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Uptake and depletion of plasma 17α-methyltestosterone during induction of masculinization in muskellunge, *Esox masquinongy*: Effect on plasma steroids and sex reversal

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Abstract

Oral administration of 17α-methyltestosterone (MT) was used to induce masculinization of sexually undifferentiated muskellunge, *Esox masquinongy*. Three groups of muskellunge (mean weight, 2.5 ± 0.6 g) were submitted to MT treatment (15 mg of MT/kg) for 60 days. An additional one group was used as a control (hormone-free diet). Food was distributed over a 10-h period by using automatic belt feeders. Blood was sampled in both control and treated fish at different intervals during and after feeding: before (0 h), at 3 h, 6 h, and cessation of feeding (10 h), and after a fast of 22 h (32 h). MT had no significant effect on growth and survival in muskellunge 6 months after the treatment. Concentrations of plasma MT increased during the feeding period and reached their maximum levels 6 or 10 h after starting feeding. This rapid increase of MT indicated a rapid absorption of this steroid. Plasma MT levels then declined and reached a radir by 22 h after cessation of feeding, suggesting that MT is rapidly metabolized and excreted. The profiles of plasma testosterone during the MT treatment did not differ significantly between control and MT-treated groups. During and after the MT treatment, the concentration of plasma testosterone did not differ significantly between control and MT-treated groups. Moreover, no sexual dimorphism of testosterone levels was observed. Six months after treatment, the sex ratio in MT-treated groups (33% males, 62% females, and 5% intersex) was opposite to control (70% and 30%, respectively) and differed significantly. This suggests that at 15 mg of MT/kg over 60 days, a paradoxical feminization took place.

1. Introduction

Muskellunge, *Esox masquinongy*, is a very popular sport fish in central North America. The sport fishery for this species in many states relies on annual artificial propagation and stocking of fingerlings to maintain the population because of the failure of natural reproduction in reservoirs [1]. Like many economically important cool-water species, muskellunge exhibit sexual dimorphic growth where females grow significantly faster and larger than males. Thus, the development of methods to produce all-female populations of muskellunge will lead toward the production of trophy fish for sport fisherman and will attract interest for commercial aquaculture.

The synthetic steroid 17α-methyltestosterone (MT) is a male-specific hormone commonly used to induce sex reversal in teleost fish [2]. However, the effect of MT is dependent on various factors such as dose, timing and duration of treatment, and mode of administration [3]. MT was successfully used to masculinize genetic and gynogenetic females into phenotypic males [4-9]. Thus, if the female muskellunge is homogametic (XX), such sex reversal would provide homogametic males (neomales) whose offspring would be 100% females.

The uptake and the depletion of MT have been reported in several species such as coho salmon *Oncorhynchus kisutch* [10], rainbow trout *Oncorhynchus mykiss* [11-13], and cichlids *Oreochromis mossambicus* [11], *Oreochromis aureus* [14,15], and *Oreochromis niloticus* [16]. Based on the results of experiments with radiolabeled hormone, it was inferred that MT is rapidly metabolized, and excreted. To our knowledge, there are no published reports on direct measurement of MT in fish blood plasma. The primary aim of the present investigation was to provide such data by determining the uptake and the depletion of MT in blood plasma of muskellunge fed MT-enriched food.

Androgens have been reported to be involved in sex determination in salmonid fish [17, 18]. However, dietary treatment with MT can inhibit in vitro testosterone production in rainbow trout [18]. Consequently, we also examined changes in blood plasma testosterone during and after the MT treatment. Moreover, we described the effects of MT treatment on the growth, survival, and sex ratio of muskellunge.

