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Sex Reversal in Tawes *Puntius gonionotus* by Oral Administration of Methyltestosterone

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ABSTRACT

An androgen, 17 alpha methyltestosterone (MT-Sigma), was orally administered to 7 or 14 days old tawes, *Puntius gonionotus* at the dosages of 0, 30, 60 and 120 mg/kg diet with treatment durations of 30 or 60 days. Growth reduction was observed during the first month in the groups treated with either 60 or 120 mg MT/kg diet. Hormone treatments did not affect the survival at 5 months. The administration of MT for 30 days enhanced sex reversal to male in some treated fish. Hormone treatment for 60 days resulted in high level of sterility regardless of dose or timing. The treatments initiated at 14 days post-hatch were more effective than those started at 7 days after hatching. The gonads of sex reversed fish showed typical anatomy of ovary but contained various stages of spermatogenic cells including spermatozoa. The sterile area in which no recognizable germ cells were detected, was observed in the sex-reversed gonads.

Key words : methyltestosterone, sex-reversal, tawes

INTRODUCTION

Tawes, *Puntius gonionotus*, has been cultured in Thailand for more than 30 years. Semi-intensive culture and integrated fish-poultry farming systems produced about 16,300 tons in 1991 (Department of Fisheries, 1993), which made it one of the top five freshwater species being cultured in Thailand. Although no systematic study has been conducted, farmers have observed that female tawes grow faster than males and reach 500 g within 8-12 months while males only grow up to 300 g. Thus, production of all-female populations would improve yields as a whole.

All-female populations will be obtained by either direct sex reversal or by breeding of developed broodstock. In practice, breeding protocol may be more desirable than direct approach which tends to produce inconsistant results (Shelton, 1986b).

If tawes have the XY sex-determining system which is generally found in cyprinids (Shelton 1986b), all-female populations would be produced if sex-reversed males (XX males) are crossed with normal XX females. This technique has been successfully practised in salmonids (Hunter *et al.*, 1983 ; Bye and Lincoln, 1986), in Nile Tilapia, *Oreochromis niloticus* (Calhoun and Shelton, 1983) and in grass carp, *Ctenopharyngodon idella* (Shelton, 1986 a).

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Type and level of hormone as well as duration and timing of treatment affect the effectiveness of sex-reversal of fish (Dunham, 1990).

Seventeen alpha-methyltestosterone has been successfully used to sex-reverse cyprinids namely gold fish (Yamamoto and Kajishima, 1968), common carp (Nagy *et al.*, 1981) and grass carp (Shelton, 1986 a).

Successful sex-reversal could be induced if suitable dosage of hormone is administered throughout the critical period of gonadal differentiation. Treatment outside this period results in incomplete or no sex-reversal (Yamamoto, 1969).

The objective of this paper was to produce sex-reversed tawes by the administration of 17 alpha-methyltestosterone and to determine the effect of different dosages, treatment durations and timing.

MATERIALS AND METHODS

Experimental fish

The fry obtained from artificial fertilization of eggs stripped from female brooders injected with pituitary gland (1.5 kg donor fish/1 kg brooders) and sperm obtained from uninjected males were stocked in the fine mesh hapas. Two days after hatching they were fed hard-boiled egg particle four times a day for 3 days. During day 4 to day 5 fish were trained to accept the commercial finely ground feed (30 % protein) which were solely fed to the fish as hormone-free feed from day 6 to initiation of MT treatment. After termination of the treatment they were fed a commercial floating pelleted ration (25 % protein) until the experiment was terminated.

Experimental groups

Series of 4 experiments were conducted in 3 replicates, in which fish were fed *ad libitum* with diet supplemented with 4 doses of 17 alpha-methyltestosterone; 0, 30, 60 and 120 mg/kg diet for the period of 30 or 60 days, beginning at day 7 or 14 days after hatching. After termination of hormonal treatment, the experimental fish were fed with the hormone-free diet until they were 150 days old.

Experimental groups were summarized as follow :

	period of MT treatment (days post-hatching)	dosage of MT (mg/kg diet)
Experiment 1	7-37	0, 30, 60 and 120
Experiment 2	14-44	0, 30, 60 and 120
Experiment 3	7-67	0, 30, 60 and 120
Experiemnt 4	14-74	0, 30, 60 and 120

Culture conditions

In each experiment the fry obtained from a single family were stocked in twelve of 100 liters aquaria at 30 fish/aquarium 3 days before the experiment began. They were fed *ad libitum* twice daily. The excess feed and fish excretion were siphoned after the second meal and 25 % of water was changed daily. One day after the termination of hormone treatment, the fish in each experiment were transfered to 12 of 1-m^3 cages suspended in a 200-m^2 pond.

The cages were cleaned every two weeks. Water was added to the pond every 2 days to maintain good water quality and water level. Water temperature was 28±2°C.

