Testicular development and serum profiles of steroid hormone levels in captive male Pacific herring *Clupea pallasii* during their first maturational cycle

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**ABSTRACT:** The present study investigates the relationship between testicular development and serum steroid hormone levels in captive Pacific herring *Clupea pallasii* during the first reproductive cycle. The maturity of the testis was divided into five periods based on histological observation. These are early spermatogenic stage (April to July), mid-spermatogenic stage (August to November), late spermatogenic stage (December to March), functional maturation stage (early April) and spent stage (late April). The pattern of seasonal change in gonadosomatic index (GSI) clearly reflected testicular maturity. 11-Ketotestosterone (11-KT) levels increased from October to a peak level (6.58 ± 1.87 ng/mL) in January, and were maintained at this level until March. In contrast, testosterone levels were consistently low, less than 1 ng/mL, at all times. These results suggest that 11-KT is the predominant androgen that controls spermatogenesis in this species. 17,20β-Dihydroxy-4-pregnen-3-one (DHP) showed a single sharp peak (3.38 ± 0.35 ng/mL) in early April of the second year, suggesting that milt production is induced by DHP as in some other teleost species.

**KEY WORDS:** 11-ketotestosterone, 17,20β-dihydroxy-4-pregnen-3-one, Pacific herring, reproductive cycle, testicular development, testosterone.

**INTRODUCTION**

Herrings, genus *Clupea*, are important species for both commercial fisheries and ecosystems, and are interesting subjects for research in biology because they are thought to be primitive teleosts. While there have been numerous studies concerning female reproductive physiology,1–6 there have been only a few studies concerning male reproductive physiology (e.g. testicular development,2 steroidogenesis5).

Androgens have important roles in various aspects of male reproduction in fish, including testicular development. In clupeids, two steroids are thought to be the predominant androgens: 11-ketotestosterone (11-KT) and testosterone (T).6,7 11-Ketotestosterone (11-KT) and testosterone (T).6,7 11-Ketotestosterone (11-KT) is detected in the blood of maturing males of many teleost species and is accepted as a major androgen inducing spermatogenesis.8 In contrast, T is thought to be the main androgen in Japanese sardine *Sardinops melanostictus* (which belongs to Clupeidae), because T is detected in the blood of maturing male of this fish but 11-KT is not.7,8 In Pacific herring *Clupea pallasii* Valenciennes both 11-KT and T were identified at similar levels in the plasma from sexually mature males.8 Thus, it is of interest to determine which steroid is the predominant androgen in the Pacific herring.

In male teleosts, the testes changes from synthesizing mainly C18 and C19 steroids to mainly C21 steroids at the time of spawning,10 and C21 steroids induce spermiation and are responsible for sperm motility.11 In some lower teleosts includ-
ing herring, 17,20β-dihydroxy-4-pregnen-3-one (DHP) has been known as the main male C21steroid.10-15 However, in the herring, the serum profile of DHP during gonadal development has not yet been investigated.

In Japan, Pacific herring is a commercially important fish for food, especially the roe, but populations have decreased dramatically during the mid-20th century. Recently, artificial reproduction and release of this species has been attempted to aid recovery; however, available information on the reproductive physiology of this fish is quite limited and not systematic. Therefore, we have conducted research on the reproductive physiology of Pacific herring in Japan, in order to obtain information for artificial fry production and resources management of this fish. In the present paper, we report the testicular development and serum levels of sex steroids and a maturation-related steroid during the first maturational cycle in captive male Pacific herring.

MATERIALS AND METHODS

Fish and sample

The Pacific herring used in the present study were artificially produced at the Japan Sea-Farming Association, Akkeshi Station, Hokkaido, Japan, in April 1993 and reared in an indoor circular tank under a natural photoperiod (Fig. 1a). Day length was longest (15.4 h) at summer solstice and shortest (9.0 h) at winter solstice. In the current study, water temperature was not regulated from April to December (8.4–17.8°C), but the temperature was kept at constant approximately 5°C from December to April (Fig. 1b), because the fish become dull to feed and often die at temperatures lower than 5°C. The average water temperature seasonally changed from May to December and the highest value (17.8°C) was recorded in early September.

Six to 12 fish were collected every month between April 1994 (16.8 ± 0.3 cm in fork length) and April 1995 (23.4 ± 0.3 cm in fork length). Each fish was anesthetized with ethyl p-aminobenzoate, then standard length and body weight measured. The fish were bled from the caudal vessel with non-heparinized syringes. Serum was separated by centrifugation at 3000 × g for 15 min at room temperature and stored at −80°C until use. The testis was dissected and weighed, and gonadosomatic index (GSI) was calculated as follows: GSI = GW × 100/BW, where GW and BW are gonad weight (g) and body weight (g), respectively.