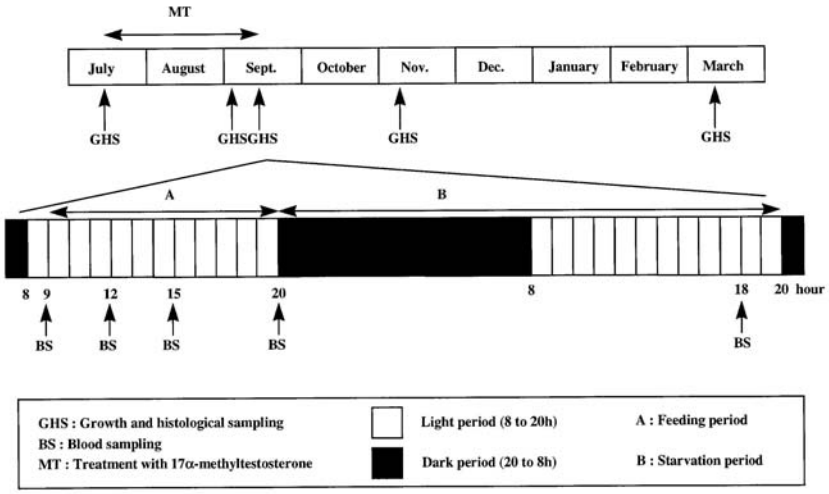

Fig. 1. Sampling procedure.

2. Materials and methods

Juveniles of mixed-sex muskellunge (n = 800; mean weight, 2.5 ± 0.6 g; mean length, 98 ± 7.5 mm), reared at Ohio's Kincaid State Fish Hatchery, were moved to the Columbus aquaculture laboratory. Fish were fed initially with freshly hatched *Artemia salina* nauplii and then weaned to artificial diet (Zeigler Brothers, Gardners, PA, USA). Fish were assigned randomly into four tanks (200 fish per tank); i.e. one tank was used as a control (hormone-free diet) and three tanks were fed MT-enriched food for 60 days. Each circular tank was supplied with continuously aerated running freshwater (60 l/h). Fish were maintained in ambient water temperature (6-21°C) and under a constant light regime of 12-h light: 12-h dark.

MT (Sigma, St. Louis, MO, USA) was dissolved in ethyl alcohol and incorporated into a commercial dry diet (New York diet 4, Zeigler Brothers) at a dose level of 0 (control diet) or 15 mg of MT/kg of food. The ethyl alcohol was allowed to completely evaporate at room temperature and the diets were stored at -4°C. The food was distributed by using automatic belt feeders over a 10-h period at a rate of 10% total tank biomass during the first 10 days and 5% thereafter. After the MT treatment (Day 60), rations were adjusted daily depending on the amount of food remaining on the tank bottom the next morning. Feeding rate averaged 5% to 10% biomass daily. Tanks were cleaned and the food residues discarded daily.

To monitor the effects of hormonal treatment on sexual differentiation, representative fish samples were obtained from each tank at different periods (Fig. 1). Fish were anesthetized with MS-222 (50 mg/l), individually weighed (± 0.1 g), and measured (total length to 1 mm). The head, tail, and viscera from each fish were removed before fixation in Bouin's solution. The vertebrae and a portion of the muscle tissue were removed before dehydration and embedding in paraffin. Cross-sections were cut at 7 μm, stained with Mayer's hematoxylin and eosin, examined, and documented by using an Olympus photographer microscope (Olympus, New Hyde Park, NY, USA).

To determine the uptake and kinetics of MT in the fish, blood samples were collected in control and treated fish at the following different intervals of feeding: on Day 55 before (0 h) and at the end of

feeding (10 h), on Day 56 after 3 h and 6 h of feeding, and on Day 57 after a fast of 22 h (32 h) (Fig. 1). Testosterone plasma levels were determined for the samples used for MT (during MT treatment) and on Day 250 (6 months after cessation of MT treatment). Blood samples were taken from the caudal vessel by using a heparinized needle and syringe. The samples were kept on crushed ice until centrifugation at 1500 × *g* for 10 min, after which plasma was removed, frozen, and stored at -20°C until radioimmunoassay (RIA). Plasma levels of MT were measured by using a commercially available RIA kit (Laboratoire d'Hormonologie, Marloie, Belgium) and the plasma levels of testosterone (T) were determined by RIA according to methods similar to those of Ottobre et al. [19]. In both cases, the steroids were previously extracted with ethyl ether. Standard and radiolabeled T were obtained from ICN Pharmaceutical (Costa Mesa, CA, USA) and NEN (Boston, MA, USA), respectively. The T antiserum was provided by the Institute of Animal Physiology (University of Agriculture and Technology, Olsztyn, Poland) and the characteristics of this antiserum have been reported previously by Dabrowski et al. [20]. Both assays were validated for use with muskellunge plasma (Table 1).