Diet preparation

Commercially available finely ground feed was used throughout the hormonal treatment period. MT was mixed with the feed by dissolving crystalline MT (Sigma) in equal volume (10 ml) of 50 % ethanol for every MT dosage. After thoroughly mixing, feed was air dried and kept in a refrigerator. The control MT free diet (0 mg/kg) was also mixed with 50 % ethanol at 10 ml/kg. During the hormone treatment period, feed was fed in a pasted form.

Measurements

A random sample of 80 % were individually weighed monthly. Survival rates were estimated at 150 days of rearing.

Histological study

At the day 150, random sample of 5 fish from each replicate were taken and pooled within each treatment (15 fish/treatment). The gonads of these fish were removed and fixed in the Bouins's solution. Sections (5 μ) were cut from paraffin embedded tissues and stained with haematoxylin and eosin. The effect of MT on gonadal development was evaluated on the basis of the germ cell type in relation to the appearance of the gonads in transverse section (Jensen and Shelton, 1983). An ovarian-type gonad contained spermatogenic tissue was classified as sex-reversed gonad. The gonads contained connective tissue stroma without germ cells were classified as undifferentiated or sterile gonads (Mirza and Shelton, 1988).

Data Analysis

The analysis of variance in completely randomized design was used to assess the survival and weight of experimental fish in each experiment. The means were compared within each experiment using Duncan's new multiple range test (Snedecor and Cochran, 1976). Sex ratios were tested for deviation from a 1:1 ratio by chi-square.

RESULTS

Effects of MT on growth and survival

The average body weight of the MT treated groups were not different (table 1). But when compared to the control (0 mg MT/kg diet), all MT treated fish (excepted the 30 mg MT/kg diet group) had inferior growth in the first month ($P<0.01$). The growth at 2, 3, 4 and 5 months did not differ from the control.

No statistical difference was observed in survival rates at 5 months in each experiment. The experiments initiated at 14-days post hatching showed higher survival rates (55.56-64.45 %) than the ones applied to 7 day-old fish (35.55-41.11 %) although no statistical comparison was made.

Histology of MT treated gonads

The testes of control fish were relatively round with a single stalk attached to peritoneum (Figure 1). They comprised numbers of lobules in which various stages of spermatogenic cells ; spermatocytes, spermatids and spermatozoa were found (Figure 2). Control females had ribbon-like ovaries attached to the peritoneum at two opposite ends (Figure 3). Previtellogenic oocytes predominated the ovaries. Few clusters of oogonia were also observed. Previtellogenic oocytes were recognized by dark stained cytoplasm and big round nucleus with distinct previtelline nucleoli.

In the groups of fish being treated for 30 days three types of gonads; testes, regressed ovaries and undifferentiated gonads were observed.

The testes of genetic males represented the control fish. The gonads of sex-reversed males were apparently distinguished from those of the genetic males by their flat, ribbon-like appearance and the presence of distinct area with no recognizable germ cells which was termed sterile area (Jonhstone *et al.*, 1978) (Figure 4, 5). The gonads contained germ cells of every spermatogenesis stage. However, the exact number of sex-reversed testes was not indicated because the shape of some testes could not be clearly identified.

A regressed ovary had filiform shape with a knob in the middle. Cross section of the filiform area (Figure 6) showed the typical anatomy of ovary with vascularized area and few unidentified cells. The cross section of the knob area (Figure 7) displayed oogonia and previtellogenic oocytes with

Table 1 Average weight (± SD) at 1-5 months and survival rate (± SD) at 5 months of experimental fish. Means in the same column of a particular experiment with different letters are significantly different ($P<0.05$).

Dosage	Average weight ± SD (gm.)					Survival (%)
	1 month	2 months	3 months	4 months	5 months	
Experiment 1 (7 day-old fry treated for 30 days)						
0	0.12±0.02 a	0.57±0.33 a	3.17±0.72 a	4.52±0.85 a	8.22±2.02 a	41.11±5.09 a
30	0.09±0.02 ab	0.43±0.23 a	2.68±0.14 a	5.52±1.54 a	11.02±2.13 a	40.00±8.82 a
60	0.05±0.01 b	0.31±0.15 a	3.10±0.78 a	5.08±0.98 a	13.98±2.71 a	41.11±11.70 a
120	0.05±0.01 b	0.47±0.21 a	2.57±0.41 a	4.51±1.48 a	8.70±2.92 a	36.66±5.77 a
Experiment 2 (14 day-old fry treated for 30 days)						
0	0.19±0.03 a	0.40±0.17 a	2.62±0.64 a	4.79±0.85 a	7.28±2.81 a	63.34±5.77 a
30	0.12±0.02 b	0.76±0.30 a	2.97±0.37 a	4.80±0.96 a	7.89±2.14 a	61.11±13.88 a
60	0.12±0.02 b	1.07±0.32 a	2.77±0.40 a	5.74±1.05 a	6.75±1.05 a	64.45±3.85 a
120	0.12±0.03 b	0.63±0.21 a	2.60±0.61 a	4.20±0.75 a	6.07±3.34 a	62.22±12.62 a
Experiment 3 (7 day-old fry treated for 60 days)						
0	0.12±0.02 a	0.26±0.14 a	0.48±0.23 a	1.51±0.17 a	2.43±0.79 a	35.55±6.94 a
30	0.08±0.01 ab	0.19±0.06 a	0.35±0.32 a	1.37±0.24 a	2.29±0.80 a	38.89±6.94 a
60	0.06±0.01 ab	0.24±0.12 a	0.49±0.19 a	1.23±0.29 a	2.31±0.76 a	36.67±12.00 a
120	0.03±0.01 b	0.22±0.09 a	0.39±0.20 a	1.23±0.46 a	2.34±0.52 a	38.89±10.18 a
Experiment 4 (14 day-old fry treated for 60 days)						
0	0.34±0.16 a	0.73±0.41 a	1.31±0.76 a	2.74±0.67 a	4.21±0.96 a	56.67±6.67 a
30	0.24±0.06 ab	0.55±0.26 a	1.00±0.56 a	2.06±0.36 a	3.06±1.25 a	55.56±10.18 a
60	0.17±0.06 b	0.54±0.33 a	0.83±0.82 a	1.87±0.33 a	3.46±1.50 a	63.33±8.82 a
120	0.15±0.08 b	0.85±0.30 a	1.01±0.38 a	2.40±0.73 a	3.65±0.91 a	64.45±13.47 a

