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ARTICLE

Safety of Feed Treated with 17α-Methyltestosterone (17MT) to Larval Nile Tilapia

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Abstract
As a synthetic androgen, 17α-methyltestosterone (17MT) is frequently used to redirect the course of sex differentiation by exposing the undifferentiated gonad to a sufficient dosage. This hormone has been widely accepted as a safe and effective treatment for sex reversal in many fish species, and it is administered to larval tilapia (3–12 d old) for ∼28 consecutive days to produce populations of >90% males. This study assessed the safety of 17MT-treated feed when administered to larval Nile Tilapia *Oreochromis niloticus* at one, three, and five times (i.e., 1×, 3×, and 5×) the proposed dosage of 9 mg 17MT/kg fish daily for 28 consecutive days. Despite elevated total ammonia nitrogen levels measured during the last 5 d of the study, environmental conditions were acceptable for rearing tilapia. Fish fed aggressively regardless of the concentration of 17MT in the feed, behavior was considered normal with no dose-related differences detected, and no mortality was observed in the 3× treatment group. Fish that were treated with five times the proposed therapeutic dosage had significant pathological changes. Based on the results of this study, the 17MT margin of safety extends to at least 3× (27 mg 17MT/kg fish daily) the proposed dosage of 9 mg 17MT/kg fish daily when administered in feed for 28 d to Nile Tilapia.

Larval stages of many teleosts contain both ovarian and testicular tissues, and sexual differentiation commences shortly after hatching or initiation of feeding (Yamamoto 1969; Donaldson and Hunter 1982; Yamazaki 1983). Various techniques have been developed to control sexual differentiation in fishes (Donaldson and Hunter 1982). These techniques have typically involved androgen or estrogen treatments to override endogenous mechanisms of sex determination in developing larval stages. Treatment regimens have generally 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 timing, dosage, and environmental conditions can influence treatment efficacy. The steroid 17α-methyltestosterone (17MT) is frequently used to redirect the course of sex differentiation by exposing the undifferentiated gonad to a sufficient dosage, and it is a male-specific hormone commonly used to induce sex reversal in teleost fishes (Hunter and Donaldson 1983).

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Gonadal differentiation in tilapia *Oreochromis* spp. occurs at 8–25 d posthatch, and these fish begin to reproduce at 3–6 months of age. Early and prolific reproduction is a restriction on commercial production of these fish. An all-male population allows for greater feed conversion and larger, more marketable fish. Oral administration of 17MT to newly hatched tilapia fry (3–12 d old) for ~28 consecutive days results in populations composed of >90% males (Green et al. 1997; Rani and Macintosh 1997; Teichert-Coddington et al. 2000). Excess androgen introduced into the early life stage of fish 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 populations of primarily male tilapia. Therefore, oral administration of 17MT is ideal for the commercial production of tilapia.

In the United States, public agencies and private industry are working to obtain U.S. Food and Drug Administration (FDA) approval of 17MT for use in aquaculture for tilapia. To obtain this approval, studies must demonstrate that this treatment regimen is safe to representative target animals. These target-animal safety studies are designed to estimate a margin of safety associated with overdosing and overexposing healthy test fish (e.g., Greenlees 1997; Gaikowski et al. 2003; Straus et al. 2012). In this study, the margin of safety of 17MT-treated feed was assessed when administered to larval Nile Tilapia *O. niloticus* at one, three, and five times (i.e., 1×, 3×, and 5×) the proposed therapeutic dosage of 9 mg 17MT/kg fish daily for 28 consecutive days.

METHODS

Nile Tilapia fry were obtained from AmeriCulture, Animas, New Mexico, and shipped to the Harry K. Dupree Stuttgart National Aquaculture Research Center (SNARC), Stuttgart, Arkansas. At SNARC, the fish were held for 4 d in two reference-population aquaria before being moved into test aquaria and allowed to acclimate for 3 d; fish weight at the start of the study was 0.032 g ± 0.008 (mean ± SD). Males and females were assumed to be present in roughly equal proportions. Fish health evaluations were conducted onsite by a fish pathologist certified by the American Fisheries Society. Reference population fish were fed nontreated tilapia starter feed (Tilapia Sex Reversal Control Feed, Rangen, Buhl, Idaho) at 15% of body weight per day (BW/d) with an automatic feeder (Fish Mate F14 Aquarium Feeders, Pet Mate, Hersham, Surrey, UK). Feed was not administered the day fish were transferred from the reference population to the test aquaria.

