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Testicular Development and Serum Levels of Gonadal Steroids during the Annual Reproductive Cycle of Captive Japanese Sardine

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Abstract Gonad and blood samples were taken throughout the year from captive males of the Japanese sardine, *Sardinops melanostictus*, and changes in serum levels of gonadal steroids were examined in relation to the annual gonadal cycle. On the basis of testicular histology, the annual gonadal cycle was divisible into four periods: immature (July–September), spermatogenesis (October–December), spermiation (January–April), and post-spawning (May–June). The pattern of seasonal changes in the gonadosomatic index (GSI) was inversely correlated with that of water temperature, and reflected the degree of testicular maturity. The serum testosterone level was relatively low during spermatogenesis (2.2–2.5 ng/m*l*), rose markedly around the time of spermiation (7.7–24.6 ng/m*l*), and became low after spawning and during immature periods (0.6–0.7 ng/m*l*). The serum 17α,20β-dihydroxy-4-pregnen-3-one level was high in males with spermatogenic or spermiating testes (0.6–1.0 ng/m*l*), but became low (0.2 ng/m*l*) during the post-spawning period and was undetectable in immature fish. Although 11-ketotestosterone was detectable in some fish, the values obtained were thought to reflect cross-reactivity of the antiserum employed with testosterone. These findings are discussed in relation to male reproduction of the Japanese sardine and steroidal regulation of spermatogenesis and spermiation in other teleosts.

Profiles of various steroids in the plasma of teleosts provide useful information on the dynamics and progress of sexual maturation. It is well known that two hormones in particular, testosterone (T) and 11-ketotestosterone (11-KT), possibly have important roles in various aspects of male fish reproduction, including maturation of the gonads and development of secondary sexual characteristics. Recently, it has been suggested that in salmonids 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) is involved in spermiation (Nagahama, 1987). However, most previous studies on the relationship between gonadal steroids and the reproductive cycle in male teleosts have used salmonids (Scott et al., 1980; Kime and Manning, 1982; Hunt et al., 1982; Fostier et al., 1982, 1984; Ueda et al., 1983, 1984a, 1985; Schulz, 1984; Dye et al., 1986; Fitzpatrick et al., 1986; Truscott et al., 1986) and some freshwater species (Lamba et al., 1983; Burke et al., 1984; Scott et al., 1984; Shimizu et al., 1985; Kobayashi et al., 1986a; Pankhurst et al., 1986; Rosenblum et al., 1987), and only a few studies have been conducted on marine fishes (Campbell et al., 1976; Wingfield and Grimm, 1977; Cochran, 1987; Pankhurst and Conroy, 1987, 1988; Ouchi et al., 1988; Zhu et al., 1989).

The Japanese sardine, *Sardinops melanostictus*, is a common, seasonally migratory, pelagic fish, being widely distributed in coastal and off shore waters around Japan, and is one of the most important fishes caught commercially in Japan for food, fish bait and manure. Aspects of its reproductive biology have been described by a number of authors (Asami, 1953; Ishida et al., 1959; Ito, 1961; Nakai, 1962; Nakai and Usami, 1962; Usami, 1964, 1972; Hiramoto, 1973). However, much of the available information has centered on female reproductive biology, and there has been little or no investigation of male reproduction.

We recently started several studies dealing with the reproduction of the Japanese sardine in coastal waters off northwest Kyushu, and have already studied the annual endocrine and gonadal cycles of female Japanese sardine (Matsuyama et al., in press). The purpose of the present study was to

follow seasonal changes in the serum concentrations of several gonadal steroids including T, 11-KT and 17α,20β-P in male Japanese sardine, and to examine any correlations between these changes and the stages of gonadal development.

Materials and methods

The fish used in the present study were captured in a set net installed off Shikanoshima I., near Fukuoka, during their annual migration to their offshore spawning area, and were transferred to the Fisheries Research Laboratory of Kyushu University, Tsuyazaki, Fukuoka Prefecture. About 800 fish were captured in April 1988 and maintained in a round 3,000*l* concrete tank (first group) until June 1989, and about 800 other fish were caught in April 1989 and kept in a square 10,000*l* concrete tank (second group). The experimental tanks containing circulating seawater were exposed to natural temperature and photoperiod conditions. The fish were fed daily with commercial food pellets. Four to nine male fish were sampled every month from the first group between October 1988 and May 1989, and from the second group between June and September 1989, amounting to a total of 69 fish (85–120 g in body weight) analyzed in the study.