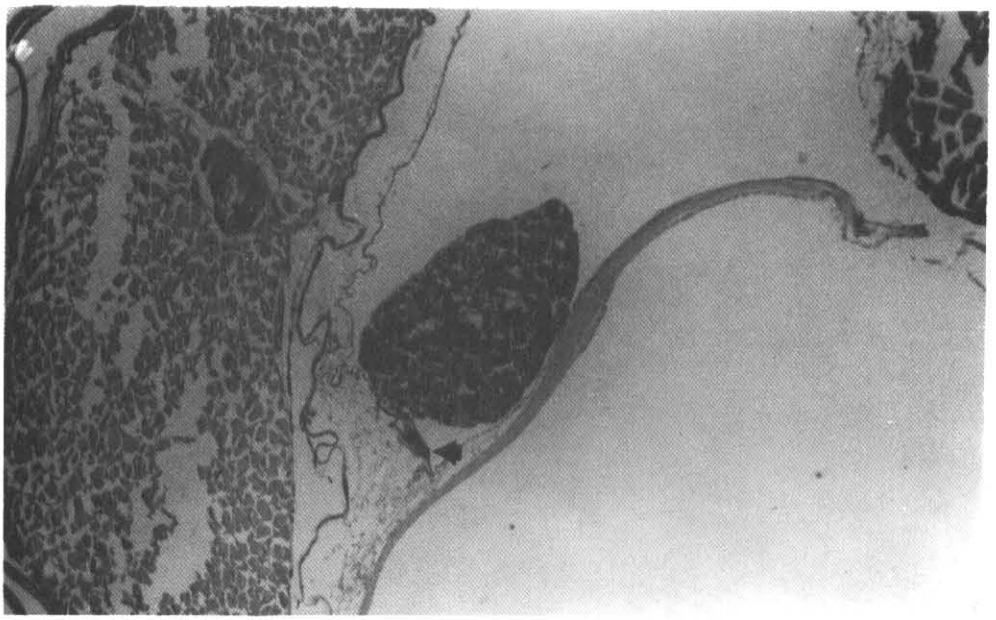


Figure 1 Testis of a control fish with a stalk (arrow) attached to peritoneum.

