



U.S. Fish & Wildlife Service

## Aquatic Animal Drug Approval Partnership

# DRUG RESEARCH INFORMATION BULLETIN

## The Safety of 17 $\alpha$ -Methyltestosterone Administered in Feed to Larval Nile Tilapia

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Larval stages of most fish contain both ovarian and testicular tissues, and sexual differentiation commences shortly after hatching or initiation of feeding (Yamamoto 1969; Donaldson & Hunter 1982; Yamazaki 1983). Techniques developed to control sexual differentiation in fishes have typically involved androgen or estrogen (i.e., steroid) treatment, which directs sexual differentiation toward males or females (Donaldson & Hunter 1982). Treatment regimens have included immersion of larval fish in water containing a steroid, incorporation of a steroid in the larval diet, or both. Results have been variable because fish species, dose, frequency, duration, and environmental conditions influence treatment efficacy.

Gonadal differentiation in tilapia (*Oreochromis* spp.) occurs at 8-25 d posthatch, and these fish begin to reproduce at 3-6 months of age. Such early reproduction is the primary impediment to their commercial production. To prevent reproduction from occurring, oral administration of the synthetic androgen 17 $\alpha$ -methyltestosterone (17MT) to newly hatched tilapia (3-12 days old) for ~28 consecutive days produces populations of >90% males (Green et al. 1997; Rani & Macintosh 1997; Teichert-Coddington et al. 2000). The excess androgen overrides endogenous hormones and directs sexual differentiation towards the formation of testes.

Orally administered 17MT is an efficacious, cost-effective, and efficient way to produce predominantly male populations of tilapia. However, its use in the U.S. for tilapia production depends on its approval by the U.S. Food and Drug Administration (FDA). Approval by FDA requires, in part, that studies be conducted to demonstrate the proposed treatment regimen is safe to target animals. As such, we conducted a study to estimate a margin of safety associated with administering 17MT-treated feed to larval Nile tilapia *O. niloticus* (mean weight at start of study, 0.032 g) at 0, 9, 27, or 45 mg 17MT per kg fish body weight per d (equivalent to 0 $\times$ , 1 $\times$ , 3 $\times$ , or 5 $\times$  the proposed maximum efficacious dosage of 9 mg 17MT per kg fish body weight per d) for 28 consecutive days.

### Methods

The in-life phase of the study was conducted at the U.S. Department of Agriculture, Agricultural Research Service, Harry K. Dupree – Stuttgart National Aquaculture Research Center (SNARC; Stuttgart, Arkansas USA) on 16 May through 15 June 2010. Completely randomized designs were used to 1) assign each exposure dose to 4 of 16 22-L aquaria (test tanks), and 2) allocate 50 fish into each test tank. Four additional tanks were stocked with 50 fish each and used as “surrogate tanks” to monitor fish growth during the study.

Each test tank contained 15.2 L of water, and inflow was set to 3.7 L per min. Water flow through each tank was provided by individual filtered aquarium pumps. During the first 23 d of the study, 20% of the water in each tank was exchanged daily to maintain adequate water quality. During the last 5 d of the study, up to 50% of the water was exchanged daily because of increased fish size and resultant increases in ammonia and nitrite concentrations. Feed samples were collected periodically

and sent to Maxxam Analytics (Burnaby, British Columbia, Canada) for analysis to verify the homogeneity and stability of 17MT in the 1×, 3×, and 5×-treated feeds and ensure the 0× (control) feed was not contaminated with 17MT.

The in-life phase consisted of a 3-d acclimation period, 28-d exposure period, and 1-d postexposure period. During the exposure period, fish were fed by automatic feeder four times per day at 15% mean body weight per d. Feed amounts administered to test fish were adjusted daily based on a projected growth curve for tilapia previously cultured at SNARC. In addition, fish in the four surrogate tanks were sample-counted weekly, and the projected growth curve was adjusted accordingly. Mortality, general fish behavior, fish feeding (appetite) behavior, water temperature, and dissolved oxygen concentration were monitored daily. Ammonia and nitrite concentrations were measured in each tank twice weekly during the first 3 weeks of the study and daily during the last week of the study. Water hardness, alkalinity, and pH were measured once per week during the study.

