AN ABSTRACT OF THE THESIS OF

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Sex differentiation in fish is a labile process that allows sex inversion in several species. The inherent capacity of fish germ cells to differentiate into oocytes or spermatocytes constitutes a key factor allowing for functional sex inversion. This thesis set out to determine the mechanism involved in steroid-induced sex differentiation of Nile tilapia, *Oreochromis niloticus,* by searching for differential expression of unique genes during the process. In addition, the studies documented the persistence of methyltestosterone (MT) in the environment after oral administration, and investigated the capabilities of short-term immersions in steroids for masculinizing tilapia fry as an alternative method. A significant leakage of MT to the rearing water and its subsequent accumulation in the sediments was detected after oral administration of the steroid. In addition, evidence was found for a significant effect of environmental conditions on the masculinizing efficacy of oral administration of steroids. Low levels of masculinization were obtained when MT was allowed to remain in the system. Results from short-term immersions in steroids indicated that

the labile period for masculinization by immersion of Nile tilapia fry reared at 28 °C occurs between 11 and 16 days post fertilization (dpf). Two three-hour immersions in trenbolone acetate at 11 and 13 dpf yielded the highest number of males. Time of immersion, length of the treatment, dosage, density, solvent vehicle, and number of immersions significantly affected the outcome of immersion trials. Heterogeneity of developmental stages, developmental rate, and sensitivity of progeny to steroids may play important roles in the efficacy of immersion treatments. The expression of unique mRNAs during the process of sex inversion was identified through the use of suppression subtractive hybridization. This technique allowed for the sequencing of 165 clones from which 61 proteins have been identified. A significant number of these genes seem related to the anabolic effects of trenbolone acetate. In addition, 12 genes were identified that are related to reproductive tissues; seven of which have unique or enriched expression in the testes. Some of the genes and protein products that have been identified are linked to gonadal development and testicular protein synthesis in other species.

Sex Determination in Nile Tilapia, *Oreochromis niloticus*: Gene Expression, Masculinization Methods, and Environmental Effects

by

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CONTRIBUTION OF AUTHORS

Dr. Martin S. Fitzpatrick was involved in the design, analysis, and writing of each manuscript. Dr. Carl B. Schreck provided laboratory facilities for the implementation of the research and participated in the writing of each chapter. Dr. Jo-Ann Leong assisted in the design, analysis and writing of the molecular biology section of the study. Dr. Marta Alonso assisted in the data collection and analysis of the data generated in the molecular biology section of the study.

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DEDICATION

This thesis is dedicated to my parents Alicia and Wilfrido; my siblings, Rita, Alicia, Oscary, Alejandro and Conchita who were constantly present in my heart. To Pedro and Melesio, who dedicated their lives to democracy and justice for a better Mexico.

DEDICATORIA

Esta tesis esta dedicada a mis padres Alicia y Wilfrido; a mis hermanas y hermanos, Rita, Alicia, Oscary, Alejandro y Conchita quienes siempre estuvieron presentes en mi corazón, y por último a Pedro y Melesio quienes dedicaron sus vidas a la busqueda de democracia y justicia por un México mejor.

Sex Determination in Nile Tilapia, *Oreochromis niloticus*: Gene Expression, Masculinization Methods, and Environmental Effects

Chapter 1

Introduction

Sex differentiation results from a cascade of events that starts with the differential expression of one or more genes whose activity is either genetically controlled (genetic sex determination) or controlled by an environmentally-sensitive process (environmental sex determination) (Spotila *et al.*, 1994). The mechanism that regulates genetic sex determination has been extensively studied in mammals, in which the underlying developmental plan gives rise to females unless male development is imposed upon it. For several years it was accepted that this male pathway was controlled by a gene present on the Y chromosome; however, the location and nature of the specific testis determining gene remained unknown until Sinclair *et al.* (1990) identified the sex-determining region Y (*Sry* gene).

Among vertebrates, fishes have the highest variability of sex determining mechanisms, ranging from polygenic systems to socially and environmentally controlled (or at least influenced) mechanisms (Yamamoto, 1969; Bull, 1983; Conover and Heins, 1986). Different models have been proposed to explain sex determination in fishes, ranging from the traditional homogametic-heterogametic system to multifactorial autosomal systems (Trombka and Avtalion, 1993). However, the specific genetic element that controls sex determination and the components of the processes downstream of genetic

sex determination remain unknown. Most researchers have focused their efforts searching for distinctive genetic material that has been found in other vertebrates (specifically in mammals) with little success--e.g. *Sry*-like sequences occur in both male and female fish; (Tiersch *et al.*, 1992)--instead of looking for the specific gene products that occur during the course of sexual differentiation in both sexes.

The plasticity observed in sexual development in fish allows for manipulation of sex differentiation--in other words, the development of functional gonads specific to the opposite sexual phenotype can be accomplished despite the presence of the genetic material that determines sex (Fitzpatrick *et al.*, 1993). The fact that sexual differentiation can be controlled by exogenous treatment with steroids in several species led Yamamoto (1969) to hypothesize that steroids may be the natural inducers of gonadal differentiation. This sensitivity to steroid-induced sex inversion has been the basis for the development of techniques for the production of single sex populations in several fish species. However, this potency does not prove that steroids are the natural inducers of gonadal differentiation; nevertheless, it provides a powerful tool for exploring the physiological process of sex differentiation.

Background

Sex determination and sex differentiation in mammals. The fundamental embryonic program for sex differentiation in mammals is inherently feminine, with diversion to an alternative male pathway possible only through the presence of the Y chromosome (Nagai, 1992). Sexual differentiation results from a cascade of events that begins with

the establishment of genetic sex. Once the gonads are completely differentiated, they dictate the fate of secondary sex characteristics. In the presence of the male determining chromosome, hormones inhibit female differentiation, and promote male development (Jost *et al.*, 1973).

The location and nature of the specific mammalian testis determining gene remained unknown until recently. During the historical search for the sex-determining gene, several sequences have been proposed as potential candidates. Wachtel and Tiersch (1994) remarked that the most likely candidates were the determinant of serological histocompatibility (*H-Y* antigen), the *Bkm* satellite DNA, the zinc finger Y gene (*ZFY*), and the sex determining region-Y (*Sry*). In mammals, *Sry* is considered to be responsible for sex determination and gonadal differentiation. The *Sry* gene gives rise to the testis-determining factor (TDF) which triggers a sequence of events that leads to the emergence of Sertoli cells surrounding germ cells and later forming the seminiferous cords, as well as the appearance of Leydig cells responsible for the secretion of testosterone (Wachtel and Tiersch, 1994). The Sertoli cells also play an important role in sex differentiation by secreting the anti-Müllerian hormone, which causes regression of Müllerian ducts (Nagai, 1992).

The identification of the *Sry* gene has led to the discovery of a new transcription factor family: the *Sox* (Sry-like genes on the X chromosome) family. One of the members of the *Sox* family, the *Sox-9* gene, has been linked to testis determination in vertebrates (Da-Silva *et al.*, 1996; Kent *et al.*, 1996). Da-Silva *et al.* (1996) proposed that *Sox-9*

plays an essential role in sex determination, probably immediately downstream of *Sry* in mammals, and acts as a critical factor during differentiation of Sertoli cells in all vertebrates. Kent *et al.* (1996) proposed that this gene might play a broad role in the development of the urogenital system. Despite the advances towards understanding the mechanism that controls sex determination and gonadal differentiation in mammals, little is known about these mechanisms in birds, reptiles, amphibians, and fishes.

Sex determination and sex differentiation in fish. As mentioned previously, fishes demonstrate a great deal of variability in sex determining mechanisms including polygenic systems as well as socially and environmentally controlled (or at least influenced) mechanisms (Yamamoto, 1969; Bull, 1983; Conover and Heins, 1986; Solari, 1994). Researchers have applied the techniques and results of the search for the *Sry* gene to fish. However, the information generated is incomplete and, in fact, contradictory. Although specific DNA sequences involved in sex determination have not been found in fishes, several authors have proposed the presence of genetic material controlling this process by indirect procedures (Avtalion and Don, 1990; Mair *et al.*, 1991a,b; Pongthana *et al.*, 1995). For example, Avtalion and Don (1990) proposed a recombination model between sex-determining genes and the centromere, defining heterogametic maternal type (WY) and homogametic paternal type (WW) based on sex ratio data of three generations of diploid gynogenetic tilapias. Pongthana *et al.*, (1995) proposed female heterogamety for the silver barb, *Puntius gonionotus*. Nanda *et al.*, (1990) found that certain repetitive sequences are associated with sex differentiation in *Poecilia reticulata* and identified a pair of chromosomes as sex-related chromosomes.

At the chromosome level, fish have been poorly studied compared to other vertebrates. Solari (1994) pointed out that from all the teleostean species studied karyologically, only about 30 have shown differentiated sex chromosomes. Ojima (1983) summarized the several gonosomal systems that have been proposed for fish: XY-XX system; ZZ-ZW system; multiple gonosomal system ($X_1X_1X_2X_2/X_1X_2Y$); and the XX/XO system. However, the proposed chromosomal patterns are not always consistent even among individuals of the same species, while in others the suggested system leads to misinterpretations (reviewed by Solari, 1994).

In the search for sex-specific DNA sequences in fish, researchers have tried to detect specific *ZFY*, *Bkm*-like fragments, human telomeric sequences, or *Sry*-related genes linked to sex determination (Lloyd *et al.*, 1989; Wachtel *et al.*, 1991; Nanda *et al.*, 1992; Fukada *et al.*, 1995). In spite of the fact that some of the mentioned fragments where found in some species, the authors indicated that they were neither sex-specific nor distinctly defined. Since *Sry* genes are present in both males and females of most nonmammalian animals, Tiersch *et al.*, (1992) considered that *Sry* and *Sry*-related genes might be involved in the mechanism that defines sex by participating at some point in the process. In support of this theory, it has been suggested that several genes may modulate sex determination in fishes (Trombka and Avtalion, 1993).

Sex determination and sex differentiation in tilapias. Among teleosts, an understanding of the mechanism of sex determination and gonadal differentiation in tilapias remains

elusive. Trombka and Avtalion (1993) stressed that four main approaches have been used to elucidate sex determination in tilapias: interspecific and intraspecific crosses, sex inversion of fry by hormonal treatments, chromosomal manipulation leading to polyploidy and gynogenesis, and karyotyping and differential staining of genetic material. These approaches led to the postulation of different models: a) the dual sex-determining system (Hickling, 1960; Chen, 1969; Jalabert *et al.*, 1971) consisting of four chromosomes (X, Y, W, Z) and based on the presence of homogametic and heterogametic sexes among species; b) the autosomal influenced system (Avtalion and Hammerman, 1978; Hammerman and Avtalion, 1979), proposed as a three gonosome (W, X, Y) model in which autosomal factors influence sex determination; c) the multifactorial autosomal system (Majumdar and McAndrew, 1983; Mair *et al.*, 1987; Lester *et al.*, 1989; Mair *et al.*, 1991a; Wohlfarth and Wedekind, 1991), stressing the effects of interactions between multiple autosomal and sex chromosomal loci; and d) the environmentally influenced system (Mair *et al.*, 1991b; Baroiller *et al.*, 1995) supported by the influence of environmental factors, such as temperature and pH on sex ratios. Recent studies have strengthened the idea that sex differentiation in tilapias is partially under the influence of temperature (D'Cotta et al., 1999; Wang and Tsai, 2000). Furthermore, D'Cotta et al. (1999) indicated that masculinization of Nile tilapia by high temperatures may be linked to the restraint of aromatase expression--a process that may inhibit ovarian development and allow testicular differentiation. Although some evidence points toward a distinctive gonosomal strategy (Tromka and Avtalion, 1993), the sex-related chromosome model fails to explain certain results showing a variety of sex ratios obtained in tilapia progeny. The autosomal and environmental

influences proposed complicate even more the understanding of sex differentiation in tilapias.

The role of sex steroids in sex differentiation in fish. Sex steroids have been proposed as the main natural inducer of sexual differentiation (Yamamoto, 1969). This hypothesis has been supported by various authors that have altered gonadal differentiation by applying sex steroids at the time at which the primordial gonads start development. However, several questions have yet to be answered: 1) are sex steroids the first and only driving force that initiates sexual differentiation? 2) are they intermediary within the cascade of events? and 3) are exogenous sex steroids overriding the strength of a natural inducer?

Supporting Yamamoto's theory, endogenous sex steroids have been found in eggs and fry of the coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*), suggesting a maternal transfer (Feist *et al.*, 1990; Feist and Schreck, 1996). Changes in steroid concentrations during early development were proposed as an indication of metabolic processing by embryos (Feist *et al.*, 1990). In mixed sex populations of coho salmon at the time of hatching, they found a bimodal distribution in the concentration of sex steroids suggesting a potential participation during sexual differentiation as a driving force. Similar results have been found in tilapia fry, which display a bimodal distribution of testosterone concentrations in mixed sex populations, but unimodal distribution in 100% male populations (Rothbard *et al.*, 1987). However, some authors have pointed out that despite the presence of sex steroids during early development,

synthesis does not necessarily precede the onset of gonadal differentiation (Fitzpatrick *et al.*, 1993). Results obtained by van den Hurk and colleagues *in vitro* (1982) suggest that rainbow trout fry gonads start androgen synthesis shortly after the onset of gonadal differentiation, while estrogens appear to be synthesized several days later.

Fitzpatrick *et al.*, (1993) reported sexually distinctive patterns of steroidogenesis during the early stages of development of rainbow trout, showing sexual differences in the gonadal steroid production by two weeks after the onset of feeding, when male gonads produced more androstenedione and testosterone than female gonads. These authors suggested that differences in steroidogenic activity during early development may influence the time at which germ cells enter meiosis. They also suggested that since interrenal steroidogenic activity precedes that for gonads by several weeks, non-gonadal sources of steroids may be important. More recently, Yeoh *et al.* (1996a,b) proposed that steroid metabolism is an integral part of embryonic and postembryonic development, demonstrating the ability of steelhead trout (*O. mykiss*) embryos to synthesize steroid glucuronides from endogenous and exogenous sources.

It is still unknown if the concept proposed by Yamamoto (1969) of steroids as the natural inducers of gonadal differentiation in fishes is accurate. Recent experiments have yielded information supporting the presence of an androgen receptor in the ovaries of juvenile coho salmon (Fitzpatrick, *et al.*, 1994). Furthermore, Gale (1996) reported the existence of an androgen receptor in the gonadal cytosol of adult and juvenile Nile tilapia, showing binding characteristics of the compound that agreed with the

established requirements for steroid receptors. Androgen receptors in the gonads suggests the possibility that this tissue is the target of exogenous steroid treatment; however, presence of androgen receptors and their possible role in undifferentiated gonads is still unknown. It is then essential, to resolve the mechanisms by which steroids exert their function during sex inversion.

Natural sex reversal in fish. Sex differentiation in fish is a labile process that allows sex inversion in several gonochoristic and sex reversal in a few hermaphroditic species (Pandian and Sheela, 1995). Although the terms "sex inversion" and "sex reversal" have been used interchangeably by many authors, for the purpose of this discussion the definitions used by Green *et al.* (1997) will be used; "sex inversion" is defined as the process where the undifferentiated gonad is directed to a particular sex, and "sex reversal" as the induction of a differentiated gonad to become that of the opposite sex. The inherent capacity of fish germ cells to differentiate into either oocytes or spermatocytes constitutes a key factor allowing for functional sex inversion. Several studies have shown that both gonochoristic and hermaphroditic species possess germ cells that can play the role of either male or female sex cells. Brusle (1983) found that germ cells can migrate from the ovary to colonize the developing testicular region in the hermaphroditic teleost *Serranus hepatus*. In subsequent studies, Brusle (1988) described the primordial germ cells of the gonochoristic mugilid *Liza aurata*, the synchronous hermaphroditic serranid *Serranus hepatus*, and the protogynous hermaphroditic labrid *Coris julis*. Each species has germ cells with the same ultrastructural characteristics in undifferentiated gonads, immature and resting (or

active) ovaries and testes, which reveals their bipotentiality to differentiate into oogonia and spermatogonia. Brusle-Sicard *et al.* (1994) obtained similar results in the protandric hermaphrodite *Amphiprion frenatus.* In adult females of some species, the sexual bipotentiality of the germ cells is retained, since after ovariectomy, females can be sexually reversed to males by steroid treatment (reviewed by Pandian and Sheela, 1995).

Sex reversal is a natural process in hermaphroditic fish. However, little is known about the mechanisms that regulate the process and the factor(s) that modulate it. In simultaneous hermaphroditic species and protogynous and protandric species (with male and female gonadal tissue present), sex differentiation takes place during early stages of gonadal development, while other protogynous species such as *C. julis*, show a very distinctive pattern; female differentiation takes place in early stages and sex reversal takes place later (even years later) inside the ovary at the time of sex reversal. This implies that sexual differentiation takes place twice in the latter species, once early during development leading to the formation of the ovaries and again during adulthood when male differentiation takes place by sex reversal (Brusle, 1987). In such scheme, little is known about the mechanisms involved, or how two supposedly opposite endocrinological events can take place in the same tissue.

Sex determination, sex differentiation, and sex reversal are closely related processes. The general idea has been that sex determination is controlled by genetic material which is established at the fusion of the gametes. This genetic information triggers a cascade

of events modulating sexual differentiation--a process commonly perceived as the development of the undifferentiated gonad and the further emergence of secondary sexual characters particular to each sex. In the presence of a natural sex reversal inducer (which may have a genetic component), it can be hypothesized that sexual differentiation is being regulated by genetic material (even if the ultimate cue is environmental), either by exerting a driving force towards a distinctive sex and/or turning other genes off. In this respect, Solari (1994) mentioned that in sequential hermaphroditic species there is a process that allows the replacement of one type of gonad by another. This replacement is controlled by a genetic switch with different programs: one that initiates the development of the first sexual condition (male or female), and another that regresses the first gonadal tissue and induces the development of the second sexual condition.

Induced sex inversion in fish. The administration of natural and synthetic steroids during early development of fish has been successfully used to induce sex inversion in several species (see reviews by Schreck, 1974; Hunter and Donaldson, 1983), and has become a common practice in the production of single sex populations to enhance productivity in the aquaculture industry. Pandian and Sheela (1995) pointed out that protocols for the masculinization of 47 species and the feminization of 31 species have been developed so far, and more research in this area is currently on its way.
Several methods of steroid administration have been developed, including systemic transfer via injection or silastic implantation, dietary supplementation, and immersion (Pandian and Sheela, 1995). Oral administration of steroids via feeding has become the

most commonly used technique worldwide. Several authors have reported successful masculinization of tilapia fry by oral administration of 17α-methyltestosterone; however, this technique is sometimes not completely successful and may expose workers and untargeted organisms in the environment to risk of steroid contamination (reviewed in Green, *et al.*, 1997).

Sex inversion by immersion of fry in steroid solutions has been extensively used in salmonids (Hunter and Donaldson, 1983). Feminization of coho salmon (*O. kisutch*) and chinook salmon (*O. tshawytscha*) has been successfully obtained by immersions in estradiol-17β (Hunter *et al.*, 1986) and ethynylestradiol-17α (Pfierrer and Donaldson, 1989; 1992), while masculinization has been obtained in coho salmon by using methyldihydrotestosterone (Pfierrer and Donaldson, 1991). Recently, Gale *et al.* (1999) provided promising results in the use of the immersion technique for masculinizing tilapia. However, the applicability of this technique at the production level remains to be demonstrated.

Until now, the conventional approach for revealing the mechanism of sex differentiation in fish has focused on searching for sexually-distinct genetic material that has been found in mammals or other vertebrates. These efforts have met with little success. The development of new techniques in molecular biology, such as suppression subtractive hybridization which has been used to identify unique mRNAs (Diatchenko *et al.*, 1996) and the production of single sex populations through steroid treatment, creates an opportunity to describe some of the major steps of sex differentiation in fish. The

subtractive hybridization technique compares two populations of mRNA and identifies differentially expressed mRNA transcripts. Jin *et al.* (1997) emphasized that this technique can be used to isolate genes that are expressed as a result of differences in developmental stages or tissues responding to exogenous stimulation. Applying this technique to androgen-immersed genotypic female tilapia, the genes turned on (or off) during sex inversion may be identified. Furthermore, once these gene products are identified, molecular probes can be developed to determine if sex-specific and tissue-specific expression occurs during natural sex differentiation.

Goals and Objectives

The goals of this study are to understand the mechanism of sex differentiation of Nile tilapia, to document the fate of masculinizing compounds in the pond environment, and to develop a reliable and safe sex inversion technique that results in the production of all male populations.

Objectives:

1) To determine the fate of the masculinizing agent 17α-methyltestosterone in the pond environment during masculinization by oral administration.
2) To determine the masculinizing efficacy of 17α-methyltestosterone oral administration under different environmental conditions.
3) To determine the period of time when Nile tilapia fry are susceptible to the masculinizing effects of immersions in synthetic steroid treatment.

4) To determine optimal conditions for successful masculinization of Nile tilapia fry by short term immersions in synthetic steroids.
5) To determine if short term immersions of Nile tilapia fry in synthetic steroids result in unique expression of messenger RNA associated to sex differentiation.

Organization of the Thesis

This thesis is organized into seven chapters. After the introductory Chapter I, Chapter II examines the fate of 17α-methyltestosterone (MT) in the pond environment. Results indicate that MT can be measured in the pond environment persisting for a short-time in the water, but remains detectable for a long-time in the sediments. In this chapter, a method based on activated charcoal is proposed for MT capture and elimination from the water used for sex inversion. Chapter III examines the masculinizing efficacy of oral administration of MT to Nile tilapia fry under different environmental conditions. In this study, treatments resulted in low masculinization in jars and aquaria that contained soil or gravel that had not been previously exposed to steroids, as well as those systems that lacked substrate. When sediments were reused, the efficacy of the hormonal treatment improved significantly. Dietary treatment with MT in systems which had charcoal filtration of water resulted in almost complete masculinization of all broods tested. Chapter IV examines the potential for masculinizing Nile tilapia by short-term immersions in synthetic steroids as an alternative to oral administration of MT. The results indicate that time of immersion, length of the treatment, dosage, density, solvent vehicle, and number of immersions significantly affected the efficacy of immersion treatment. The labile period for masculinization of Nile tilapia fry by

immersion reared at 28 °C lasts 5 to 6 days and the masculinizing efficacy of the steroids was: trenbolone acetate > methyldihydrotestosterone > norgestrel > methyltestosterone. Chapter V investigates the potential interactions between treatment factors as well as the effects of multiple immersions in steroids during masculinization of Nile tilapia using short-term immersions. The results of this study suggest that the combination of immersions at 11 and 13 days post-fertilization (dpf) in steroids resulted in more males than those at 13 and 14 dpf. The influence of density, length of immersion time, steroid dosage, and their interactions was explored using a fractional factorial design. Despite significant masculinization results, the fractional factorial experiment indicates that masculinization rates are highly variable and the combination of dosage, duration and density does not show a consistent masculinization pattern. Chapter VI examines whether specific mRNAs activated after sex inversion with the synthetic steroid trenbolone acetate. Results obtained from mRNA collected 16 hrs after treatment suggest that short-term immersions of tilapia fry in trenbolone acetate induce the expression of unique genes. This study allowed the identification of 12 genes related to reproductive tissues; seven of which have unique or enriched expression in the testes. Chapters II through V are all presented in manuscript form according to the specifications of the journal "Aquaculture". Chapter VI is presented in manuscript form according to the specifications of the journal "General and Comparative Endocrinology". Chapter VII is a general discussion of the results found in this study.