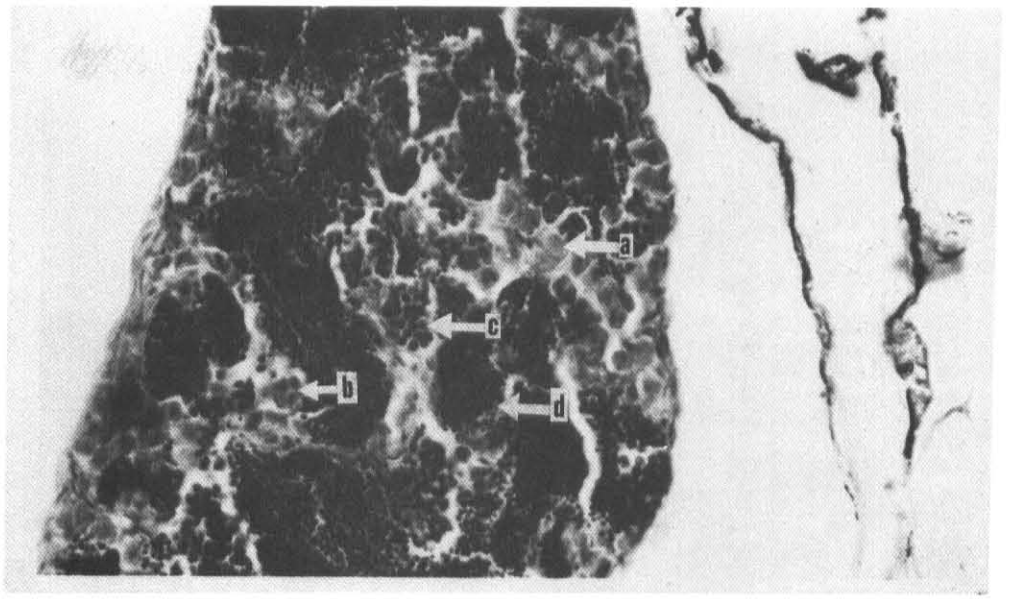


Figure 2 Enlarged view of Fig. 1 showing primary spermatocytes (a), secondary spermatocytes (b), spermatids (c) and spermatozoa (d).

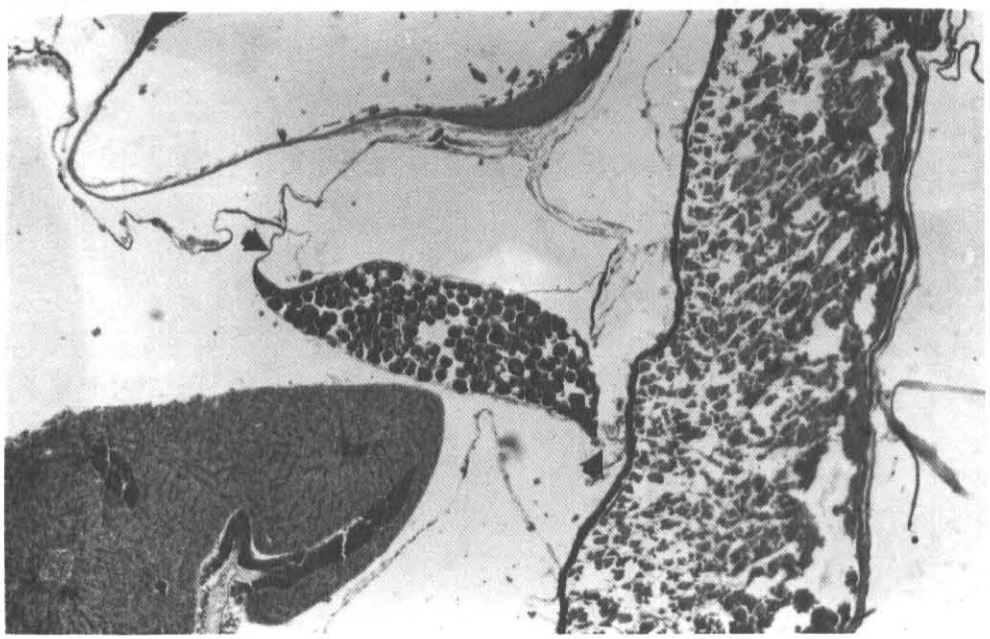


Figure 3 Control ovary attached at the two opposite ends (arrow) to peritoneum.

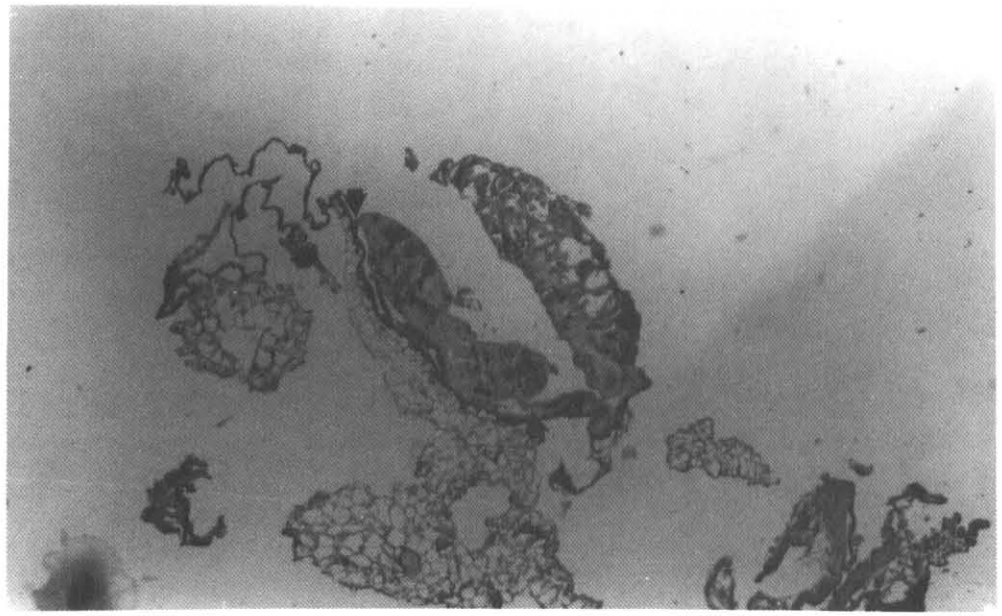


Figure 4 Sex reversed testis which was ribbon-band like, attached to peritoneum at the two opposite ends (arrow).