The fish were anesthetized with 50 ppm MS222 and measured, after which blood samples were taken from the caudal vessels. The blood was then centrifuged and the serum stored at −30°C until steroid analysis. Serum levels of T, 11-KT and 17α,20β-P were measured by radioimmunoassay (RIA). Details of the steroid RIAs used have been described previously (Young et al., 1983; Ueda et al., 1985). The antiserum against 11-KT crossreacts with 11-KT and T at 100% and 1.9%, respectively.

The testes were dissected out and weighed, and the gonadosomatic index (GSI) was calculated as follows; GSI = GW·100/BW, where GW and BW represent gonad weight (g) and body weight (g), respectively. The testes were fixed in Bouin's solution, embedded in paraffin, sectioned at 5 μm, and stained with Mayer's hematoxylin and eosin for histological examination. Immature testes obtained in the summer were fixed in Karnovsky's fluid, post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer (pH 7.2), and embedded in epoxy resin after dehydration. Sections were cut at a thickness of 1 μm, and stained with a 1% solution of toluidine blue in 0.1 M phosphate buffer.

Maximum and minimum water temperatures were recorded daily. Natural day length was calculated from the times of sunrise and sunset at Fukuoka.

Data were analyzed statistically using Duncan's multiple range test, and Student's *t* test.

Results

Seasonal changes in water temperature, photoperiod and GSI. Seasonal changes in day length and water temperature at Tsuyazaki during the study period are shown in Fig. 1. The monthly average for water temperature changed with a fairly regular seasonal pattern. Water temperature was highest (26.5°C) in August, and lowest (11.0°C) in March. Day length was longest (14.3 h) in June, and shortest (9.9 h) in December.

Seasonal changes in GSI are also shown in Fig. 1. The average GSI in males was lowest in September (0.16), then increased gradually, following the progress of spermatogenesis, to peak in February (7.55), but fell to a low level after the spawning season. The pattern of seasonal changes in GSI (Y) was inversely correlated with water temperature (X) as follows; $Y = -0.3967X + 10.07$, $r = -0.864$, $P < 0.05$, and reflected the degree of testicular maturity as described later, but no correlation was evident between day length and GSI ($P > 0.05$).

Seasonal changes in testicular maturity. Testicular maturity in the Japanese sardine was divided into five stages as shown in Table 1 and Fig. 2.

Immature testes predominantly occupied by mitotic spermatogonia were classified as stage I (Fig. 2-1). In the testes at stage II, the first appearance of spermatozoa was evident (Fig. 2-2). The actively spermatogenic testes at stage III contained all gamete stages, but with spermatozoa predominating (Fig. 2-3). Spermiating testes (Fig. 2-4) were divided into two substages according to their GSI values, i.e. IV-a (GSI < 5.0) and IV-b (GSI ≧ 5.0). Spent testes containing residual spermatozoa in the lobule lumen and/or sperm duct were classified as post-spawning, stage V (Fig. 2-5).

Monthly changes in testicular maturity composition in captive Japanese sardine are shown in Fig. 3. All fish were immature (stage I) in August, and spermatogenesis (stage II) occurred in autumn (September–November). Spermatogenesis (stage III) proceeded from October to December. With the advance of spermatogenesis, spermiating testes (stage IV) displayed an increased percentage of oc-