Table 1
Radioimmunoassay characteristics of steroid hormones in muskellunge

Characteristics	T	MT
Within-assay coefficient of variation (%) (n = 6)	0.54	3.50
Between-assay coefficient of variation (%) (n = 3)	2.04	10.0
Accuracy (coefficient of regression)	0.986	0.985
Sensitivity (pg/ml)	1	2
Recovery of extraction (%)	97	89
Parallelism	Serial dilutions of plasma samples showed parallelism with the standard curve between 25 and 100 μl	

T, testosterone; MT, 17α-methyltestosterone.

The MT antiserum cross-reacted with testosterone (12.6%), with dihydrotestosterone (2.8%), and also with some metabolites of MT identified by Cravedi et al. [13] such as 17α-methyl-5β-androstan-17β-ol-3-one (48%) and 17α-methyl-5α-androstan-3-17β-diol (12.6%). According to the RIA for MT, the concentration of MT in the plasma of fish not supplemented with MT was negligible. As such, cross-reactivities with circulating androgens is thought to have little impact on our estimates of MT concentrations.

The cross-reactivity of T antiserum at 50% binding was determined to be 6.7% with MT. Because levels of MT in MT-treated fish are often quite high, this level of cross-reactivity could confound estimates of plasma testosterone. Thus, it was necessary to correct the values of T obtained for the concentrations of MT present in each sample. Separate standard curves were developed by using the T antiserum and increasing doses of T and MT. The equations representing these standard curves were then determined.

T: $y = 3.335 - [0.9774 \times \log(T)]$

MT: $y = 4.5789 - [0.8041 \times \log(MT)]$

These equations were solved simultaneously by subtraction in the following manner:

$$3.335 - [0.9774 \times log(T)] = 4.5789 - [0.8041 \times log(MT)]$$

$$log(T) = (-1.2439 + [0.8041 \times log(MT)])/0.9774$$

$$T = antilog\{(-1.2439 + [0.8041 \times log(MT)])/0.9774\}$$

By substituting the previously determined concentration of MT for each sample into this equation, it was possible to determine the amount that MT contributed to each estimate of T. This amount was then subtracted from each estimate of T to obtain the final value. A similar strategy for accounting for cross-reactivity of a prostaglandin RIA was used by Silvia et al. [21].

Results are expressed as mean ± SD or SE values. Weight, length, MT, and T concentrations were first analyzed by one-way analysis of variance for changes that might have occurred during the experimental period. Then, each parameter was compared simultaneously among all treatments by using two-way analysis of variance to test for treatment effects. The x^2 test was used for analysis of alterations in sex ratios. Intersex fish were combined with females in the groups treated with 17α-methyltestosterone so that only fish that were completely sex-reversed were recognized in the x^2 test as having been affected by the treatment. Homogeneity of variance was confirmed for all data before analysis.

3. Results

3.1 Growth and survival

On Day 44 (September), we found that one MT-treated group (MT-2) grew more slowly ($P < 0.01$) than the other groups (control and MT-1 and MT-3) (Fig. 2). Higher mortality than in other groups was also recorded in MT-2 (17% vs. 11%). The cause of this differences in survival was not identified. Fifty-five days after the treatment ended (Day 115, November), the weights and lengths of the fish from the MT-treated groups did not differ significantly ($P > 0.05$) from those of the control group (Fig. 2). However, a significant difference ($P < 0.01$) was observed between two MT-treated groups (MT-1 and MT-2). These differences disappeared 6 months after cessation of treatment (Day 250, March), when no significant differences ($P > 0.05$) in size between the different groups were found (Fig. 2). At the end of the experiment (Day 250, March), specific growth rate was similar in all groups averaging 1.03 ± 0.02%/day (Table 2). After some mortality before Day 44 in Group MT-2, survival of muskellunge was high in all groups.