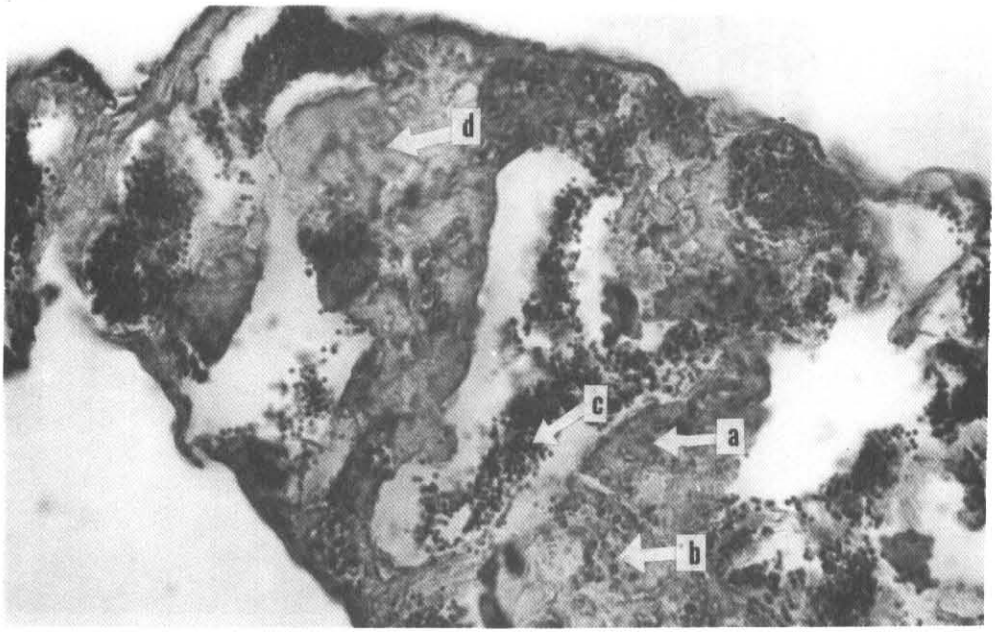


Figure 5 Enlarged view of Fig. 4, showing spermatocytes (a), spermatids (b), spermatozoa (c) and sterile area (d).

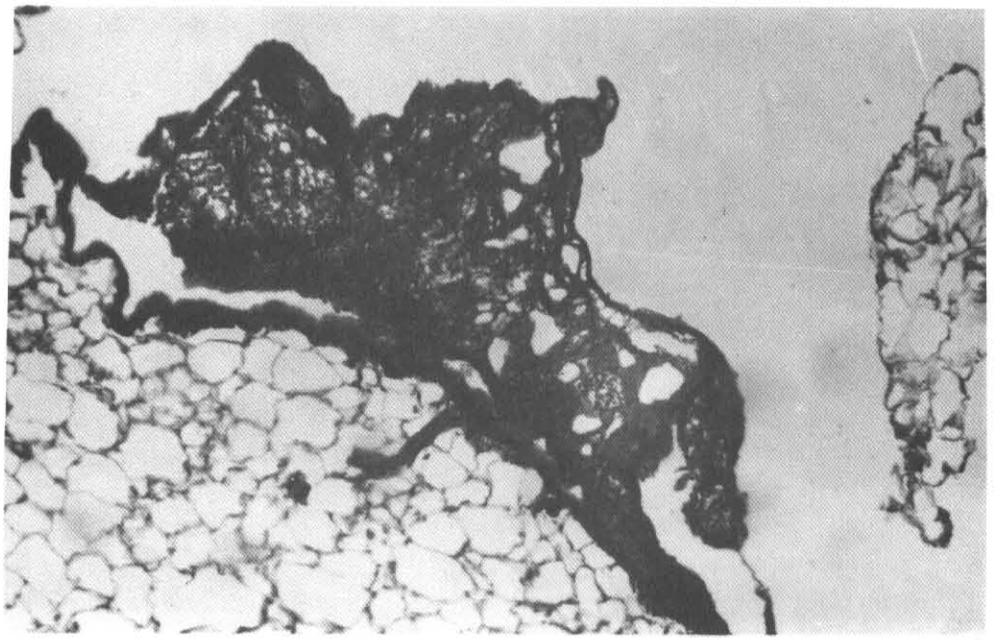


Figure 6 Filiform area of a regressed ovary showing typical anatomy of ovary and vascularized area.

