproductive process, particularly spermatogenesis and serum sex-steroid profiles of male Japanese huchen. The present study was conducted using huchen as a new system for the analysis of mechanisms controlling spermatogenesis to understand the physiological changes that occur both before and during puberty. During this period, we studied the testicular development, histological changes, and serum steroid profiles of 11-KT, 17α,20β-DP, and E_2. For this purpose, fish were sampled from the prepubertal stage (testes contained only primitive spermatogonia) to the first sexually mature stage (testes filled with spermatozoa, milt could be stripped by abdominal massage). The in vitro study was carried out on prepubertal fish to check the ability of various hormones and steroids to induce spermatogonial proliferation in cultured testicular fragments.

MATERIALS AND METHODS

Fish

Three to six male huchen were sampled monthly starting from the prepuberty stage (December 1997) until the spawning season (March 1999). In March 1999, sampling was carried out weekly. The fish were reared at the Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University, in outdoor ponds supplied with river water under natural water temperature and photoperiod and were fed ad libitum with commercial salmon food (Trout Fodder; Oriental Yeast Co., Ltd., Tokyo, Japan). During the period of study, the lowest water temperature (1.8°C) and the highest (18.7°C) were recorded on January 7, 1998 and August 21, 1998, respectively (Fig. 1A). The fish were killed immediately by a blow to the head, and blood samples were collected from the caudal

FIG. 1. Monthly changes in water temperature (**A**), total body length (**B**), and body weight (**C**) during the sampling period of male Japanese huchen from the prepubertal stage (42 mo of age) to the spawning time (57 mo of age). Values are displayed as mean ± SEM. During March 1999, sampling were carried out weekly, so the value represents the mean of all samplings.

vein with a syringe, transferred to centrifuge tubes, and placed on crushed ice for 1–2 h. Blood samples were centrifuged at 3000 rpm for 15 min. The serum was then collected and stored at −20°C until hormone assays were performed. Body weight and total body length were recorded. The testes were excised and weighed for calculation of the gonadosomatic index (GSI; gonad weight/body weight × 100).

Histology

Histological analysis of spermatogenic activity was performed to correlate testicular development with steroid production. A small piece taken from the midportion of the testis of each male huchen was fixed in Bouin solution, dehydrated through an ascending series of ethanol, and embedded and blocked in paraffin. Then, 5-μm sections were cut and stained with hematoxylin and eosin. Sections were analyzed under light microscopy and classified into several distinct spermatogenic stages according to the method of Grier [8]. From each male huchen, five standard histological grids were chosen randomly, and the number of the cysts of each germ cell type was counted and expressed as a percentage of the total number of germ cell cysts. From October 1998 until the end of study period, counts could not be done, because testicular sections contained spermatozoa either in cysts or free in lobular lumens or testicular ducts. Therefore, the values of each germ cell type were calculated based on the relative area of each type in photographs. Light micrographs of testicular sections stained with hematoxylin and eosin at different stages of development (see Fig. 3) were prepared from plastic sections (HistoResin Plus; Heraeus Kulzer GmbH, Heidelberg, Germany) according to the manufacturer's instructions.

Radioimmunoassay

After two extractions of steroids with diethyl ether, serum levels of steroids were measured by RIA according to the method of Ueda et al. [27] and Sakai et al. [29] for 11-KT, the method of Young et al. [35] for 17α,20β-DP, and the method of Kagawa et al. [36] for E_2.

Testicular Organ Culture

The organ culture technique employed was the floating method described by Miura et al. [37, 38], with some modifications. Briefly, eight immature male huchen were killed by a blow to the head, and their tails were cut. All the following operations were carried out under sterile conditions. The fish were then sterilized in 70% (w/v) ethanol, and the testes were excised and cut into 1 × 1 × 0.5-mm fragments. For each tested treatment, two testicular fragments of each fish were placed on elder pith (treated with 100% ethanol, washed with distilled water, autoclaved, dried, and covered with a nitrocellulose membrane) in a 24-well plastic culture dish. Each elder pith was floated separately in a culture well containing 1 ml of culture medium. The basal culture medium consisted of Leibovitz-15 medium supplemented with 0.1 mM aspartic acid, 0.1 mM glutamic acid, 1.7 mM L-proline, 0.5% (w/v) bovine serum albumin fraction V (Sigma, St. Louis, MO), 1 mg/L bovine insulin, and 10 mM Hepes, adjusted to pH 7.4 with 1 M NaOH. Steroids were first dissolved in ethanol, except for cortisol, which was dissolved in distilled water, and then all hormones were diluted with the culture medium. The same concentration of ethanol was added to the cortisol- and nonhormonal (control)-treated fragments. Testicular fragments were cultured in a medium containing one of the following hormones: 11-KT, testosterone, 5β-dihydrotestosterone (5β-DHT), androsterone, androstenedione, dehydroepiandrosterone (DHEA), cortisol, E_2, 17α,20β-DP, and salmon pituitary glycoprotein fraction (SPG, rich in gonadotropin (GTH)-II and contaminated with GTH-I and probably with thyroid stimulating hormone (TSH) at a dose of 10 ng/ml, then incubated for 15 days in humidified air at 12°C. The medium was changed after 1 wk of culture.