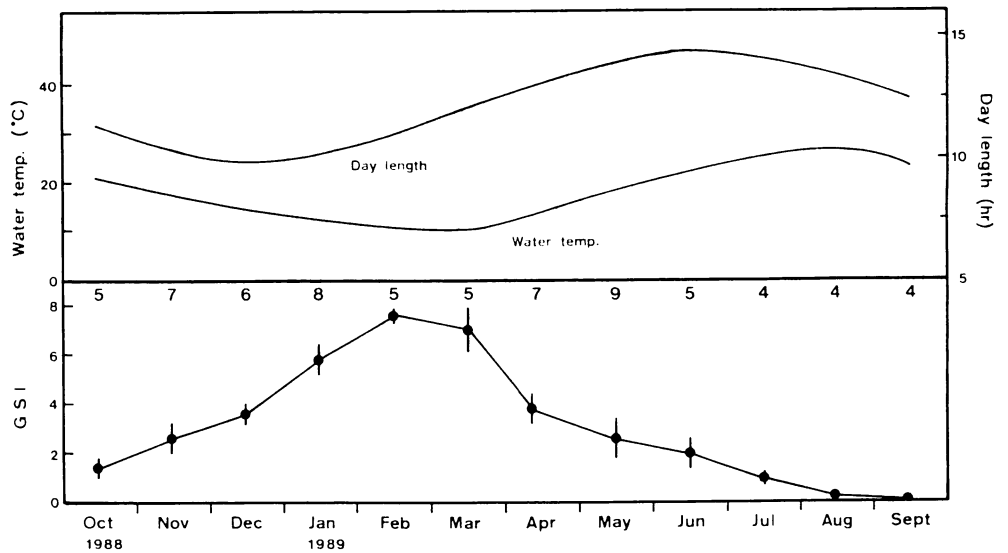

Fig. 1. Seasonal changes in water temperature, day length and gonadosomatic index (GSI) of male Japanese sardine, *Sardinops melanostictus*. Each GSI value is mean±SEM of four to nine samples.

currence, and all fish were in spawning condition between January and March. Fish in spent condition (stage V) appeared from April to July.

Thus, on the basis of testicular histology, seasonal changes in testicular condition of captive Japanese sardine, i.e. spermatogenesis (October–December), spermiation (January–April), post-spawning (May–June) and immature (July–September), were elucidated (see Fig. 3).

Seasonal changes in steroid hormone levels. Seasonal changes in serum levels of T and 17α,20β-P in males are shown in Fig. 4.

The serum T level in males exhibited seasonal changes which were generally similar to the respective changes in GSI. Serum T remained at low levels during the immature (July–September) and sperm-

Table 1. Maturity stages of testis in *Sardinops melanostictus*.

Maturity stage of testis		Histological appearance
I	Immature	Testicular lobules are predominantly occupied by mitotic spermatogonia. The number of spermatocytes steadily increases with time, but spermatids and spermatozoa are absent.
II	Early spermatogenesis	Cells at all stages of spermatogenesis are present; a small number of spermatozoa have appeared in the lobular lumina. The numbers of spermatocytes and spermatids continue to increase, concomitant with a reduction in the number of spermatogonia.
III	Late spermatogenesis	Cells at all stages of spermatogenesis are present; testes contain a large number of spermatozoa, but no spermatozoa are present in the sperm duct.
IV-a	Early spermiation	Lobular lumen and sperm duct are fully filled with spermatozoa, and other gamete stages are present in small quantities. Small amount of viscous milt can be expressed. GSI<5.0.
IV-b	Active spermiation	Lobular lumen and sperm duct are fully filled with spermatozoa. By the end of this stage, spermatocytes and spermatids have been replaced by spermatozoa. Fully flowing milt is expressed from some fish at this stage. GSI$\geqq 5.0$.
V	Post-spawning	Residual spermatozoa remain in the lobular lumina, but no milt can be expressed from the testes.