3.2 Uptake and depletion of MT

In two MT-treated groups (MT-1 and MT-2), concentrations of MT in blood plasma increased during the feeding and reached their maximum levels 10 h after onset of feeding (Fig. 3). At 32 h after the onset of feeding (22 h after cessation of feeding), concentrations of MT in those two groups declined significantly ($P < 0.01$) and were similar to those observed before feeding. In the third MT-treated group (MT-3), the profile of blood plasma MT was different and the maximum level of MT was observed 6 h after feeding. The presence of MT in the plasma of the control group (< 1 ng/ml) may be explained by the cross-reactivity of the MT antibody with T (12.6%) and dihydrotestosterone (2.8%) (Fig. 4).

3.3 Sex ratio

Fish from the three MT-treated groups were combined for the analysis of sex ratio, because no significant difference was observed between the three tanks treated with MT. Histological examinations of gonad cross-sections before MT treatment revealed that 65% of fish had undifferentiated gonads and 35% were females (Table 3; Fig. 5A and B). During the treatment (Day 44), the sex ratio in control and MT-treated groups was close to 1:1. In the MT-treated group, however, the male gonads consisted

mainly of vacuolized connective tissue (Fig. 5C). Fifty-five days after treatment (Day 115, November), the percentage of females in the MT-treated group declined whereas the percentage of intersex fish (with presence of testicular and ovarian tissues) increased (Fig. 5D and F). Six months after the treatment (Day 250, March), the percentages of females and males in the control group were similar to those observed before the treatment, assuming that all undifferentiated gonads become testes in fish of this size ([32]; Table 3; Fig. 5A and E). The sex ratio of the MT-treated group (62:33, females/males) was opposite to the control group (30:70) and this difference was significant.

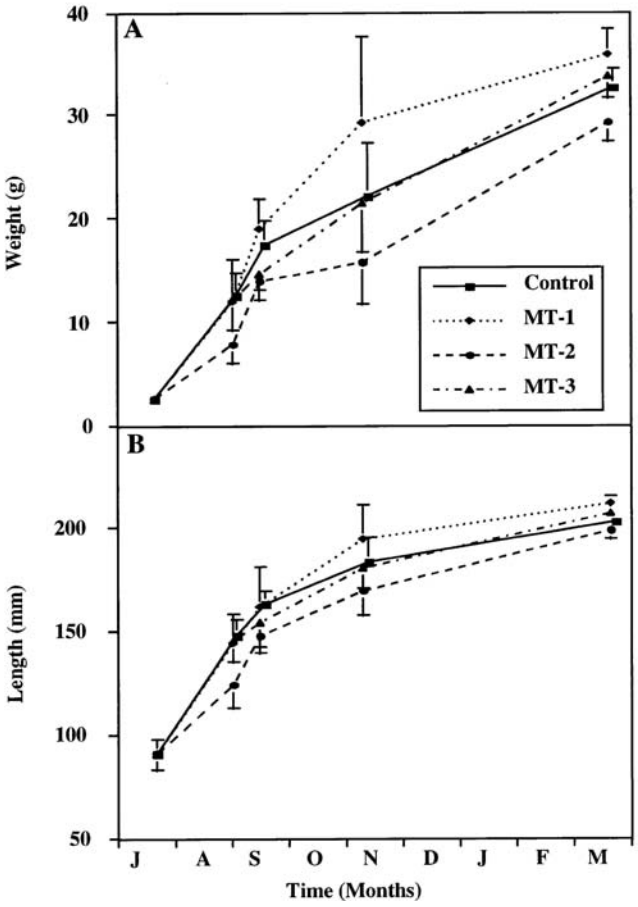

Fig. 2. Effects of oral administration of 17α-methyltestosterone (MT) for 60 days on the weight (A) and length (B) of muskellunge in control and MT-treated groups. Data are presented as mean ± SD values (*n* = at least 10 per time point). Error bars ≤ the data point symbols are not shown.