Chapter 2

Masculinization of Nile Tilapia, *Oreochromis niloticus*: I. Fate of the Masculinizing Agent 17α-Methyltestosterone in Model Pond Environments[1]

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Abstract

The following study tested the hypothesis that 17α-methyltestosterone (MT) persists in the environment after its use for masculinizing Nile tilapia, *Oreochromis niloticus*. Fry were treated with different doses of MT for four weeks beginning at the initiation of feeding. Model ponds consisted of 3.7 l jars or 50 l aquaria containing either 0.5 or 5 kg of soil, respectively. Water and soil samples were taken before the onset of treatment and weekly beginning on the last day of treatment (water samples were also taken weekly during the four week treatment period). Concentrations of MT were determined by radioimmunoassay, which revealed that the levels of immunoreactive MT in the water peaked between approximately 1 and 3.6 μg/l about 14 to 28 days after the onset of feeding. When sediments were present, the concentration of MT in water decreased to background level by 35 days after the onset of feeding (1 week after the end of treatment with MT-impregnated food). In contrast, the levels in the soil were 2.2 to 16 μg/kg at 29 days after the onset of feeding with MT-impregnated food and remained detectable in the soil at between 0.8 and 1.6 μg/kg through 49 days (3 weeks after ending treatment with MT-impregnated food) in one experiment and around 1.9 μg/kg at 113 days after the end of MT administration in another experiment. The concentrations of MT measured in water and sediments varied between experimental environments. These results suggest that MT persists in sediments for at least three and a half months after cessation of MT treatment, which raises the possibility that unintended exposure to MT may occur. When charcoal filtration was added to the tanks, MT levels in water were significantly lower than those systems without filtration (i.e. 12 *vs.* 5,000 pg/ml on day 29 after initiation of feeding). Charcoal filtration can be an efficient system for reducing MT from the water.

Introduction

The administration of natural and synthetic steroids during early development of fish has been successfully used to induce sex inversion in several species (see reviews by Schreck, 1974; Hunter and Donaldson, 1983), and has become a common practice in the production of single sex populations to enhance productivity in the aquaculture industry. Among the techniques developed, oral administration of steroids via feeding is most common. In tilapia culture, the production of all-male populations through treatment of fry with 17α-methyltestosterone (MT) impregnated food has become a widespread practice. All male populations have greater growth potential because less energy is shunted toward reproduction and no competition with younger fish occurs (Green *et al.*, 1997). Despite the success of this technique, significant "leakage" of MT into the pond environment may occur from uneaten or unmetabolized food. This leakage poses a risk of unintended exposure of hatchery workers as well as fish or other non-target aquatic organisms to anabolic steroids if MT persists in the environment after treatment of tilapia fry. Furthermore, in some countries, pond sediments are dredged and sometimes used to prepare soil for crop production thereby spreading the risk of exposure to MT to terrestrial systems and to other aquatic systems.

Surprisingly, despite the wide use of MT for masculinizing tilapia in aquacultural facilities, no published literature exists on MT levels in water or sediments from systems that use this sex inversion protocol and little is known about the fate of this potent synthetic steroid in the pond environment. A few studies have been dedicated to detect MT or its metabolites in body tissues of the sex-inverted fish (Goudie *et al.* 1986a and 1986b; Cravedi *et al.*, 1989; Curtis *et al.*, 1991); however, almost no effort has been made to determine if MT or its metabolites can dissociate from the

impregnated food and accumulate in the pond environment. Some researchers have warned about unintended effects of steroid administration, such as fish to fish transfer of steroids (Budworth and Senger, 1993), biased sex ratios in untargeted organisms (Gomelsky *et al.*, 1994; Abucay and Mair, 1997; and Abucay *et al.*, 1997), and paradoxical feminization (Piferrer and Donaldson, 1991; Piferrer *et al.*, 1993; Rinchard, *et al.*, 1999; Eding *et al*, 1999). If MT is being added to the food in such amounts that efficiently masculinizes fish despite steroid loss to the environment, then determining the fate of MT in semi-closed systems such as ponds will yield important information on both safety and efficacy of MT use for masculinization. To determine if MT separates from the food and potentially remains within the pond environment, the following study was undertaken using model pond systems as microcosms.

Materials and Methods.

Fish rearing and breeding. Studies were conducted at the Oregon State University's Fish Performance and Genetics Laboratory, Corvallis, OR. Adult Nile tilapia, *Oreochromis niloticus*, were separated by sex in 1,100 l tanks supplied with constant flow of recirculating water. Breeding families were placed in 200 l aquaria (one male to three females), and checked every 2 hours for spawning activity. Once breeding occurred, the other fish were removed and the brooding female was left to incubate the progeny. On 10 days post-fertilization (dpf), fry were collected from the tank (if the female released the fry from her mouth) or from the female, that was forced to release the fry. Water temperature in all systems was maintained at 28 ± 1°C.

Experimental designs. Model ponds were set up 2 days before the expected time of fry release. Only fry from individual broods were used in each experiment. Experiments I and II were conducted to determine the fate of MT in water and

sediments in model ponds. To test for possible long-term accumulation of MT, sequential feeding trials were conducted in aquaria (Experiment III). Experiment IV was designed to determine the effects of MT elimination from water by charcoal filtration.

Single Trial in Jars (Experiment I). Each experimental unit consisted of a 3.8 l jar in which 500 g (3 cm deep) of packed soil were placed. The surface area of each experimental unit was 156 cm^2. The soil was obtained from one of the dry Soap Creek ponds (Oregon State University) located north of Corvallis (Class: Clay; 55.4% clay, 9.9% sand, 34.9% silt). Each model pond contained 3 l of dechlorinated tap water (16.5 cm deep). Fry were randomly assigned to the model ponds at a stocking rate of 47 fry/container (1 fry/3.3 cm^2 which corresponds to a recommended stocking rate for masculinization of 3,000 fry/m^2 [Popma and Green, 1990]). The following experimental groups were included: MT fed fish (at 60 mg/kg food), control fed fish (EtOH-treated food), MT food with no fish present (at 60 mg/kg food), and control food with no fish present. All treatments were triplicated.

Single Trial in Aquaria (Experiment II). Each model pond consisted of a 50 l aquarium and all aquaria were stocked with fry. Aquaria contained either: a) soil (fed MT or control food); b) gravel (fed MT); c) no substrate (fed MT). Five kg (3 cm deep) of packed soil were placed in each of the two tanks containing soil (a) and five kg (2 cm deep) of gravel were placed in the tank containing gravel (b). Soil was obtained from a meadowed hill near Corvallis (Class: Clay; 53.8% clay, 22.6% sand, 23.8 silt). The surface area of each experimental unit was 1,858 cm^2. Each model pond contained 40 l of dechlorinated tap water (22.8 cm deep). Fry were randomly assigned to the model ponds at a stocking rate of 200 fry/tank. Each treatment

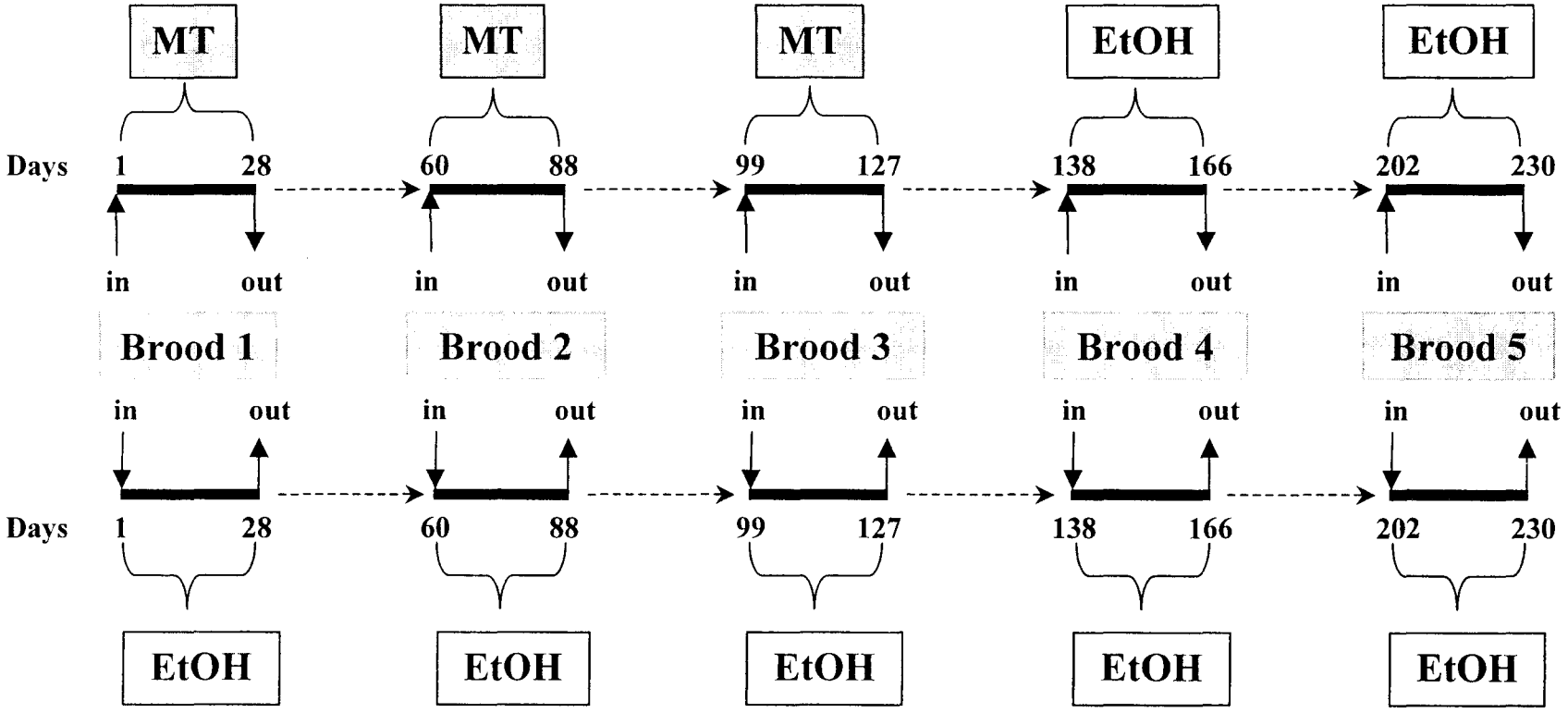
consisted of one tank, because the stocking rate utilized almost all the fry from the single brood.

Sequential Trials in Aquaria (Experiment III). Each experimental unit consisted of a 50 l aquarium (2 replicates per treatment). Five kg of packed soil from a meadowed hill (3 cm deep) were placed in each tank, and tanks were filled with 40 l of dechlorinated tap water (22.8 cm deep). Figure 1 depicts this experimental design. In the MT treatment tanks, three sequential separate broods of fry were fed with MT-treated food. Thirty-two days elapsed between the first and the second MT feeding trials and 11 days elapsed between the second and the third trials. Following the last MT treatment trial, two sequential separate broods of fry were fed EtOH-treated diet in the MT treatment tanks with 11 days elapsing between the last MT trial and the first EtOH-treated diet and 36 days elapsing between the first EtOH-treated diet and the second. The positive control treatment consisted of a group of fish from the same brood fed EtOH-impregnated diet at each feeding trial. The number of fish per replicate for each trial were: 175, 100, 120, 200, and 120, respectively). At the end of the feeding treatment, fish were removed to a grow-out facility.

Aquaria with Charcoal Filtration (Experiment IV). Each experimental unit consisted of a 50 l aquarium without sediments. Each tank was equipped with a filtration system (Whisper® 1; Tetra/Second Nature). This system has an average filtration capacity of 6 l/min and uses a mechanical filtration system based on a Bio-bag® cartridge made of thick floss and contains on average 19 grams of Ultra-Activated™ carbon. Two treatments were included: MT fed fish (at 60 mg/kg food) and control fed fish (EtOH-treated food). Experimental treatments were triplicated; n = 50 fish per replicate for each trial. Bio-bags were cleaned with pressurized tap water twice a week. After the

Figure 1. Schematic representation of the experimental design for evaluating the effects of MT accumulation in sediments (Experiment III). Two tanks were assigned to the MT treatment and two to the control treatment. Nile tilapia fry from a single female (brood) were randomly assigned to each tank at 15 dpf. Fish were introduced to the tanks ("in" arrow) and fed with MT or EtOH vehicle impregnated food for 28 days (indicated by solid line). After treatment, water was removed from the tanks, the fish were taken out of the treatment tank ("out" arrow), and moved into a grow-out system. Sediments were kept and tanks refilled with clean water. Dashed arrows indicate the time elapsing between the end of one feeding trial and the beginning of the next. The time at which each trial was conducted is indicated by the numbers above lines. After three groups of fry were fed MT in the treatment tanks, two consecutive broods were fed control feed (EtOH).

Treatment group



Control Group

Figure 1

28 days of steroid administration, the fish were removed from the experimental tanks into the grow-out system. Water and Bio-bags were sampled for MT.

Feeding and water quality. MT-impregnated food was made by spraying crushed flaked (Natureboy™) food with MT dissolved in EtOH; control food was made by spraying crushed flaked food with EtOH. All food was allowed to dry overnight in a forced-air hood. Fry were initially fed with Hatchfry Encapsulon™ (Argent Chemical Laboratories) until 15 dpf. In all experiments, fry were fed MT (60 mg/kg) or control diet for 4 weeks (from 15 to 43 dpf). Feeding rate was at 20% per calculated body weight for the first 14 days of treatment and then 10% per calculated body weight from days 15 to 28 of treatment. Water temperatures in the model ponds were maintained at 28 ± 2°C, except for the first week of Experiment III when water temperatures were 25 ± 1 °C. Temperature was monitored daily; pH, ammonia, nitrites, and dissolved oxygen were checked weekly. Half the water was exchanged twice per week throughout the period of dietary treatment.

Water and sediment sampling and processing. Twenty five milliliters of water were collected with pipettes at each sampling time, placed into 50 ml Falcon tubes and stored at -20°C until analysis for MT. At each sampling time, one (Experiment I) or eight (Experiments II and III) random soil core samples were collected with 1.9 cm diameter PVC pipes (Experiment I) or 0.5 cm diameter acrylic tubes (Experiments II and III), placed in whirl-pak® bags; excess water was collected into a separate 50 ml Falcon tube (Experiment I), or poured off (Experiments II and III) and the bags stored at -20°C until analysis. The excess water (called 'interface' hereafter) samples were stored frozen at -20°C. All samples were collected in the mornings before any feeding had taken place. For analysis of MT concentration, 1.0 ml of each water and interface sample and 0.2 g of each soil sample were extracted in 8 ml of diethyl ether, then the

extract was collected into new tubes after the aqueous phase was snap frozen in liquid nitrogen. The extraction procedure was repeated and the combined ether extracts were dried down in a SpeedVac. Each dried extract was reconstituted in 1 ml of phosphate-buffered saline containing gelatin. Aliquots of the reconstituted extracts were removed to 12x75 mm tubes for determination of MT concentration by radioimmunoassay (RIA). The RIA methods followed the procedure outlined in Fitzpatrick et al. (1986 and 1987). Antisera specific to MT (UCB-Bioproducts SA) and ^{3}H -MT (Amersham) were generously donated by Dr. Gordon Grau of the Hawaii Institute of Marine Biology. Standards of known concentration of MT were made in EtOH and used in each assay to generate a standard curve. The assay was validated by demonstration of parallelism between serial dilutions of several samples and the standard curve, and by demonstration of low cross-reactivity with testosterone (6.5%), dihydrotestosterone (2.8%), nortestosterone (0.8%), and eight other steroids that showed < 0.1% crossreactivity. Extraction efficiency for MT for the RIA was checked by adding a known amount of ^{3}H-MT to water, soil, and interface samples (n=6 for each), and then extracting the samples as described above. Once each of these tubes was reconstituted in 1 ml of Phosphate-buffered saline containing gelatin, 0.5 ml was removed from each and the amount of radioactivity counted by scintillation spectroscopy (extraction efficiencies were 80.4% for water, 78.2% for soil, 66.9% for interface, 47.8% for Bio-bag, and 25.9% for filter charcoal). To check efficiency of the assay, sediment samples were spiked with 100 pg of MT and processed for RIA as above. The calculations performed indicated that 100% of the steroid added was measured by the assay. The lowest detectable value by the RIA was 2.5 picograms per tube. Each sample was run in duplicate.

Leakage timing: To determine the pattern of MT dissociation from the food (leakage) in a 50 l tank with sediments (Experiment III), water samples were collected at 0, 1, 5,

15 and 30 minutes after MT-feeding. Samples were collected 2 inches below the surface and special attention was made to avoid collecting any food particles in the sample. A similar sampling was performed in a tank with no sediments, but had charcoal filtration (Experiment IV) at times 0, 1, 5, 10, 25, 60, 120, and 900 minutes.

Background levels analysis. Control samples analyzed by RIA had a significant level of background, with particularly high amounts in sediments. To determine the characteristics of this background, sediment samples from different treatments were fractionated by using High Performance Liquid Chromatography (HPLC). Fractions were collected every 30 seconds (n = 60/sample) and analyzed by RIA. The analyzed sediments were collected from the following treatments: 1) sediments from a model pond before initiation of treatment; 2) sediments from control treatment after 28 days of feeding; 3) sediments from MT treatment after 28 days of feeding. To determine the radiochemical purity of the labeled MT (^{3}H-MT) and evaluate the time at which ^{3}H-MT elutes from the HPLC gradient, 50 µl of ^{3}H-MT stock solution (~ 170,000 CPM) were fractionated at 30 sec intervals using HPLC. The amount of tritium recovered in each fraction was counted on a Packard scintillation spectrophotometer. Sediment samples for HPLC were extracted in diethyl ether as described above, reconstituted in 1 ml of methanol, vortexed, and filtered through 0.45 µm Acrodiscs (Gelman Sciences, Ann Arbor, MI). The methanol was evaporated, and the dried extract reconstituted in 150 µl of mobile phase and injected onto the HPLC. The HPLC methods followed the procedure outlined in Huang *et al.* (1983) and modified by Feist *et al.* (1990). The HPLC analysis was performed using a Waters System consisting of a 600 controller, 717 autosampler, 996 photodiode array detector, a Dell Dimension V400c computer, Millenium PDA software, and a reverse phase C18 column (flow rate 0.4 ml/min). We used an isocratic mobile phase of water:methanol:acetonitrile:isopropanol (62:28:5:5) followed by a linear gradient

(3.3%/min) of water:methanol:butanol (35:45:20) for 30 minutes monitored at a wide variety of wavelengths, but specifically analyzed at 254 and 280 nm. This system allows for the separation of 18 steroid standards with detection limits of 3 ng for each steroid.

Concentrations of MT in water, soil, and interface at the various sample times were not compared statistically because of the limited sample size (n = 1 - 3 per date) and because the goal of the study was descriptive (presence/absence).

Results

Background levels. Injection of standards through HPLC showed that the elution time for MT lies between minutes 22 and 23 (MT elution time hereafter). The radiolabeled MT showed a single large peak of counts at the MT elution time, indicating the radiochemical purity of ^{3}H-MT. This peak accounted for 75% of the total radioactivity analyzed. A much smaller peak appeared at minute 2 (3.7% of the total counts), before any steroid elution time; this second peak is characteristic of counts produced by free tritium. The other 21.3% of the counts was evenly distributed among the rest of the fractions (Fig. 2a). Background values measured by RIA after HPLC fractionation of a pre-treatment control sediment sample indicated that the antibody crossreacted with an unknown compound that appears in the HPLC fractions at the same time as MT does. The background detected in the control sample at this time accounted for 50.2% of the total MT measured, the rest of the immunoreactivity was evenly distributed among the other fractions (Fig. 2b). The HPLC analysis of sediments collected from a control treatment with fish fed with ethanol-impregnated food showed two distinct small peaks: one in fractions collected at minute 21 (37.9% of total) and the other at the MT elution time (40.4% of total), with the rest of the

Figure 2. HPLC radiochromatogram of ^{3}H-Methyltestosterone (a) and immunoreactivity of the MT antibody expressed as pg/g of MT after HPLC fractionation of samples from pre-treatment sediments (b), sediments collected after 28 days of feeding control food (c), and sediments collected after 28 days of feeding MT-impregnated food (d). U1-U5 indicate unknown compounds detected in sediments from the MT-treatment tank.