Histology

Testis was fixed in Bouin’s solution, embedded in paraffin, sectioned at 6 µm, and stained with Delafield’s hematoxylin and eosin. The microscopic images (×400) randomly chosen from three fields within each cross-section of the testis were captured by video. A sheet of tracing paper was attached to the monitor for each image. The areas occupied by the different spermatogenic cells (e.g. spermatogonia, spermatocytes, spermatids and spermatozoa) were traced, cut and weighed. The percentage of each type was expressed as the ratio of the area occupied by a particular spermatogenic cell stage to the total area of all spermatogenic cells.

Enzyme immunoassay for steroid hormone

Serum T, 11-KT and DHP levels were measured by specific enzyme-linked immunoassay (ELISA) according to the method of Asahina et al.16 The values under the detection limit (T, 50 pg/mL; 11-KT, 25 pg/mL; DHP 50 pg/mL) were each considered as limit values for statistical analysis.
Statistics

All data are presented as mean±SEM. The data were analyzed by one-way ANOVA followed by Fisher’s Protected Least Significant Difference Test (Fisher’s PLSD test) using the Statview 4.5 program for Macintosh (Abacus Concepts, Inc. Berkeley, CA, USA) (P<0.05).

RESULTS

Gonadosomatic index

Seasonal changes in GSI are shown in Fig. 1c. The GSI values remained very low from April to June, but then increased gradually from 0.38% in July to 1.64% in September. From October, they rapidly increased, and reached 13.8% in November: these high values were maintained until March. In early April, they increased temporarily to 17.2%, then decreased dramatically to a low level (3.12%) in late April.

Testicular maturity

The relative abundance of spermatogenic cells in the testis is shown in Table 1. The percentage of spermatogonia was high from April to June, but then decreased gradually to zero in November. A high percentage of spermatocytes was observed from July to September, but they then decreased in October. Spermatids and spermatozoa were first observed in July and both increased gradually to September, and then increased rapidly in October. Afterwards, the percentage of spermatozoa increased while the percentage of spermatids decreased, until finally spermatozoa represented >95% of the testis.

Testicular maturity of each fish can be divided into the following five stages based on the spermatogenic activity.

Early spermatogenic stage (Fig. 2a): Testis is occupied by spermatogonia and spermatocytes.

Mid-spermatogenic stage (Fig. 2b): Spermatocytes rapidly increase (more than 50%) and account for the majority of testicular cysts. Spermatids and spermatozoa appear but they do not exceed 30%.

Late spermatogenic stage (Fig. 2c): The percentage of spermatocytes decreases to less than 30%. Spermatids and spermatozoa rapidly increase and account for the majority of testicular cysts.

Functional maturation stage (Fig. 2d): The percentage of spermatozoa accounts for more than 95% in the total area in histological sections.

Spent stage (Fig. 2e): Testis reduces its volume dramatically. Residual spermatozoa occupy a high percentage of the testis, but not spermatocytes and spermatids. A few spermatogonia are observed.

All fish were in the early spermatogenic stage from April to June. The mid-spermatogenic stage was observed from July and October, and the late spermatogenic stage was from October to January. The functional maturation stage was observed from December, and all fish were in this stage in March and early April. In late April, all fish were in the spent stage.

Table 1  Monthly changes in each stage of male germ cells of male Pacific herring

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>n</th>
<th>SG</th>
<th>SC</th>
<th>ST</th>
<th>SZ</th>
</tr>
</thead>
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<tr>
<td>26 Apr. '94</td>
<td>5</td>
<td>73.4</td>
<td>26.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 May '94</td>
<td>5</td>
<td>34.3</td>
<td>65.7</td>
<td></td>
<td></td>
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<tr>
<td>29 Jun. '94</td>
<td>4</td>
<td>63.6</td>
<td>36.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 Jul. '94</td>
<td>5</td>
<td>10.0</td>
<td>87.1</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>30 Aug. '94</td>
<td>5</td>
<td>8.0</td>
<td>84.8</td>
<td>4.2</td>
<td>3.0</td>
</tr>
<tr>
<td>28 Sep. '94</td>
<td>5</td>
<td>2.2</td>
<td>83.9</td>
<td>7.6</td>
<td>6.3</td>
</tr>
<tr>
<td>26 Oct. '94</td>
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<td>0.2</td>
<td>26.2</td>
<td>32.5</td>
<td>41.1</td>
</tr>
<tr>
<td>28 Nov. '94</td>
<td>5</td>
<td>4.1</td>
<td>26.0</td>
<td>69.9</td>
<td></td>
</tr>
<tr>
<td>26 Dec. '94</td>
<td>5</td>
<td>3.3</td>
<td>5.7</td>
<td>91.0</td>
<td></td>
</tr>
<tr>
<td>31 Jan. '95</td>
<td>5</td>
<td>1.0</td>
<td>1.7</td>
<td>97.3</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>7 Apr. '95</td>
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<tr>
<td>26 Apr. '95</td>
<td>5</td>
<td>100.0</td>
<td></td>
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</tbody>
</table>

Numerals indicate the percentage of area occupied in the section of testis.

SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa.

Serum steroid hormone levels

Serum 11-KT levels (Fig. 3a) remained low (<0.1 ng/mL) from April to September. The level increased from October to a peak level (6.58±1.87 ng/mL) in January, and remained kept high until March, then rapidly decreased to a low level (<0.1 ng/mL) again in early April.

