17-Alpha Methyltestosterone Clinical Field Trials - INAD 11-236

Year 2006 Annual Summary Report on the Use of 17-Alpha Methyltestosterone in Field Efficacy Trials

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Summary

Various techniques have been developed for the control of sexual differentiation in a variety of fish species, including the use of the androgen, 17-alpha Methyltestosterone (MET). Specifically, this compound has been used effectively in the U. S. under compassionate INAD Exemption #11-236 on early life stage tilapia to produce predominately male populations (i.e., sex reversal). In calendar year 2006 (CY06) the efficacy of MET was evaluated in 240 trials involving approximately 24.2 million early life stage tilapia for sex reversal. Trials were conducted at 1 USGS Co-op Unit, and 11 commercial tilapia culture facilities. The compassionate study protocol under which treatments were administered allowed the investigator to administer MET at a dosage of 9 mg MET/kg of fish biomass for 28 consecutive days (60 mg MET/kg feed when fed at a rate of 15% body weight). Overall, results from trials conducted in CY06 indicated that treatment appeared either effective (in approximately 72% of the trials) or were characterized as inconclusive (in the remaining 28% of the trials). Based

on results from CY06, it appears that MET is an effective compound for sexual reversal of early life stage tilapia.

Introduction

Larval stages of many teleost species contain both ovarian and testicular tissue, and sexual differentiation commences shortly after hatching or after the initiation of feeding (Yamamoto,1969; Donaldson and Hunter, 1982; Yamazaki, 1983). Various techniques have been developed for the control of sexual differentiation in a variety of fish species (Donaldson and Hunter, 1982). These techniques have typically involved the use of either androgen or estrogen treatment to override the endogenous mechanisms of sex determination in developing larval stages and direct sexual differentiation toward the production of either males or females (i.e., sex reversal). Treatment regimens have generally involved immersion of larval stages in water containing a steroid, incorporation of a steroid in the larval diet, or both. As numerous factors such as dosage, timing, duration, and environmental conditions often influence efficacy, results have been somewhat variable.

Although the gonadal tissue of tilapia remains undifferentiated at hatch, tilapia generally attain sexual maturity by three to six months of age, and begin to immediately reproduce. This somewhat precocious reproduction behavior is the primary impediment to the development of successful strategies for the commercial production of tilapia. In

response to this problematic issue, strategies for monosex male culture have been evaluated including: 1) manual separation of the sexes by visual examination; 2) hybridization; and 3) sex reversal/direction via steroid administration.

1. Manual separation of the sexes via visual examination (or hand sexing) has been found to be a tedious and time consuming process, prone to significant human error, and less productive than other methods. Currently, hand sexing of tilapia is practiced by only a limited number of fish farmers in underdeveloped countries.

2. Hybridization involves the crossing of two different species of tilapia that can result in the production of 95 -100% male offspring. The most frequently used crosses suitable for aquaculture production are *Tilapia nilotica* (F) x *T. hornorum* (M); *T. mossambica* (F) x *T. hornorum* (M); and *T. nilotica* (F) x *T. aurea* (M). However, there are limitations to hybridization that include behavioral incompatibilities between two species that lead to significantly decreased fingerling production; the high potential for the contamination of the broodfish populations; and the high costs associated with the sophisticated equipment and techniques necessary to ensure and confirm broodfish purity. Hybridization is currently practiced on only a limited basis worldwide.

3. Gonadal differentiation in tilapia typically occurs between 8 to 25 days post-hatch, dependent upon environmental conditions. It has been demonstrated that the oral administration of the synthetic androgen 17-alpha methyltestosterone (MET) to newly hatched tilapia fry (3 -12 days old) for ~28 consecutive days results in populations comprised of greater than 90% males (Green et al., 1997; Rani and Macintosh, 1997; and Teichert-Coddington et al., 2000). The excess androgen that is introduced into the early life stage fish overrides endogenous hormones and directs sexual differentiation towards the formation of testis. The use of orally administered MET has been shown to be an efficacious, cost-effective, and efficient methodology to produce populations of male tilapia.

Of the three above-described methods/strategies for the monosex culture of male tilapia, oral administration of MET is the most effective and economic option, and is best suited for successful tilapia production in the United States.

Purpose of Report

The purpose of this report is to summarize the results of calendar year 2006 (CY06) MET field efficacy trials conducted under INAD #11-236. Furthermore, it is expected that data from these trials will be used to enhance the existing MET effectiveness database that has been established from previous years studies for the purpose of developing an appropriate label claim for the use of MET in aquaculture.

Facilities, Materials, and Treatment Procedures

1. Facilities

One USGS Co-op Unit and 11 commercial tilapia culture facilities used MET for sex reversal of early life stage tilapia. Mean water temperature during all trials was 81.3 °F, and water temperature ranged between 75.8 - 88.3 °F at the different testing facilities.