5-Bromo-2-Deoxyuridine Labeling

Labeling of 5-bromo-2-deoxyuridine (BrdU) was carried out according to the manufacturer's instructions, with minor modification. Because BrdU is incorporated into replicating DNA, detection of the proliferating cell is possible. Testicular fragments were incubated with BrdU (1 μl/well) for the last 24 h in culture. After culture, testicular fragments were fixed in Bouin solution, and 5-μm paraffin sections were cut and stained immunohistochemically. Sections were then counterstained with hematoxylin. The number of immunolabeled germ cells was counted and expressed as a percentage of the total number of germ cells (BrdU index; immunolabeled germ cells/total germ cells × 100). For each tested hormone, three randomly chosen, standard histological grids from different sections of each animal were viewed with a light microscope.

Statistics

All data except serum steroid concentrations were analyzed using a one-way ANOVA followed by Duncan New Multiple Range Test (SuperAnova; Abacus Concepts, Inc., Berkeley, CA). Steroid data of 11-KT, 17α,20β-DP, and E_2 did not follow a normal distribution; therefore, these data were analyzed by a nonparametric statistical test (Kruskal-Wallis ANOVA), followed by the Bonferroni multiple-comparison test. In all cases, significance was accepted at $P < 0.05$. Data are presented as the mean ± SEM.

RESULTS

Body Size

Throughout the sampling period, total body length gradually increased from 37.2 ± 1.2 cm at 42 mo of age (beginning of the sampling) to 49.9 ± 1.1 cm at 54 mo of age (December 1998). From January to March 1999, body length remained at a relatively constant range of 47–49.5 cm. During the same period, average body weight increased from 477 ± 51.7 g to 1267.5 ± 44.4 g at 42 and 54 mo of age, respectively. For both body length and body weight, the differences between values obtained during the prepuberty and early spermatogenic stages were significantly lower than those obtained during the active spermatogenic stage (Fig. 1, B and C). This indicates that fish may have to reach a certain size before puberty can begin.

Testicular Development and Serum Steroid Profiles

The mean GSIs remained very low (0.01%–0.05%) from December 1997 to May 1998. With the onset of the active spermatogenesis stage, as evidenced by histological data, the GSI increased rapidly, reaching the highest value (1.4%) in September 1998. From November 1998 until March 1999, the GSI remained relatively constant, but the values were significantly lower than in September and October 1998 (Fig. 2A).

The serum level of 11-KT was positively associated with germ cell development (Fig. 2B); its average increased from 0.9 ± 0.4 ng/ml in the immature stage to 4.15 ± 0.38 ng/ml with the appearance of late-type B spermatogonia (March 1998). In the active spermatogenic stage (testes containing all germ cell types), the average level was 12.7 ± 0.42 ng/ml. It increased gradually through spermiogenesis (sperm production) to reach the highest value of approximately 100 ng/ml at 2 wk before spawning time, then declined to 54.5 ± 11.7 ng/ml at the beginning of milt production. Serum levels of E_2 showed high variations not only between months but also among individuals (Fig. 2C). Despite the high variation in E_2 levels, the hormone was detected during the entire process of spermatogenesis, spermiation, and at the spawning time. The highest value (333 pg/ml) was detected at the beginning of mitosis (April 1998), and at the end of March 1999 (spawning time), the level was 55.5 pg/ml. Serum concentrations of 17α,20β-DP were not detectable throughout the spermatogenic stages, except for a small peak (209 pg/ml) detected at the active mitosis stage and the beginning of meiosis (appearance of spermatocytes and spermatids). In March 1999, when sampling was carried out weekly, the level increased dramatically, reaching a peak value of 7.5 ng/ml (Fig. 2D) and indicating that this steroid is highly correlated with the final maturation stage.