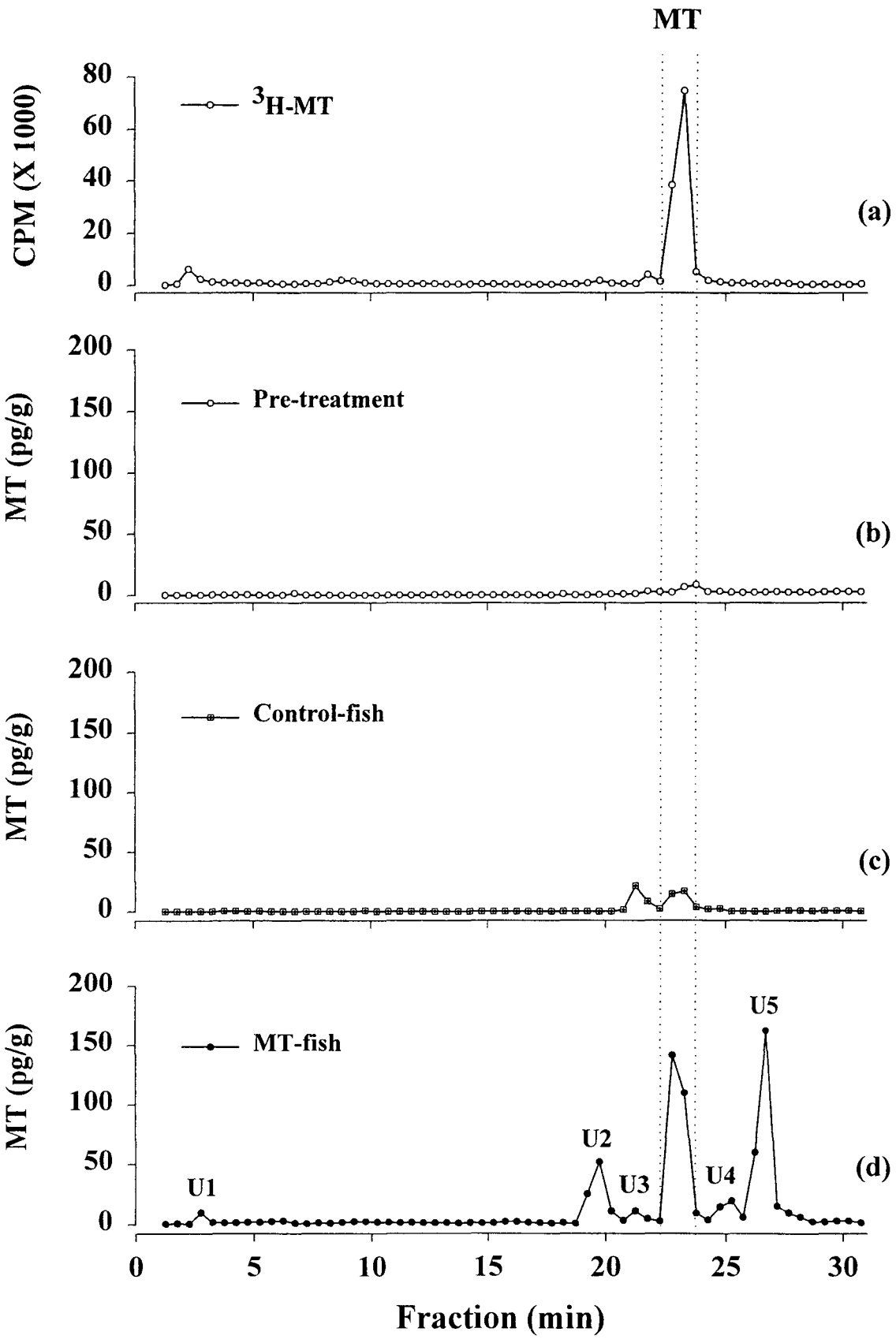

MT
CPM (X 1000)
80
60
40
20
0
^{3}H-MT
(a)
MT (pg/g)
200
150
100
50
0
Pre-treatment
(b)
MT (pg/g)
200
150
100
50
0
Control-fish
(c)
MT (pg/g)
200
150
100
50
0
MT-fish
U1
U2
U3
U4
U5
(d)
0
5
10
15
20
25
30
Fraction (min)

Figure 2

immunoreactivity (21.7%) evenly distributed among the rest of the fractions (Fig. 2c). Levels of MT summed across all fractions from pre- and post-treatment control sediments averaged 399 pg/g ± 27.3 (SE). This value was similar to those obtained from the RIA of whole sediment samples from similar dates (mean = 386.3 pg/g ± 47.3). A different profile was obtained from sediments collected in a tank with fish fed MT (Fig. 2d). Three large peaks were detected at minutes 18 (unknown 2; 11.9% of total), 22-23 (MT; 35.3% of total), and 26 (unknown 5; 34.3% of total). Three small peaks were also detected at minutes 2 (unknown 1; 0.3% of total), 21 (unknown 3; 2.6% of total), and 25 (unknown 4; 5.4% of total). When compared to the elution time of the standards, the peak that appears at minute 21 in sediments from both control and MT-fed fish treatments coincides with the elution time of the testosterone standard. All other peaks did not correspond to any of the remaining standards. The sum of the steroid-like peaks accounted for 89.5% of the total MT detected. The sum of all fractions provide an estimate of MT of 5,559 pg/g while direct measurement of soil samples without fractionation estimated 10,365 pg/g, suggesting that 46% of the MT is lost during the microfiltration process. Based on the HPLC data, hereafter we will refer to the total immunoreactivity to the antibody as "MT concentrations", understanding that part of the immunoreactivity may be due to crossreactivity with other steroids and/or metabolites.

Leakage timing: The use of MT-impregnated food resulted in rapid leakage of MT into the water and subsequent deposition into the sediments. Within 1 minute of adding MT-impregnated food to the water, the levels of MT in the water reached nearly 100 pg/ml. Values increased to 150 pg/ml within 5 min and to 170 within 15 min of feeding. At 30 minutes, levels of MT in the water were around 180 pg/ml (Fig. 3). Since the fish received ¼ of a day's ration (nominally 17 μg of MT) the detected

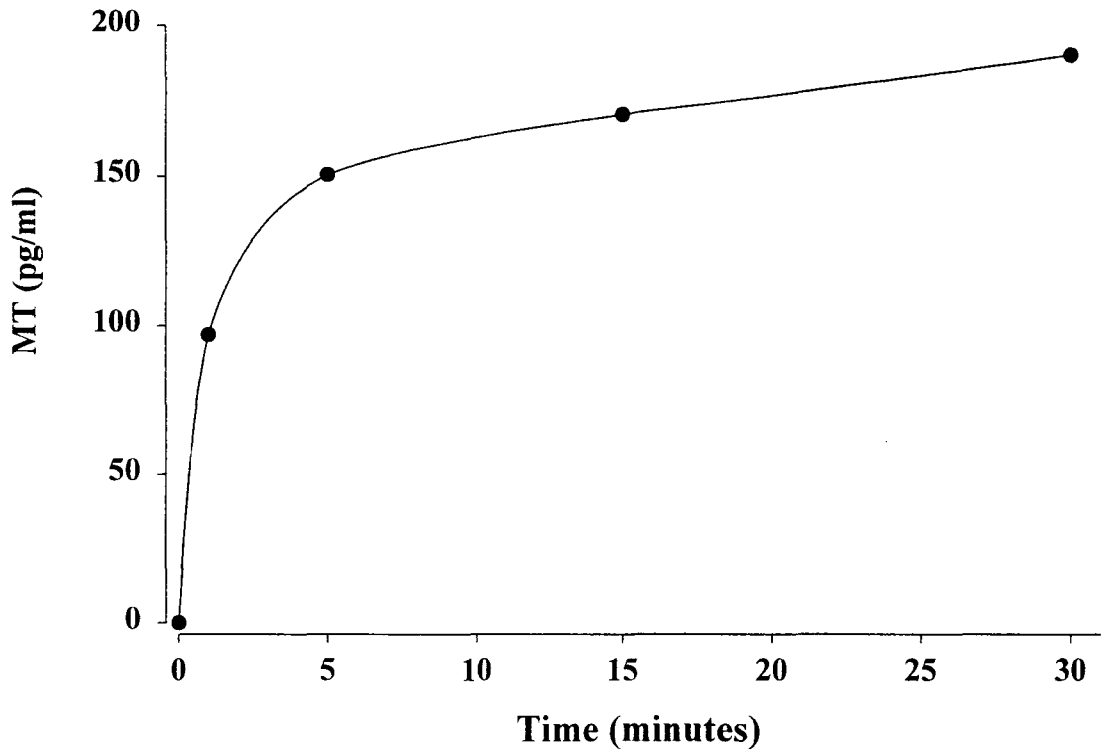


Figure 3. Time course of the concentration of methyltestosterone (MT; single observations) in water before (time 0) and after administration of MT-impregnated food in a tank that had sediments.

value at 30 minutes (7.2 μg in the tank) represents 42.3% of the total steroid administered.

Single Trial in Jars (Experiment I). All sampled components (water, sediments and interface) had MT background levels that remained relatively stable throughout the experiment. Water, sediments and interface from the model ponds containing fish and fed control food had a background average of 24 ± 9 pg/ml, 282 ± 91 pg/g, and 77 ± 12 pg/ml, respectively (Fig. 4a). A similar pattern was observed in the ponds that had no fish and control food was added (data not shown). Figure 4b depicts mean values measured from the model ponds containing fish fed with MT-impregnated food. Mean MT levels (803 ± 253 pg/ml), in the water showed a pronounced increase after 8 days of MT administration and remained elevated for the next seven days, before dropping to 185 pg/ml on day 22. MT levels in water (45.7 ± 6.9 pg/ml) decreased to near background one day after the end of MT feeding and from this day onward, water values were similar to background levels (16 ± 4 pg/ml). Sediment samples had 1408 ± 500 pg/g at the end of the MT feeding period. These values remained elevated for three weeks after cessation of MT administration. A similar pattern was observed in the interface, with levels averaging 188 ± 70 pg/ml. In jars that contained no fish but received the same amount of MT (Fig. 4c), the average level of MT in the water was 2,668 ± 710 pg/ml at the end of week one, 2,284 ± 954 pg/ml at the end of week four, and returned to pretreatment levels by week five. Mean levels of MT in the soil for this treatment was 5,198 ± 1,566 pg/g of soil at the end of week four, increased to 26,605 pg/g at the end of week five, and remained around 9,000 pg/g through weeks six and seven. Variation in the levels of MT measured in the soil samples was considerably higher than the variation detected in water and interface samples for all treatments. For this experiment, fry were fed a calculated total amount of 488 μg of MT. The estimated amount of MT remaining in the sediments at the end of feeding

Figure 4. Background levels of MT (Mean ± SE) in water, sediments and interface in model ponds containing fish fed with control food (a) and MT concentration (Mean ± SE) in water, sediments and interface from model ponds that received MT-impregnated food and contained fish (b), and tanks that received MT-impregnated food but contained no fish (c).

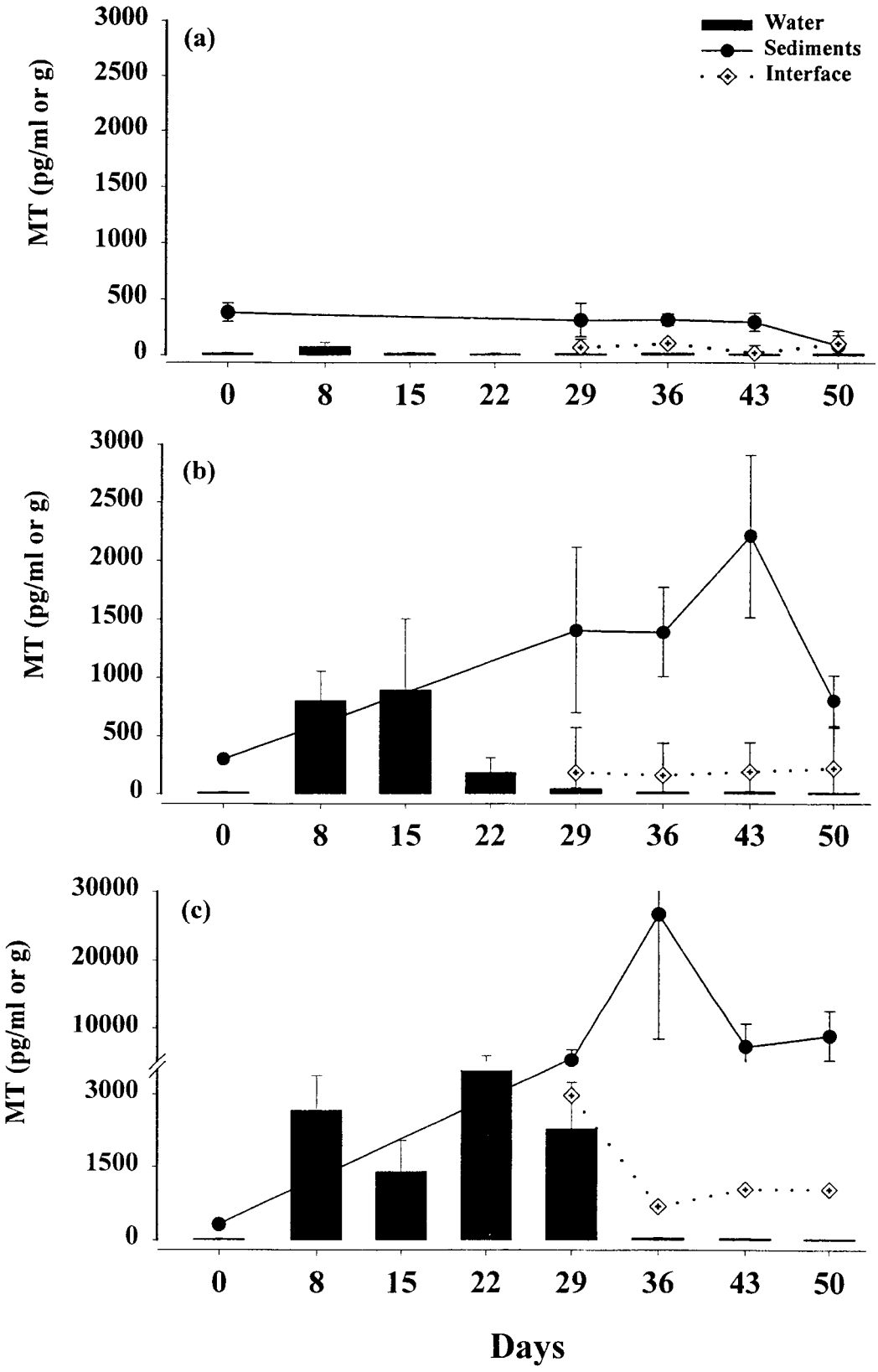

(a)
(b)
(c)
Water
Sediments
Interface
MT (pg/ml or g)
3000
2500
2000
1500
1000
500
0
30000
20000
10000
0
8
15
22
29
36
43
50
Days

Figure 4

was 0.7 μg, corresponding to 0.15% of the total steroid administered. In the absence of fish, 2.6 μg of MT were detected in the soil at the end of feeding (0.53% of the total MT administered). Three weeks after cessation of feeding, 0.90% (4.4 μg) of the total MT administered was still detectable in the soil from this treatment.

Single Trial in Aquaria (Experiment II). Background levels of MT in sediments (433 ± 42.4 pg/g) were higher in this type of soil (meadowed hill) than in the previous experiment (soap creek ponds); however, the values had a similar pattern. Background levels of MT in water samples from this experiment were similar to those obtained previously (15.2 ± 1.7 pg/ml). MT concentration in sediments at the end of MT feeding was 6,097 pg/g. Thereafter, MT remained high for two more weeks, dropped to 2810 pg/g three weeks after cessation of the treatment, and remained unchanged for the remaining two weeks of the experiment (Fig. 5a). MT levels in the water were relatively low in all model ponds where fish were subjected to MT feeding during the first three weeks (102, 70, and 216 pg/ml in tanks with soil, gravel and no substrate, respectively; Fig. 5b). However, significant peaks of MT in water were detected one day after the last MT feeding in all treatments (4,971, 3,616, and 504 pg/ml for no substrate, soil and gravel, respectively). Two weeks after cessation of feeding, MT values in water dropped to background levels in the tanks with soil and gravel. In the tank with no substrate, MT dropped about 50% every week, reaching 138 pg/ml five weeks after cessation of treatment. In this experiment, a calculated total amount of 2,078 μg of MT was used, and the estimated amount of MT remaining in the soil at the end of feeding was 30 μg, which corresponds to 1.5% of the total steroid administered. Similar values were detectable eight weeks after cessation of feeding.

Figure 5. Changes in methyltestosterone (MT) concentration in sediments (a) from tanks containing fish that received control food (solid circles) or MT-impregnated food (open circles) for 28 days, and water (b) from tanks that had soil and received control food (solid circles) or MT-impregnated food (open circles), tanks with fish that received MT-impregnated food and had gravel as substrate (open diamond) or no substrate (solid triangle).

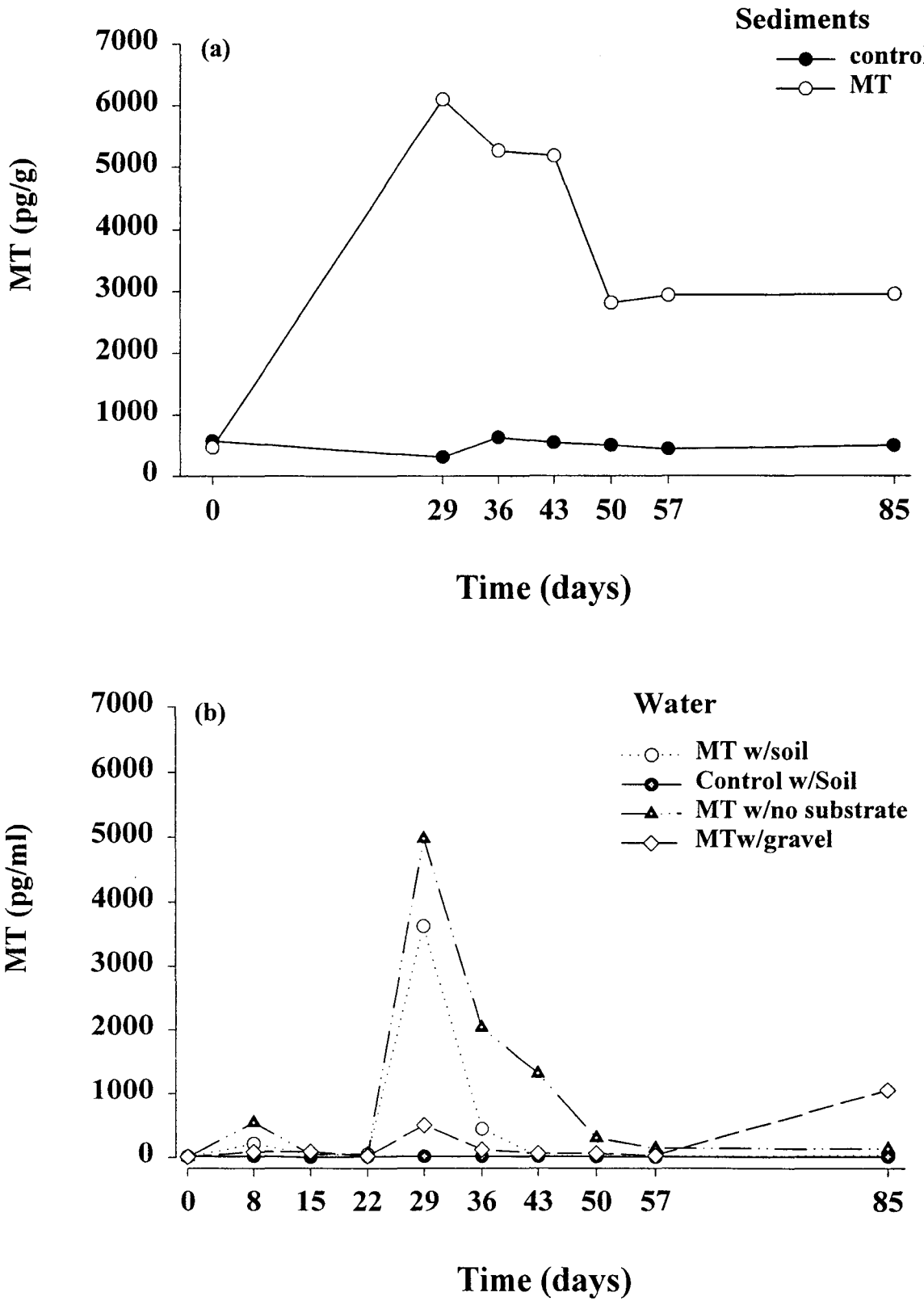
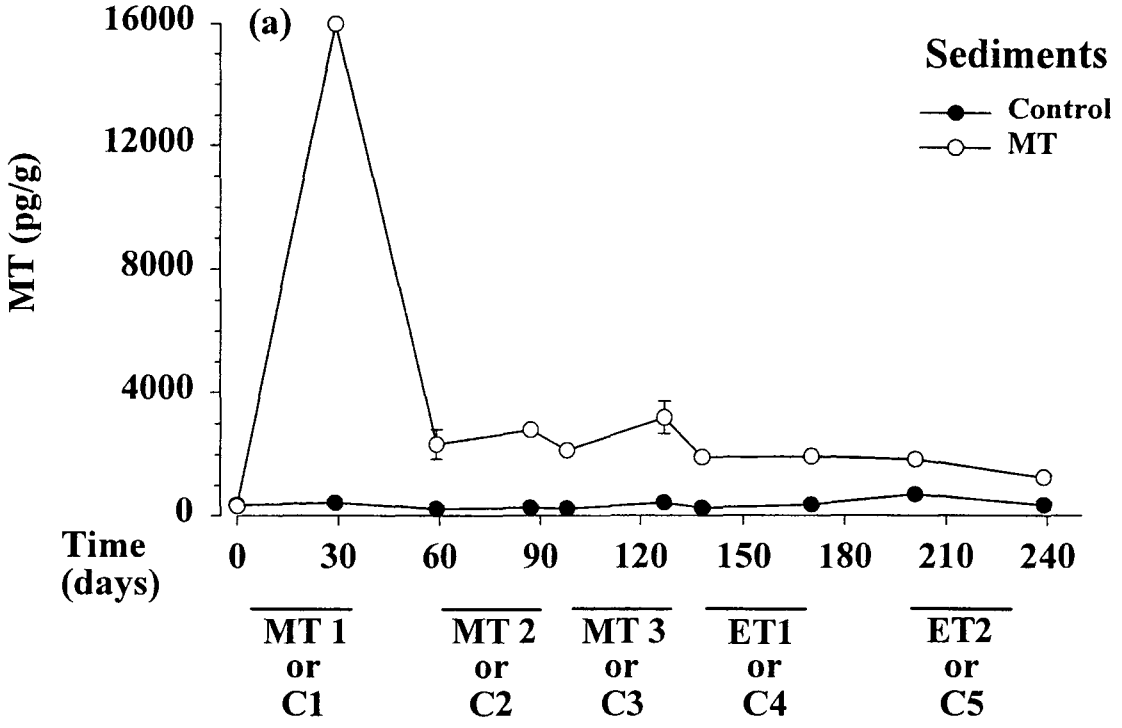

Sediments
control
MT
(a)
MT (pg/g)
7000
6000
5000
4000
3000
2000
1000
0
0
29
36
43
50
57
85
Time (days)
Water
MT w/soil
Control w/Soil
MT w/no substrate
MTw/gravel
(b)
MT (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
0
8
15
22
29
36
43
50
57
85
Time (days)

Figure 5

Sequential Trials in Aquaria (Experiment III). Background levels in this experiment were similar to those recorded in Experiment II. Mean levels of MT in the soil were elevated to 15,976 pg/g after one feeding cycle, then decreased to 2,332 after 58 days of treatment and remained elevated thereafter between 1,265 and 3,193 pg/g through 3 months after the conclusion of the last MT feeding cycle (Fig. 6a) including the time during which the control-fed fry were raised in these tanks. Mean levels of MT in the water were elevated for the first 60 days of the experiment, ranging between about 234 and 1,179 pg/ml (Fig. 6b). MT levels in water dropped to background and remained near background through the rest of the experiment. In this experiment, a calculated total amount of 7,223 μg of MT was used, and the estimated amount of MT remaining in the sediments at the end of feeding was 15.96 μg, which corresponds to 0.22% of the total steroid administered. Four months after cessation of MT administration, an estimated 6.3 μg of MT were still present in the soil.