Larval Nile Tilapia were randomly stocked into 16 test aquaria filled with recirculating well water at 50 fish per aquarium. An additional four aquaria were stocked identically and used as "surrogates" to monitor fish growth during the exposure period. Diets (treatment groups) were randomly assigned to four replicate aquaria per treatment. Nontreated control feed was administered to the surrogate aquaria, and the fish in these aquaria were weighed weekly to calculate proper feeding quantities in the test aquaria. Treatment groups were 0×, 1×, 3×, or 5× (0, 9, 27, and 45 mg 17MT/kg fish daily, respectively) the proposed therapeutic dose of 9 mg 17MT/kg fish daily for 28 consecutive days.

Static test aquaria ($n = 20$) were glass (22 L), and each was filled with 15 L of water and contained a biofilter (Tetra Whisper 10, Blacksburg, Virginia). The laboratory in which the study was conducted was equipped with overhead lights set on a timer to produce a 12 h light : 12 h dark cycle. Test and surrogate aquaria were cleaned daily. Approximately 20% of the water in each aquarium was removed daily and replaced to maintain adequate rearing conditions.

Water temperature and dissolved oxygen (DO) concentration were measured with a YSI Pro20 dissolved oxygen and temperature meter (YSI Environmental, Yellow Springs, Ohio), alkalinity and hardness were measured by titration using standard methods (APHA 2005), and pH was measured with an Orion Research 720A meter (Thermo Electron Corporation, Beverly, Massachusetts). Total ammonia nitrogen (TAN) and nitrite (NO_2) concentrations were measured with a HACH colorimeter (Hach, Loveland, Colorado). The TAN and NO_2 levels became elevated near the end of the study due to fish growth and increased feeding, and it was necessary to change 50% of the water daily during the last few days.

Rangen prepared 5-kg batches of Tilapia Sex Reversal Feed with the appropriate dose of 17MT (Spectrum Chemical Manufacturing, Gardena, California), and one 20-kg bag of nontreated tilapia starter feed (0×). Samples were collected from each batch of feed for dose verification analyses to confirm that (1) the concentration of 17MT in each batch of treated feed was within ±20% of the target concentration, (2) 17MT was mixed homogenously throughout each batch of treated feed, (3) 17MT was not present in nontreated feed, and (4) the 17MT concentration in each batch remained stable throughout the study. Analysis was performed by Maxxam Analytics (Burnaby, British Columbia) using HPLC.

During the 28-d treatment period, feed was administered to fish at a rate of 15% BW/d using automatic feeders. The amount of feed was adjusted daily based on a projected growth curve for tilapia previously cultured at SNARC. The fish in the test tanks were assumed to grow at a similar rate as fish in the surrogate tanks and adjusted feed amounts were delivered to test tanks based on surrogate fish size. Separate feed containers were filled each week with the appropriate amount of feed to be delivered during the week and stored in a freezer until use.

Prior to distributing fish in test aquaria, 30 fish were sampled from the reference population (15 fish/aquaria) for general necropsy and 10 of these fish were randomly selected for histological evaluation; due to the small size of these fish, they were examined under a microscope to assess general health. On the day after the last treatment, 20 live fish were taken from each test aquaria, measured for length and weight, and assessed for general health using standard necropsy procedures. Fish

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health evaluations included a visual examination of (1) skin for discoloration and presence and severity of dermal lesions, (2) gills for pallor, and (3) internal tissues (liver, spleen, and kidney) for signs of gross lesions and abnormalities. Ten of the 20 fish from each test aquarium were randomly selected, placed in individual containers, and processed for histological evaluation. Over the course of the in-life phase, nine fish died or became moribund. A fish health evaluation was performed on each of these fish, and then they were processed for histological evaluation.