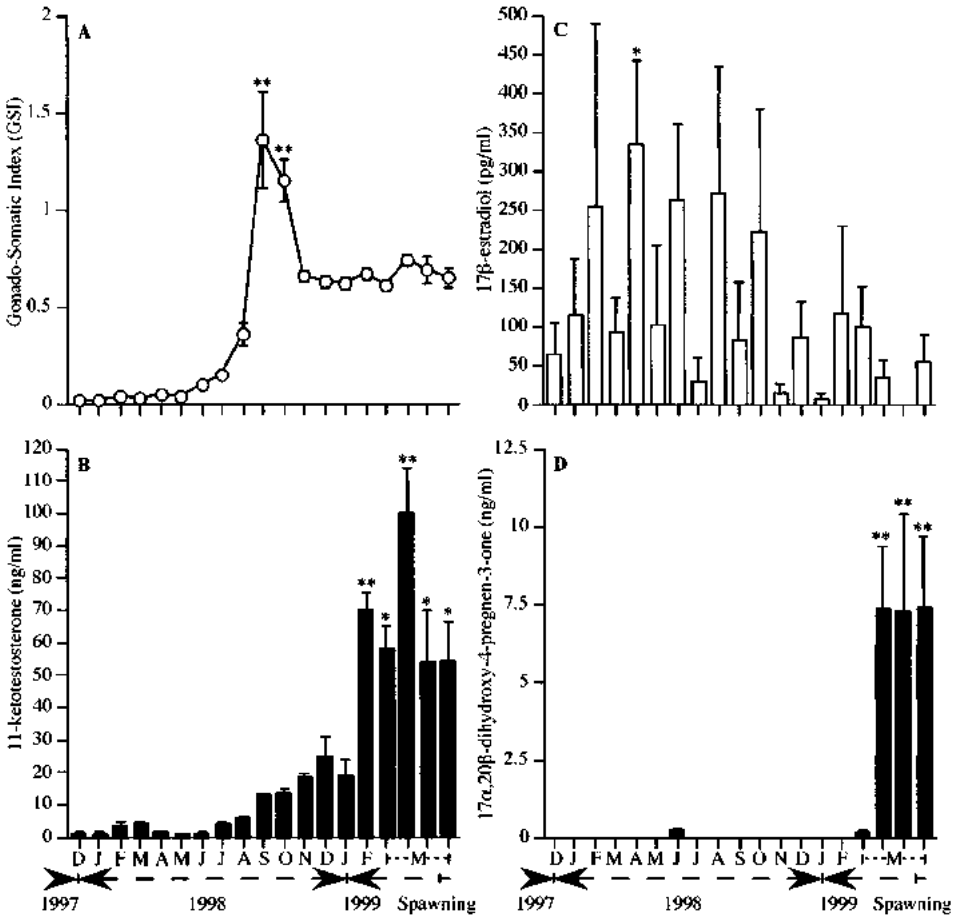


FIG. 2. Monthly changes in GSI (**A**), serum steroid profiles of 11-KT (**B**), E_2 (**C**), and 17α,20β-DP (**D**) in male Japanese huchen from the prepubertal stage to the time of spawning. During March 1999, blood sampling were carried out weekly to detect any hormonal changes before the beginning of milt production. Values are displayed as mean ± SEM. The GSI for March 1999 represents the mean of all fish sampled during that month. A star (or stars) above a bar indicate a significant value. $*P < 0.05$, $**P < 0.01$.

Spermatogenesis and Histology

Histologically, the testis of Japanese huchen is of the unrestricted spermatogonial-type according to the terminology of Billard et al. [6] and Grier et al. [7]. During the prepubertal stage, the testes consisted mainly of connective tissue, interstitial cells of Leydig, and the primitive (type A and early type B) spermatogonia, which occurred with Sertoli cells as solid cords within the testicular lobules (Fig. 3A). In March 1998, a few late-type B spermatogonial cysts became apparent in some individual testicular lobules. In June 1998, spermatocytes started to appear for the first time, and very few cysts of spermatids were observed, indicating the initiation of puberty. By August 1998, the testicular lobules had increased in size and were filled with cysts of late-type B spermatogonia, spermatocytes, and spermatids, and occasionally, a few isolated clusters of spermatozoa were observed in some individuals (beginning of spermiogenesis). The testes underwent very rapid growth and development from September to October 1998, and each testicular lobule contained all germ cell types (Fig. 3B): type A spermatogonia (13%), late-type B spermatogonia (19%), spermatocytes (17%), spermatids (21%), and spermatozoa (30%). In November and December 1998, more than 60% of germ cells were spermatozoa (Fig. 4). From January 1999 onward, the testicular sections were filled with spermatozoa either in the testicular lumen or in the sperm duct (Figs. 3C and 4), and no other germ cell types were observed, except type A spermatogonia, which are the starting point for the next spermatogenic cycle. At the end of March 1999, milt could be stripped by abdominal massage for the first time, indicating that puberty had been completed.

In Vitro Spermatogonial Proliferation

Immature testicular fragments were cultured in L-15 medium with or without one of the following hormones: 11-KT, testosterone, 5β-DHT, androsterone, androstenedione, DHEA, cortisol, E_2, 17α,20β-DP, and SPG at a dose of 10 ng/ml. After 15 days of culture in testicular sections taken from 11-KT-, E_2-, 17α,20β-DP-, and SPG-treated fragments, BrdU was incorporated into numerous spermatogonial cells, compared with the control and all other tested hormones, indicating that DNA was synthesized (Fig. 5). The effect of these hormones on the incorporation of BrdU (as a proliferating marker) by the testicular germ cells was examined by calculation of the BrdU index. The BrdU index of the testicular fragments incubated with 11-KT, 17α,20β-DP, E_2, and SPG was significantly ($P < 0.01$) higher than those of the initial, control, and all other hormonal-treated fragments (Fig. 6A). The highest value (34.5% ± 1.7%) was detected in 11-KT-treated fragments, but this value was not significantly different from those of 17α,20β-DP-, E_2-, and SPG-treated fragments. A slight, but statistically significant, stimulation was observed in testosterone-treated fragments compared with that in the control, but the values in other hormonal treatments were not significantly different from those in the control and with testosterone. On the other hand, two types of immunoreacted germ cells were observed in testicular sections taken from either 11-KT-, 17α,20β-DP-, or SPG-treated fragments