2. Test article used

The MET used during the reporting period was 17 beta-hydroxy-17-methylandrost-4-ene-3-one. All MET medicated feed used in INAD trials was manufactured and supplied by Rangen Inc, P.O. Box 706, Buhl, ID. The source of MET used by Rangen Inc. to prepared medicated feed was supplied by Hawkins, Inc. Pharmaceutical Group, 3000 East Hennepin Ave, Minneapolis, MN.

3. Treatment regimen

As described in the Study Protocol, Investigators were allowed to only use MET at a dosage of 9 mg MET/kg of fish biomass for 28 consecutive days (i.e., the industry standard; equivalent to 60 mg MET/kg feed when fed at a rate of 15% body weight; approximately 97% of trials were conducted using this treatment regimen). However, the treatment regimen administered in the remaining 3% of the trials deviated from the protocol-mandated use. In these trials, fish were fed

at 5.92 - 9 mg MET/kg of fish biomass for 14 - 27 days. In most cases in which deviations occurred, Investigators were made aware of the deviation and informed that adherence to the protocol is a vital element to the aquaculture INAD process.

Fish Species Involved in CY06 Trials

1. Species of fish treated

Tilapia were the only fish species treated during CY06. Treated fish ranged in length from 2 - 14 mm. Tilapia strains that were treated included:

1. Nile tilapia *Oreochromis niloticus*
2. Tilapia aurea *O. aureus*
3. California mozambique *O. mossambica*
4. Red Hybrid Tilapia *O. mossambica x O. urolepis*

Data Collected

1. Primary response variable (microscopic examination of gonadal tissue reports or gross examination of features characteristic of mature males/females)

The efficacy of MET medicated feed to produce populations of tilapia comprised of > 90% males was the primary indicator of clinical field efficacy trials success. The sex of individual fish was determined by evaluation of gonadal tissue

according to procedures of the gonadal squash technique as described by Guerrero and Shelton, 1974. Investigators were required to send 60 fish to the U. S. Fish and Wildlife Service's Aquatic Animal Drug Approval Partnership Program's research office in Bozeman, MT where gonads were examined and classified as testis, ovary, or ovotestis (gonads containing both ovarian and testicular tissue). However, in CY06, samples were sent from a limited number of Investigators, and subsequent microscopic examination of gonadal tissue reports were available for only 3 of 240 (1%) trials. Investigators were also allowed to perform a gross visual examination of external features characteristic of either male or female tilapia of fish that had reached or were near reaching sexual maturity. Results from gross visual examinations were submitted with 169 of 240 (70%) of the trials. Overall, a report of sex determination was submitted for 172 of 240 (72%) of the trials.

2. Secondary response variables

Secondary parameters include general observations on fish behavior and response to routine culture activities. Secondary parameters of interest, such as feeding activity, feed consumption, apparent level of stress, negative fish behavior, and mortality.

Discussion of Study Results:

1. General observations on the efficacy of MET to produce populations comprised of greater than 90% male fish (Note: Table 1 provides a summary of all trials characterized as effective; Table 2 provides a summary of all trials characterized as inconclusive; Table 3 provides summary data for all trials; and Table 4 provides a summary of all trials conducted during CY06 under INAD #11-236).

A. Efficacy at 9 mg/kg bw/day for 28 days

Fish were treated the industry standard dose for 28 days in 232 trials (Tables 1 & 2). Treatments were determined to be effective by either microscopic examination of gonadal tissue or by the Investigators visually evaluating the treated fish a few months after the treatment period ended. Effectiveness of some of the treatments were characterized as inconclusive because (1) Investigators did not determine fish sex while fish were on station, or (2) young fish were sold to a commercial grow out facility before they could be sexed. Results from all trials in which sex was determined using microscopic or gross visualization techniques showed that MET treatments was effective (i.e., such techniques were used to determine sex of fish in 166 trials). Treatment effectiveness was characterized as inconclusive in the remaining 66 trials because sex determination was not performed by the Investigator.

B. Efficacy at 9 mg/kg bw/day for 14 - 27days

Fish were treated at the industry standard for 14 - 27 days in six trials (Tables 1 & 2). Investigators noted that the full 28 day treatment of feed was not met primarily due to running out of feed or mechanical break downs at the facilities. Treatments were determined to be effective by the Investigators visually evaluating the treated fish a few months after the treatment period ended. Effectiveness of some of the treatments were characterized as inconclusive because (1) Investigators did not determine fish sex while fish were on station, or (2) young fish were sold to a commercial grow out facility before they could be sexed. Results from all trials in which sex was determined using gross visualization techniques showed that MET treatments was effective (i.e., such techniques were used to determine sex of fish in four trials). Treatment effectiveness was characterized as inconclusive in the remaining two trials because sex determination was not performed by the Investigator.

C. Efficacy at 5.92 - 9 mg/kg bw/day for 28 days under Pivotal Research Protocol MT-05-EFF

Fish were treated at 5.92 and 9 mg/kg bw/day for 28 days in 2 trials (Table 1). The Investigator noted that both studies were supposed to be conducted at the industry standard for 28 days; however, it was discovered after the feed assay results were completed that one study was actually at 5.92 mg/kg bw/day. Sex of fish was determined by the Investigator who performed histology on the treated fish a few months after the treatment period. Please refer to pivotal submission

numbers MT-05-EFF.3-01 and MT-05-EFF.2-02 for more details on these studies. Treatments were considered effective in both trials.