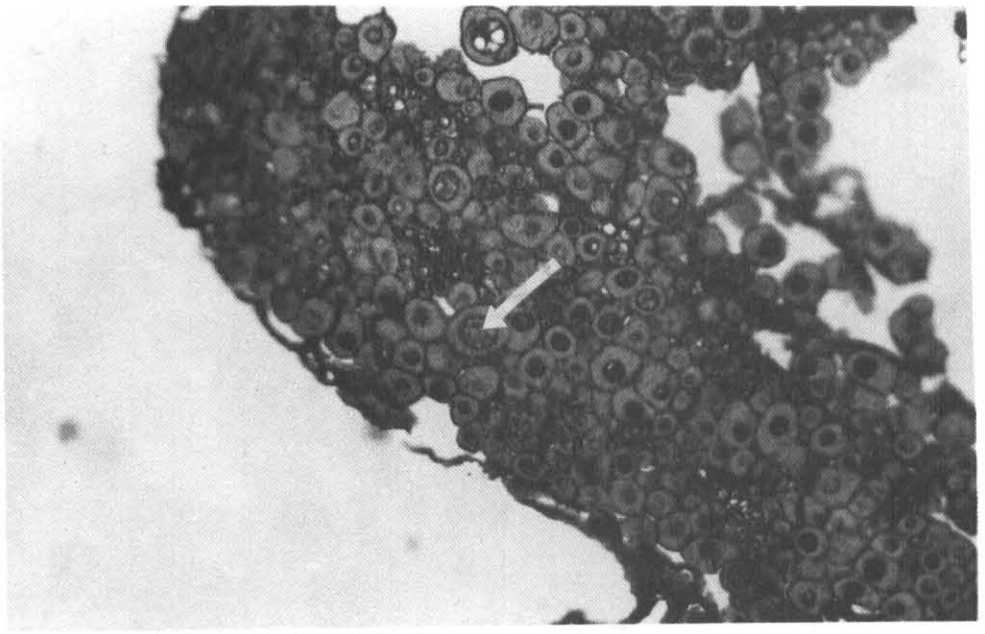


Figure 7 Knob found in a regressed ovary showing regressed nucleus of previtellogenic oocyte.

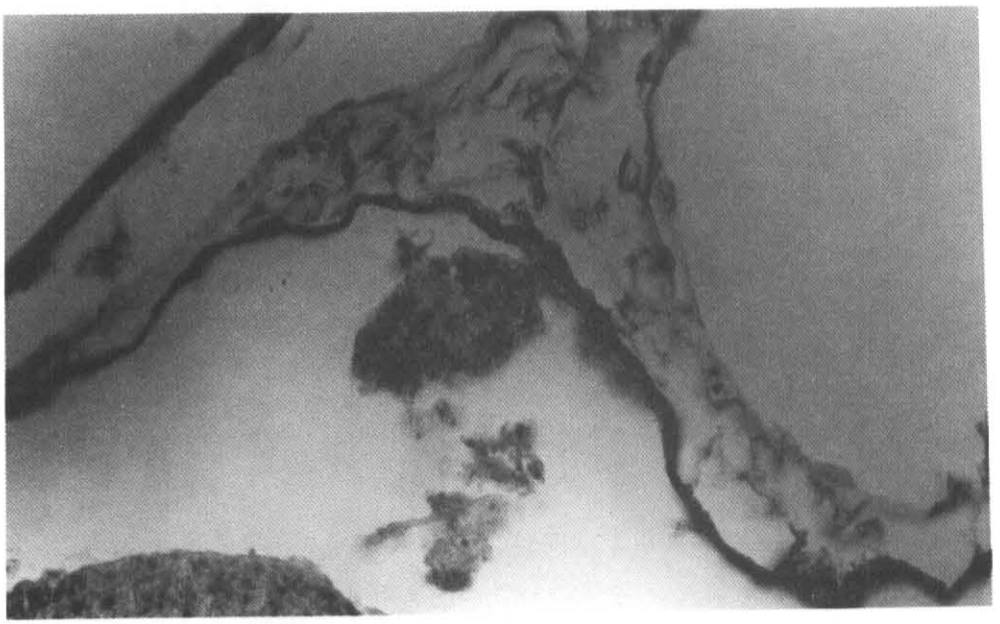


Figure 8 Undifferentiated gonad (testis).

pale cytoplasm and regressed nucleus.

The cross section of undifferentiated gonads showed the typical anatomy of either testes (Figure 8) or ovaries without recognizable germ cells.

In the groups treated with MT for 60 days, two types of gonads were observed ; genetic male testes and undifferentiated gonads. No sex-reversed testes and ovaries were found.

Effects of MT on the proportion of sexual types

The chi-square test revealed that the sex ratio of control groups did not deviate from the ratio of 1:1. The sex ratio of the groups treated for 30 days significantly deviated from 1:1 ratio while the ones treated for 60 days showed no significant chi-square value due to small number of distinct sexual type (Table 2).

The thirty-days-administration of MT at the dosage of 30, 60 or 120 mg/kg diet to fry of 7 days old resulted in 66.7, 73.3 and 80.0 % of males respectively. Besides, 26.7 % sterile gonads, a regressed ovary (6.7 %) were observed in the group treated with 30 mg MT/kg diet. The sterile gonads

Table 2 Sex ratio and percentage of sexual types (total number of 15 in each treatment). Percentage of male follow by asterisk means atleast one sex reversed testes were apparently observed. Chi square values followed by asterisks mean the sex ratio was significantly different from 1:1 (< 0.01).