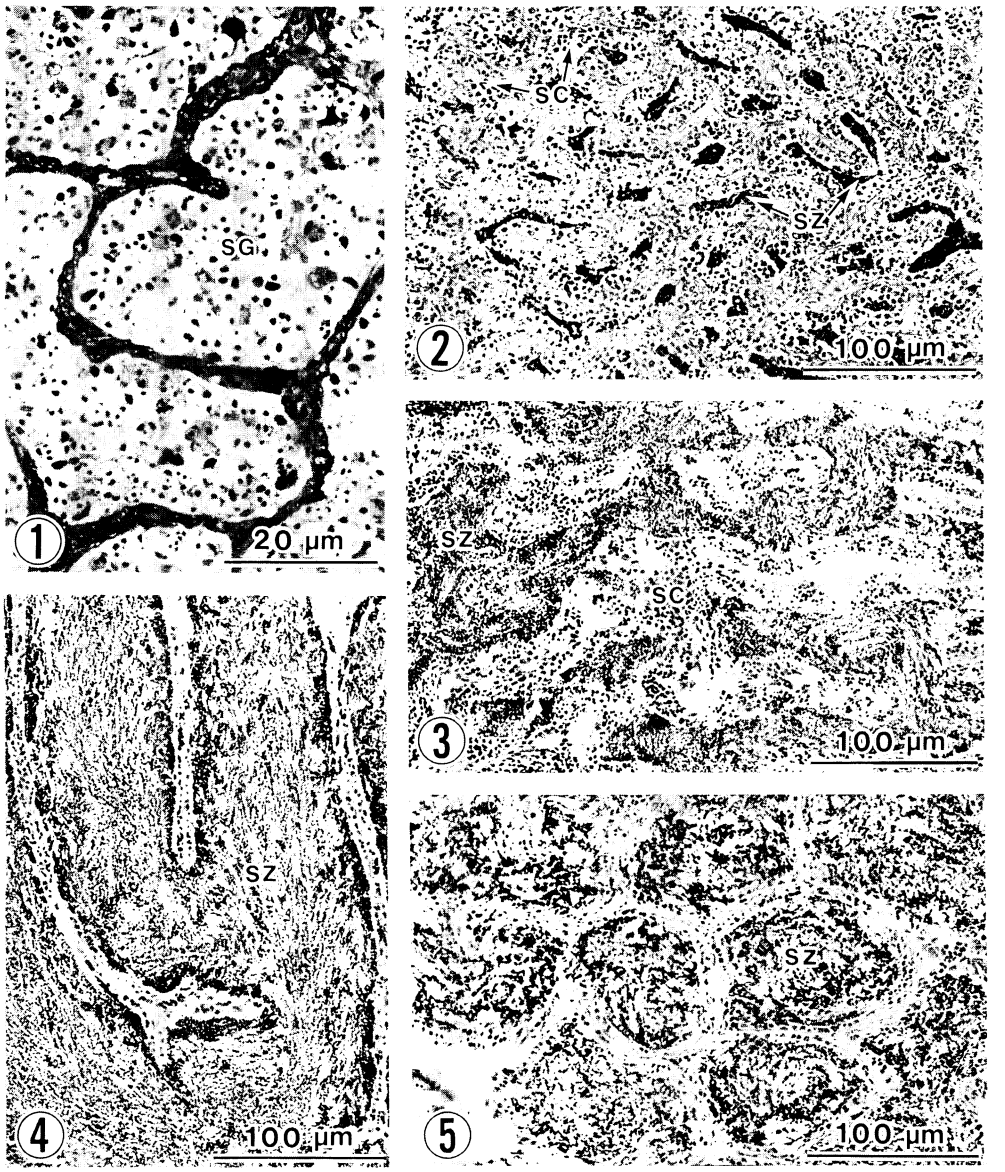

Fig. 2. Micrographs of sections of Japanese sardine testes at the following stages: 1, immature (August); 2, early spermatogenesis (October); 3, late spermatogenesis (December); 4, active spermiation (February); 5, post-spawning (May). SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa. See Table 1 for histological description of each testis stage.

atogenesis (October–December) periods, the lowest value (0.54 ± 0.28 ng/m*l*) occurring in August. This was followed by a marked rise in January ($P < 0.01$) and maintenance of high levels during spermiation (January–April), the peak level (26.76 ± 4.10 ng/m*l*) occurring in February, with subsequent decline to a low level in the post-spawning period (May–June). Serum 17α,20β-P was detectable at a relatively high level (0.60–1.16 ng/m*l*) during November to March with no significant changes ($P > 0.05$), but remained low (< 0.3 ng/m*l*) at all other times, and was undetectable in August. Although 11-KT was recorded in some fish with high serum T levels, the values of 11-KT in such fish were less than 2% of the T levels