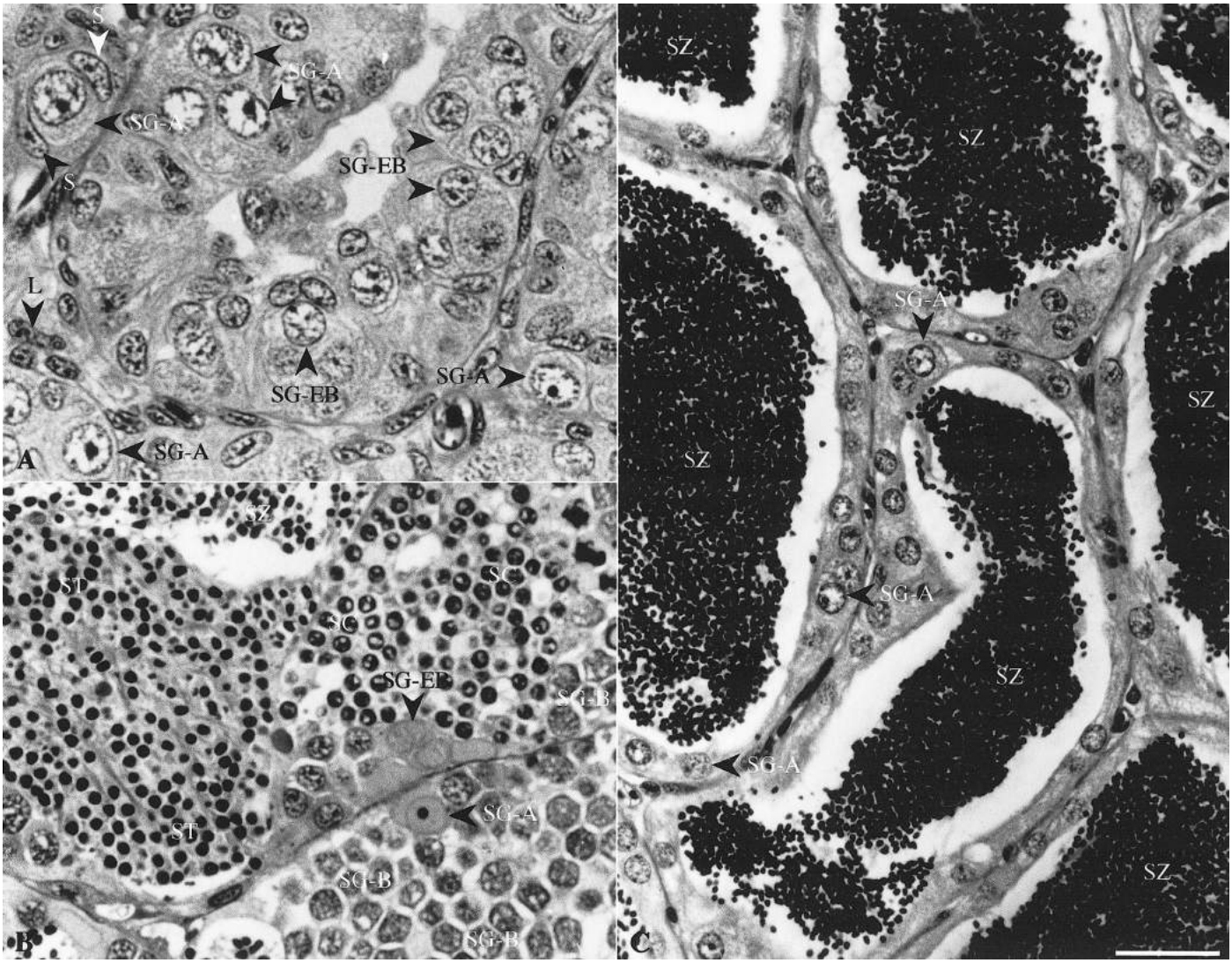


FIG. 3. Light micrographs of testicular sections stained with hematoxylin and eosin at the prepubertal stage (**A**), active spermatogenesis stage (**B**), and mature stage (**C**) of development. L, Leydig cells; SC, spermatocytes; S, Sertoli cell; SG-A, type A spermatogonia; SG-EB, early type B spermatogonia; SG-B, late-type B spermatogonia; ST, spermatids; SZ, spermatozoa. Bar = 50 μm.

(Fig. 5, B and C): large, primitive stem cells and small, dark-stained with condensed nuclei, proliferated germ cells. In E_2-treated fragments, both positively and negatively immunoreacted germ cells were of type A and early type B spermatogonia (Fig. 5D). The percentage of proliferated germ cells (late-type B spermatogonia) in testicular sections taken from fragments incubated with 17α,20β-DP was significantly higher than those of 11-KT- or SPG-treated fragments (Fig. 6B).

DISCUSSION

To our knowledge, the present study provides the first information on the spermatogenic cycle and steroid profiles of 11-KT, 17α,20β-DP, and E_2 from the prepubertal stage through the first sexually mature stage in male Japanese huchen. It is also, to our knowledge, the first attempt to induce in vitro spermatogonial proliferation in this species.

In fish, all spermatogenesis occurs in cysts that are groups of germ cells dividing in synchrony and surrounded by Sertoli cells [6, 8, 10, 39]. This process was divided by Bellvé et al. [3] and Schulz et al. [4] into three major phases: mitosis (spermatogonial proliferation and differentiation), meiosis (formation of spermatids), and spermiogenesis (sperm production). In the present study, testicular architecture remained unchanged until the fish reached 45 mo of age, indicating that huchen is a late-maturing species. This feature makes huchen a good animal system for the analysis of mechanisms controlling spermatogenesis, and the role played by steroid hormones in the early stages of spermatogenesis can also be investigated. The GSI showed three different phases associated with germ cell types during the cycle. It was very low during the prepubertal stage and reached the maximum value during the active spermatogenic stages (September–October 1998), when the testes contained all germ cell types, including spermatozoa. In fish, the GSI often reaches a maximum just after the onset of meiosis at the spermatocyte stage, then generally declines during spermiogenesis due to the elimination and phagocytosis of the spermatid cytoplasm by Sertoli cells [39]. By the end of spermiogenesis (December 1998), the spermatozoa were released from the cyst into the lobular lumen, and the lumen then progressively filled with spermatozoa, resulting in the release of spermatozoa into the sperm duct (spermiation). During the later part of the sper-