Dosage (mg/kg)	Male (%)	Female (%)	Undifferentiated (%)	Sex ratio (male:female)	Chi square value
Experiment 1 (7 day-old fry treated for 30 days)					
0	60.0	40.0	0.0	1:0.7	0.60
30	66.7 *	6.7	26.7	1:0.1	7.36**
60	73.3 *	0	26.7	1:0	11.00**
120	80.0 *	0	20.0	1:0	12.00**
Experiment 2 (14 day-old fry treated for 30 days)					
0	46.7	53.3	0	1:1.14	0.06
30	66.7 *	0	33.3	1:0	10.00**
60	80.0 *	0	20.0	1:0	12.00**
120	80.0 *	0	13.3	1:0	13.00**
Experiment 3 (7 day-old fry treated for 60 days)					
0	53.3	46.7	0	1:0.9	0.06
30	26.7	0	73.3	1:0	4.00
60	13.3	0	86.7	1:0	2.00
120	6.7	0	93.3	1:0	1.00
Experiment 4 (14 day-old fry treated for 60 days)					
0	46.7	53.3	0	1:1.1	0.06
30	6.7	0	93.3	1:0	1.00
60	20.0	0	80.0	1:0	3.00
120	0	0	100	-	-

were also found at 26.7 and 20.0 % in the groups treated with 60 and 120 mg MT/kg diet, respectively.

In the experiment 3 and 4, in which MT of the same dose level were administered for 60 days beginning at 7 and 14 days post- hatching, no sex-reversed testes or ovaries were found. Most of the fish inspected had undifferentiated gonads (Table 2).

DISCUSSION

MT applied at 30 mg/kg did not affect the growth of tawes while the dosage of 60 or 120 mg/kg reduced the growth during the first month of administration. The low dosage of MT was reported to accellerate growth while the high dosage depressed growth (Dunham, 1990). In gold fish, growth depression was observed in the fish treated with MT at concentration higher than 10 mg/kg while at low concentration (1 mg/kg) growth enhancement was achieved (Yamazaki, 1976). The growth of common carp received 1.0-5.0 mg/kg MT was accellerated, while the treatment of 10 mg/kg MT resulted in reduced growth (Lone and Matty, 1980).

Growth retardation may be a result of catabolic action of the hormone and decrease in appetite of the fish (Lone and Matty, 1980). Yamazaki (1976) reported that different growth rate of each group of MT treated gold fish closely paralleled the amount of food consumed.

Administration of MT did not affect the survival rate of tawes. Similar results were reported in common carp (Nagy *et al.*,1981). A contradictory result was reported in *Clarias macrocephalus* where high mortality was observed in the MT-treated group (Na-Nakorn, unpublished data). In channel catfish, *Ictalurus punctatus*, Simone (1990) reported the degeneration of the renal tubules and many of the renal corpuscles of MT treated fish which might lead to mortality.

In these experiments, although sex ratios of the treated fish might not be conclusive evidence that sex-reversal has occured due to the small number of fish being inspected, sex reversal has been histologically shown by the presence of sex-reversed testes.

Treatments with MT beginning at 14 days post-hatch with the duration of 30 days could completely reverse sex of tawes. The same treatments applied to 7-day-old fish resulted in the incomplete sex reversal as indicated by the presence of a regressed ovary in the groups treated with 30 mg MT/kg diet. MT administered to fish at 7 days post-hatch might be too early, hence prevented the period of 30 days to encompass gonadal differentiation which was found to occur in the 21-day-old tawes (Na-Nakorn, unpublished data). The treatments started at 14 days post-hatch seemed to be suitable. The so-call sensitive period, during which sex reversal can be induced, could not be indicated in these experiments. It was shown to be approximately 8-98 days after hatching in common carp (Nagy *et al.*, 1981).

However, the sizes of fish being treated is also important because size determines the stages of gonadal differentiation. MT treatment according to size was found to be effective in common carp in which 100 % sex-reversed males (genetic females) were achieved from the treatment initiated with fry of 7, 13 or 19 mm and ended when they reached the length of 21, 39 or 57 mm, respectively. (Nagy *et al.*, 1981). Variability of sizes which indicated the individual rate of gonadal morphogenesis might accounted for variable results observed in the fish received the same treatments.

The effects of long treatment duration on sterility, which indicated by the number of undifferentiated gonads in the fish being treated for 60 days was apparently shown in these experi-

ments. A dose of MT administered for longer period resulted in more undifferentiated gonads. Recent investigation on the 1-year-old, 60-days-MT-treated tawes indicated more than 80 % of sterile fish characterized by a swelling and transparent gonad filled with clear fluid. When the fluid was used to fertilize freshly stripped eggs of the same species, no fertilization was observed (Na-Nakorn, unpublished data).