Aquaria with Charcoal Filtration (Experiment IV). MT levels in the water from tanks where fish were fed with steroid-impregnated food were slightly higher than background values obtained from the control tanks (Fig. 7). Background levels averaged 3.07 pg/ml ± 1.0 while MT-fed tanks averaged 7.3 ± 1.9 pg/ml. The highest values observed were 10.9 and 11.8 pg/ml at 7 and 22 days after initiation of feeding, respectively. Figure 8 depicts MT levels in the water immediately after feeding and the following hours. MT concentrations in the water increased to about 130 pg/ml one minute after feeding, and remained elevated for 60 minutes. Within two hours, MT levels decreased to 64 pg/ml and background levels were detected by next morning (15 hours after feeding). RIA for MT in filter bags and charcoal indicated an average of 5.6 ng of MT per gram of charcoal and 5.3 ng of MT/g of bag.

Figure 6. Changes in methyltestosterone (MT) concentration (mean ± SE) in sediments (a) and water (b) from tanks that housed 5 consecutive broods. In MT-treatment tanks, three broods received MT-impregnated food for 28 days each (MT1, MT2 and MT3); after the last MT-fed brood, two new consecutive broods received EtOH-treated food (ET1-ET2). In control tanks, all brood received EtOH-impregnated food (C1-C5).


(a)
Sediments
Control
MT
MT (pg/g)
16000
12000
8000
4000
0
Time (days)
0
30
60
90
120
150
180
210
240
MT 1 or C1
MT 2 or C2
MT 3 or C3
ET1 or C4
ET2 or C5

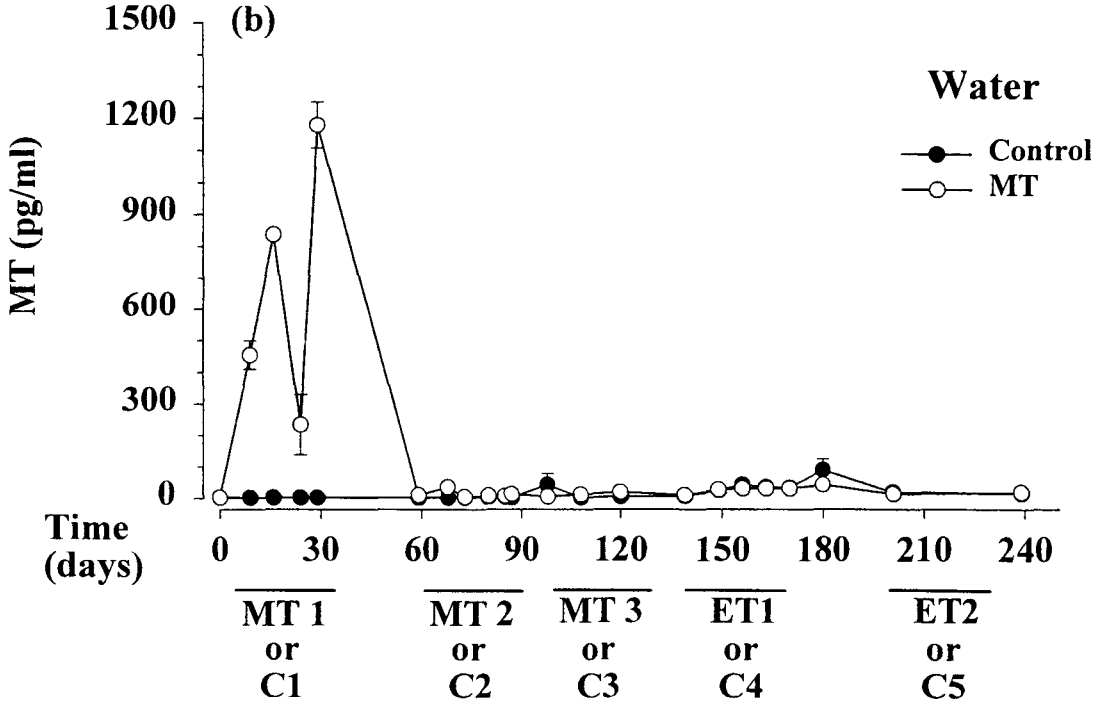

(b)
Water
Control
MT
MT (pg/ml)
1500
1200
900
600
300
0
Time (days)
0
30
60
90
120
150
180
210
240
MT 1 or C1
MT 2 or C2
MT 3 or C3
ET1 or C4
ET2 or C5

Figure 6

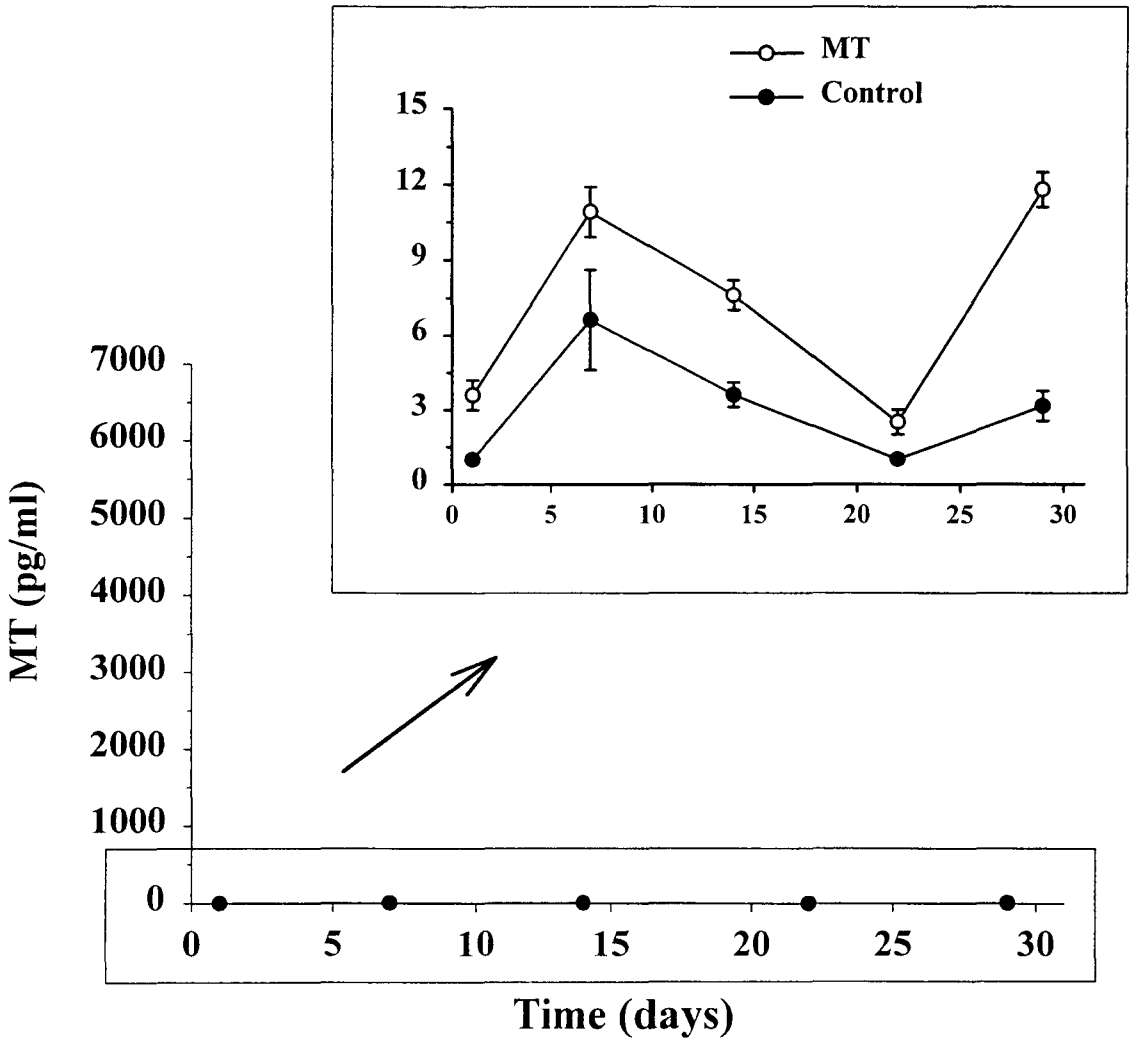


Figure 7. Changes in methyltestosterone (MT) concentration (mean ± SE) in water from tanks with charcoal filtration and containing fish that received control food (solid circles) or MT-impregnated food (open circles) for 28 days. The figure in the bottom is shown in a scale that is comparable with other experiments. The arrow points to a graph that depicts same data drawn in reduced scale to provide better definition.

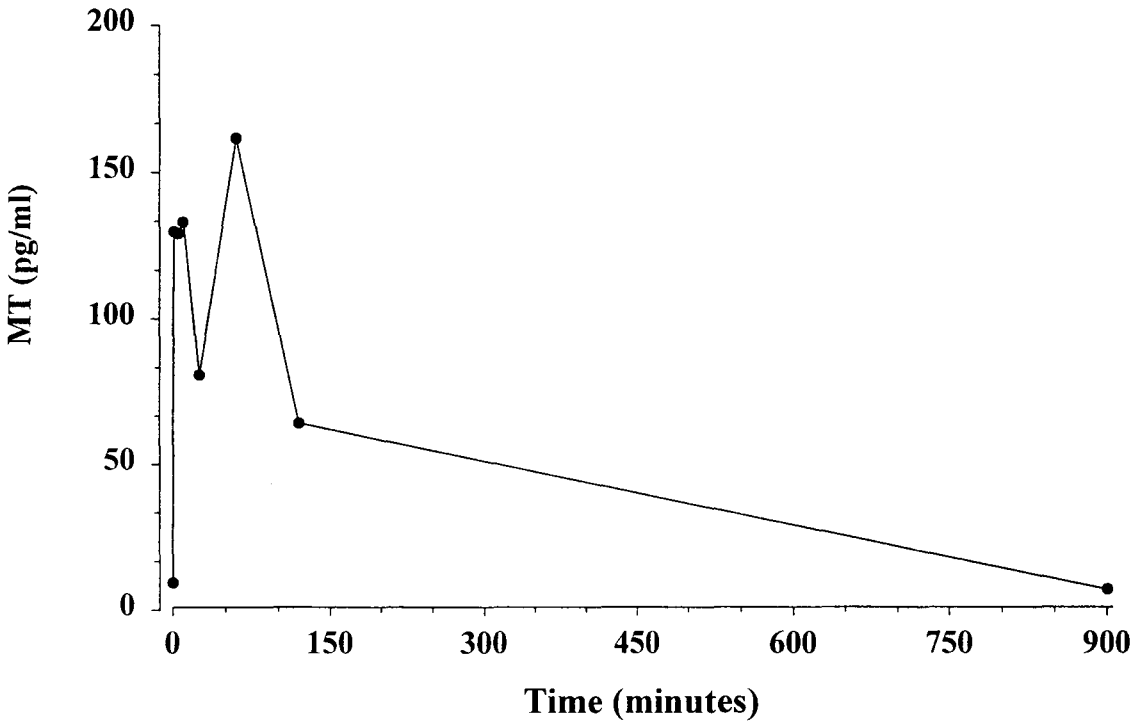


Figure 8. Time course of the concentration of methyltestosterone in water before (time 0) and after administration of MT-impregnated food in a tank that had activated charcoal filtration. Samples were taken after the last feeding of the day, minute 900 represents sample taken the next morning (15 hours after feed administration).

Discussion

We have demonstrated that considerable amounts of MT leak into the environment during dietary treatment. Apparently, MT dissociates from the treated food quickly. As soon as the treated food enters in contact with the water, the highly hydrophobic molecules of the steroid may react to the unfavorable conditions and the potential for crystal formation is high. High values of MT in the water remained for at least 30 minutes. These results indicate that even after the fry consume all the food, relatively high levels of MT are present in the water. Furthermore, since all samples were taken in the mornings before feeding began, the detection of MT in the water during the period of steroid administration indicates that MT remained high15 hours past the last feeding. Therefore, it is possible that in closed or semi-closed systems, the fish are exposed to the steroid not only via feeding but also through immersion, since the route of entry can be cutaneous and/or respiratory.

The relatively fast disappearance of MT from water in treatments where substrate was present (soil or gravel) suggests that MT precipitates or is taken up by the soil once feeding ceases and the fish are removed. When no substrate was present, MT levels in water decreased significantly, but remained above background levels for up to 3 weeks after cessation of MT feeding. In the absence of fish, MT levels in water were higher than those obtained when fish were present, with a 10-fold difference in concentrations remaining at the end of this experiment. These findings suggest that the presence of fish and substrate acts to remove MT from the water. Furthermore, tanks containing gravel as substrate showed a faster removal rate of MT from water. In our aquarium systems, we mix gravel with activated carbon and zeolite (Ammo-Carb®, Aquarium Pharmaceuticals Inc.), and these materials readily bind steroids.

In contrast to the rapid disappearance of MT in water, the levels of MT in the soil remained detectable for as long as four months after cessation of MT administration. The persistence of MT in the sediments for such a considerable time after the end of treatment poses a potential exposure risk to workers and non-target fish and other organisms. Potentially, tilapia may disturb sediments when they build nests or in search for food, leading to resuspension of MT from the soil into the water column. Thus, "rotating" the pond use from fry production to rearing or breeding will not reduce the risk of re-exposure. The fast decline of MT from elevated to moderate values during the early stages of the sequential feeding experiment has been described as a typical process in contaminated soils. Once the soil becomes "enriched" strains of organisms that can metabolize the compound develop and after a lag phase, the rate of biotransformation increases (Walker et al., 1997).

Detection of MT by radioimmunoassay in water, sediments, and interface proved to be a useful method. The fact that a background amount is consistently measured in all substrates analyzed indicates that there are other compounds crossreacting with the antibody; however, this background was relatively low. Background measurement is a common feature of RIA, and in this case we speculate that it may be caused by the crossreactivity of the antibody with phytosterols or animal steroids and their metabolites deposited in the soil via urine and feces. Antibody crossreactivity in RIA has been described in detail (Chard, 1995), and the molecular basis of crossreactivity in antibody-antigen reactions involving steroids is explained in Arevalo et al. (1993).

The results obtained from the HPLC analysis from the control treatment also indicate that the antibody recognizes another compound produced when fish, but not MT, have been present in the system. This chemical may have been excreted by the fish, or may be present in the food used to feed the fry. Excretion of steroids and metabolites in

animal feces and urine is well known (Palme et al., 1996), and the presence of steroids in fish food has been previously documented (Feist and Schreck, 1990).

The fractionation by HPLC of sediment samples from the treatment exposed to MT feeding indicates that the total amount of MT measured by the RIA corresponds to the sum of MT and four distinct compounds that crossreacted with the antibody. The high levels and elution times obtained from unknowns 2, 4 and 5 suggest that these compounds may be MT metabolites. In this regard, Cravedi et al. (1993) found that rainbow trout (*Oncorhynchus mykiss*) forced-fed ^{3}H-MT excreted eleven MT metabolites over a 24 hour period, with unchanged MT accounting for only 27% of the total fractions recovered. Since our antibody only recognized four potential metabolites, it is possible that more steroidogenic compounds are present in the MT-treatment tanks.

However, it can not be assumed that steroid transformation in the pond environment is solely accomplished by the fish present in the system since biotransformation of steroids can also be accomplished by bacteria and fungi (Datcheva et al., 1989; Ahmed et al., 1996; Oppermann et al., 1996). This process is so well known that microbial hydroxylation of steroids has been employed as a method for preparation of hydroxysteroids used in research as well as for product development (Holland, 1999). Despite this, little research has been reported regarding bacterial transformation of steroids in the water environment. In a recent paper, Panter et al. (1999) suggested that non-estrogenic steroid metabolites were transformed to an estrogenically active form by minimal bacterial activity, which elevated plasma vitellogenin and reduced gonosomatic index in male fathead minnows (*Pimephales promelas*). This suggestion was based on observations of the fish; however, the authors presented no data to confirm bacterial biotransformation of the steroid.

Several researchers have pointed out that steroid metabolites and conjugates are equally or more potent than their parent compound (Somogyi et al., 1976; Pelissero, 1993; Sundaram, 1995). Recently, Bhavnani (1998) reported that in postmenopausal women, the 17-keto derivatives of some estrogens are metabolized to the more potent 17β-reduced products, showing nearly 10-times higher activity with some ring B unsaturated estrogens. Therefore, the relatively low values of MT and metabolites detected after the end of the experiments do not minimize the potential risks posed by using MT-impregnated feed during aquacultural operations. It is well known that many chemicals can exert biological functions in very low concentrations (parts per million or parts per billion). Concentrations of picograms per milliliter or gram may have a significant biological meaning in the context of androgen and estrogen levels in whole fish embryos and larvae. For example, Feist et al. (1990) found that the concentration of sex steroids from whole body extracts of fish during the stage of sexual differentiation ranged between <100 - 900 pg/g for testosterone, 11-ketotestosterone, androstenedione, 17α-hydroxy-20β-dihydroprogesterone, and 17 β - estradiol.

The need to avoid exposure of untargeted organisms when steroids are administered in aquatic systems should be of concern. In one of the few studies on the fate of steroids used in experiments involving live fish and recirculating systems, Budworth and Senger (1993) reported that testosterone injected into rainbow trout, *Oncorhynchus mykiss*, leaked out of the fish's body and eventually reached other fish present in the system. Recent studies have reported that exposure of untargeted organisms to MT can result in biased sex ratios. Gomelsky *et al.* (1994) found significant masculinization of common carps, *Cyprinus carpio*, exposed to water used in MT-impregnated feeding trials. They also reported that the masculinizing effects of MT were stronger in recirculating systems than in tanks with flow-through water. These

observations suggest that MT (and/or its metabolites) can persist in the water at concentrations capable of causing sex inversion. Incidental sex reversal in tilapias has been reported recently (Abucay and Mair, 1997 and Abucay *et al.* 1997). These authors indicated that in aquaria and concrete tanks, sex ratios are significantly biased when non-target fish are housed in the same tank where groups of fish are fed with MT.

Paradoxical feminization has been identified as a potential problem during steroid treatment. It has been reported that paradoxical feminization of fish may occur when using MT under certain circumstances (Piferrer and Donaldson, 1991; Piferrer *et al.*, 1993; Rinchard, *et al.*, 1999; Eding *et al*, 1999). This process is thought to be caused by the enzymatic aromatization of testosterone to estradiol and it has been documented that potent synthetic androgens (such as MT) are aromatizable (LaMorte, 1994). However, the mechanism of paradoxical feminization by MT has not been elucidated.

The hydrophobic characteristics of steroids are of special interest when analyzing the potential deleterious effects of contaminants. The hydrophobicity of these chemicals allows passive transport through the skin of mammals (including humans) and can be taken from the water by fish (National Research Council, Committee on Hormonally Active Agents, 1999). Furthermore, lipophilic compounds are readily adsorbed to the surfaces of clay and organic matter and often have long half lives in soil (Walker et al., 1997). The lipophilic nature of steroids is likely to promote the binding of these chemicals to lipid-containing particles present in the system. Therefore, steroid deposition in sediments increases availability to detritivorous and/or omnivorous species via dietary exposure and gastrointestinal absorption.

The problems with contamination of water and sediments are not only related to the immediate contact of the animal with the contaminated media, as many effects are related to bioaccumulation and the transfer of the contaminants and their metabolites through the food web (Kime, 1998; National Research Council, Committee on Hormonally Active Agents, 1999). Therefore, it is important to evaluate if the use of MT in aquacultural facilities requires preventive measurements such as filtration or biodegradation of the steroid and its metabolites in water and sediments. It is time to take any necessary protective measures, either by demonstrating that steroids and their metabolites are not a health hazard for humans or the environment, or by removing these compounds from the farm effluents.

Apparently the filtration system used in Experiment IV efficiently removed MT from the tanks. Our results indicate that a significant amount of MT was detected in the Bio-bag and the charcoal and close to background levels were detected in the water throughout the experiment. Considering that the filters were cleaned with pressurized tap water twice a week, these results show that the filtration actively traps MT and may provide substrate for biotransformation of the steroid. In a recent paper, Green and Teichert-Coddington (2000) calculated MT effluents using a model farm and a series of assumptions and recommended that farms should be managed to maintain MT concentrations under 1µg/l mainly through diluting farm effluents. It has been well documented that dilution is not the solution to contamination of water and sediments in streams and rivers and well intended recommendations might be misleading. The aquaculture industry is already involved in the use of Best Management Practices (BMP) in attempts to respond to increased environmental and regulatory pressures (Boyd and Schmittou, 1999). If usage of MT is not approached with preventive measures, the use of steroids for sex inversion in aquaculture may become another environmental concern for the industry. The use of model ponds as

microcosms has the advantage of providing quantifiable information and the RIA has proven to be a reliable method for MT detection and measurement. We recommend the use of activated charcoal filtration systems to eliminate excess MT and potentially increase masculinization. Studies are needed to determine if MT can be added to the treatment food by means that prevent steroid dissociation. This may allow the reduction of the amount of MT used.

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Chapter 3

Masculinization of Nile Tilapia *Oreochromis niloticus*: II. Efficacy of the Masculinizing Agent 17α-Methyltestosterone in Different Environments[1]

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Abstract

The use of steroid-treated feeds for the production of all-male populations in tilapia (*Oreochromis* spp.) culture is widespread. Masculinization of tilapia fry by oral administration of 17α-methyltestosterone is considered the most successful method employed; however, under certain conditions this technique is sometimes not completely effective. The objective of this research was to investigate the masculinizing efficacy of methyltestosterone under conditions that allow the steroid to persist in the system and determine the effects of water filtration. We evaluated hormonal treatment in jars and aquaria that either contained or lack sediments, and used charcoal filtration in aquaria that did not have sediments. We found low masculinization in both jars and aquaria that contained soil or gravel that had not been treated with steroids, as well as those systems that lacked substrate. When sediments were reused, the efficacy of the hormonal treatment improved significantly (99.5-100% males) in the second and third brood treated in tanks with the same sediment. Instances of gonadal asymmetry and intersexuality were found in tanks that had previously been used for MT treatment. Charcoal filtration of water resulted in almost complete masculinization all three broods tested (100, 98 and 100% males, respectively). Apparently, the recommended dose of MT for masculinizing tilapia is higher than needed and a significant portion of it separates from the food and remains either in suspension in the water for the short term or persists in the sediments over the long term. We recommend the use of activated charcoal filtration systems to eliminate excess MT and potentially increase masculinization, and to prevent potential risks of unintended exposure due to MT contamination of water and soils at farms.