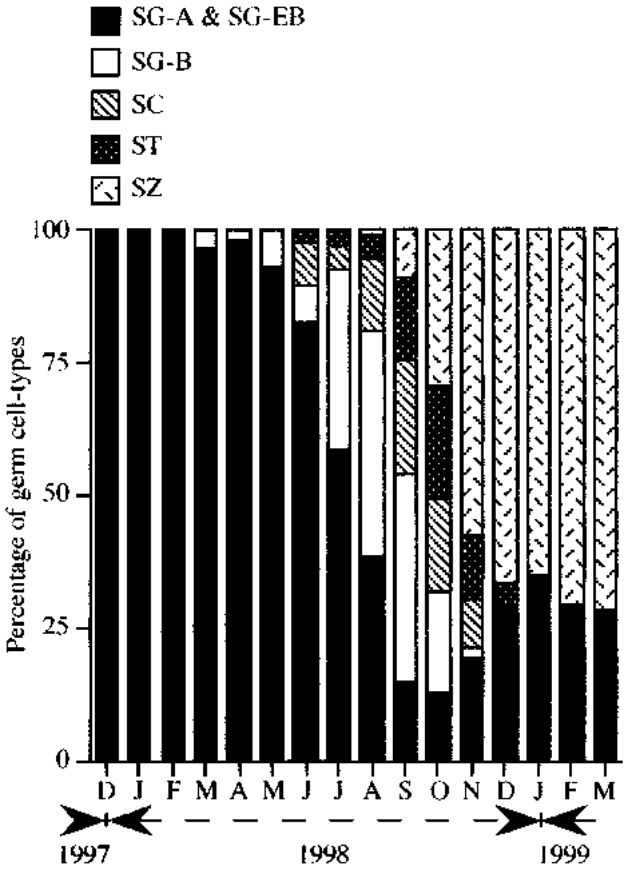


FIG. 4. Monthly changes in germ cell types as a percentage of total germ cells in male Japanese huchen from the prepubertal stage to the time of spawning. Values are displayed as the mean. From October 1998 until March 1999, when counts could not be done because testicular sections contained spermatozoa free in either lobular lumen or testicular duct, values of each germ cell type were calculated based on the relative area of each type in photographs. During March 1999, when sampling was carried out weekly, the value of each germ cell type represents the mean of all fish sampled during the month.

miating period, when the testis contained mainly spermatozoa and a few cysts of other germ cell types, the GSI decreased and remained at an approximately constant value until the spawning time. The GSI values reported here were lower than those reported for striped bass [2], rainbow trout, mirror carp, and Baltic salmon [39–41]. From these data, we can concluded that, despite the completion of puberty, more than one reproductive cycle is required to reach full sexual maturation (adulthood) in this species.

The histological changes and spermatogenesis stages were correlated with the serum androgen levels. Serum levels of 11-KT were very low before the initiation of spermatogonial proliferation, but they rose slowly with the appearance of late-type B spermatogonia, indicating that 11-KT is necessary for the initiation of spermatogenesis, as previously reported in Japanese eel [33]. With the advancing spermatogenic stages, the level continued to increase gradually, reaching 12.7 ng/ml with the appearance of spermatozoa, and it remained high during the active spermiogenesis stage. In a number of teleost species, the levels of androgens are high during the later stages of spermatogenesis but decline significantly after the onset of spermiation [16, 18, 42, 43]. The coincidence of the presence of spermatozoa, either in the testicular cysts or the lobular lumen and sperm duct, and the elevated levels of 11-KT suggests a direct relationship between this steroid and sperm production. The level remained high throughout the release of spermatozoa into the sperm duct, reaching the highest value 2 wk before the beginning of spawning and then decreasing slightly with the onset of milt production. This is in agreement with reports in some species of elevated levels of androgens during spermiation [2, 15, 17, 41, 44–46], suggesting that 11-KT plays a role during spermiation and milt production. Human chorionic gonadotropin injection has induced spermatogenesis and spermiogenesis in the Japanese eel, and this was accompanied by a marked increase in serum levels of androgens, particularly 11-KT [24, 47, 48]. The elevated levels of 11-KT in the present study were first associated with the increase of the GSI, but as the GSI declined with the initiation of active spermiogenesis, levels of 11-KT continued to rise, to a peak of approximately 100 ng/ml in March 1999. From this evidence, we can suggest that the production of this androgen depends essentially on the activity of the Leydig cells rather than on the size of the testis; a similar suggestion was made for the protogynous fish *Thalassoma duperrey* by Hourigan et al. [19]. Elevated androgen levels before spawning may regulate spermatogenesis and spermiogenesis [6, 10, 11, 16], could be responsible for the release of mature spermatozoa from the testicular cysts into the lobular lumen and sperm duct in male rainbow trout [21], or may have an important role in maintaining viability of spermatozoa during the prolonged period of sperm storage [49]. Moreover, in vitro studies of 11-KT production by testicular fragments at different stages of development have revealed that the capacity of testicular fragments to produce this steroid in response to gonadotropin is high during the later stages of spermatogenesis but rapidly declines after the onset of spermiation [29].