Histology.—Upon completion of fish health evaluations, the 10 fish selected from each test aquarium were placed in Davidson's fixative solution for 48–72 h, and then transferred into 70% ethyl alcohol. If fish were small enough, they were processed whole; if fish were larger, the head section was removed and they were embedded in two separate Omnisette tissue cassettes (Fisher Scientific, Fair Lawn, New Jersey) as follows: (1) the head of larger fish was removed posterior to the gills and cut in half longitudinally, and both halves were placed into a cassette, (2) the muscle wall was removed from the left side of the body and the entire body section was placed into another cassette, (3) cassettes were infiltrated with paraffin with a Leica ASP 300 advanced smart processor (Leica Microsystems, Nussloch, Germany), (4) samples were embedded in paraffin blocks with a Leica EG 1160 tissue embedding system, (5) blocks were sectioned (5 μm thick) on a Leica RM2255 rotary microtome, and (6) sections were mounted on glass microscope slides, stained with hematoxylin and eosin (H&E), and protected with a cover slip.

Of the 10 fish sampled from each aquarium for histology, eight were designated for evaluation of gill, liver, anterior kidney, and posterior kidney. Tissues evaluated in the remaining two fish included those four tissues as well as brain, heart, muscle, skin, spleen, pyloric intestine, and rectal intestine. A histopathologist evaluated each tissue for cellular changes or lesions that might provide evidence of 17MT-induced toxicity. Lesions were scored using a six-point ordinal severity scale (Straus et al. 2012); tissue lesions in fish from the 0× or 5× treatment groups were examined and compared first. If lesions were detected in one or more tissues of the 5× group that were characterized as marked or severe and not observed in the 0× group, then all fish from the 3× group were examined for that specific tissue. If such lesions were detected in one or more tissues of the 3× group, then all fish from the 1× group were examined for that specific tissue. Because differences were detected among histological evaluation scores as stated above, fish from all treatment groups were evaluated histologically.

The histologist used a Zeiss light microscope (Carl Zeiss International, Jena, Germany) to evaluate fish tissues and documented features associated with lesions (e.g., inflammation, edema), whether lesions were scattered or focal, and whether lesions were considered to be artifacts. For data analysis purposes, a dichotomized histological scoring scheme was developed in which histology scores of 0, 1, 2, or 3 were considered nonpathological and given a score of 0. Histology scores of 4 or 5 were considered pathological and were given a score of 1.

Data analysis.—For each of the treatment groups, mortality and histology data were analyzed using SAS Proc Glimmix (SAS version 9.1.3, SAS Institute, Cary, North Carolina). Treatment effects were tested at $\alpha = 0.10$ significance level. All other data were summarized or statistically analyzed, or both, using Microsoft Office Excel (2003 version), SYSTAT 12.02 (Systat Software, Chicago, Illinois), or SigmaPlot 11.2 (Systat Software, San Jose, California). Mean fish TL and body weight for treatment groups at the end of the study were analyzed by ANOVA. A pairwise multiple comparison test was used if a significant difference was detected by ANOVA. Treatment effects were tested at $\alpha = 0.05$ significance level.

RESULTS

Mortality was observed in the 1× and 5× treatment groups; the single mortality in the 1× group was considered incidental (i.e., cannibalism). Although mean cumulative mortality in the 5× group (9%) was greater than in the other treatment groups (mean cumulative mortality ranged from 0.0 to 0.5%), differences were not significant.

Fish behavior was characterized as normal in the 0×, 1×, and 3× treatment groups. Behavior of fish in the 5× group was also characterized as normal, but these fish did not consume feed as aggressively. During the first 24 d of the study, fish fed actively and consumed virtually all feed offered. However, in the last 5 d of the study, fish in most aquaria, including fish in surrogate tanks, consumed less than 100% of the feed (often consuming only 75% of the feed). In two aquaria of the 5× treatment group, less feed was consumed during the last 5 d possibly because the amount of feed offered to fish was not adjusted to account for mortality (feed was preweighed in 1-week increments). As a result, fish were fed at rates ranging from 15.3 to 19.2% BW/d.

Over the course of the in-life phase, mean TL and weight of fish in all treatment aquaria increased. Mean (± SD) TL and weight of the 30 fish collected from the reference population before the start of the study was 9.8 ± 0.65 mm and 0.012 ± 0.003 g, respectively. A significant difference was detected in mean TL and weight among the four treatment groups (n = 80 for each group) at the end of the study. Based on results from a pairwise comparison test, fish from the 1× group (53 ± 5.07 mm and 2.52 ± 0.78 g) were significantly larger than fish in the 0× (49 ± 5.18 mm and 2.11 ± 0.70 g) and 5× (47 ± 4.67 mm and 1.64 ± 0.50 g) groups. Fish in the 0× and 3× (50 ± 3.85 mm and 2.15 ± 0.48 g) groups were significantly larger than fish in the 5× group. Size of fish in the 3× group was not significantly different from the 0× and 1× groups.