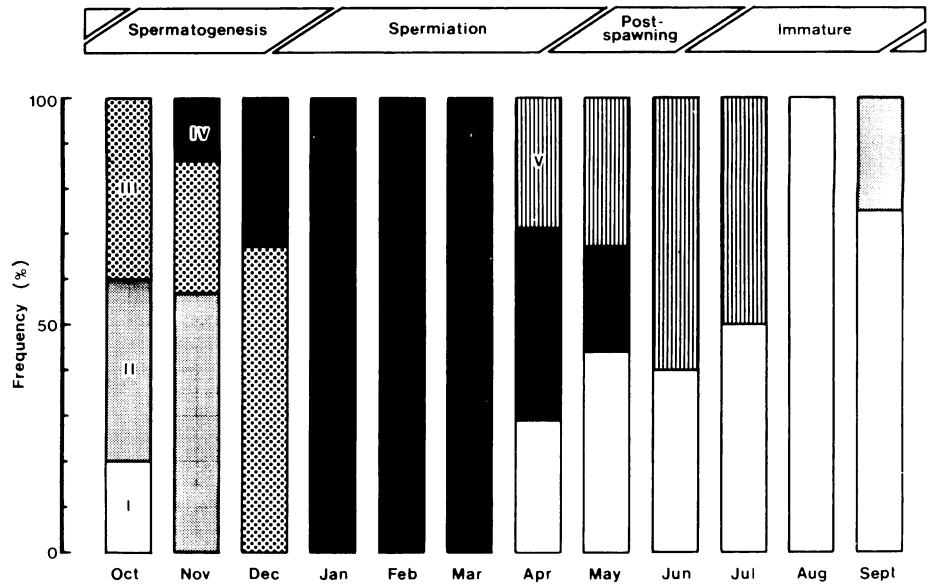

Fig. 3. Monthly changes in proportional frequencies of testicular maturity stages in the Japanese sardine. See Table 1 for histological description of each testis stage.

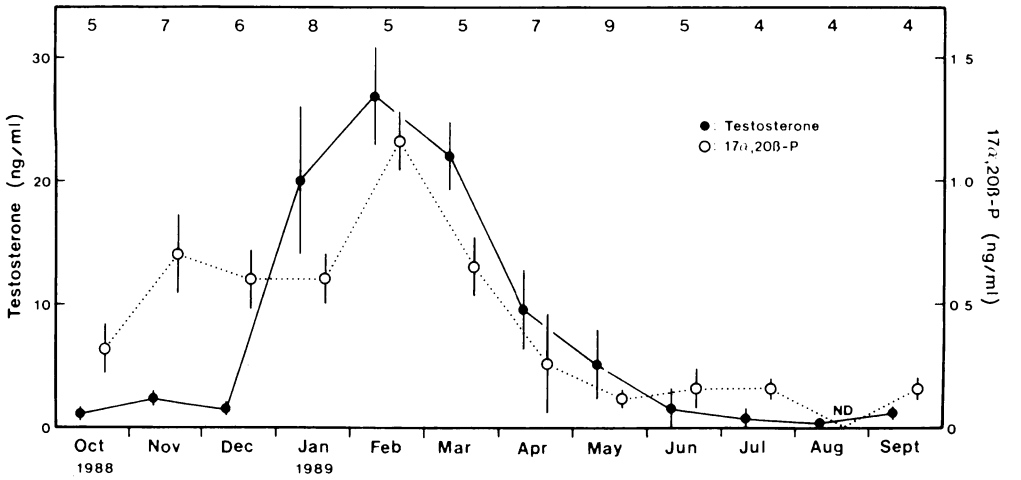

Fig. 4. Seasonal changes in serum levels of testosterone and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) in male Japanese sardine. Values are mean$\pm$SEM of four to nine samples. ND, not detectable.

and consequently were thought to reflect cross-reactivity of the antiserum with T, as noted in the Materials and methods above. Therefore, 11-KT was not detected in the serum of any male fish throughout the year.