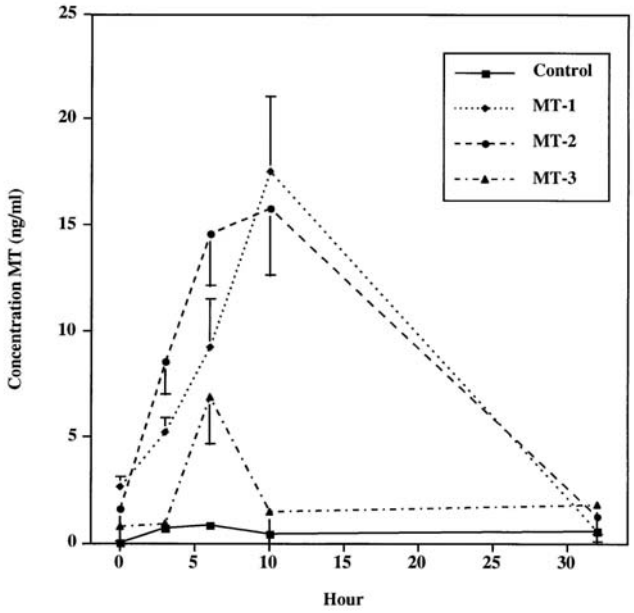

Fig. 3. Changes in blood plasma 17α-methyltestosterone (MT) in control and MT-treated groups. Data are presented as mean ± SEM values (n = 4 or 5 per time point, for treated and control groups, respectively) and are standardized to the onset of feeding MT (time 0; i.e. 9 h on Fig. 1). Error bars ≤ the data point symbols are not shown.

3.4. Blood plasma testosterone levels

The profiles of plasma T during the MT treatment (Days 55-57, September) did not differ significantly between control and MT-treated groups ($P > 0.05$) (Fig. 4). Although between Days 55 and 57 the concentrations of plasma T were twice as high as in MT-treated groups in comparison with the control group (Table 4), great variation among individual fish resulted in no significant difference. Six months after the MT treatment (Day 250, March), the levels of plasma T were significantly ($P < 0.05$) lower than those between Days 55 and 77 (Table 4).

Table 2
Effect of oral administration of MT for 60 days on specific growth rate (SGR) and survival rate of muskellunge

Parameters	Treatment			
	Control	MT-1	MT-2	MT-3
SGR[a] (%/day)	1.03	1.06	0.98	1.04
Survival rate (%)	95	97	77	85.5

MT, 17α-methyltestosterone.
[a] SGR = {[(ln final weight − ln initial weight)/days] × 100}.

Table 3
Effect of oral administration of MT for 60 days on sex ratio of muskellunge (% of analyzed fish)

Dates	Treatment	Undifferentiated	Male	Female	Intersex
July (Day 0)	Prior (n = 20)	65	0	35	0
September (Day 44)	Control (n = 18)	0	50	50	0
	MT-treated (n = 41)	0	51	46	3
November (Day 115)	Control (n = 10)	0	60	40	0
	MT-treated (n = 14)	0	57	14	29
March (Day 250)	Control (n = 20)	0	70	30	0
	MT-treated (n = 21)	0	33*	62*	5

MT, 17α-methyltestosterone.
* Significantly different from the control sex ratio observed at the beginning of the experiment ($P < 0.05$).

4. Discussion

Differences in growth and 17α-methyltestosterone plasma concentrations were observed between three MT-treated replicates (Figs. 2 and 3). Those variations might be explained by the feeding behavior and territoriality of the muskellunge. This species is an active predator, and therefore, individual variability in the acceptability of the dry diet is an expected result. The use of belt feeders provides a continuous food supply for the muskellunge during the feeding period, which is necessary because muskellunge will not pick up pellets on the bottom of the tank. As reported by Casselman [22], Northern pike (*Esox lucius*), another esocid fish fed ad libitum with minnows, also have shown individual variation in feeding rate. Moreover, a direct correlation existed between the rate of food consumption and swimming activity. The establishment of hierarchies in feeding among the fish and the number of dominant muskellunge within each tank might also reflect the variability of dry food intake among the three MT-treated groups. These factors could not be controlled and reflect specificity of the species.