Introduction

All-male populations are used in tilapia (*Oreochromis* spp) aquaculture because the culture of mixed sex populations often results in precocious maturation and early reproduction (Schreck, 1974; Mires, 1995). Early maturation shunts energy to gonadal rather than somatic growth. In addition, reproduction in ponds may lead to the harvest of many unmarketable fry. Individuals in mono-sex populations have increased somatic growth rate due to the avoidance of energy losses associated with gonadal development and reproduction. Furthermore, all-male tilapia populations are desirable because males achieve a larger final size than females (MacIntosh and Little, 1995).

One of the most common techniques for producing mono-sex populations is steroid-induced sex inversion (Hunter and Donaldson, 1983). This involves the administration of synthetic androgens or estrogens to differentiating fry. The steroids act as sex-inversion agents by functionally masculinizing or feminizing individuals in the population. Several methods of steroid administration have been developed, including systemic transfer via injection or silastic implantation, dietary supplementation, and immersion (Pandian and Sheela, 1995). Worldwide, oral administration of steroids via feeding has become the most commonly used technique.

In tilapia aquaculture, the use of steroid-treated feeds for the production of all-male populations is widespread (MacIntosh and Little, 1995). Several authors have reported successful masculinization of tilapia fry by oral administration of 17α-methyltestosterone (MT; reviewed in Green et al., 1997; Pandian and Sheela, 1995). However, it has been demonstrated that this technique is not completely successful (Nakamura and Iwahashi, 1982; MacIntosh and Little, 1995). Factors such as temperature (Shelton, 1981; Varadarj et al., 1994), photoperiod (Varadarj et al., 1994),

and high doses of steroid (Yoshikawa and Oguri, 1978; Green et al., 1997) may interfere with the masculinizing efficiency of the steroid. It has been proposed that failure of MT at high doses is due to paradoxical feminization, which results in the inadvertent production of feminized rather than masculinized populations. This phenomenon is thought to be caused by the aromatization of the synthetic androgen to a feminizing estrogenic compound (Piferrer and Donaldson, 1991). Despite the lack of a clear understanding of the mechanism, paradoxical feminization has been reported when using MT under certain circumstances (Piferrer and Donaldson, 1991; Piferrer *et al.*, 1993; Rinchard, *et al.*, 1999; Eding *et al*, 1999). These authors suggest that the fish are exposed to an overdose of androgen that eventually is converted to estrogens in concentrations high enough to have feminizing effects.

Overexposure to the androgen can occur by ingestion of treated food or by assimilation through skin and gills due to exposure to the steroid in the water. In our laboratory, we have demonstrated that MT leaks out of the impregnated food into the water environment, remaining high overnight (Contreras-Sánchez et al., Chapter II). When this leakage occurs in closed systems, the fish may be exposed to a constant hormonal immersion for as long as the fish are fed with the MT-impregnated food. If this holds true, the amounts of MT used in open or flow-through systems has to be high enough to account for the steroid loss and still be able to masculinize the fish that ingest the food. In order to standardize the protocol for tilapia sex inversion, the usage of 60 mg of MT per kilogram of food has been recommended as optimal (Popma and Green, 1990). We tested the working hypothesis that the recommended amount of MT (60 mg/kg) for tilapia masculinization in closed systems results in reduced masculinization under conditions that allow the steroid to reside in the system.

Materials and Methods.

Fish rearing and breeding. Studies were conducted at the Oregon State University's Fish Performance and Genetics Laboratory, Corvallis, OR. Adult Nile tilapia, *Oreochromis niloticus*, were separated by gender in 1,100 l tanks supplied with constant flow of recirculating water. Breeding families were placed in 200 l aquaria (one male to three females), and checked every 2 hours for spawning activity. Once breeding occurred, the other fish were removed and the brooding female was left to incubate the progeny. On 10 days post-fertilization (dpf), fry were collected from the tank (if the female released the fry from her mouth) or from the female (which was forced to release the fry). Water temperature in all systems was maintained at 28 ± 1°C.

Experimental designs. Tanks were set up 2 days before the expected time of fry release. Only fry from individual broods were used in each feeding trial. Experimental designs are similar to those presented in Contreras et al. (Chapter II); however, more trials were added here. For clarity, all experiments are described.

Jars with Sediments (Experiments Ia and Ib). These experiments were designed to test the efficacy of oral administration of MT in small, closed systems. Each experimental unit consisted of a 3.8 l jar in which 500 g (3 cm deep) of packed soil was placed. The surface area of the sediment in each experimental unit was 156 cm^2. In Experiment Ia, soil was obtained from one of the dry Soap Creek (Oregon State University) ponds located north of Corvallis (Class: Clay; 55.4% clay, 9.9% sand, 34.86% silt). For all other experiments that involved sediments, soil was obtained from a meadowed hill near Corvallis (Class: Clay; 53.8% clay, 22.6% sand, 23.8 silt). Each model pond contained 3 l of dechlorinated tap water (16.5 cm deep). Fry were randomly assigned

to the model ponds at a stocking rate of 47 fry/container (1 fry/3.3 cm^2 which corresponds to a recommended stocking rate for masculinization of 3000 fry/m^2 [Popma and Green, 1990]). The following experimental groups were included for Experiment Ia: MT fed fish (at 60 mg/kg food), Control fed fish (EtOH-treated food). An additional group (MT at 120 mg/kg food) was included in Experiment Ib. All treatments were triplicated; n = 47 fish per replicate.

Aquaria with Different Substrates (Experiment II). The following experiment was designed to determine if different substrates affect the efficacy of oral administration of MT. Each experimental unit consisted of a 50 l aquaria and all aquaria were stocked with fry. Aquaria contained either: a) soil from a meadowed hill (fed MT or control food), b) gravel (fed MT), c) no substrate (fed MT). In each of the two tanks containing soil (a), 5 kg (3 cm deep) of packed soil were placed. The surface area of each experimental unit was 1,858 cm^2. In the tank containing gravel (b), five kg (2 cm deep) of gravel were placed. Each model pond contained 40 l of dechlorinated tap water (22.8 cm deep). Because of the size of the experimental units, the stocking rate used was based on the total number of fry in the brood. Fry were randomly assigned to the model ponds at a stocking rate of 200 fry/tank.

Aquaria with Sediments, Sequential Broods (Experiment III). To test for possible biological effects of residual MT in sediments, sequential feeding trials were conducted in aquaria. After feeding three broods with MT in sequence, two broods in sequence were fed control diet in the same tank. A positive control group fed EtOH-treated food was present at each feeding trial. In essence, for each of the five sequential feeding trials, progeny of a single brood were equally distributed into each of four 50 l aquaria; two aquaria were assigned to the MT-treatment and two as controls (2 replicates per treatment). Five kg of packed soil (3 cm deep) were placed

in each tank, and tanks were filled with 40 l of dechlorinated tap water (22.8 cm deep). In the MT treatment tanks, three sequential separate broods of fry were fed with MT-treated food. Thirty-two days elapsed between the first and the second MT feeding trials and 11 days elapsed between the second and the third trials. Following the last MT treatment trial, two sequential separate broods of fry were fed EtOH-treated diet in the MT treatment tanks with 11 days elapsing between the last MT trial and the first EtOH-treated diet and 36 days elapsing between the first EtOH-treated diet and the second. The positive control treatment consisted of a group of fish from the same brood fed EtOH-impregnated diet at each feeding trial. The number of fish per replicate for each trial were: 175, 100, 120, 200, and 120, respectively).

Aquaria with Charcoal Filtration (Experiment IV). This experiment was designed to evaluate the effects of water filtration on the masculinizing efficacy of MT oral administration. Each experimental unit consisted of a 50 l aquaria without sediments. Each tank was equipped with a filtration system (Whisper® 1; Tetra/Second Nature). This system has an average filtration capacity of 6 l/min and uses a mechanical filtration system based on a Bio-bag® cartridge made of thick floss containing an average of 19 grams of Ultra-Activated™ carbon. Three separate broods (IVa, IVb, and IVc) of fry were treated with MT-impregnated food. Each MT-treated group was paired with a different group of fish from the same brood that was fed control diet. All experiment treatments were triplicated; n = 50 fish per replicate for each trial.

Feeding and water quality. Protocols for diet preparation, feeding regimes and water quality are described in part I of this study (Contreras–Sánchez, et al., Chapter II).

Growth measurements, sex identification and histology. Fish were grown to a size where differences in the gonad were identifiable under 100X magnification (usually

after 60 dpf). At this time, fish were killed with an overdose of anesthetic (methanesulfonate; MS-222) and weighed to the nearest 0.01 g. Sex ratios were determined by examination of gonads using squash (100X) preparations after Wright's staining (Humason, 1972). In addition, 10 fish per replicate were sampled in experiments III c and d and experiment IVc (brood I) for histological analysis. One gonad from each sampled fish was fixed in 10% buffered formaldehyde solution and embedded in paraffin. Serial sections (6-8 μm) were taken and stained with hematoxylin and eosin. Descriptions of tilapia gonadal differentiation outlined by Nakamura and Nagahama (1989, 1995) and Hines et al. (1999) were used to identify gonadal stages and potential treatment effects on gonadal development. Classification of the gonadal development was done using the stages proposed by Grier and Lo Nostro (2000).

Statistical analysis. When the size of the brood allowed replication, data were pooled because there was no evidence of tank effects within treatments (Chi square test or ANOVA). Sex ratio and mortality data were analyzed using Chi square test estimated in GraphPad Prism™. The mean final weights of sampled fish were analyzed for differences between groups using multivariate ANOVA including density and sex as possible confounding variables. For all analyses, differences were considered statistically significant when the p-value (P) was less than 0.05. Data are presented as means ± standard errors.

Results

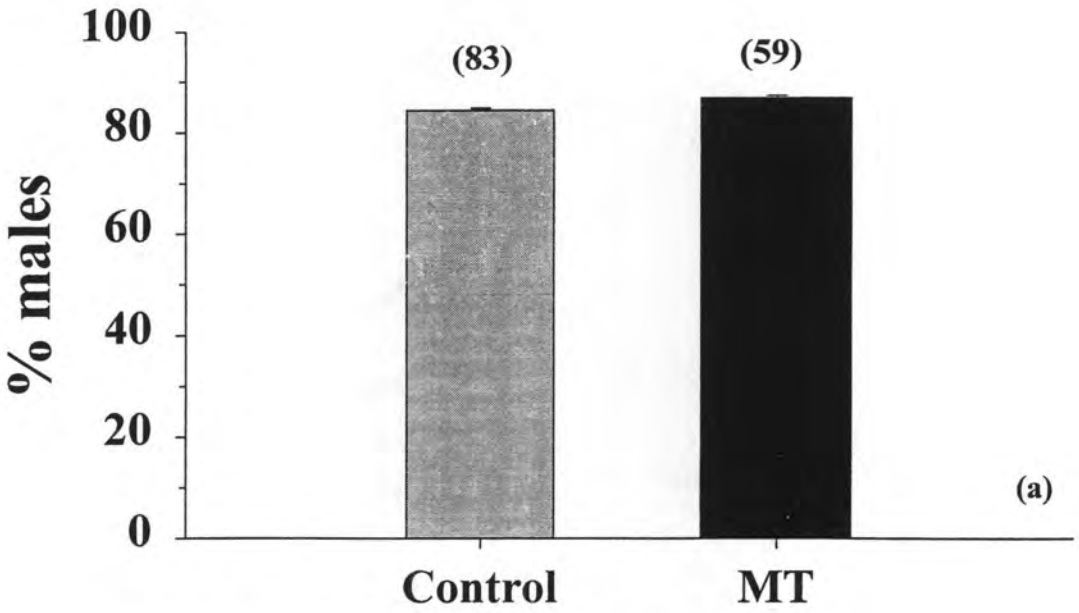
The sex ratios from the control treatments indicate that from the eleven broods used in this study, nearly 50% had sex ratios significantly different from the expected 50:50 ratio; three broods had significantly more males than females and two had

significantly more females. Six of the broods used in the experiment were at or near the expected 50:50 ratio.

Jars with Sediments (Experiments Ia and Ib). No significant masculinization was observed in Experiment Ia. The sex ratio of the control group was biased toward males with a mean of 84.3 ± 0.5% males while the MT fed group averaged 86.7 ± 0.4% males (Fig. 9a). Average mortality for this experiment was 41% for the controls and 47% for the MT fed group. In Experiment Ib the control progeny averaged 51.4 ± 3.5% males. Fry fed with 60 mg/kg of MT had 63.8 ± 1.1% males which was not significantly different from the controls. Fish fed with twice the amount of MT had 45.7 ± 6.2% males which was significantly less ($P = 0.012$) than the fish fed with 60 mg/kg of MT, but was not significantly different from controls (Fig. 9b). Mortality in Experiment Ib was 44% for the control group, 21% for fish fed 60 mg/kg of MT and 12% for the group fed 120 mg/kg of MT. Significant differences in mortality were found between the control and the MT treatments ($P < 0.01$).

Aquaria with Different Substrates (Experiment II). Significant masculinization was achieved in all MT treatments; however, the maximum number of males obtained did not reach 50% of the population. The control group was significantly biased towards females with a mean of 10.4% males. Fish fed with MT in a tank with sediments had 47.0% males at the end of the experiment, and similar results were obtained when fish were treated in presence of gravel as substrate (48.4% males). Fish fed MT in a tank that had no substrate had 19.3% males (Fig. 10) which was significantly higher than controls, but significantly lower than the other two MT treatments. Mortality in this experiment was similar between treatments with an average of 7.8 ± 0.9%.

Figure 9. Mean percentage of males (+ SE) from fish fed with MT-impregnated food (60 mg/kg) and EtOH-impregnated food (control) in Experiment 1a (a). In Experiment 1b (b) fish were fed EtOH-impregnated food (control), MT-impregnated food (60 mg/kg), and twice the recommended dose of MT (120 mg/kg). Experimental units were 3.8 l Jars with sediments. Each treatment was triplicated. Number of fish sampled is indicated in parentheses. Common letters indicate treatment values that are not significantly different from each other.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Control
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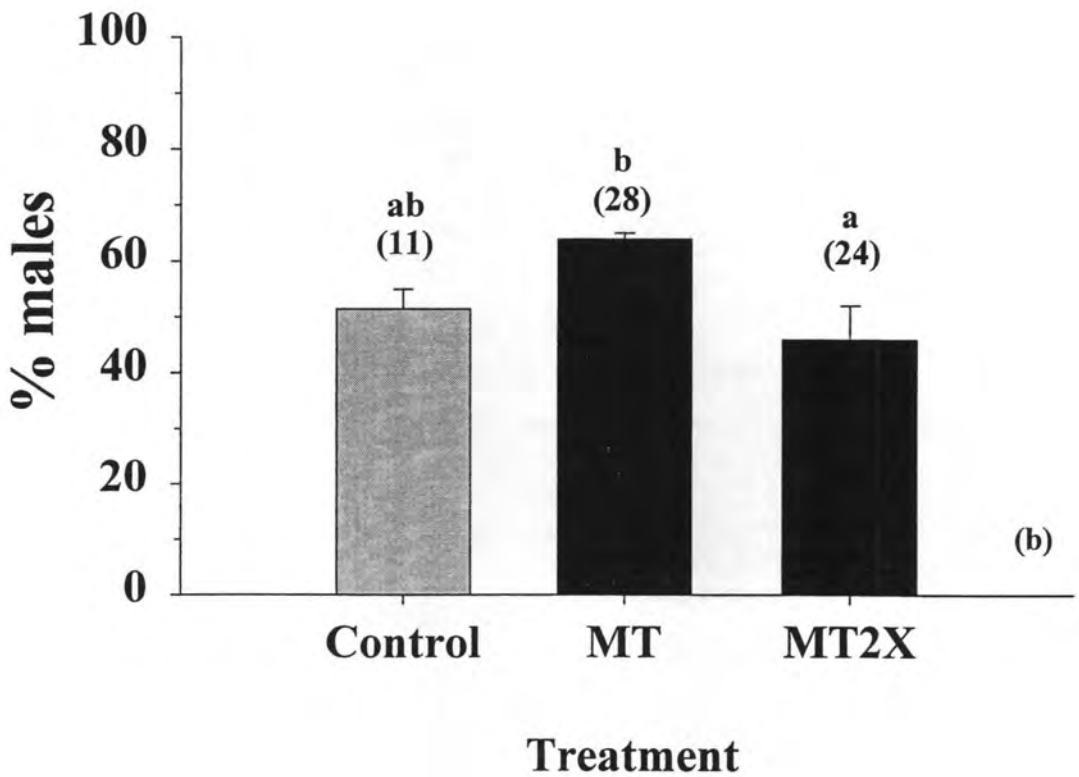
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Treatment

Figure 9

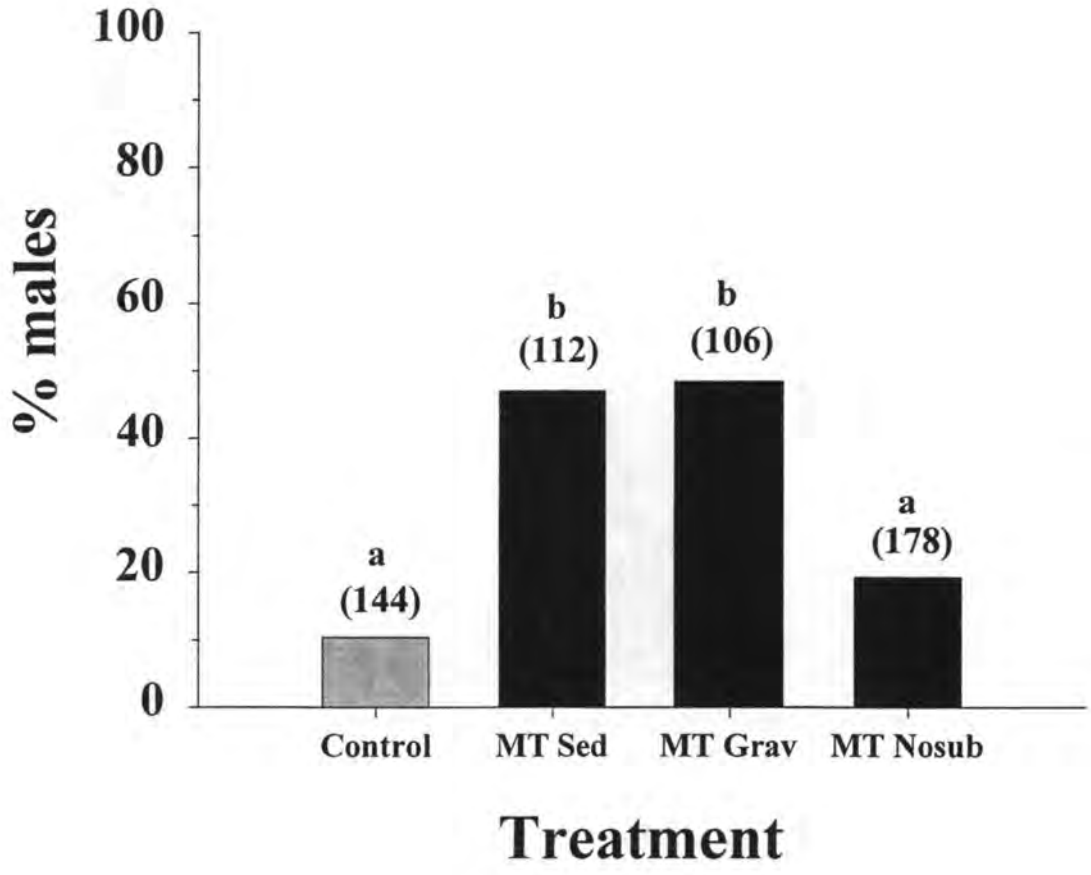

Figure 10. Percentage of males from fish fed with EtOH-impregnated food in a tank with sediments (control), MT-impregnated food in a tank with sediments (MT Sed; 60 mg/kg), MT-impregnated food in a tank with gravel (MT Grav; 60 mg/kg), and fish fed with MT-impregnated food in a tank without substrate (MT Nosub; 60 mg/kg) in Experiment II. Experimental units were 50 l tanks. Number of fish sampled is indicated in parentheses. Common letters indicate treatment values that are not significantly different from each other.

Aquaria with Sediments, Sequential Broods (Experiment III). Significant masculinization was achieved in all of the broods fed MT in this experiment; however the efficacy of the steroid-treated food was low for the first brood and increased significantly in subsequent broods. The progeny of the first brood had a female biased sex ratio (20.5 ± 2.8% males; Figure 11), with the fish fed with MT having a slightly but significantly higher percentage of males (31.8 ± 9.5% males; $P = 0.005$). The second brood had a male biased sex ratio (83.8 ± 0.3% males). Significant masculinization was achieved with MT feeding (99.5 ± 0.5% males; $P < 0.001$). Complete masculinization was obtained with the progeny from the third brood where the control group was composed of 50.2 ± 3.2% males and the MT group resulted in 100% males ($P < 0.001$). Mortality was low in all three trials showing no significant differences between treatments (3.9 ± 1.2, 6.6 ± 3.6, and 13.9 ± 6.4%, respectively). The subsequent groups of fish fed control diet in the tanks having previously received MT treatment did not show any statistically significant treatment effects on sex ratios. Fish held in the control tanks had 53.7 ± 3.1 and 57.1 ± 3.3% males respectively and those held in the tanks formerly receiving MT had 56.1 ± 3.1 and 60.2 ± 3.3% males; however, instances of intersex fish were observed--two in the first and one in the second trials fed EtOH-treated diet, in tanks previously treated with MT (Figure 12). Five females in the first trial but none in the second EtOH-fed groups in the MT-treated tanks had one gonad significantly smaller; the differences were extreme in two cases; in the other three, both gonads, while bilaterally asymmetrical appeared much smaller than normal (Fig. 13). No intersex or lack of bilateral symmetry in gonad size were observed in any fish from the control diet tanks.