Steroid hormone levels in fish at different stages of maturity. Data on serum levels of T and 17α,20β-P, and GSI were lumped according to testicular maturity stage, and are shown in Fig. 5. The average GSI in males was lowest (0.48 ± 0.08) in immature fish (stage I), increased during spermatogenesis (stage III, $P<0.01$), peaked (7.56 ± 0.23) at the spermiation stage (stage IV-b), and then fell markedly to a low level (1.13 ± 0.25, $P<0.01$) in post-spawning fish (stage V).

Changes in the serum T level, which were lumped

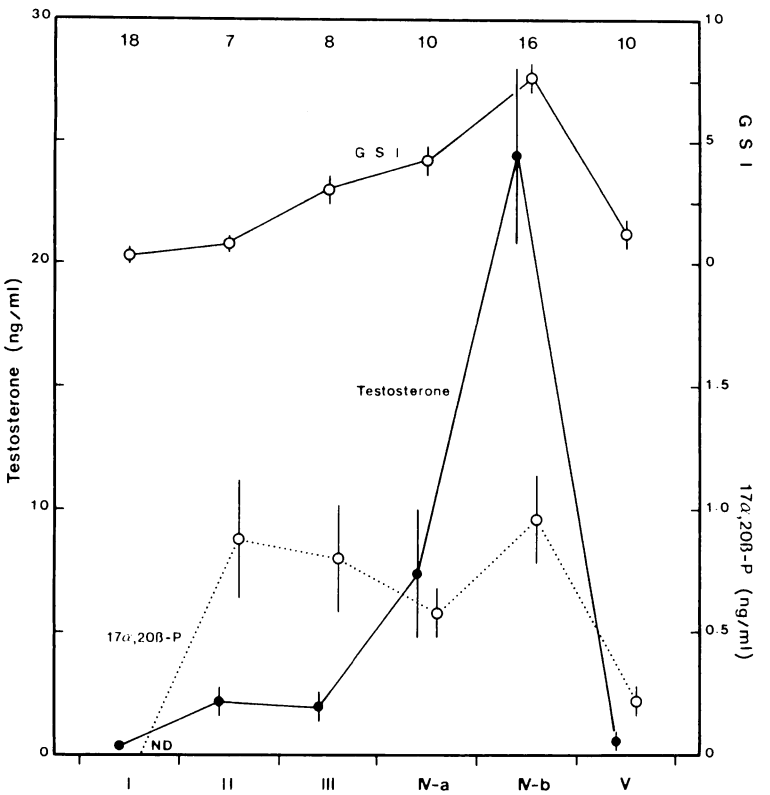

Fig. 5. Changes in gonadosomatic index (GSI), and serum testosterone and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P) levels in male Japanese sardine at various testicular maturity stages. Testicular stages are as in Table 1. Values are mean±SEM of seven to eighteen samples. ND, not detectable.

according to the testicular maturity stage, were lowest (0.55±0.18 ng/m*l*) in immature fish at stage I, high (7.67±2.64 ng/m*l*) at stage IV-a, with an apparent increase in the spermatogenesis stages (stages II and III, 2.46±0.40 and 2.23±0.48 ng/m*l*, respectively, $P<0.01$), and rose markedly to a peak (24.61±3.69 ng/m*l*, $P<0.01$) at stage IV-b, thereafter falling to a low level (0.72±0.12 ng/m*l*, $P<0.01$) at stage V. The serum 17α,20β-P level was below the assay detection limit (0.06 ng/m*l*) in immature fish (stage I), between 0.6 and 1.0 ng/m*l* during spermatogenesis (stages II, III) and spermiation (stages IV-a, IV-b) with no significant changes ($P>0.05$), and then fell markedly to a low level (0.21±0.06 ng/m*l*) in post-spawning fish (stage V).