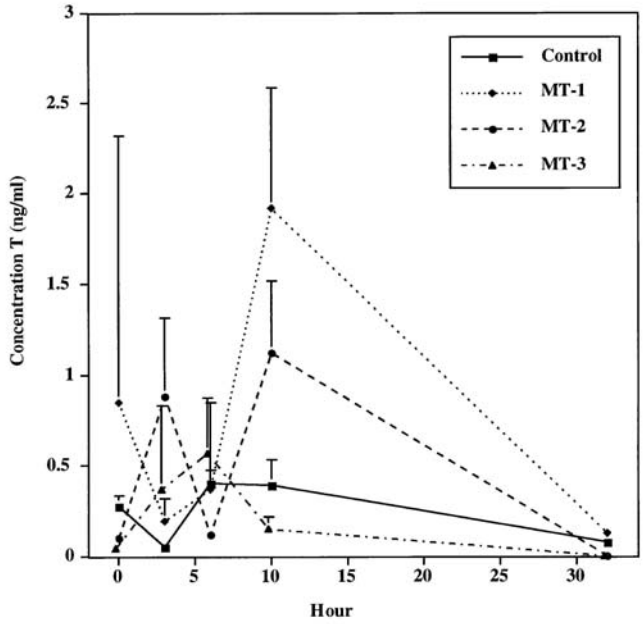

Fig. 4. Changes in blood plasma testosterone (T) in control and 17α-methyltestosterone (MT)-treated groups. Data are presented as mean ± SEM values (n = 4 or 5 per time point, for treated and control groups, respectively) and are standardized to the onset of feeding MT (time 0; i.e. 9 h on Fig. 1). Error bars ≤ the data point symbols are not shown.

The anabolic effects of MT on growth have been reported in several teleost fish such as common carp *Cyprinus carpio* [23,24], coho salmon [10], rainbow trout [25], and cichlids [26,27]. However, the magnitude of the response to MT depends on many factors including age, size, developmental stage, temperature, salinity, dietary factors, treatment duration, season, and method of application [28]. Kuwaye et al. [26] also emphasized that, in some cases, it has remains unclear as to whether MT affects growth directly or through its ability to reverse sex. However, our study clearly showed that, in muskellunge, oral administration of MT at a dose of 15 mg/kg of food did not significantly increase the growth during or after the MT treatment. Specific growth rates of control and hormone-treated groups were similar (Table 2) and comparable with those obtained by Brecka et al. [29] where muskellunge was fed semipurified diets containing 37% or 40% protein (specific growth rate, 0.98% and 1.18%/day, respectively). Although treatment with a synthetic steroid induces, in general, higher mortality than in control [30], the survival rate of muskellunge was not affected by the MT treatment.

The changes of blood plasma MT in muskellunge are consistent with the findings in salmonids, ictalurids, and cichlids fishes [10-12,14-16]. The appearance of MT in the circulation of muskellunge within 3 to 6 h indicates a rapid absorption of the steroid. Diminished intake may accelerate peak of MT in blood (Fig. 2). Then, plasma MT levels declined and reached a nadir by 22 h after cessation of feeding. In blue tilapia fed a single meal of diet containing 30 μg of radiolabeled MT, Goudie et al. [15] observed that the radioactivity was the highest in all tissues 6 to 12 h after the feeding. Radioactivity declined nearly 90% by 4 days and only 0.5% of original radioactivity remained after 21 days. In coho-salmon-fed [^{3}H]MT, maximum uptake in blood and nonexcretory tissues was reached between 2 and 8 h after feeding. Then, after 10 days of feeding the hormone-free diet, the concentration of radioactive substances was reduced to < 2 ng/g in all tissues [10]. Thus, it appeared that the direct measurement of MT allowed us to show that this androgen is rapidly metabolized in muskellunge. The excretion of MT occurred primarily as free metabolites in water and as glucuronide conjugates in feces [12]. Moreover, the minor importance of urinary excretion in fish may be related to preferential elimination across the gill and to the significant role played by the bile in steroid metabolism. Cravedi et al. [12] reported that in rainbow trout ≈20% of the ingested radiolabeled MT was eliminated in the gallbladder at 72 h. This elimination occurred after biotransformation of the parent compounds into conjugated metabolites, probably in the liver.