At the end of the in-life phase, all test fish were collected from test tanks and euthanized. Necropsies were performed on the first 20 fish collected from each tank. Of these 20, 10 were randomly selected and used for histology, of which two were randomly selected for evaluation based on a “long” list of tissues (gill, liver, anterior kidney, posterior kidney, brain, heart, muscle, skin, spleen, pyloric intestine, and rectal intestine). A “short” list of tissues (gill, liver, anterior kidney, and posterior kidney) were evaluated from the remaining eight fish. Tissues were examined for lesions that might provide evidence of 17MT-induced toxicity and scored by using the following scale: none, normal, mild, moderate, marked, or severe. Tissues from the 0× and 5× exposure groups were examined and compared first. If lesions were detected in one or more tissues of the 5× exposure group that were (a) marked or severe, (b) not observed in the 0× exposure group, and (c) appeared to be 17MT-induced, then all fish representing the 3× exposure group were examined for that (or those) specific lesion(s). If such lesions were detected in one or more tissues of the 3× exposure group, then all fish representing the 1× exposure group were examined for that (or those) specific lesion(s). A dichotomized histological scoring scheme was developed in which lesions scored as marked or severe were considered pathological (biologically important) while all other scores were considered nonpathological (not biologically important).

Cumulative mortality was statistically compared among exposure groups at the end of the exposure period with a general linear mixed model in SAS PROC GLIMMIX (logit link;  $P < 0.1$ , two-sided; tank = experimental unit).

A separate analysis was done for each tissue in which pathological lesions were detected in fish exposed to 17MT and nonpathological lesions were observed in the control fish. Each analysis was performed with a general linear mixed model in SAS PROC GLIMMIX (logit link;  $P < 0.1$ , two-sided; tank = experimental unit).

## Results and Discussion

Mortality occurred in the 1× and 5× exposure groups only (Table 1). Differences in mean percent cumulative mortality among exposure groups were not significant ( $P = 0.3058$ ), although mortality in the 5× exposure group (9%) was greater than that observed in the other three exposure groups. General fish behavior was characterized as normal. During the first 23 d of the exposure period, fish consumed 100% of the feed ration offered. During the last 5 d of the study, fish in some tanks consumed only 75% of the feed, and in two of the 5× exposure tanks, fish consumed as little as 50% of the daily feed ration offered.

Lesions of concern were observed most frequently in fish in the 5× exposure group and included (1) protein in blood in the gill, heart, liver, and kidney, (2) proteinaceous material in gill tissue, and (3) moderate to moderately severe hypertrophy, degeneration, and necrosis of cardiac muscle cells in heart tissue (observed in the 3× and 5× group fish). As such (and based on criteria described in the research protocol), it was necessary to statistically compare histology data between only the 5× and 0× exposure groups. The protein in blood and proteinaceous material were observed in some fish in all but the 0× group and were considered treatment-related. Moreover, the severity of this finding increased in a dose-dependent manner. However, the differences between pathological lesions in the 0× and 5× exposure groups were not statistically different. Lysis of muscle cells suggested the heart was the target tissue of 17-MT. Tissue damage was most severe in the atrium of the heart. Gill epithelial lifting and liver vacuolation observed were attributed to the elevated concentrations of ammonia and nitrite in the water during the last 9 d of the study.

Based on feeding test fish at 15% BW, mean 17MT doses administered to the 1×, 3×, and 5× exposure groups were 88%, 89%, and 93% of respective target doses (Table 1).

Mean concentrations of ammonia (<0.2 mg per L) and nitrite (<0.1 mg per L) were considered low during the first half of the study. During the last 9 d of the study, mean daily concentrations of ammonia ranged from 0.3 mg per L to 6.7 mg per L. The overall mean concentration of ammonia in all test tanks during this 9-d period was 3.0 mg per L, and the mean concentration of ammonia in tank water in each of the four exposure groups ranged from 2.6 mg per L (in the 3× group) to 3.2 mg per L (in the 1× group). Although the ammonia and nitrite concentrations were considered elevated, other

investigators (Redner & Stickney 1979; Daud et al. 1988; Atwood et al. 2001) found such concentrations to be within a range tolerated by tilapia.

Overall mean water temperature and DO concentration among test tanks were 28.0°C (range, 26.9 to 28.8°C) and 6.7 mg per L (range, 4.0 to 7.6 mg per L). Overall mean water hardness (131 mg per L as CaCO<sub>3</sub>), alkalinity (231 mg per L as CaCO<sub>3</sub>), and pH (8.3) in test tanks were within the broad range considered acceptable for rearing healthy Nile tilapia.

Based on the results of this study, we concluded the margin of safety associated with administering 17MT-treated feed to larval Nile tilapia reared at a water temperature of approximately 28°C extends to at least 27 mg 17MT per kg fish BW per day when administered for 28 consecutive days.

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**Table 1. Mean cumulative mortality of test fish at the end of the 28-d exposure period, and 17MT dose-verification results.**

Exposure Group	Mean cumulative mortality (%)	Dose verification		
		$\mu\text{g}$ 17MT per g feed	mg 17MT per kg fish BW per dfish d	% of target dose
0×	0.0	0.0	0.0	n/a
1×	0.5	52.8	7.9	88
3×	0.0	160.3	24.1	89
5×	9.0	279.8	42.0	93