Serum levels of 17α,20β-DP were undetectable during the different stages of spermatogenesis, except for a slight increase during the active mitosis stage and the beginning of meiosis (appearance of spermatocytes and spermatids), suggesting that 17α,20β-DP could enhance mitosis and spermatocytes to enter meiosis. In rainbow trout, a similar observation was reported by Scott and Sumpter [46], who suggested that the plasma levels of 17α,20β-DP were not associated with any of the germ cell stages and found that median values of 17α,20β-DP coincided with a time when spermatocytes and spermatids were still present in the testes. In March 1999, the level in the present study increased dramatically, reaching the highest value (7.5 ng/ml), and at this time, the milt could be stripped by abdominal massage for the first time, supporting the idea that 17α,20β-DP is the principal hormone related to the final maturation stage. In the plasma of spermiating rainbow trout, approximately the same concentration of 17α,20β-DP has been reported by Campbell et al. [50], and in male masu salmon, the plasma levels of this steroid are much lower during the early breeding season (0.5–1.0 ng/ml) than during the active breeding season (9.0–10.0 ng/ml) [22]. It has been reported that a rapid shift in the steroidogenic pathway from androgen to progestogen production occurs in the testes around the time of spermiation in several teleost species [29, 51, 52], and 17α,20β-DP levels have been reported to be high during spermiation in the plasma or serum of several salmonid species [18, 21, 25–27, 41, 42, 44, 46, 53], common carp [51, 52], goldfish [54], and white sucker [55]. Furthermore, in rainbow trout, an in vitro study of steroid production by testicular fragments at different stages of spermatogenesis has revealed that great amounts of 17α,20β-DP are synthesized during gonial mitotic activity,

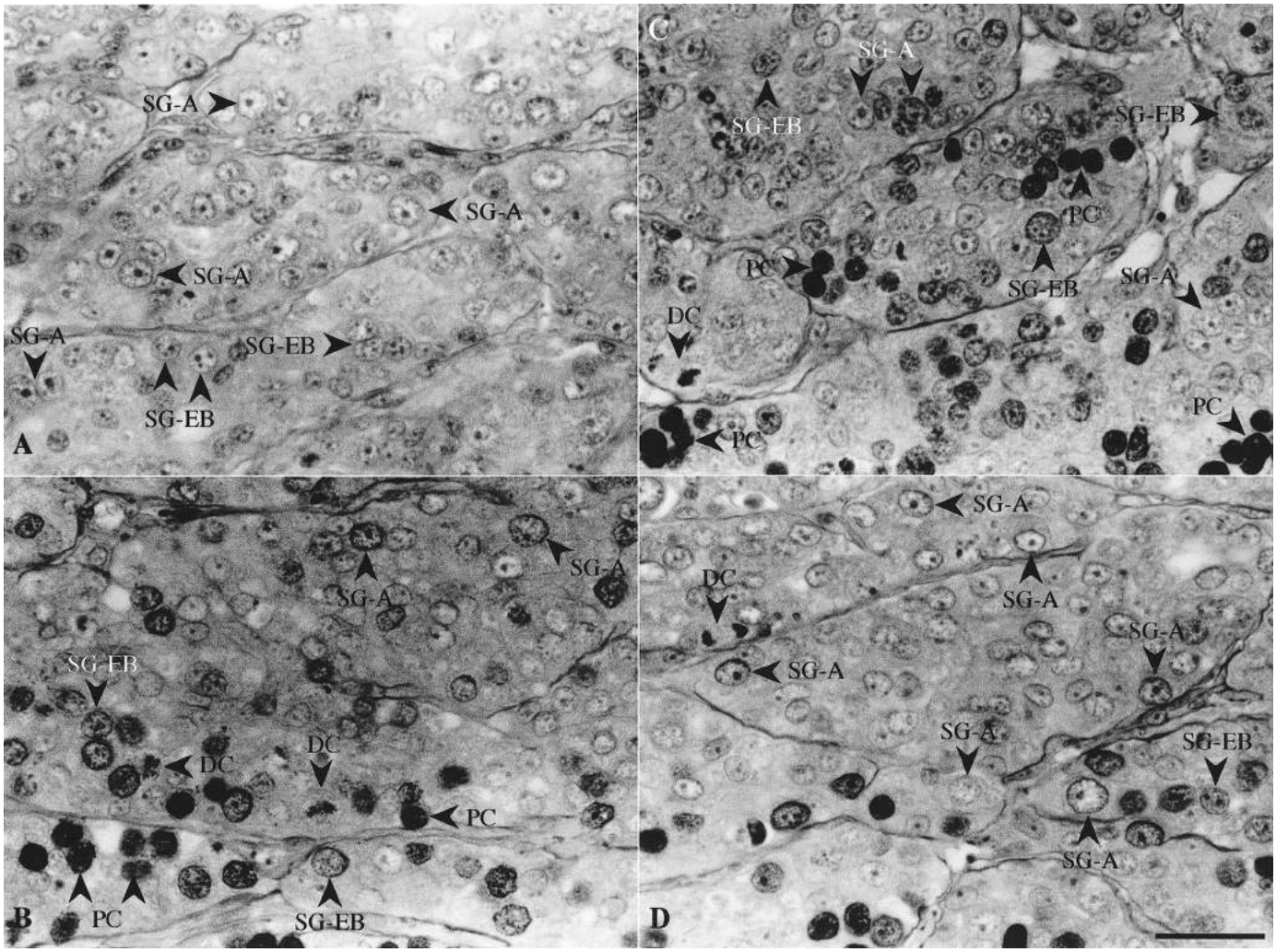


FIG. 5. Immunoreaction of BrdU in testicular sections from fragments cultured in L-15 medium alone (**A**) or with 11-KT (**B**), 17α,20β-DP (**C**), or E_2 (**D**). DC, Dividing cell; PC, proliferating cell; SG-A, type A spermatogonia; SG-EB, early type B spermatogonia. Bar = 50 μm.