It is difficult to indicate that high dosage of MT or long term of treatment caused sterility in 30-days-groups. The duration of 30 days might not be too long since our preliminary study revealed that cytological sex differentiation of tawes was first detected at 63 days post-hatch (Na-Nakorn, unpublished data). The treatment started at 14 days after hatching and continued for 30 days did not encompass sex differentiation period. Thus, the duration of 30 days might be a minimal treatment period for efficient sex-reversal of tawes. High dose of MT and long treatment duration were frequently applied simultaneously and resulted in high percentage of sterile fish. Rainbow trout, in which low level of MT was needed for successful sex reversal, 50 mg MT/kg treated for 5 months caused the depression of gonadal differentiation (Yamazaki, 1976). In common carp, the administration of 220 mg MT/kg for 131 days caused sterilization.

The dosage of 30-120 mg/kg might be too high, thus caused sterilization of treated tawes. In the groups of tawes treated for 30 days the number of undifferentiated gonads did not vary with dosage. But in the 60 days-groups, the number of undifferentiated gonads increased with the increasing level of MT appied to the fish. This may indicate the more significant influence of treatment duration than that of dosage level.

However, conclusion concerning this matter should not be made by direct comparing the dosage being used in these experiments with those previosly reported. The species difference as well as difference in culture conditions of experiment especially temperature certaily influence the results (Nagy *et al.*, 1981; Yamazaki, 1976; Dunham, 1990)

Despite of sterility observed in experiment 2, MT treated for 30 days and initiated at 14 days after hatching yielded desirable amount of sex-reversed males. Lower dosage of MT should be studied in order to prevent sterility. Sizes of fish being treated must be considered together with age to ensure the optimum timing and duration of treatment. Functional sex-reversed male was expected since spermatozoa were histologically detected.

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LITERATURE CITED

Bye, V.J. and R.F. Lincoln. 1986. Commercial methods for the control of sexual maturation in rainbow trout (*Salmo gairdneri* R.). Aquaculture 57 : 299-310.

Calhoun, W.E. and W.L. Shelton. 1983. Sex ratios of progeny from mass spawnings of sex-reversed broodstock of *Tilapia nilotica*. Aquaculture 33 : 365-372.

Department of Fisheries. 1993. Freshwater fish farm production 1991. Fisheries Policy and Planning Division, Bangkok. 90 p.

Dunham, R.A. 1990. Production and use of monosex or sterile fishes in aquaculture. Reviews in Aquatic Sciences 2 : 1-17.

Hunter, G.A., E.M. Donaldson, J. Stoss, and J. Baker. 1983. Production of monosex female groups of chinook salmon (*Oncorhynchus*

tshawytscha) by the fertilization of normal ova with sperm from sex reversed females. Aquaculture 33 : 355-364.

Jensen, G.L. and W.L. Shelton. 1983. Gonadal differentiation in relation to sex control of grass carp, *Ctenopharyngodon idella* (Pises : Cyprinidae). Copeia 3 : 749-755.

Johnstone, R., T.H. Simpson, and A.F. Youngson. 1978. Sex reversal on salmonid culture. Aquaculture 13 : 115-134.

Lone, K.P. and A.J. Matty. 1980. The effect of feeding methyltestosterone on the growth and body composition of common carp (*Cyprinus carpio* L.). General and Comparative Endocrinology 40 : 407-424.

Mirza, J.A. and W.L. Shelton. 1988. Induction of gynogenesis and sex reversal in silver carp. Aquaculture 68 : 1-14.

Nagy, A., M. Bercsenyi, and V. Csanyi. 1981. Sex reversal in carp *Cyprinus carpio* by oral administration of methyltestosterone. Canadian Journal of Fisheries and Aquatic Science 38 : 725-728.

Shelton, W.L. 1986 a. Broodstock development for monosex production of grass carp. Aquaculture 57 : 311-319.

Shelton, W.L. 1986 b. Control of sex in cyprinids for aquaculture. Page. 179-193. In Billard, R. and J. Marcel. Aquaculture of Cyprinids, INRA, Paris.

Simone, D.A. 1990. The effects of the synthetic steroid 17-alpha-methyltestosterone on the growth and organ morphology of the channel catfish (*Ictalurus punctatus*). Aquaculture 84 : 81-93.

Snedecor, G.W. and W.G. Cochran. 1976. Statistical Methods. (6th ed). Iowa State Univ., Ames, Iowa.

Yamamoto, T. 1969. Sex differentiation. pp. 117-175. *In* Hoar, W.S. and D.J. Randall (Editors). Fish Physiology Vol III, Academic Press, New York.

Yamamoto, T. and T. Kajishima. 1968. Sex hormone induction of sex reversal in the goldfish and evidence for male heterogamety. The Journal of Experimental Zoology 168 : 215-222.

Yamazaki, F. 1976. Application of hormones in fish culture. Journal of the Fisheries Research Board of Canada 33 : 948-958.