Discussion

In most temperate-zone teleosts previously studied, water temperature and/or photoperiod have been proposed as the principal environmental factors regulating the timing of reproduction (Lam, 1983). In the present study, changes in water temperature were inversely correlated with changes in GSI, but no correlation was found between day length and GSI. GSI values peaked in February (7.55) and maintained a constant level in March (7.04) when the water temperature fell to a minimum (11.3℃ in February and 11.0℃ in March). All males had spermiated testes at this time. This suggests that decreasing water temperature may be the main cue for the initiation of testicular recrudescence in this species. Immature testes appeared in April and the proportion of individuals with such testes increased concomitant with rising water temperature. All males had immature testes in August when the average water temperature was highest (26.4℃), suggesting that high temperature may induce regression of the testis in this species. These results suggest that water temperature may play a role in both initiation

and termination of the spawning season in both males and females (Matsuyama et al., in press) of the Japanese sardine.

The testes of the Japanese sardine pass through successive stages of maturation during their annual cycle (Fig. 3). Five stages of testicular development were histologically distinguished, and there was a good correlation between changes in GSI and histological events (Fig. 5). Spermiating fish were observed over a relatively long period between November and May, and all fish examined between January and March had spermiated testes. Ovarian histology indicated that the spawning period of females reared together with male fish in the present study, was from January to March (Matsuyama et al., in press), a period coinciding with spermiation in males. These long spawning periods in females and males suggest that this species is multiple spawner, and in fact, this is supported by a histological study of seasonal gonadal changes in a natural spawning population of the Japanese sardine off northwest Kyushu (Matsuura et al., in press).

In the present study, the serum levels of T showed seasonal changes corresponding to testicular stage. The highest level of serum T in male Japanese sardine was lower than that of salmonids (35–150 ng/ml, Kime and Manning, 1982; Baynes and Scott, 1985; Dye et al., 1986; Fitzpatrick et al., 1986; Truscott et al., 1986), but higher than those of some other freshwater (3–12 ng/ml, Lamba et al., 1983; Burke et al., 1984; Scott et al., 1984; Kobayashi et al., 1986a; Pankhurst et al., 1986; Rosenblum et al., 1987) and marine species (0.25–15 ng/ml, Wingfield and Grimm, 1977; Cochran, 1987; Pankhurst and Conroy, 1987; Ouchi et al., 1988; Zhu et al., 1989).

In many male teleosts, T is typically elevated during spermatogenesis, and falls at the onset of spermiation (Fostier et al., 1983). This may be due to the conversion of androgen precursors into the production of 17α,20β-P (Scott and Baynes, 1982) or to the synthesis of downstream metabolites of T. For example, 11-KT is synthesized by the testes in a wide range of teleost species (Fostier et al., 1983) and commonly peaks in plasma prior to spawning, after T has begun to decline (Campbell et al., 1976; Scott et al., 1980; Hunt et al., 1982; Kime and Manning, 1982; Baynes and Scott, 1985). Thus, previous data from other teleosts support the concept that T possibly plays a role in the initiation and probable maintenance of spermatogenesis. However, the pattern of seasonal changes in serum T levels in male Japanese sardine was different from those in the species cited above. In the present study, the level of serum T showed a slight increase during spermatogenesis in comparison with that of immature fish, but began to increase markedly with the appearance of spermiation. It peaked in actively spermiating fish, and then fell markedly to a low level in the post-spawning period (Fig. 5). This suggests that in male Japanese sardine the physiological role of T may be related to spermiation rather than to spermatogenesis.

In several teleost species examined to date, 11-KT is considered to be the principal androgen, even though blood levels of the steroid are often lower than those of T. In the present study, however, the low serum 11-KT levels detected in some males were not physiologically significant, but simply reflected cross-reactivity of the antiserum empolyed with T. The high levels of serum T during spermiation and the lack of serum 11-KT at this time suggest the possible presence of unmeasured steroid(s) which may act physiologically as sexual hormone(s) in spermatogenesis in the Japanese sardine.

Recently, Asahina et al. (1985) found that the testes of the urohaze-goby *Glossogobius olivaceus* produced 5α-reduced androgens in vitro, but almost no 11-KT or T, and suggested that 5α-dihydrotestosterone, one of the major androgens produced by the testes of the urohaze-goby, might act as the main androgen in this fish (Asahina et al., 1989). These results suggest the possibility of species differences in major androgens among male teleosts. We have found a lack of serum T in female Japanese sardine throughout the year, including the spawning season, suggesting the possibility of alternative pathway(s) for estradiol-17β biosynthesis other than via T (Matsuyama et al., in press). The lack of T in females, and of 11-KT in males as described here, is of considerable interest, and warrants further in-vitro studies on steroid metabolism in the gonads, in order to identify the steroids which act physiologically as sexual hormones in this species.