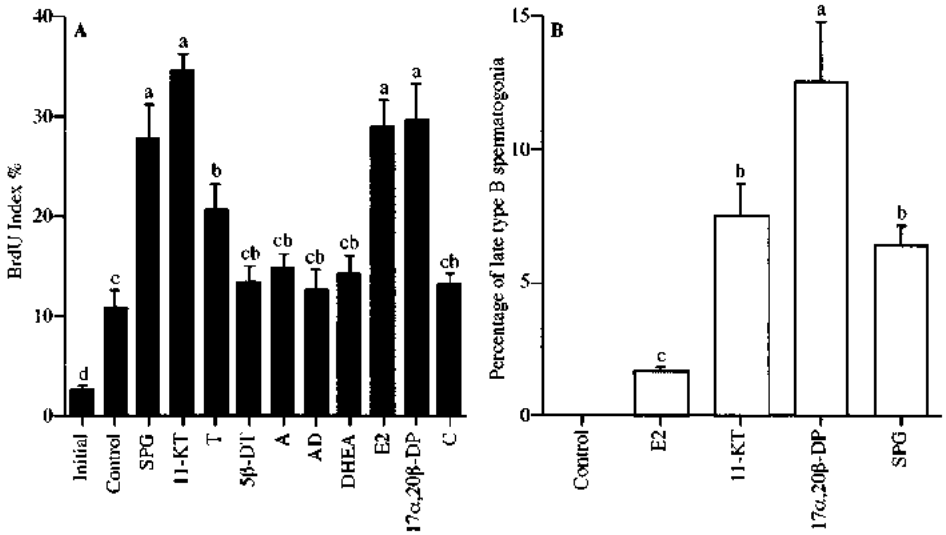


FIG. 6. Effects of various steroids and hormones on in vitro spermatogonial proliferation. Testicular fragments from immature males were cultured in L-15 medium in the presence or absence of one of the following steroids and hormones: 11-KT, testosterone (T), 5β-DHT, androsterone (A), androstenedione (AD), DHEA, cortisol (C), E_2, 17α,20β-DP, and SPG at a dose of 10 ng/ml. Fragments were then incubated for 15 days in humidified air at 12°C. The proliferation was assessed using incorporation of BrdU (a proliferation marker) as described in *Materials and Methods.* **A**) BrdU index. The number of positively immunoreacted germ cells is expressed as a percentage of the total number of germ cells. **B**) Percentage of the proliferated germ cells (late-type B spermatogonia). The number of proliferated germ cells is expressed as a percentage of the total number of germ cells. Results are given as the mean ± SEM. Values with the same lowercase letter(s) are not significantly different ($P < 0.05$).

at the time when spermatocytes enter meiosis and at the onset of spermiation, in which the synthesis of this steroid predominated over that of C19 steroids [56]. Those authors suggested that 17α,20β-DP may act on some steps of mitotic cycle and meiotic processes of male germ cells. Other in vitro studies have also revealed that the activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), which is the enzyme involved in the conversion of 17α-hydroxyprogesterone to 17α,20β-DP, is consistently high in the testes throughout the period of spermatogenesis and spermiation in rainbow trout and amago salmon [25, 28, 29]; those investigators also reported that the capacity of testicular fragments to produce this steroid was low during spermatogenesis but increased sharply at the time of spermiation. In salmonids, Nagahama [11] and Miura et al. [22] suggested that gonadotropin stimulates the testicular somatic cells to produce 17α,20β-DP from a precursor (probably 17α-hydroxyprogesterone in the presence of 20β-HSD secreted by spermatozoa), which acts to increase sperm duct pH, which, in turn, increases intrasperm cAMP, allowing the acquisition of sperm motility. A similar suggestion has also been reported for Japanese eel by Miura et al. [24]. In contrast, in rainbow trout, the various testicular cell preparations that are mostly devoid of sperm secrete 17α,20β-DP regardless of gonadotropin stimulation [53, 57, 58]. From our results, we can suggest that 17α,20β-DP is essential not only for the final maturation stage, but that it also probably plays a role in the spermatogenic process during either mitosis or meiosis. This steroid may also play a role in the regulation of spawning behavior, as evidenced by the dramatic increase in its levels during the spawning season. Further investigations for elucidating and confirming the role of 17α,20β-DP during spermatogenesis are required.

In most vertebrates, E_2 circulates in males at low or undetectable levels; however, the enzyme that catalyzes the conversion of androgen to E_2, aromatase, can be present near E_2 targets in the brain [59] and in testicular germ cells [60]. The present study showed high variations in E_2 levels not only between months but also among individuals as measured each month. The highest value of E_2 was observed during mitosis, indicating the involvement of estrogen in spermatogenesis, especially during the early stages of mitosis, and supporting previous results reported in Japanese eel [31]. However, E_2 was detected throughout the entire process of spermatogenesis and spermiation, indicating that estrogen may play a role in spermatogenesis and testicular functions. In the elasmobranch *Squalus acanthias*, the presence of testicular aromatase and of binding sites for E_2 in lobules with gonia and with meiotic cells suggests a paracrine role of E_2 during the early stages of spermatogenesis [61, 62]. Estrogen levels declined before the time of spawning, whereas levels of 17α,20β-DP increased. Thus, E_2 could be involved in the regulation of 17α,20β-DP production in males [46, 63]. After hCG injection, the average serum level of E_2 in male Japanese eel was 490 pg/ml, and this level did not show any significant changes during the whole process of hCG-induced spermatogenesis [31]. Interestingly, in 2- to 3-mo-old hypogonadal mice treated with slow-release estradiol implants, full and qualitatively normal spermatogenesis was induced, suggesting that estrogens exert paracrine actions within the testis to promote spermatogenesis [32]. These results and the widespread expression and identification of estrogen receptors (α and β) within several cell types, including Leydig cells, Sertoli cells, and some testicular germ cells [31, 64–66], as well as aromatase within germ cells of the mouse testis [60], point toward a physiological role for estrogen in many aspects of testicular functions.