Serum T levels were measured in only three of the months. The levels were 125.2±30.3 pg/mL (n=8) in August, 893.8±84.7 pg/mL (n=8) in January, and 103.5±32.7 pg/mL (n=6) in late April of the second year.

Serum DHP showed a low level (<0.6 ng/mL) from April to March, rapidly increased in early April (3.38±0.35 ng/mL), and was followed by a sharp decrease to low levels (0.68 ng/mL) in late April (Fig. 3b).
DISCUSSION

The present study histologically demonstrates the first maturation cycle of the testis in 1-year-old male Pacific herring. From April to June, spermatogonia gradually decreased and spermatocytes increased, indicating the beginning of meiosis. From August to November, spermatids and spermatozoa increased as a result of active progression of meiosis and spermiogenesis. Similar gonadal growth has been reported in the Atlantic herring Clupea harengus. From December to March, the GSI showed a tendency to gradually decrease, which is probably related to cytoplasmic reduction of germ cells during spermiogenesis. In early April, GSI values showed a temporal increase, suggesting milt production by hydration of the testis just prior to sperm release. In late April, GSI values rapidly decreased due to sperm release at spawning, and only residual spermatozoa occupied the testis. From these results, the annual reproductive cycle of male Pacific herring was divided as follows: early spermatogenic period (April to July), mid-spermatogenic period (from August to November),
late spermatogenic period (from December to March), functional maturation period (early April) and spent period (late April). The process of maturation in male Pacific herring in the present study coincided with the process of ovarian development in females, which were kept in the same tank. In particular, there was a correlation between milt production and final oocyte maturation (our unpublished data, 1995). This would seem to facilitate the synchronous spawning of males and females.

It is known that serum concentrations of 11-KT increase with spermatogenesis in many teleosts, including salmonid species. In the Japanese eel Anguilla japonica, in vitro experiments have shown that 11-KT induces all stages of spermatogenesis. However, in male Japanese sardine Sardinops melanostictus serum T not 11-KT increases according to testicular development, suggesting that T, not 11-KT, has a role as the primary androgen in this fish. In the Pacific herring in Canada, both T and 11-KT were detected in abundance in male blood during the late stage of spermatogenesis.

In the present study, we also measured serum concentration of T and 11-KT in the Pacific herring. The serum levels of T were measured during the three periods: mid-spermatogenesis (August), late spermatogenesis (January), and spent (late April of the second year). The serum T levels were significantly higher in January than in the other months; however, the levels were low, being less than 1 ng/mL. In contrast, serum levels of 11-KT were generally higher than those of T, in particular, being seven times higher than T levels in the late spermatogenic period. From the results of the present study, we suggest that 11-KT is the predominant androgen of the Pacific herring.

In the study of Carolsfeld et al. the plasma levels of not only T but also 11-KT were high in the spermiogenic period compared with the present study (about 20 times in T and four times in 11-KT). The herring were captured in the prespawning season, maintained in a net pen, and they spawned later than about 2 months after the spawning of wild herrings. On the contrary, the herrings of the present study are artificially produced and have been reared since birth. They spawned the same period as wild herring in the aquarium. Therefore, we cannot compare both sets of data; however, we conclude that at least our data is within an physiologically normal range.

17,20β-Dihydroxy-4-pregnen-3-one has been identified as the maturation-inducing steroid (MIS) of some females. In the female Pacific herring, DHP is suggested to be MIS. It is known that serum DHP levels elevate during the period of milt production in male teleosts. Ueda et al. demonstrated that injection of DHP induced milt production in the amago salmon Oncorhynchus rhodurus and goldfish Carassius auratus. Recent studies indicate that DHP induces acquisition of sperm motility through elevation of pH in the seminal plasma. Also, in the Pacific herring in Canada, substantial amounts of DHP are found in the seminal fluid and its serum levels are elevated coincidently with milt production. In the present study, serum DHP levels showed a marked increase during the functional maturation period concomitant with temporal increases of GSI values. Thus, DHP appears to promote milt production in the male Pacific herring.

Annual reproductive cycles of teleosts are thought to be controlled by external environmental factors such as water temperature and photoperiod. In the male Pacific herring, spermatogenesis gradually proceeded during spring and summer, and rapidly progressed during autumn, when the water temperature was decreasing and the day length was shortening. These results suggest that low temperature and short-day photoperiod stimulates the progression of spermatogenesis. In the functional maturation period, day-length became long; however, the water temperature did not show any changes due to heating.
of the aquarium in the present study. Therefore, the long-day photoperiod seems likely to be the environmental cue for initiating milt production in this fish. In the female Pacific herring reared in the same tank, final oocyte maturation and ovulation occurred during the same period of milt production (our unpublished observation, 1995). Therefore, we suggest that some ‘communication’, such as pheromonal cues (Stacy et al. 1986), exists between males and females to synchronize the timing of maturation in both sexes for mass synchronous spawning.

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