All fish sampled from the reference population showed mostly normal tissues, except that moderate lifting of gill epithelium and moderately severe vacuolation of liver hepatocytes was common. Nearly all moribund fish collected during the

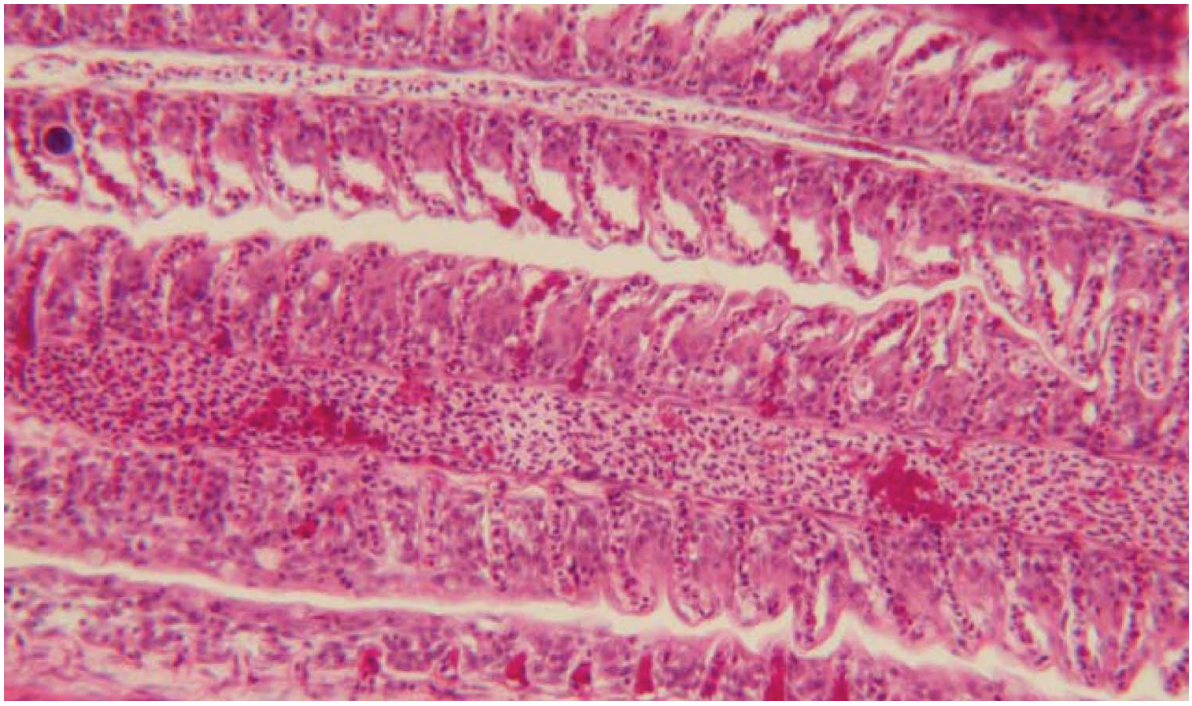

FIGURE 1. Proteinaceous material (dark orange–red) in blood vessels of gill tissue, clearly visible in filament blood (just below center from right to left) of the 5× treatment group in the study on the safety of 17MT to larval Nile Tilapia. [Figure available in color online.]

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study had gill lesions, and filamentous bacteria were observed in H&E-stained sections. No bacterial pathogens were observed on any live fish sampled.

Fish sampled from all treatment groups had mostly normal brain, skeletal muscle, skin, spleen, and intestine tissues. Lesions that were characterized as marked or severe and considered pathological were detected in the gill, liver, anterior kidney, posterior kidney, and heart of fish from all groups. The following tissue changes were common: (1) gill tissue had moderate to moderately severe edema, mild proliferation of epithelium, and, in some fish, low numbers of eosinophilic granular cells, (2) liver tissue had moderate to moderately severe vacuolation and mild nuclear pleomorphism of hepatocytes, and (3) kidney tissue had minimal to moderate hyaline droplet degeneration and necrosis of tubule epithelium, numbers of regenerating (developing, immature) tubules, and scattered eosinophilic granular cells.