From the annual cycle results mentioned above, we found that testicular development and spermatogenesis in Japanese huchen were associated with steroid levels of 11-KT, 17α,20β-DP, and E_2. To understand the role of each of these steroids in spermatogenesis, immature testicular fragments (containing only primitive spermatogonia) were cultured with 10 ng/ml of each tested hormone. Synthesis of DNA is an indicator for mitosis and spermatogonial proliferation; therefore, the proliferative response of spermatogonia was determined by incorporation of BrdU (a proliferation marker) into germ cells. We found that 10 ng/ml of 11-KT, E_2, 17α,20β-DP, and SPG were sufficient to induce DNA synthesis in spermatogonia, indicating that mitotic divisions were promoted by the addition of each of these hormones. Spermatogonial proliferation was induced by 11-KT, 17α,20β-DP, or SPG, as evidenced by the appearance of late-type B spermatogonia. The effects of 11-KT could be mediated through Sertoli cells, which this androgen activates to produce other growth factors, such as activin B, that, in turn, act on spermatogonia to induce mitosis, leading to the formation of spermatocytes [11, 12, 33, 37, 67]. In cultured testicular fragments of Japanese eel, hCG induces the entire process of spermatogenesis from premitotic spermatogonia to spermatozoa within 24 days [38]. This hCG-induced spermatogenesis is accompanied by a marked activation of Leydig cells and Sertoli cells and by a rapid increase in levels of 11-KT. Our results indicated that 10 ng/ml of 11-KT could induce only spermatogonial proliferation in Japanese huchen, whereas the same concentration could induce the full process of spermatogenesis in Japanese eel in vitro within 21 days [37]. Furthermore, cultured testicular fragments of Japanese eel with 100 ng/ml of recombinant human insulin-like growth factor (IGF)-I in combination with 10 ng/ml of 11-KT can induce spermatogenesis from spermatogonial proliferation to spermiogenesis, whereas each of them alone cannot [68]. In contrast, testosterone, 11-KT, E_2, and 17α,20β-DP do not influence the proliferation of trout mitotic germ cells, whereas IGF-I does [69, 70]. These variable results suggest a species-related variation in the initiation and control of gametogenesis. Thus, depending on the species, sex steroids would be more or less involved in the control of spermatogenesis (i.e., the molecules required for the mitotic phase of spermatogenesis differ among species), as has been previously suggested [48, 70]. It is well established that, in vertebrates, spermatogenesis is controlled by pituitary gonadotropin and testicular androgens. So, the effect of SPG, which is rich in gonadotropins, on spermatogonial proliferation seems to be mediated through the stimulation of Leydig cells to produce androgens.

The variability of the E_2 levels throughout the first wave of spermatogenesis in this species, however, makes it difficult to establish the role of estrogens in spermatogenesis, but the effect of E_2 on the spermatogonial renewal has been confirmed by the in vitro experiment. Recently, it was also reported that, in vitro, estradiol could induce proliferation of isolated rat fetal gonocytes [71], primary spermatogonia in the frog *Rana esculenta* [72, 73], and spermatogonial stem cell renewal in Japanese eel [31]. These effects were inhibited by an estrogen antagonist, suggesting that estrogen effects are mediated by the binding of estrogen to its receptors, either through Sertoli cells or directly on germ cells. Thus, estrogens could participate in the autocrine and/or paracrine regulation of spermatogenesis. The 17α,20β-

DP promoted DNA synthesis, as evidenced by the incorporation of BrdU into germ cells. Our results indicated that 17α,20β-DP was detected during the mitotic phase of spermatogenesis in vivo and enhanced DNA synthesis in vitro. Therefore, 17α,20β-DP could play an important role in spermatogenesis, especially mitosis, and in the initiation of meiosis in addition to its well-established role during the final maturation stage. Furthermore, large amounts of 17α,20β-DP were synthesized in vitro by rainbow trout testicular fragments during gonial mitotic activity, when spermatocytes enter meiosis, as reported by Dépêche and Sire [56]. Although a slight stimulation was observed in testosterone-incubated fragments, the value of positively stained spermatogonia was significantly lower than those of 11-KT, E_2, 17α,20β-DP, and SPG. Stimulation observed in testosterone-incubated fragments could be due to the conversion of this androgen into 11-KT or E_2 by the endogenous enzymes.

We conclude that 1) 11-KT is necessary for the initiation of spermatogenesis and sperm production, and it probably plays a role in spermiation, 2) 17α,20β-DP is essential for the final maturation stage, could play a significant role in mitosis and meiosis, and probably participates in the regulation of spawning behavior, and 3) estrogen is an indispensable male hormone that plays a physiological role in some aspects of testicular function, especially in mitosis. The three steroids, 11-KT, E_2, and 17α,20β-DP, as well as SPG significantly induced DNA synthesis, thence spermatogonial renewal and/or spermatogonial proliferation. Further investigations for elucidating and confirming the role of 17α,20β-DP, E_2, and the androgenic steroid hormones in spermatogenesis, especially during the early stages using *H. perryi* as a new animal system, are required.

ACKNOWLEDGMENTS

We thank Dr. John Bower from the Training Ship Laboratory, Hokkaido University, for his critical reading of the manuscript. We also thank Mr. Toshitsugu Higashino, Division of Marine Biosciences, Hokkaido University, for his assistance during sampling and technical help with RIA.

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