In the present study, 17α,20β-P was detected in the serum of male Japanese sardine. The serum levels of 17α,20β-P were significantly high (0.6–1.0 ng/ml) throughout the periods of spermatogenesis and spermiation (Fig. 5). The highest level of serum 17α,20β-P in male Japanese sardine was lower than those in salmonids (Scott and Baynes, 1982; Ueda et al., 1983, 1984a; Baynes and Scott, 1985; Dye et al., 1986; Fitzpatrick et al., 1986; Truscott et al., 1986),

the white sucker *Catostomus commersoni* (Scott et al., 1984) and the blue cod *Parapercis colias* (Pankhurst and Conroy, 1987), similar to those in the goldfish *Carassius auratus* (Kobayashi et al., 1986b) and the goldeye *Hiodon alosoides* (Pankhurst et al., 1986), and slightly higher than that of the red sea bream *Pagrus major* (Ouchi et al., 1988).

A hormonal role for 17α,20β-P in male fish has not yet been completely established. It has been reported, mainly in salmonids, that plasma 17 α,20 β-P shows markedly high levels during spermiation (Scott and Bynes, 1982; Ueda et al., 1983, 1984a; Baynes and Scott, 1985; Dye et al., 1986; Fitzpatrick et al., 1986; Truscott et al., 1986), and there is considerable evidence that 17α,20β-P may be associated with control of the ionic composition of seminal fluid in trout; Baynes and Scott (1985) found that the plasma level of 17α,20β-P was highly correlated with the ratio of $K^+ : Na^+$ in seminal fluid. Recent in-vitro experiments have demonstrated that both testicular fragments and sperm of spermiating rainbow trout produce 17α,20β-P (Ueda et al., 1983, 1984b), and that intraperitoneal injection of 17α, 20β-P into the amago salmon *Oncorhynchus rhodurus* and the goldfish induces spermiation in both species (Ueda et al., 1985). Furthermore, in male goldfish, it has been shown that plasma levels of 17α,20β-P increase only during spawning behavior (Kobayashi et al., 1986b), and are elevated concomitant with increased milt production, following injection of human chorionic gonadotropin (Kobayashi et al., 1986c). From these results, 17α,20β-P is considered to be a hormone that induces spermiation and milt production in some teleosts. Although the association between 17α,20β-P and final maturation of sperm, which has been observed in other species, was not established conclusively in the present study, the significantly high levels of this steroid during spermatogenesis and spermiation are believed to indicate its involvement in sperm formation and maintenance in the Japanese sardine. Further experiments using in-vivo injection or in-vitro methods will be required in order to understand the physiological roles of 17α,20β-P and the androgens described above, in male reproduction of this species.

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飼育下におけるマイワシ雄の生殖年周期および性成熟に伴う血中ステロイドホルモン量の変動

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屋外コンクリート水槽で飼育したマイワシ *Sardinops melanostictus* 雄の生殖年周期と，性成熟に伴う血中ステロイドホルモン量の変動を調べた．精巣の組織学的観察に基づき，マイワシ雄の生殖年周期は，未熟(7–9 月)，精子形成(10–12 月)，排精(1–4 月)，排精終了(5–6 月)の 4 期に区分された．精巣の発達および退縮は水温の変化によく対応していた．血中テストステロン量は排精時に急激に増大したが (7.7–24.6 ng/ml)，硬骨魚の主要アンドロゲンである 11-ケトテストステロンは周年認められず，マイワシではテストステロンが精子形成，特に排精時に重要な役割を果たすことが示唆された．17α,20β-ジヒドロキシ-4-プレグネン-3-オンの血中量は精子形成および排精過程を通して一定した高い値を示し (0.6–1.0 ng/ml)，サケ科魚類やキンギョで報告されたような，排精との関連性は特に認められなかった．

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