Aquaria with Charcoal Filtration (Experiment IV). Significant masculinization was achieved in each one of the three broods used in this experiment ($P < 0.001$). The control groups for each one of the broods were 59.1 ± 4.5; 50.0 ± 2.5 and 41.5 ± 5.1%

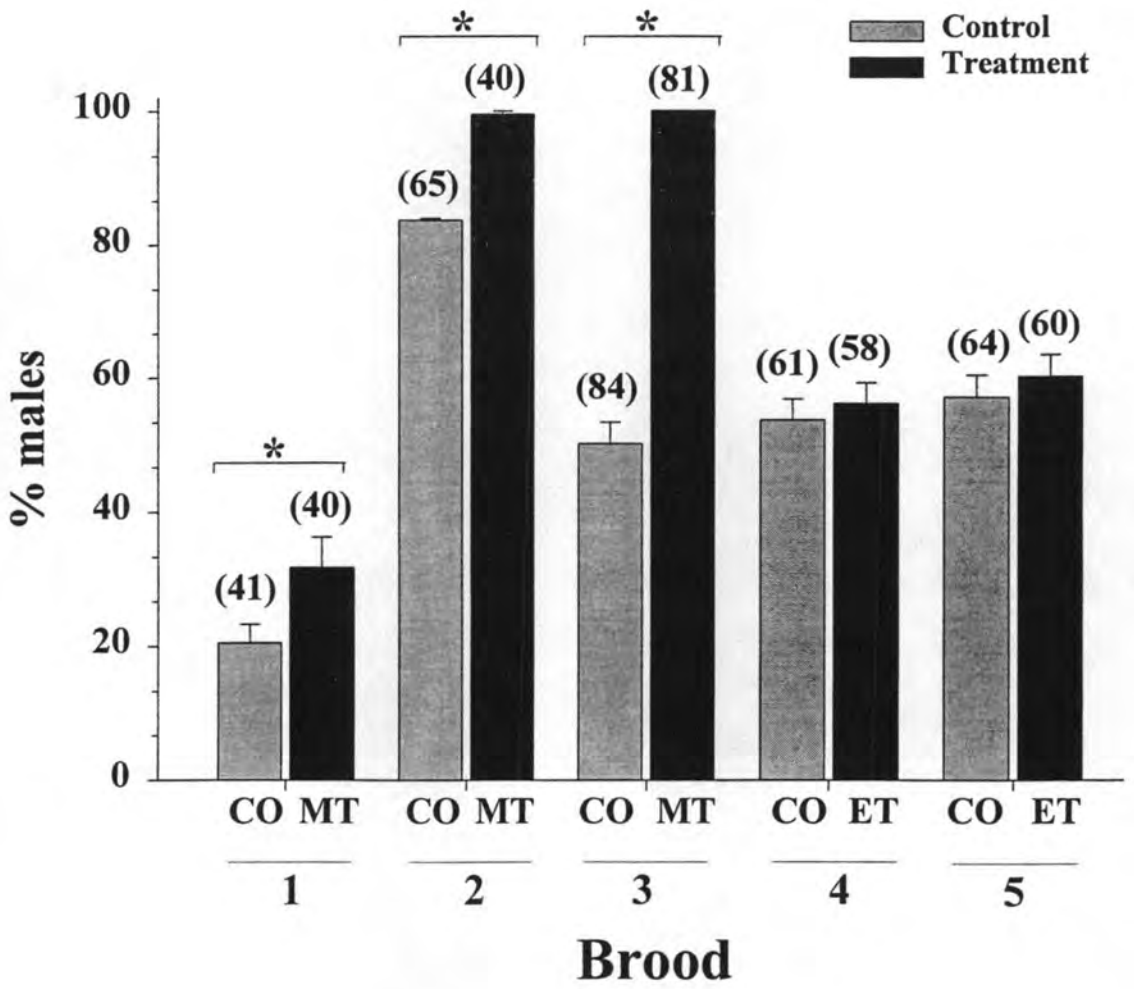

Figure 11. Percentage of males (mean ± SE) from fish fed with EtOH-impregnated food (CO), MT-impregnated food (60 mg/kg; MT), and fish fed with EtOH-treated diet in the MT treatment tanks (ET) in Experiment III. Experimental units were 50 l tanks with sediments. Each treatment had two replicates. Number of fish sampled is indicated in parentheses. Asterisks indicate significant differences between treatments for each brood.

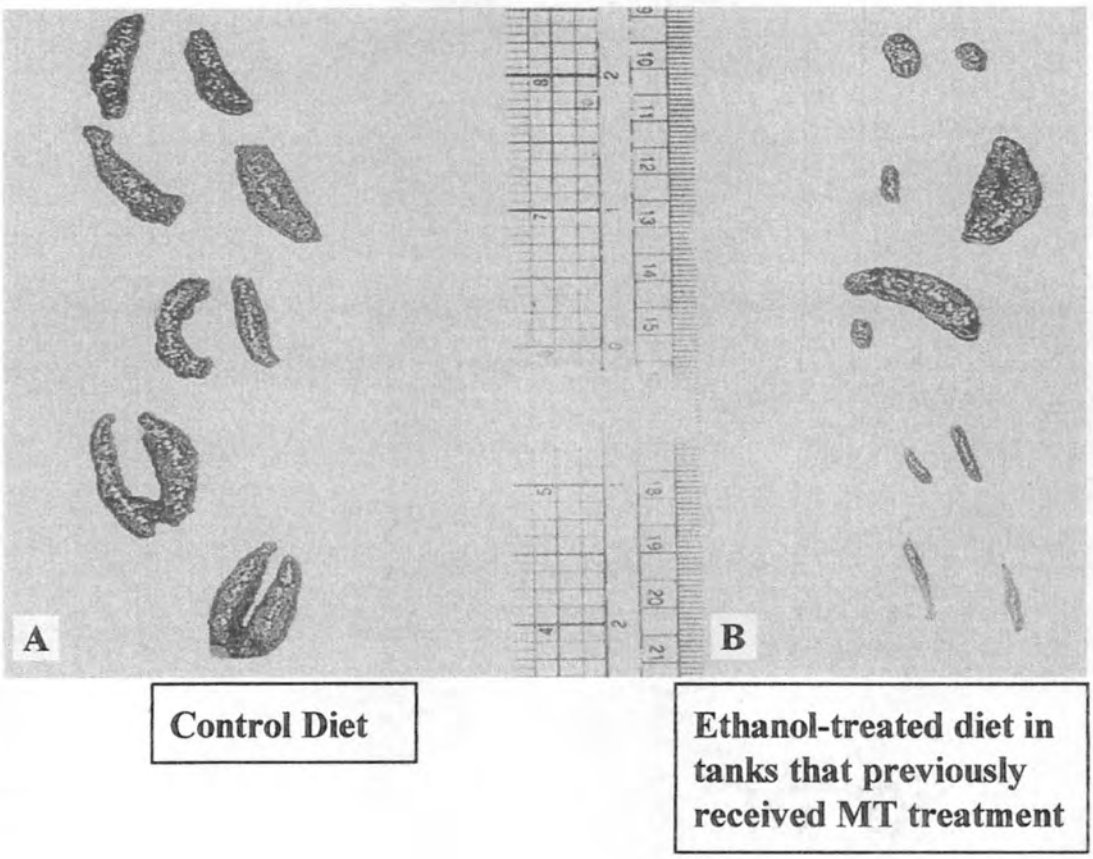

Figure 12. Ovaries from Nile tilapia females fed EtOH-treated diet (control diet) in the positive control tanks (A) and ovaries from females fed EtOH-treated diet in tanks that previously received MT-treatment showing significant discrepancy in gonadal size and bilateral symmetry (B).

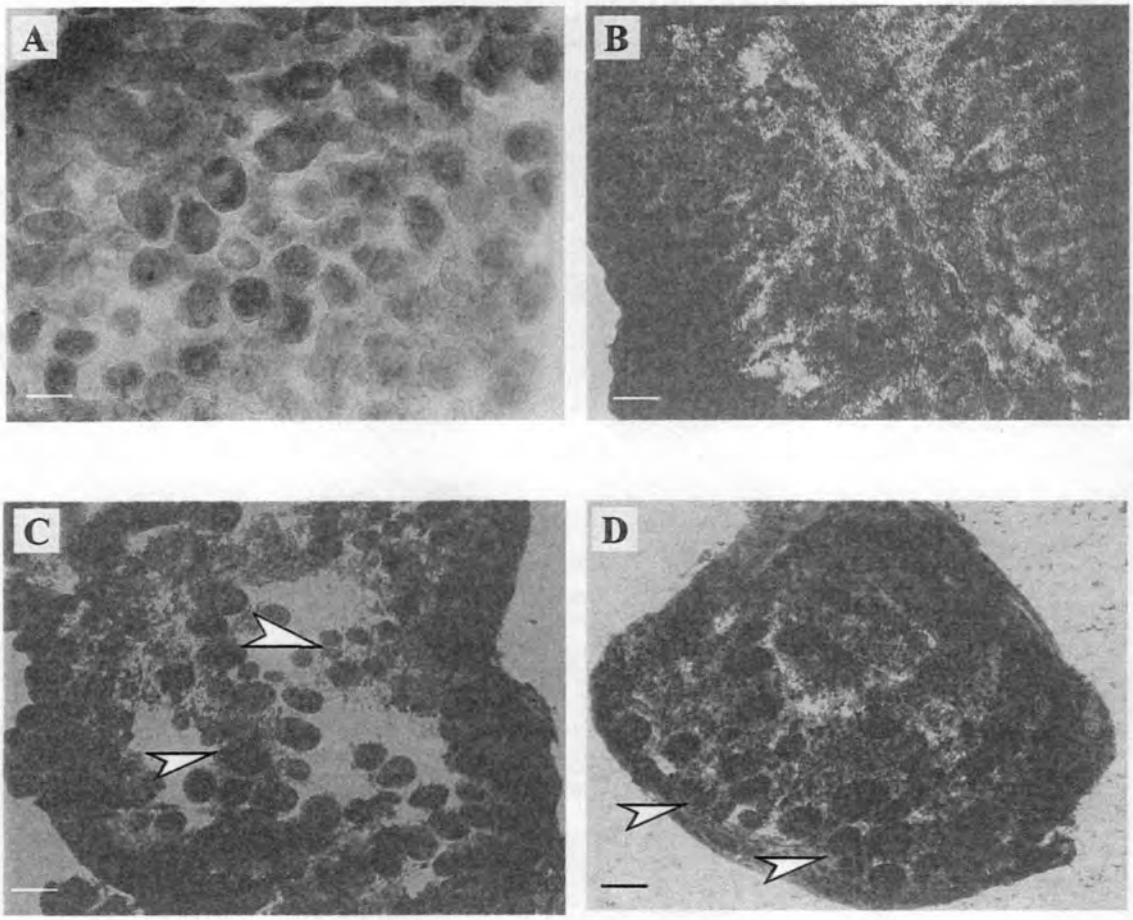

Figure 13. Gonads from fish in control tanks and from tanks previously treated with MT. All fish were fed EtOH treated diet. (A) Ovary from a control female showing normal development and (B) testis from a control male showing normal development. Intersex gonads from fish fed EtOH-treated food and reared in tanks that previously received MT treatment (C and D). Photographs were taken after squash method using Wright's stain. Arrows indicate scattered oocytes surrounded by testicular tissue; Bar, 100 μm.

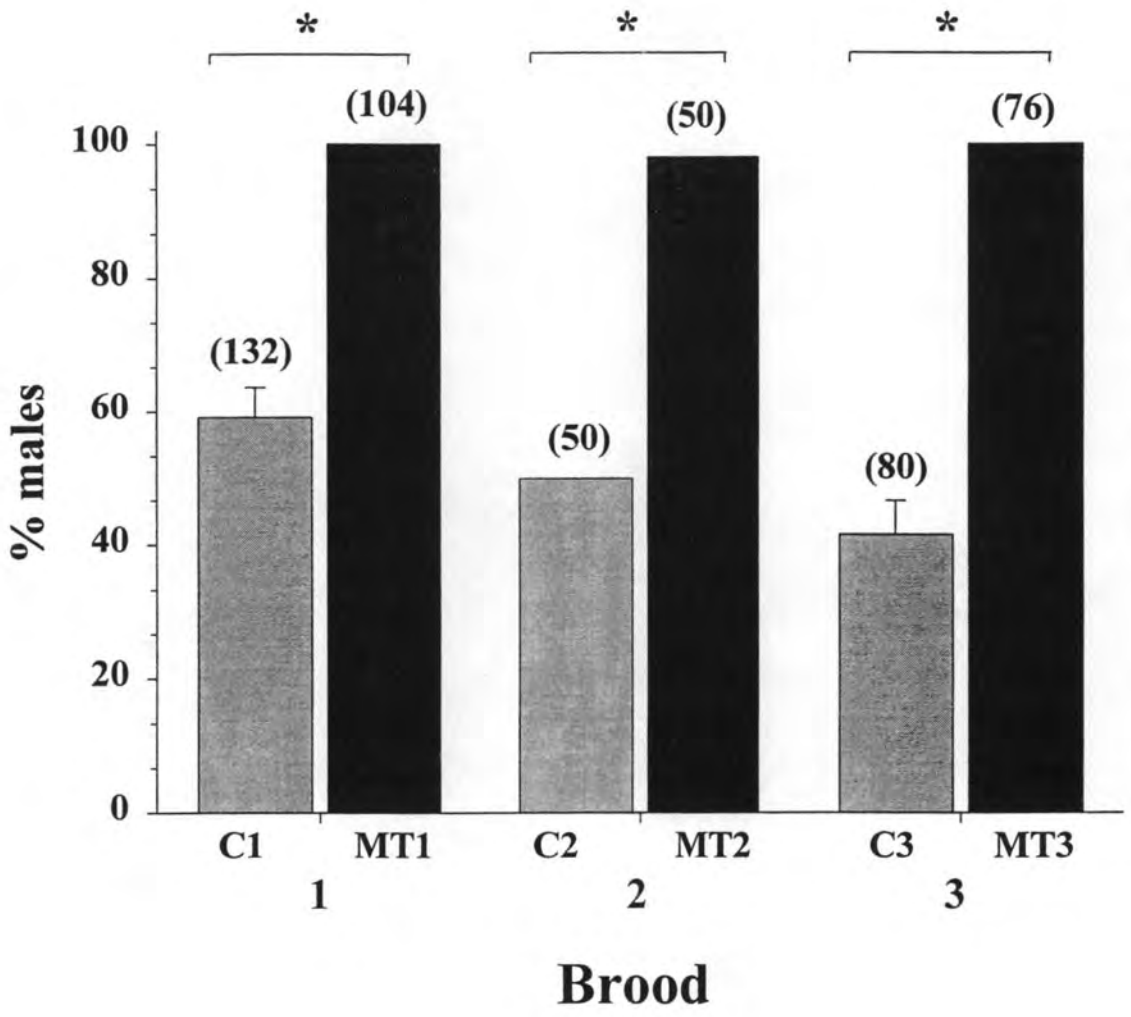

Figure 14. Percentage of males (mean + SE) from fish fed with EtOH-impregnated food (C1-C3) or MT-impregnated food (60 mg/kg; MT1-MT3) in 50 l tanks without sediments. The water in each tank was filtered with activated charcoal. Each treatment was duplicated. Numbers of fish sampled are indicated in parentheses. Asterisks indicate significant differences between treatments for each brood.

males, respectively. The MT-fed groups resulted in 100.0, 98.0 and 100.0% males respectively (Fig. 14). Different mortalities between treatments were observed in the first brood; control tanks had 12.7 ± 8.2% while MT-fed tanks had 30.7 ± 13.0%. Problems in the grow-out system forced us to sample the second brood earlier than scheduled, mortalities in this trail were not recorded. Mortalities in the third brood were 13.3 ± 7.4% for the control group and 36.7 ± 12.2% for the MT-fed group. Statistically significant differences were found between these two groups ($P < 0.01$).

Growth. Table 1 summarizes final growth and mortality from all experiments. No significant differences in final weight were found in eight of the ten broods measured. In the two cases (Experiments Ib and II) where significant differences were found, density and sex were found to be significant confounding variables ($P < 0.001$ in both cases).

Histology. The histological analysis of gonads from control and MT treated fish showed no differences in the gross anatomy of the gonads (Fig. 15). According to the recent classification of testicular developmental stages proposed by Grier and Lo Nostro (2000), the histological analysis of all testes sampled indicated that males were in the mid-maturation class (characterized by a discontinuous germinal epithelium near the ducts, while a continuous germinal epithelium is near the testis periphery) and the late maturation class (characterized by the discontinuous germinal epithelium located near the tip of the lobule). All males analyzed from MT-feeding trials showed normal developing testes. No signs of intersexuality were observed in the gonads analyzed histologically.

Water Quality. In general, water quality was maintained within standards for tilapia culture. Mean values for dissolved oxygen, pH, ammonia, and nitrites were 7.6 ± 0.2

Table 1. Mean final weight (in grams) and final percent mortality (± SE) of fish fed EtOH-impregnated food or MT-impregnated food (60 mg/kg) under different conditions. The days post-fertilization at which final weights were measured (dpf), the number of fish sampled (n) and statistical significance when all experiments were compared (N. S. = not significant $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) are indicated for each treatment. Treatments are described in the Materials and Methods section (C = Control, MT = 17 α-methyltestosterone, MT2X = fish fed double dose of MT, n.a. = statistical analysis not performed. Lower case letters indicate different experiments.

Experiment	dpf	mean wt (g) ± SE	(n)	S. S.	Mortality (%) ± SE	S. S.
Ia	64	C = 1.57 ± 0.17	(83)	N.S.	C = 42.3 ± 11.4	N.S.
		MT = 1.80 ± 0.25	(59)		MT = 47.7 ± 8.4	
Ib	74	C = 3.82 ± 0.63	(11)	**	C = 44.0 ± 6.2	N.S.
		MT = 1.99 ± 0.17	(28)		MT = 20.6 ± 11.2	
		MT2x = 2.27 ± 0.30	(24)		MT2x = 12.1 ± 4.3	
II	82	C soil = 1.97 ± 0.06	(144)	**	C soil = 10.0	n.a.
		MT soil = 2.98 ± 0.27	(112)		MT soil = 6.0	
		MT gravel = 2.98 ± 0.27	(106)		MT gravel = 7.5	
		MT no substrate = 0.72 ± 0.33	(178)		MT no subs = 8.0	
IIIa	130	C = 23.70 ± 0.85	(41)	N.S.	C = 4.5 ± 0.5	N.S.
		MT = 23.02 ± 0.99	(40)		MT = 3.3 ± 2.7	
IIIb	128	C = 29.60 ± 1.74	(65)	N.S.	C = 2.3 ± 0.5	***
		MT = 31.10 ± 0.25	(40)		MT = 10.9 ± 6.4	
IIIc	170	C = 34.7 ± 1.90	(84)	N.S.	C = 14.6 ± 12.1	N.S.
		MT = 39.9 ± 2.60	(81)		MT = 13.3 ± 10.0	
IIId	142	C = 33.4 ± 2.50	(61)	N.S	C = 9.2 ± 6.7	
		MT = 39.1 ± 3.10	(58)		MT = 28.8 ± 8.8	N.S.
IIIe	118	C = 17.98 ± 0.66	(64)	N.S.	C = 0.0 ± 0	
		MT = 20.37 ± 0.78	(60)		MT = 1.6 ± 0.8	N.S.
IVa	78	C = 3.80 ± 0.45	(132)	N.S.	C = 12.7 ± 8.2	*
		MT = 3.98 ± 0.21	(104)		MT = 30.7 ± 13.0	
IVc	58	C = 11.35 ± 0.60	(80)	N.S.	C = 30.0 ± 15.3	**
		MT = 12.61 ± 1.17	(76)		MT = 6.2 ± 5.0	

Table 1

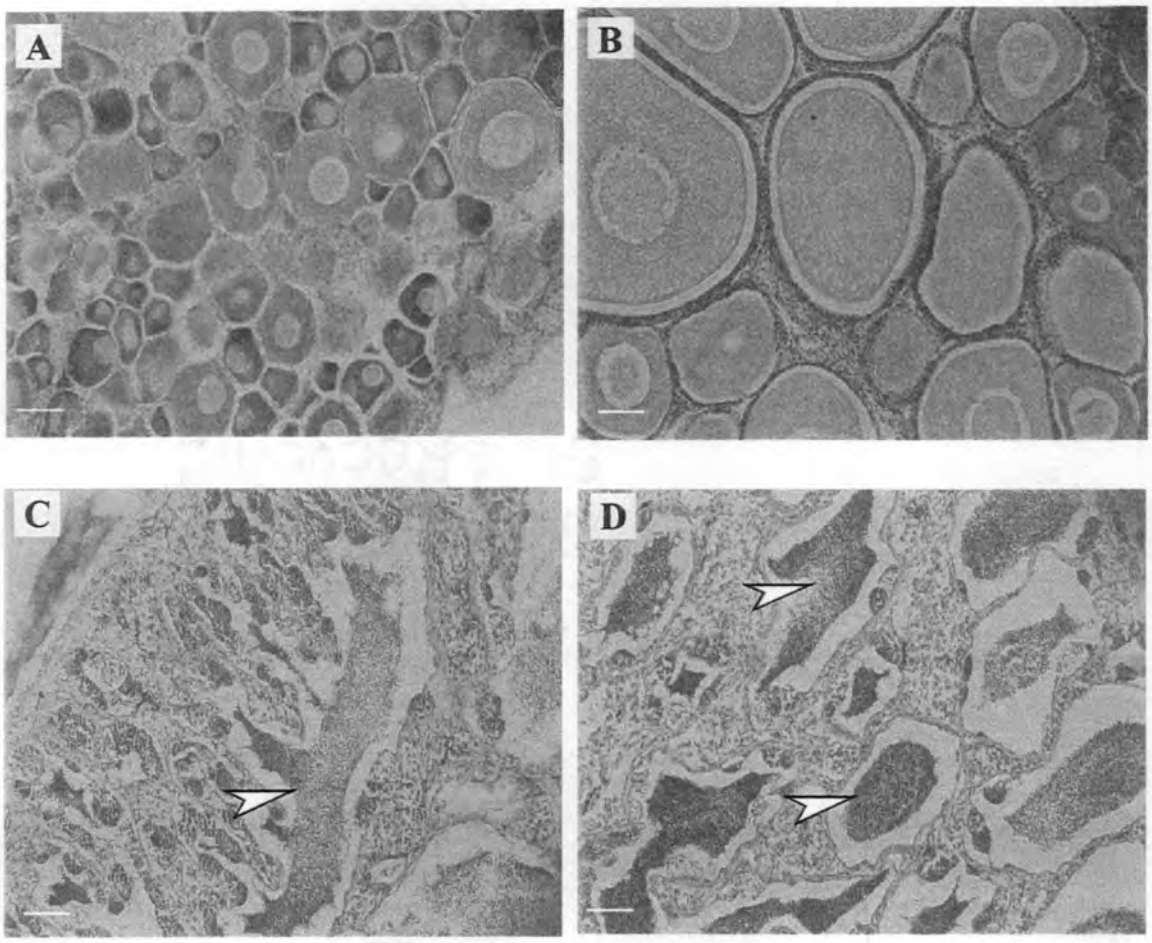

Figure 15. Gonadal histology of Nile tilapia fry at 78 days post-fertilization (dpf) from control females (A and B) and MT-treated males (C and D). All sections are sagittal. (A) Ovary during early stages of maturation showing vitellogenic oocytes. (B) Ovary in advanced stage of vitellogenesis [note enlarged oocytes]. (C) Testis from a juvenile male, the germinal epithelium displays several stages of germ cell development. (D) Testicular tissue from a maturing male showing large amounts of sperm concentrated in the center of tubules (indicated by arrows); Bar, 100 μm.

mg/l, 6.7 ± 0.1, 0.01 ± 0.01 mg/l, and 0.31 ± 0.28 mg/l, respectively. The only instances of elevated ammonia and nitrites were during the last week of experimental feeding in jars (Experiments Ia and Ib), when ammonia reached values higher than 0.02 mg/l and nitrites were between 1 and 2.0 mg/l.