2. Observed Toxicity

No toxicity or adverse effects relating to MET treatment were reported in any of the trials conducted in CY06.

Summary of Study Results

MET was administered at 9 mg/kg bw/day for 14 - 28 days on four different species of tilapia and involved approximately 24.2 million fish. Treated fish ranged in length from 2 - 14 mm. Water temperature during treatment ranged from 75.8 - 88.3 °F, with a mean treatment temperature of 81.3 °F. Overall results in all cases in which microscopic or gross visual exams were performed to determine sex of fish showed that treatment appeared effective. However, such techniques to determine sex were only used in 72% of trials. Consequently, treatment results in the remaining 28% of the trials were characterized as inconclusive. No evidence of toxicity or adverse effects related to MET treatment was reported in any of the trials. The trials conducted under the pivotal research protocol MT-05-EFF included use of control fish, detailed histology reports documenting the male to female sex ratio during the post-treatment periods, and will likely be accepted by CVM as pivotal or supportive. Data from the other studies will be considered as ancillary data because of a general lack of quality control criteria essential for pivotal or supportive studies, such as use of untreated control fish, dose

verification, replication, and randomization. None-the-less, the data described above should provide useful corroborative data to support a label claim for MET for the sex reversal in tilapia. It is anticipated that additional ancillary efficacy data will continue to be collected under INAD #11-236 until such a time that the INAD is terminated. In future trials conducted under INAD #11-236, efforts will be directed towards the generation of high quality data.

References

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Table 1. Summary of CY06 MET Treatment Results - Effective Trials

Hatchery	Number of Trials	Tilapia Species	Fish Size (mm)	Number of Treated Fish	Treatment Duration (Days)	Dose (mg/kg fish bw)	Temp. (°F)
Americulture	44	Nile Tilapia	9.00	2,221,000	27 - 28	9	82 - 84
Canyon Farm - SeaPac of Idaho	22	Red Hybrid Tilapia	3.00	755,500	28	9	80 - 88
Canyon Farm - SeaPac of Idaho	1	Red Hybrid Tilapia	3.00	1,600	28	5.92	88.3
Kent SeaTech Corp	59	California mozambique	14.00	289,844	28	9	78.0
Pacific Aquafarms	28	California mozambique	5.00	2,056,547	27 - 28	9	78 - 84.2
Simaron Fresh Water Fish Inc.	18	Nile Tilapia Red Hybrid Tilapia	8.00	3,079,604	28	9	75.8 - 88.3

Table 2. Summary of CY06 MET Treatment Results - Inconclusive Trials

Hatchery	Number of Trials	Tilapia Strain	Fish Size (mm)	Number of Treated Fish	Treatment Duration (Days)	Dose (mg/kg fish bw)	Temp. (°F)
Aquasafra	51	Nile tilapia Tilapia aurea	6.00	8,798,000	24 - 28	9	79 - 83
Arizona Mariculture Associates	1	Nile tilapia	5.00	5,400,000	28	9	?
Aurea, Inc.	1	Tilapia aurea	?	18,360	14 - 28	9	76.0
Canyon Farm - SeaPac of Idaho	2	Red hybrid tilapia	3.00	57,000	28	9	80 - 88
Canyon Springs Fish Farm	2	Red hybrid tilapia	2.00	110,000	28	9	80.0
Guam Aquaculture Development and Training Center	4	Nile tilapia	5.00	845,310	28	9	?
Lake Geneva Fisheries	5	Nile tilapia	10.00	500,000	28	9	78.0
North Auburn Fisheries Research Unit	2	Nile tilapia	9.66	42,250	28	9	76.5

Table 3. Summary Data Regarding Summary of CY06 MET Treatment Trials

Total Fish Treated:	**24,175,015**
Number of fish treated in efficacious trials	8,404,095
Number of fish treated in inconclusive trials	15,770,920
Total number of trials:	**240**
Efficacious trials	172 (72%)
Microscopic evaluation	3 of 172 trials
Gross visual examination	169 of 172 trials
Inconclusive trials	
No sex determination techniques used	68 (28%)

Pivotal Study:

Study Numbers: 11-236-06-91 & 11-236-06-92

Treatment Regimen Used:

5.92 mg/kg bw/day for 28 days	1 trial
9 mg/kg bw/day for 28 days	233 trials
9 mg/kg bw/day for 14 - 27 days	6 trials

Treatment Water Temperature (°F):

Temperature Range	75.8 - 88.3
Mean Temperature	81.3

Size of Treated Fish:

Size Range	2 - 14 mm

Tilapia Strains Treated:

Tilapia

Nile tilapia *Oreochromis niloticus*
Tilapia aurea *O. aureus*
California mozambique *O. mossambica*
Red Hybrid Tilapia *O. mossambica x O. urolepis*