Table 4
Levels of plasma testosterone (pg/ml) in muskellunge during (Days 55 to 57) and after (Day 250) MT treatment in control and MT-treated groups

Sex	Days 55 to 57 (September) during treatment		Day 250 (March) 6 Months after MT treatment	
	Control	MT	Control	MT
Male	232 ± 121* (n = 10)	427 ± 141* (n = 22)	22 ± 5† (n = 14)	25 ± 9† (n = 7)
Female	321 ± 110* (n = 10)	600 ± 216* (n = 19)	30 ± 10† (n = 6)	67 ± 19† (n = 13)
Intersex	-	56 (n = 1)	-	97 (n = 1)

Data are mean ± SE values. Mean values with the same superscript designation are not significantly different from each other (P > 0.05). MT, 17α-methyltestosterone.

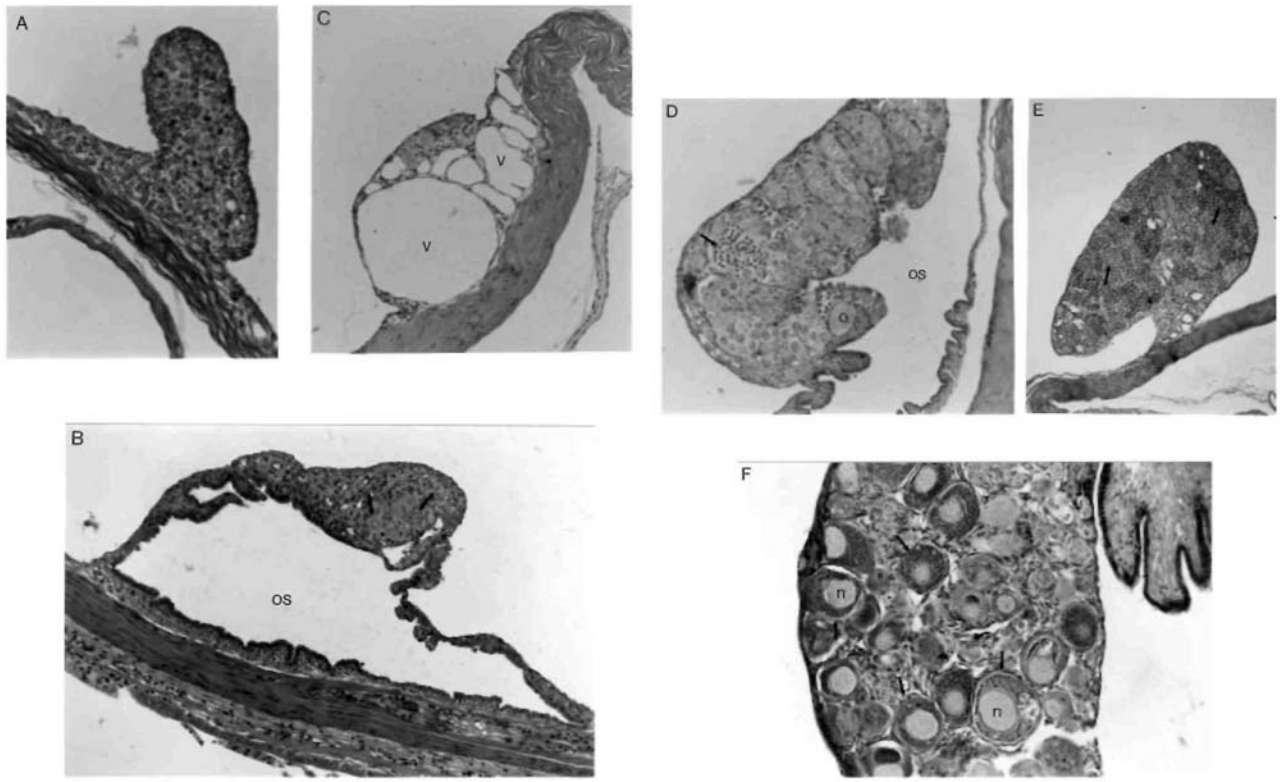

Fig. 5. Cross-sections of muskellunge gonads. (A) Undifferentiated gonad connected to the dorsal wall from a fish sampled before 17α-methyltestosterone (MT) treatment (×300). (B) Ovary from a fish sampled before MT treatment (×300). Note the presence of the ovarian sac (os) and lobules containing individual primary oogonia (arrows). (C) Testis consisted mainly of vacuolized tissue (v) from a fish during MT treatment (×300). (D) Intersex gonad from a fish during the MT treatment with presence of ovarian sac (os), oocytes (o), and spermatogenic tissue (arrow) (×300). (E) Testis containing spermatogenic tissues (arrows) from a fish after MT treatment (×300). (F) Ovary with primary oocytes (arrows) containing nucleus (n) from a fish after MT treatment (×300).

Yamamoto [31] reported that complete and functional sex reversal should occur if the administration of sex hormones is started at the undifferentiated gonad stage and is continued through the subsequent stages of sexual differentiation. In muskellunge, the anatomical differentiation of the female gonad occurs when fish size ranges between 119 and 151 mm and precedes the one of the male (192 to 229 mm) [32]. The size of muskellunge at the beginning of this experiment was 98 mm, and histological examination before the experiment revealed that some fish were already differentiated as females. During the MT treatment, the sex ratio of the MT-treated groups was similar to the control group. However, in the MT-treated groups, the male gonads consisted mainly of vacuolized connective tissues. In sea bream, *Dicentrarchus labrax*, gonads of this type have been classified by Blasquez et al. [33] as partially sterile gonads. The appearance of partially sterile fish denotes inhibitory effects of MT on gonadogenesis after prolonged treatment [33,34]. These inhibitory effects of MT were confirmed by an increase in the percentage of fish with intersex gonads 2 months after the treatment began and also by a change in the sex ratio to 62:32:5 (female/male/intersex) 6 months after the treatment. Thus, oral administration