Discussion

The results of the present study show that the efficacy of MT feeding on masculinizing Nile tilapia fry varies significantly according to the environment. Little or no masculinization is obtained when fish were fed with MT in small containers that lack filtration and had a significant amount of solids in suspension. The inability to obtain 95-100% masculinization is troubling but is not uncommon in production facilities around the world. The concentrations of MT were measured in the diets by RIA (data not shown) and established to be at the target dosage of 60 mg/kg of food. Therefore, the lack of masculinization was not due to improper diet preparation. The fish used in all experiments fed actively and food was not observed at the bottom of the model ponds, indicating that poor feeding was not a likely explanation either. Two possible explanations of these results are: 1) the conditions in the system were so stressful that the mechanism that allows sex inversion in tilapias was blocked while energy was diverted to handle the allostatic load; or 2) the amount of MT in such a closed system was constantly high providing enough steroid to serve as substrate for the aromatase enzyme to transform it into estrogen, thereby inhibiting masculinization or enhancing feminization.

The effects of stressful conditions and the interactions between stress and sex steroids have been studied primarily in mammals and birds. Studies involving steroid interactions in the brain and gonadal functions of different species have linked sex and

stress hormones in different ways. In a recent review, McEwen (1994) stated that during early development sex hormones play an important role in mediating the sexual differentiation of the brain which accounts for sex differences in the hippocampus. At the same time, the hippocampus is vulnerable to the deleterious action of hormones secreted during the stress response--which may promote atrophy of apical dendrites resulting in gender-related differences. Impairment of sex hormone action by stressful conditions has been reported extensively in humans and other vertebrates, this blockage is consider to be caused by the interaction of stress hormones and the hypothalamic-pituitary-adrenal axis with the hormones responsible for normal reproductive activity (Crump and Chevins, 1989; Schenker et al, 1992). It has been proposed that stress may inhibit gonadal steroidogenesis in fishes (Pankhurst and Van Der Kraak, 2000). Hobby et al. (2000) suggested that changes in sex steroid binding proteins and cortisol competition for the binding protein binding sites may be responsible for stress-induced decreases in plasma levels of estradiol observed in several species following stress. Despite all the links proposed between stress and sex hormones, there is no evidence suggesting that the level of disruption caused by these interactions can affect sexual differentiation.

The fact that a significant level of masculinization was also not achieved when fish were treated in larger experimental units and potentially less stressful conditions (Experiments II and IIIa) leads us to speculate that the variability in MT efficacy may be linked to the amount of MT in the tank environment. We have demonstrated that MT levels in the water were high until cessation of treatment (Contreras-Sánchez et al., Chapter II). Even though masculinization rates due to MT treatment were below what has been reported by other researchers (see Green *et al.*, 1997), these values were higher when a substrate was present (either soil or gravel). Interestingly, the fish fed with MT but kept in tanks with no substrate had significantly lower masculinization

than those with either soil or gravel. Levels of MT in tanks where fish were fed MT but no substrates were present tended to remain higher for a longer period (Contreras-Sánchez et al., Chapter II); therefore, the fish may have been exposed to higher doses of MT. Furthermore, there is a coincidence between a decline of MT in the water and an increase in masculinization rates when broods are subsequently treated in tanks (Experiment III; Contreras-Sánchez et al., Chapter II). In Experiment III, a significant level of masculinization was obtained from dietary treatments with MT. After the first cycle of MT treatment, nearly complete masculinization was achieved in subsequent broods exposed to MT treatment. Therefore, some 'conditioning' of the soil with MT may be necessary to optimize masculinization. These results suggest that the sediments are dynamically interacting with the MT suspended in the water. Subsequent groups of fish fed control diet in the tanks formerly receiving MT treatment did not show any statistically significant effect on sex ratios; however, instances of intersex fish were observed in each of the subsequent cycles of feeding control diet. This suggests that level of MT contamination in the soil after three cycles of feeding was not sufficient to alter sex ratios; nevertheless, some biological effects likely occurred.

The low levels of masculinization obtained when tilapia fry were fed twice the recommended dose of MT or when fish were held in tanks without substrates suggests that masculinization is being inhibited or that paradoxical feminization is occurring. Even though the mechanism has not been demonstrated, overexposure of fish to MT could result in paradoxical feminization due to a hypothesized conversion of MT into estrogen by the enzymatic action of aromatase. Paradoxical feminization has been reported in channel catfish, *Ictalurus punctatus* (Goudie et al., 1983; Davis et al., 1990); african catfish, *Clarias gariepinus,* (Eding *et al*, 1999); rainbow trout, *Oncorhynchus mykiss* (Solar et al., 1984); coho salmon, *O. kisutch* (Piferrer and

Donaldson, 1990); chinook salmon, *O. tshawytscha* (Piferrer et al., 1993); muskellunge, *Esox masquinongy* (Rinchard, *et al.*, 1999); and Mozambique tilapia, *Orechromis mossambicus* (Varadaraj et al., 1994). Varadaraj et al., (1994) indicated that paradoxical feminization of Mozambique tilapia occurred when fish were fed *ad libitum* for 11 days with as little as 10 mg/kg of MT at 38 °C. In that experiment water in the aquaria was exchanged daily, thereby possibly reducing the exposure of the fish to MT. It is also possible that enzymatic activity involved in the conversion of MT to estrogens might be enhanced by the elevated temperatures. In coho and chinook salmon, paradoxical feminization was observed when the fish were immersed for only two hours in concentrations of 6.4 or 10.0 mg/l of MT (Piferrer and Donaldson, 1990; Piferrer et al., 1993). The evidence suggests that MT is being actively converted to an estrogenic compound, and that the amount required to cause paradoxical feminization will vary according to the species and the conditions under which the organisms are treated.

All three masculinization trials in aquaria with activated carbon filtration resulted in significantly high levels of masculinization. Two broods produced 100% and one 98% males. When MT levels were measured in this system, we found that the hormone levels were significantly lower when compared with a similar system without filtration (i.e. 12 *vs.* 5,000 pg/ml on day 29 after initiation of feeding; Contreras-Sánchez et al., Chapter II). These results support the possibility that the lack of masculinization under certain conditions is caused by an overexposure to MT. This overexposure is due to the combined effects of MT in the feed plus that in the water. We have shown that MT leaks out of the food and stays in the water for at least several hours. However, when charcoal filtration was included, we found that MT concentrations started decreasing within 2 hours and reached background levels overnight (Contreras-Sánchez et al., Chapter II).

Histological analysis of gonads from MT-treated tilapia indicated development of normal testes. Considering that a significant proportion of the fish may have been sex-inverted, the results suggest that the masculinized fish were potentially functional. However, the analysis of a larger number of samples by the squash method at 60-80 dpf revealed that a small proportion (<1%) of the individuals had intersex gonads, characterized by a normal-looking testis with few oocytes dispersed along the gonad. Since we did not observe this phenomenon in fish sampled after 140 dpf, it is possible that these oocytes become atretic and may not interfere with the normal development of the testicular tissue. Intersex gonads in Nile tilapia were reported by Nakamura and Nagahama (1982) when feeding with 50 or 100 mg/kg of MT for 30 days, but only if the hormone administration started 5 or 10 days after capture.

We found no relevant differences in final weight between control and MT-fed fish; in the only instances when significant differences occurred, the statistical analysis showed that the growth was biased by differences in density and sex proportions. A common misconception regarding hormonal influences on growth involves biased statistical analysis. Due to the masculinization effects of MT, sex must be included in the analysis as a potential confounding variable because after a significant number of days, males will be larger in size than females. Therefore, it is possible that some differences in growth are due to biased sex ratios and not to the potential anabolic effects of the hormonal treatment.

This is the first laboratory study that has demonstrated that reducing MT from the water significantly increases MT masculinizing efficiency. It is possible that the recommended dose of MT for masculinizing tilapia is higher than needed and a significant portion of it separates from the food and remains in suspension in the water. We recommend the use of activated charcoal filtration systems to eliminate

excess MT and to potentially increase masculinization. Studies are needed to determine if MT can be added to the treatment food by means that prevent steroid dissociation. If so, this may allow for the reduction on the amount MT used. At the same time, these preventive measures may help decrease the risks of MT contamination of water and soils surrounding the farms that employ it.

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Chapter 4

Masculinization of Nile Tilapia, *Oreochromis niloticus*, by Immersion in Synthetic Steroids: I. Effects of Timing, Duration, Dosage and Density[1]

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Short title: Factors involved in masculinization of tilapia by hormonal immersion

Keywords: Masculinization, Immersion, Methyldihydrotestosterone, Trenbolone Acetate, Nile tilapia, *Oreochromis niloticus.*

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Abstract

The effects of short term immersions of fry in the synthetic steroids trenbolone acetate (TA), 17α-methyldihhydrotestosterone (MDHT), norgestrel (NG), and 17α-methyltestosterone (MT) on masculinization of Nile tilapia, *Oreochromis niloticus*, were investigated. Time of immersion, length of the treatment, dosage, density, solvent vehicle, and number of immersions significantly affected the outcome of immersion trials. The labile period for masculinization by immersion of Nile tilapia fry reared at 28 °C lasts 5 to 6 days. Some masculinization occurs with immersions beginning at 11 days post-fertilization (dpf); the degree of masculinization increases with immersions at 12 dpf; reaches maximal effectiveness at 13 and 14 dpf; and by 15 dpf, the response to steroid administration starts to decline. Masculinizing efficacy of the hormones was as follows: TA > MDHT > NG > MT. Two three-hour immersions on 308 and 364 Celsius Temperature Units (11 and 13 dpf at 28 °C) yielded the highest number of males. EtOH or DMSO as delivery vehicle did not differ in the percentage of males produced by immersions in TA; however, slightly fewer males were obtained if DMSO was used to deliver MDHT in comparison to EtOH delivery. Significant variability in the masculinization efficiency of the treatments was observed and may indicate that the labile period for androgen influence of gonadal development of Nile tilapia fry is very short, and therefore the chances of missing the sensitive period is high. The susceptibility of different broods to androgen-induced masculinization was also found to be highly variable.

Introduction

Masculinization of tilapia (*Oreochromis* spp.) for the production of single-sex populations continues to be an important tool for aquaculturists to avoid unwanted reproduction--which prevents the shunting of energy away from growth towards

gamete production--and to produce the sex with the larger growth potential (MacIntosh and Little, 1995; Green *et al.*, 1997). Aquaculturists usually administer hormones to fish through the diet, but this method often requires long term administration of the steroid, and poses the risk of contamination by the uneaten or unmetabolized hormone that eventually reaches the pond environment. In previous studies, we have demonstrated that a substantial amount of the methyltestosterone administered with the food quickly appears in the tank water and remains in the sediments for at least four months (Contreras-Sánchez et al., Chapter II). Therefore, techniques that can offer significant masculinization of fry, involving short-term treatments and present little or no risk to the environment or hatchery workers may be advantageous.

Immersion of tilapia fry in steroid solutions has been proposed as a way to avoid the use of hormone-impregnated foods. This technique has proven to be very effective in salmonids (Piferrer and Donaldson 1989; Feist *et al.* 1995); but remains largely experimental in tilapia culture. Early studies on tilapia fry immersions in androgens involved protocols that required treatments for 1 to 5 weeks (Varadaraj and Pandian 1987; Torrans *et al.* 1988). Recently, Gale *et al.* (1999) reported that immersion for just three hours in 17α -methyldihydrotestosterone (MDHT) on 10 and 13 days post-fertilization (dpf) resulted in significant masculinization of Nile tilapia, *O. niloticus*, indicating the potential for this technique. However, there was evidence that significant variability resulted between experiments and no information has been published addressing the length of the labile period at which gonadal differentiation can be directed toward male development. A number of studies have been devoted to determining the labile period in several species of salmonids (reviewed in Piferrer and Donaldson, 1993). While most experiments have focused on attaining high numbers of

individuals of one sex or the other using multiple-day immersion protocols, few have approached the delimitation of the labile period by single immersion protocols.

Piferrer and Donaldson (1993) recommended the optimization of immersion protocols by addressing qualitative (type of hormone and treatment timing) and quantitative (dosage, duration, and number of immersions required) variables. In the present study, we have undertaken a series of experiments to expand the knowledge of tilapia masculinization by immersions. We have sought to determine the optimal levels for those factors that most likely influence the masculinizing effects of the immersion protocol. We first focused on the effects of density, dosage, hormones and solvents used as vehicles, day of treatment, and length of the treatment (this chapter). We then considered the effects of multiple immersions and the variability in the efficacy of the immersion protocol observed between trials when using what we considered the best treatments (Chapter V).

A variety of androgens--especially synthetic androgens--are effective masculinizing agents (Hunter and Donaldson 1983); however, there may be differences in their potency. We studied the efficacy of methyltestosterone (MT), 17α – methyldihydrotestosterone (MDHT), trenbolone acetate (TA), and norgestrel (NG), because these steroids have been reported as good masculinizing agents in several species of fish. MT has been used extensively in tilapia sex inversion by oral administration and has resulted in very efficient masculinization of three salmonid species and Nile tilapia (Baker et al., 1988; Piferrer et al, 1993; Feist et al., 1995; Gale et al., 1999). MDHT was first used for masculinizing salmonids (Piferrer and Donaldson, 1991), and recently Gale et al. (1999) reported successful masculinization of Nile tilapia by short immersions in this steroid. TA is a potent anabolic and androgenic steroid that has been widely used in the cattle industry. In aquaculture, TA

was first used by Galvez et al., (1995) in masculinization trials of channel catfish, *Ictalurus punctatus*, by oral administration. Later, Galvez et al. (1996) successfully masculinized blue tilapia, *O. aureus*, by feeding TA-impregnated food for 28 days. NG has been used for masculinizing very-female biased (95.4%) populations of swordfish, *Xiphophorus helleri* (Marañon-Herrera et al., 1999), by oral administration. As many studies indicate, the response of fish to synthetic steroids seems to be more robust than to natural steroids, which presents advantages such as lower cost and slower bio-degradation rate (reviewed in Pandian and Sheela, 1995).

Materials and Methods

Fish rearing and breeding. Studies were conducted at the Oregon State University's Fish Performance and Genetics Laboratory, Corvallis, OR. Adult Nile tilapia were separated by sex in 1,100 l tanks supplied with a constant flow of recirculating water. Adult fish were fed three times a day with floating pellets (Silver Cup Fish Feed; Nelson and Sons, Inc). Breeding families were placed in 200 l aquaria (one male to three females), and checked every 2 hours for spawning activity. Once breeding occurred, the other fish were removed and the brooding female was left to incubate the progeny. Water temperature in all systems was maintained at 28 ± 1°C.

Experimental design. Because efficacy of masculinizing treatments is dependent on developmental stage (Hunter and Donaldson, 1983), only fry from individual broods were used in each experiment to ensure as much between-fry similarity as possible. Temperature in the spawning tank was monitored every 30 minutes with an Optic Stowaway® data recorder (Onset Computer Corp.), and the average temperature was estimated between time of spawning to time of fry capture. Celsius Temperature Units (CTU) where estimated by multiplying the mean water temperature by the number of

days or estimated hourly when needed (i.e. 10 days at 28 °C = 280 CTU). At 280 CTU post-fertilization, fry were collected from the tank (if the female released the fry from her mouth) or from the female and randomly assigned to experimental units. Immersions were carried out in 3.8 l glass jars and the number of replicates per treatment was based on the total number of fry in each brood. We used 280 CTU as a reference because Gale et al. (1999) obtained 90-100% masculinization by immersing the fry on days 10 and 13 post-fertilization after maintaining the brooding females at a mean temperature of 28 °C. In our study, we also wanted to establish possible correlations between developmental stages and the time at which masculinization can be induced, therefore CTU were used for tracking temperature-related development.

Treatments consisted of immersions in either steroid or vehicle [ethanol (EtOH) or dimethyl sulfoxide (DMSO) depending on the experiment], which were mixed with dechlorinated water 1 min before addition of fry. Ethanol was used as vehicle in experiments 1-6, while DMSO was used only in experiment 7. Steroids were obtained from Sigma Chemical Company (St. Louis, Missouri) and stored at 4 °C in stock solutions of vehicle (1 mg/ml). For treatments, 500 µl of EtOH or DMSO vehicle per liter of treatment water (0.05%) were used.

Effects of Day of Immersion (Experiments 1a-1c). This series of experiments was designed to delimit the labile period for masculinization.

Experiment 1a. Fry at a density of 33 fish/l in each replicate were immersed in TA for three hours at 336, 364, 392, or 420 CTU (12, 13, 14 or 15 dpf), at a concentration of 500 µg/l. Fish in two EtOH control groups were immersed at 336 or 420 CTU, respectively. Each experimental group was triplicated.

Experiment 1b. Fry at a density of 33 fish/l in each replicate were immersed in TA for three hours at 336, 364, or 392 CTU (12, 13, 14 dpf), at a concentration of 500 µg/l.

Fish in the EtOH control group were immersed at 336 CTU. Each experimental group was triplicated. The number of fry in the brood limited the number of treatments in this experiment.

Experiment 1c. Fry at a density of 33 fish/l in each replicate were immersed in TA for twelve hours at 350, 364, 378, or 392 CTU (12.5, 13, 13.5 or 14.5 dpf), at a concentration of 500 µg/l. Control groups were immersed in EtOH at the same times. Each experimental group consisted of two replicates.

Effects of Density (Experiment 2). The objective of this experiment was to determine effects of different densities on the efficacy of the masculinizing agent MDHT. Fry were immersed for two hours in 500 µg/l of MDHT at 280 and 364 CTU (10 and 13 dpf at 28°C) using 33, 67, 100 or 200 fish/l in each replicate. Fish in the control group were immersed in 0.5 ml/l of ethanol vehicle (EtOH) using 33 fish/l in each replicate. Each experimental group was triplicated with the exception of the density of 200. The number of fry in the brood allowed only one experimental unit for this treatment.

Effects of Single and Double Immersions (Experiment 3). The goal of this experiment was to determine differences in the masculinizing efficacy between single and double immersions. Fry were immersed for two hours at 280, 310, or 364 (10, 11 or 13 dpf), or twice at 280 and 364 CTU in 500 µg/l of MDHT at a density of 33 fish/l in each replicate. Fish in the EtOH control group were immersed at 280 and 364 CTU. Each experimental group was triplicated.

Effects of Different Steroids (Experiments 4a-4b). This series of experiments tested the masculinizing potency of different synthetic steroids.

Masculinizing Effects of TA, MDHT, NG, and MT (Experiment 4a). Immersion treatments consisted of 33 fish/l immersed at 364 CTU for 2 h in either TA, MDHT,

NG, or MT, or 3h in TA, MDHT, or NG (no MT treatment was included because the size of the brood limited the number of treatments). All steroid immersion concentrations were at 500 µg/l. Fish in the EtOH control group were immersed at 364 CTU for 3 h. Each treatment was triplicated.

Masculinizing Effects of TA and MT (Experiment 4b). Immersion treatments consisted of 33 fish/l immersed at 292 CTU for 48 hours in either MT or TA; at 310 CTU for two or four hours in TA; and two immersions at 310 and 364 CTU each for 2 hours in either MT or TA. All steroid immersion concentrations were at 500 µg/l. Control groups consisted of fry immersed for four hours in water containing EtOH vehicle at 310 CTU. All treatments consisted of two replicates.

Effects of Double Immersions -- day 11 and 13 (Experiment 5). This experiment was designed to test the efficacy of combining immersions during the period of early responsiveness (11 dpf) with immersions during the period of peak responsiveness (13 dpf). Fry were immersed for three hours in 500 µg/l of TA at 364, or 308 and 364 CTU (11 and 13 dpf) using 33 fish/l in each replicate. Fish in the control group were immersed in water containing EtOH vehicle at 308 and 364 CTU. Each experimental group was triplicated.

Effects of Steroid Dosage (Experiment 6). The objective of this experiment was to assess the effects of steroid dosage. Fry (33 fish/l) were immersed for three hours in TA or MDHT at 308 and 364 CTU (11 and 13 dpf) in 500, 750, or 1,000 µg/l of hormone (for TA) and 500 or 1,000 µg/l (for MDHT). Stock solutions were made to maintain the volume of EtOH vehicle constant. Control fish were immersed in water containing EtOH vehicle. Due to the size of the brood, each experimental group consisted of one experimental unit.

Effects of EtOH and DMSO as administration vehicle (*Experiment 7*). The goal of this experiment was to determine if masculinization is influenced by the type of vehicle used to deliver the steroid. Fry were immersed for three hours in 500 µg/l of TA or MDHT diluted in either EtOH or DMSO at 308 and 364 CTU (11 and 13 dpf) using 33 fish/l in each replicate. Fish in the control groups were immersed in 0.5 ml/l of either EtOH or DMSO at the same time. Each experimental group was triplicated.

For each experiment, fry were collected after each immersion, jars were thoroughly cleaned, and then fish were reallocated in fresh dechlorinated tap water. After 5-7 days, fry were transferred to 75 l fiberglass tanks in a recirculating system. Water temperature in this grow-out system was maintained at 28 ± 2°C. Fish were fed to satiation 4 times a day with Natureboy Basic Food Flakes (Rexotic Products, Portland, OR, USA). At 60-70 dpf, fish were killed with an overdose of the anesthetic methanesulfonate (MS-222) and sex ratios were determined by examination of gonads using squash (100X) preparations using Wright's stain (Humason, 1972). Ten fish per replicate were sampled in experiment 3 for histological analysis. One gonad from each sampled fish was fixed in 10% buffered formaldehyde solution and embedded in paraffin. Serial sections (6-8 µm) were taken and stained with hematoxylin and eosin. Descriptions of tilapia gonadal differentiation outlined by Nakamura and Nagahama (1989, 1995) and Hines et al. (1999) were used to identify gonadal stages and potential treatment effects on gonadal development. Classification of the gonadal development was done using the developmental stages proposed by Grier and Lo Nostro (2000).