Histological changes that were specific to the treatment groups were as follows. A few fish from those sampled from the 1× group had minimal to mild amounts of proteinaceous material in the heart atrium (i.e., eosinophilic deposits were not seen in blood vessels of any fish in this group); hypertrophy (mild to moderate) of cardiac muscle cells was also confined to the heart atrium. Fish sampled from the 3× group had intravascular deposits of proteinaceous material, except for fish in one aquarium; 10 of the 3× group fish showed changes similar to 5× group fish. Fish sampled from the 5× group had intravascular deposits of proteinaceous material, which was the most remarkable finding; accumulation of this material was most pronounced in blood vessels of the gills (Figure 1). Moderate to severe hypertrophy, degeneration, and necrosis of cardiac muscle cells were seen in heart tissue of fish from the 5× group (Figures 2, 3). Mild to moderately severe accumulations of intracellular protein in hepatocytes was evident. Skeletal muscle appeared normal in all fish examined in the 5× group.

Tissues in which lesions were considered pathological are summarized in Table 1. Statistical results indicated that significant differences were not detected in the severity of lesions between the 5× and 0× treatment groups.

Mean concentrations of 17MT in the treated feeds (Table 2) were within ±20% of the target dose. All individual feed samples analyzed from each batch of feed were less than the target feed concentration by 6.7–11.9%, but were also within ±20% of the target dose. No 17MT was detected in the 0× control feed.

Mean water temperature among test tanks was 28.0°C, and individual measurements ranged from 26.9°C to 28.8°C. Mean DO concentrations among test aquaria was 6.7 mg/L, and individual measurements ranged from 4.0 to 7.6 mg/L. All DO concentrations measured in test aquaria were in a range considered acceptable for rearing tilapia. Mean water hardness (131.1 ± 2.84 mg/L as $CaCO_3$), alkalinity (231.3 ± 4.07 mg/L as $CaCO_3$), and pH (8.3 ± 0.24) in test aquaria were also within the range considered acceptable for rearing tilapia.

Mean concentrations of TAN (<0.2 mg/L) and NO_2 (<0.09 mg/L) were considered low during the first half of the