of MT at a dose of 15 mg/kg food for 60 days induced paradoxical feminization in muskellunge. This phenomenon, previously reported in the African catfish *Clarias gariepinus* [35], channel catfish *Ictalarus punctatus* [36], rainbow trout [37], coho salmon [38], and cichlid *O. mossambicus* [39], results either from a high dose of androgens or from a prolonged period of androgen treatment. Piferrer and Donaldson [38] suggested that paradoxical feminization may be due more to aromatization than to inhibition of in vivo synthesis of androgens. However, these authors also stressed

that in some species, aromatization and inhibition of in vivo synthesis of androgens could be the causative factor. Thus, the use of nonaromatizable androgens in the diet such as 17α-methyldihydrotestosterone may result in masculinization rather than feminization of fish.

The presence of high concentrations of T in the plasma of control and MT-treated fish during the MT treatment indicated the steroidogenic capabilities of muskellunge juveniles undergoing sex differentiation [32]. However, no sexual dimorphism of plasma T levels were found during or after the MT treatment (Table 4). As reported in several teleost fish [40-42] and in mammals [43], T production is possible only after the onset of gonadal differentiation. In muskellunge, this event did not occur shortly after hatching, as reported by Fitzpatrick et al. [44] in coho salmon, but later when the fish size ranged between 90 and 151 mm. In contrast to the rainbow trout in which the production of gonadal steroid was inhibited by the MT treatment [18], the levels of plasma T in muskellunge did not seem to be affected by the MT treatment because no significant difference was found between control and MT-treated groups. Nevertheless, future studies must clarify the involvement of the endocrine system in the process of sexual differentiation in muskellunge by determining the profiles of sex steroids (testosterone, estradiol-17β, and 11-ketotestosterone) through the period of sexual differentiation and by correlating these levels with gonadal development.

In summary, we presented actual concentration of MT in fish blood and addressed the question of direct impact of synthetic steroid on production of T in juvenile differentiating fish. The dose of MT, commonly regarded as low to cause sex reversal in fish, resulted in vacuolization of testes, and possibly in paradoxical feminization in muskellunge. It may also be concluded that a dietary supplementation of synthetic androgen, because of a variable intake of individual fish, may result in the profoundly different response, and other means of treatment need to be explored.

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