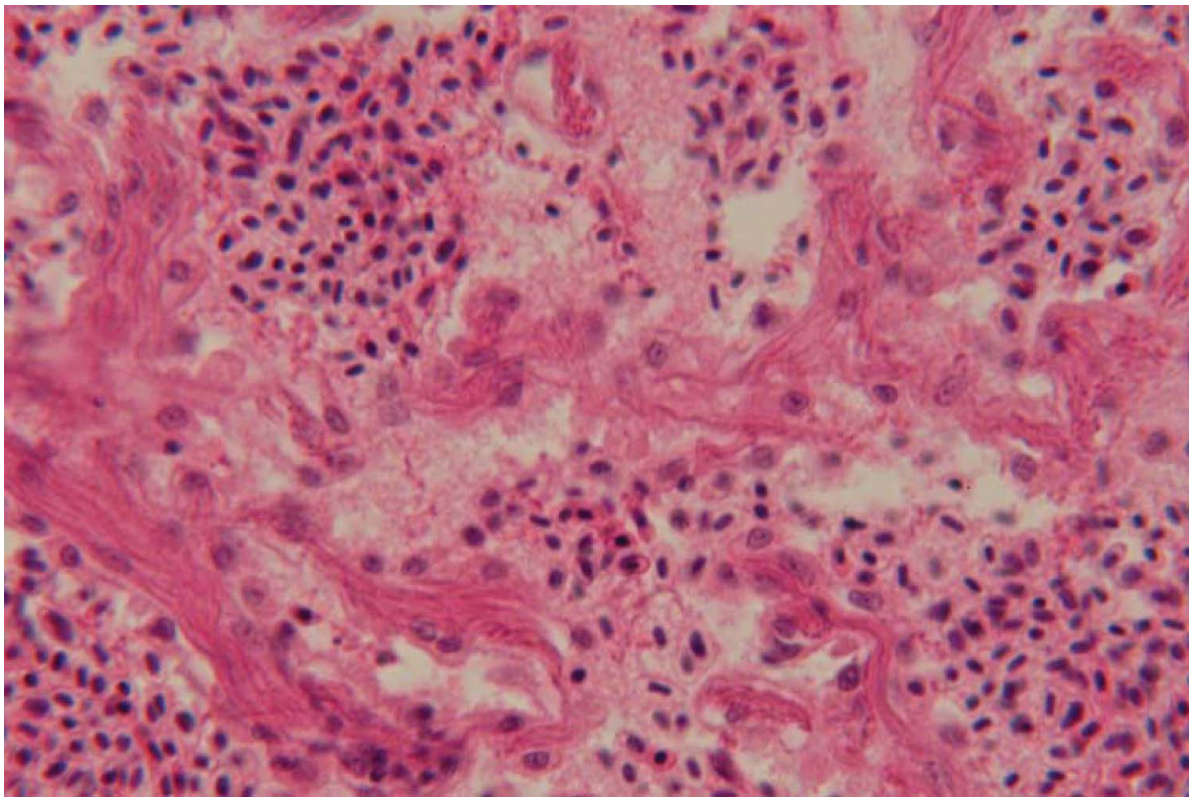

FIGURE 2. Hypertrophy and lysis of cardiac muscle cells of the 5× treatment group in the study on the safety of 17MT to larval Nile Tilapia. [Figure available in color online.]

TABLE 1. Treatment group histology scores of marked (4) or severe (5) fish tissues considered pathological in the study on the safety of 17MT to larval Nile Tilapia.

	Histology score	
Tissue	4	5
Gill		
Epithelial lifting	0×	None
Edema	0×, 1×, 3×, 5×	None
Proliferation	1×, 5×	5×
Proteinaceous material	5×	None
Protein in blood	5×	None
Liver		
Vacuolation	5×	None
Protein in hepatocytes	5×	None
Posterior Kidney		
Degeneration	0×, 3×	None
Necrosis	0×, 3×	None
Protein in blood	5×	None
Anterior Kidney		
Protein in blood	5×	None
Heart		
Degeneration	3×, 5×	None
Protein in blood	3×, 5×	None

study. During the last 9 d of the study, mean daily concentrations of TAN ranged from 0.27 to 6.70 mg/L. The overall concentration of TAN in all test aquaria during this 9-d period was 2.97 mg/L ($n = 7$ sample days), and the mean concentration of TAN in tank water in each of the four treatment groups ranged from 2.59 (3× group) to 3.23 mg/L (1× group).

Water quality was suitable for holding and rearing healthy tilapia, despite elevated TAN levels during the last 5 d of the study. Therefore, survival of test fish, general and feeding (appetite) behaviors, fish health evaluations, and 17MT-induced gross and microscopic lesions evident in test fish were used to estimate a margin of safety.

DISCUSSION

Based on the results of this study, the 17MT margin of safety extends to at least 3× (27 mg 17MT/kg fish daily) the proposed dosage of 9 mg 17MT/kg fish daily when administered in feed for 28 d to Nile Tilapia. Fish fed aggressively regardless of the concentration of 17MT in the feed offered, behavior was considered normal with no dose-related differences detected, and no mortality was observed in the 3× treatment group.

A reason that the margin of safety was not recommended to extend the standard dosage to 5× was the presence of protein in blood of the gill, heart, liver, and kidney and proteinaceous

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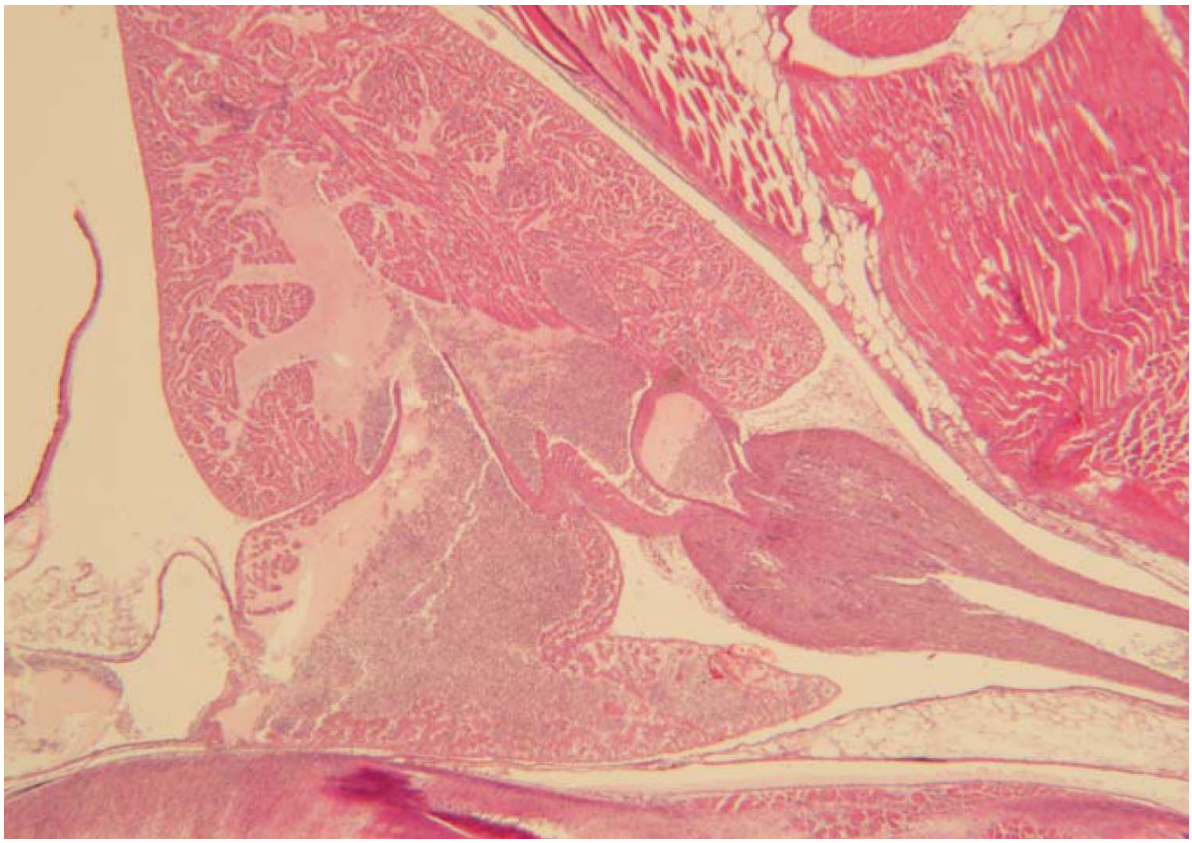

FIGURE 3. Ventricle (top), bulbous (left), and atrium (bottom) chambers of the heart of the 5× treatment group in the study on the safety of 17MT to larval Nile Tilapia. [Figure available in color online.]

material in gill tissue. These lesions were observed in some fish in all treatment groups and were considered 17MT-related, as this finding was not present in the 0× group (control) and the severity of this finding increased in a dose-dependent manner. However, the differences among pathological lesions in the 0× and 5× groups were not significant. Some of the observed lesions (primarily in the gill and liver) were attributed to the elevated concentrations of TAN and NO_2 in the water during the last 9 d of the study.

In the present study, nontreated control feed (i.e., 0×) was administered to fish in the surrogate aquaria; the mean weight of fish in these aquaria was used for the adjustment of feed in the test aquaria. Based on the significant growth difference of the fish in the different treatment groups, the use of the mean weight of fish in the surrogate aquaria for the calculation of feed for the test aquaria resulted in a slightly different amount given to test fish than required. Feed amounts were not adjusted for mortality that occurred during the week for which feed had been aliquoted, resulting in the 5× group being offered more feed (based on weight). Based on the mean weight of fish in the surrogate tanks on the final day of the study (1.69 ± 0.11 g), the larger fish from the 0×, 1× and 3× groups were offered less feed; this weight difference could be attributed to the treated fish not being stressed by handling as were the surrogates.

Based on a mean water temperature of 28°C and pH of 8.3, approximately 12.5% of the total TAN in an aqueous solution is

TABLE 2. Mean concentrations of 17MT in the treated feeds and analytical results for each dose in the study on the safety of 17MT to larval Nile Tilapia.

Treatment group	Target feed concentration[a]	Actual feed concentration[a]	Difference	Target dose to fish[b]	Actual dose to fish[b]	Difference
1×	60	52.8	−11.9%	9	7.9	−12.2%
3×	180	160.3	−10.9%	27	24.1	−10.7%
5×	300	279.8	−6.7%	45	42.0	−6.7%

[a]μg 17MT/g feed.
[b]mg 17MT/kg fish daily.

un-ionized ammonia (NH_3; Avault 1996). As a result, during the last 9 d of the study, mean daily concentrations of NH_3 ranged from 0.04 to 0.84 mg/L. The overall concentration of NH_3 in all test tanks during this period was 0.37 mg/L, and the mean concentration in each of the four treatment groups ranged from 0.32 (3× group; 2.59 mg/L TAN) to 0.40 mg/L (1× group; 3.23 mg/L TAN) NH_3.

Although the NH_3 and NO_2 concentrations were elevated, others have found the concentrations were within a range tolerated by tilapia. Redner and Stickney (1979) found that the 48-h LC50 for Blue Tilapia *O. aureus* was 2.46 mg/L NH_3, and in fish acclimated to low concentrations of NH_3, 100% survival was achieved when fish were exposed to 3.4 mg/L NH_3 for 48 h. Daud et al. (1988) reported that the NH_3 LC50 values at 48, 72, and 96 h in Red Tilapia (*O. mossambicus* × *O. niloticus* hybrids) fry were 6.6, 4.1, and 2.9 mg/L, respectively. Atwood et al. (2001) found that the 96-h LC50 value for small Nile Tilapia (4.4 g) was 81 mg/L NH_3, but that the 96-h LC50 value for large Nile Tilapia (90.7 g) was 8 mg/L NH_3. However, El-Shafai et al. (2004) found that concentrations of NH_3 ranging from 0.07 to 0.14 mg/L negatively affected growth of Nile Tilapia fed fresh duckweed. Some of the histological findings of note (e.g., gill edema and proliferation) were also observed in fish from the 0× or 1× treatment groups of the present study and were therefore not considered to be 17MT related.

During the last 9 d of the study, mean daily concentrations of NO_2 ranged from 1.4 to 4.1 mg/L. The overall concentration of NO_2 during this period was 2.88 mg/L, and the mean concentration in each of the four treatment groups ranged from 2.41 (3× group) to 3.33 mg/L (1× group). In future studies, procedures should be established to minimize buildup of NH_3 and NO_2 concentrations, such as transferring fish from smaller aquaria to larger aquaria, using filtering systems designed to remove nitrogen compounds, or exchanging aquaria water with fresh water more frequently.

The heart may be a target organ of 17MT pathologies including lysis and hypertrophy of cardiac cells (degeneration and necrosis), which could lead to decreased cardiac function. In the present study, lysis of muscle cells suggests that the heart is a target tissue of 17MT, and damage was most severe in the atrium of the heart (Figure 1). Such findings would be consistent with one of the manifestations of methyltestosterone use or abuse in humans. Given that the heart damage was also treatment-related and dose-dependent in the present study, it was suspected that the intravascular eosinophilic material might have been myoglobin, a muscle breakdown protein (Jeff Wolf, Pathology Manager and Veterinary Pathologist, Experimental Pathology Laboratories, Sterling, Virginia, personal communication). It was conjectured that the material might have been some sort of lipoprotein, possibly linked to the liver's metabolic changes due to the presence of 17MT (Hugh Ferguson, Professor of Pathology, St. George's University, Grenada, West Indies, personal communication). Hornung et al. (2004) reported that 17MT aromatizes to 17α-methylestradiol and contributes to the estrogenic effects in Fathead Minnow *Pimephales promelas* after exposure to the androgen, resulting in significant vitellogenin production in exposed fish.

Gill epithelial proliferation was observed and is often induced by exposure to irritants (e.g., ammonia) or is known to be an artifact of sampling. In this case, elevated TAN and NO_2 levels near the end of the study might have increased susceptibility of moribund fish to bacterial colonization.

This study is considered to be a valid test of the safety of 9 and 27 mg 17MT/kg fish daily administered in feed to Nile Tilapia for 28 d. In spite of the elevated TAN levels measured during the latter stages of the study, the testing was conducted under environmental conditions that were acceptable for rearing tilapia. Fish used to establish the reference population were larval Nile Tilapia that were approximately 10 d old (about the time when treatment would be started in industry to produce predominantly male populations of fish), and the 0-mg 17MT/kg fish daily test fish served as satisfactory controls.

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