Statistical analysis. Data were checked for evidence of tank effects within treatments (Fisher's test or ANOVA). Whenever no significant differences between replicates were found, data were pooled. Sex ratio and mortality data were analyzed using Chi square tests. When significant differences between all treatments were found, pairwise

comparisons were performed using Fisher's exact test, with exact p-values (a more conservative test than the chi-square test for small sample sizes) estimated in GraphPad Prism™ (GraphPad Software Inc.). The mean final weights of sampled fish were analyzed in Statgraphics Plus, release 3.0 (Statistical Graphics Corp.) for differences between groups using multivariate analysis of variance, including mortality and sex as possible confounding variables. For all analyses, differences were considered statistically significant when the p-value (P) was less than 0.05.

Results

Effects of Day of Immersion (Experiments 1a-1c). All three broods had female-biased sex ratios (7-22% males). In experiment 1a, the control groups immersed in EtOH at 336 and 420 CTU had mean values of 22.9 and 23.1% males. Significant masculinization was obtained in TA immersions conducted at 336, 364 and 392 CTU ($P < 0.05$; Fig. 16a). A three hour immersion at 336 CTU resulted in 35.1% males, while immersions at 364 and 392 CTU produced almost the same percentage of males (42.2 and 45.1% respectively). These three groups showed no statistical difference ($P > 0.05$). Immersions at 420 CTU had a mean value between the previous treatment and the controls (34.0% males). When compared statistically, the group immersed at 420 CTU was not significantly different to the group immersed at 364 CTU, nor different from the controls.

In Experiment 1b, the control group was composed of 18.1% males (Fig. 16b). Significant masculinization was achieved in each immersion treatment ($P < 0.001$). Fish immersed in TA at 336 CTU had a higher proportion of males (46.5%) than the control group. The groups immersed at 364 or 392 CTU produced similar percentages

Figure 16. Effect of treatment timing on masculinization of tilapia by single immersions in TA. Graph shows mean percentage of males (± SE) obtained after single immersions in TA or EtOH. Fish were immersed for 3h in 500 µg/l of TA at 336, 364, 392, or 420 CTU or 0.5 ml of EtOH vehicle at 336 or 420 CTU (a); at 336, 364 and 392 CTU (b); or for 12 h in either TA or EtOH at 350, 364, 378, and 392 CTU (c). Each treatment was triplicated. The numbers of fish sampled per treatment were 89-94 for experiment 1a; 99-101 for experiment 1b; and 55-90 for experiment 1c. Common letters indicate treatment values that are not significantly different from each other (1a and 1b) or from the appropriate control (1c).

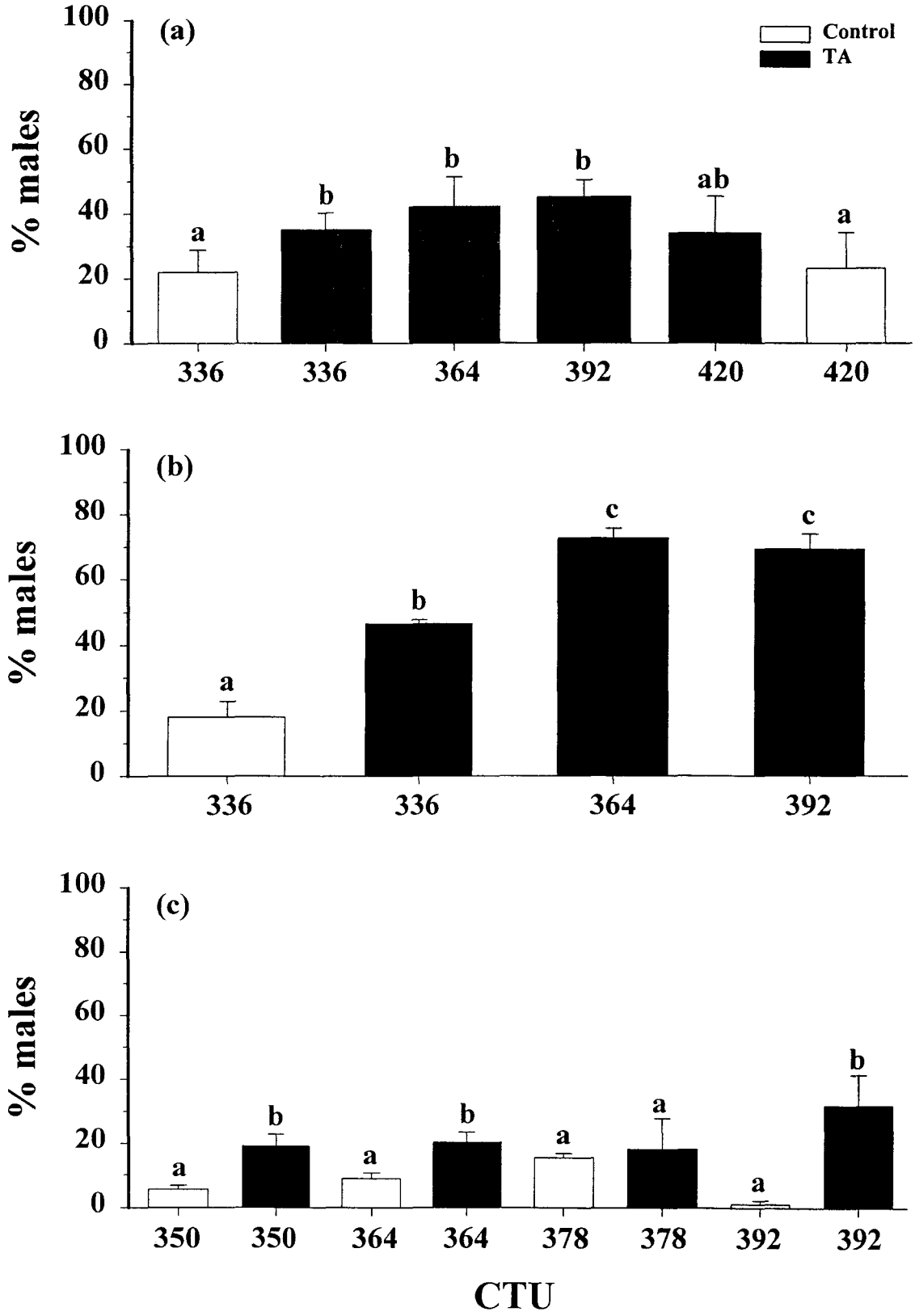

(a)
Control
TA
% males
100
80
60
40
20
0
a
b
b
b
ab
a
336
336
364
392
420
420
(b)
% males
100
80
60
40
20
0
a
b
c
c
336
336
364
392
(c)
% males
100
80
60
40
20
0
a
b
a
b
a
a
a
b
350
350
364
364
378
378
392
392
CTU

Figure 16

of males (72.7 and 69.3%) which were significantly higher than the controls ($P < 0.0001$) and the group immersed at 336 CTU ($P < 0.001$).

All control groups from Experiment 1c showed a low percentage of males (1.1 – 15.3%; Fig. 16c). Significantly higher numbers of males were obtained in groups immersed at 350, 364, or 392 CTU when compared with their respective control group ($P < 0.01$ in each case). The group immersed at 378 CTU was not significantly different than its respective control group (18.0 vs. 15.3% males).

Effects of Density (*Experiment 2*). The masculinizing effects of MDHT were significantly affected by the density at which the fish were immersed (Fig. 17). The proportions of males in treatments with densities of 33, 67 and 100 fish/l were 80.3, 71.7 and 71.7% respectively. The one sided Fisher's exact test indicated that these values were significantly higher than the control (56.7%) group ($P = 0.004$, 0.043 and 0.043, respectively). The proportion of males in the only experimental unit of the treatment with 200 fish/l (64.5%) was not significantly different from the control group. Statistically significant differences where found when the MDHT treatments were compared against each other ($P < 0.001$): the proportion of males in the group immersed at a density of 33 fish was significantly higher than any other treated group; however, no significant differences were found between the groups immersed at densities of 66 and 100 fish/l.

Effects of Single and Double Immersions (*Experiment 3*). Single immersion in 500 µg/l of MDHT at 364 CTU resulted in 79.3% males (Fig. 18), which was not significantly different from fish receiving two immersions at 280 and 364 CTU (82.9% males). Each of these treatments had a significantly higher proportion of males than the EtOH control group (56.6% males; $P < 0.001$). No significant

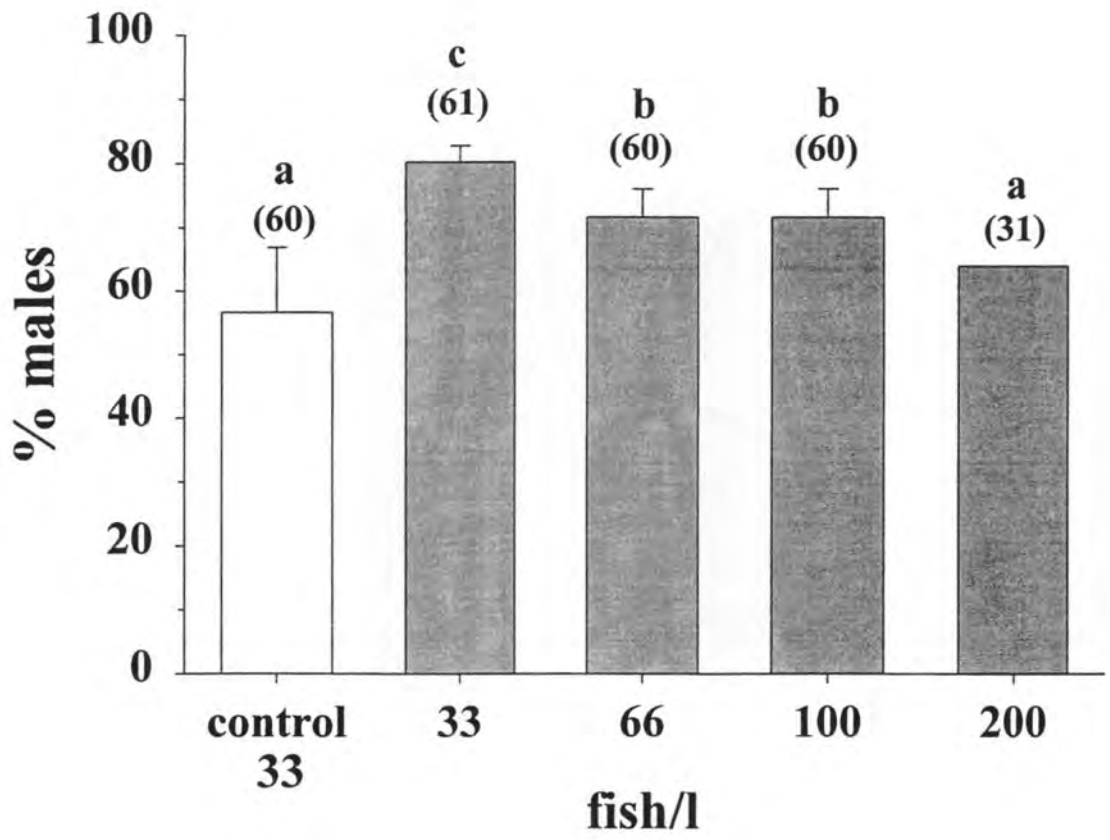


Figure 17. Effects of fish density on the mean percentage of males (± SE) produced by immersion in 500 µg/l of MDHT. Treatments using 33 (including controls), 66, and 100 fry were triplicated, while the treatment with 200 fry had one tank. The numbers of fish sampled are provided in parentheses. Common letters indicate treatment values that are not significantly different from each other.

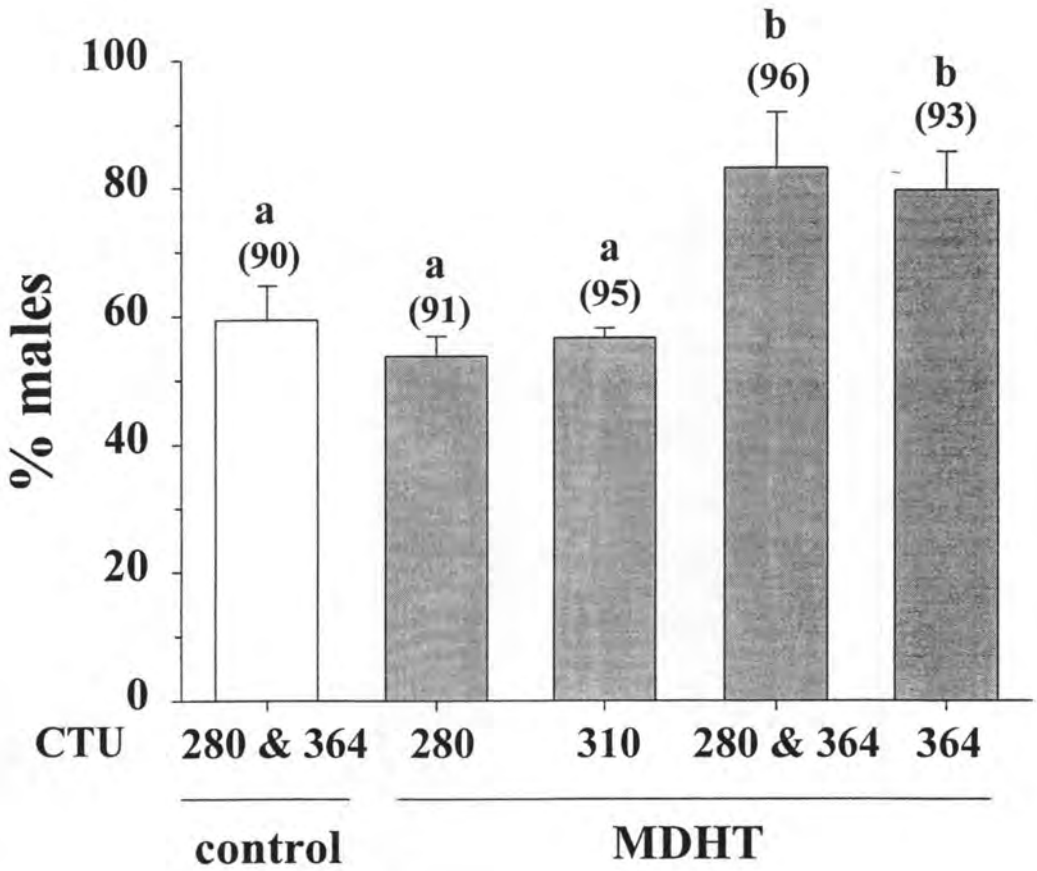


Figure 18. Masculinizing effects of single and double immersions in 500 µg/l of MDHTon Nile tilapia fry. Graph depicts mean percentage of males (± SE) in groups treated at different times (indicated by CTU). Each treatment was triplicated. Data from replicates were pooled in each treatment. The numbers of fish sampled are provided in parentheses. Common letters indicate treatment values that are not significantly different from each other.

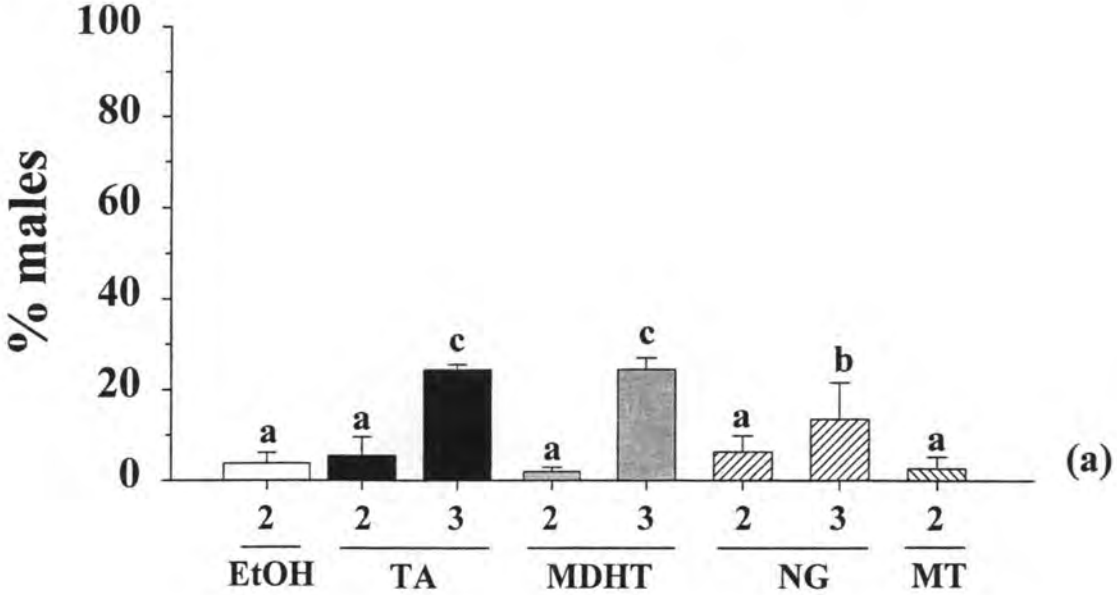
masculinization effects were observed in groups immersed in MDHT at either 280 or 310 CTU.

Effects of Different Steroids (Experiments 4a-4b). In both experiments, the control progeny had a very female-biased sex ratio. In Experiment 4a (Fig. 19a), the control group was composed of 3.8 ± 2.4% males. Significant masculinization ($P < 0.01$) was obtained when fish were immersed for 3 hours in 500 µg/l of TA (24.4% males), MDHT (24.5% males), or NG (13.5% males). In contrast, no significant masculinization was observed when the fry were immersed in the same hormones or MT for 2 hours (5.5, 1.9, 6.3, and 2.6% males, respectively).

In Experiment 4b, the control group had female-biased sex ratio (15.6% males; Fig. 19b). No significant differences were found between the control group and the groups immersed in TA for 2 hours at 310 CTU (20.0% males), or immersed in MT for 2 hours at 310 and 364 CTU (20.0% males). Single immersions in MT (26.7% males) and TA (37.3% males) for 48 hours at 292 CTU resulted in higher proportions of males, although MT produced results that were only slightly significant ($P = 0.049$ compared with 0.002 for TA). Immersions in TA for 4 hours at 310 CTU and 2 hours at 310 and 364 CTU produced significantly higher proportions of males (64.4 and 91.9% males, respectively; $P < 0.0001$).

Effects of Double Immersions -- day 11 and 13 (Experiment 5). Significant masculinization was achieved in groups immersed either once at 364 CTU or twice at 308 and then again at 364 CTU in 500 µg/l of TA (Fig. 20). However, the efficacy of the immersion was higher for the fry immersed twice (88.6% males) than that observed for the fry immersed only at 364 CTU (65.4% males). The control group in this experiment had 49.5% males.

Figure 19. Effects of different steroids and duration of exposure on masculinization of Nile tilapia. Figure depicts mean percentage of males (± SE) produced after immersion in TA, MDHT, NG and MT for 2 or 3 h (a) and immersion in TA and MT at different CTU and for different durations (hours immersed) (b). Treatments shown in Figure 19a consisted of 3 replicates each and 97-103 fish sampled in each treatment. Treatments in Figure 19b had 2 replicates and 60-63 fish sampled in each treatment with the exception of immersions in MT at 310 and 364 CTU which had 30 fish sampled in each replicate. Data from replicates in each treatment were pooled. Common letters indicate treatment values that are not significantly different from each other.


100
80
60
40
20
0
% males
a
a
c
a
c
a
b
a
(a)
2
2
3
2
3
2
3
2
EtOH
TA
MDHT
NG
MT

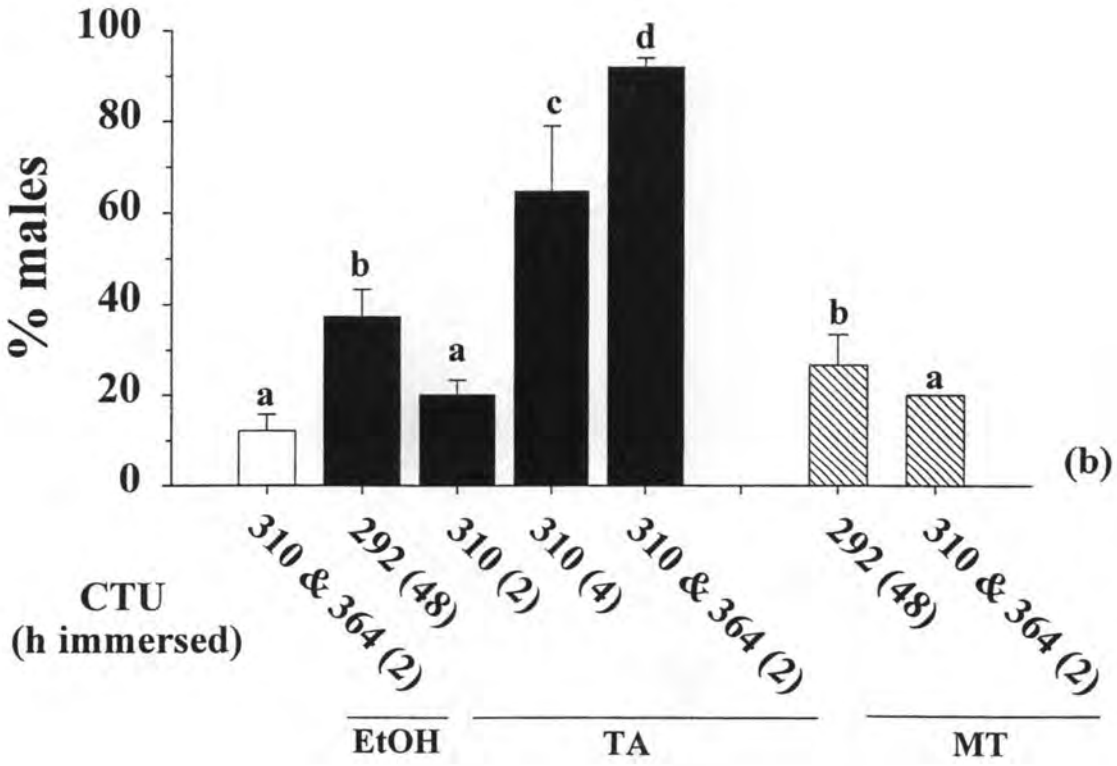

100
80
60
40
20
0
% males
a
b
a
c
d
b
a
(b)
310 & 364 (2)
292 (48)
310 (2)
310 (4)
310 & 364 (2)
292 (48)
310 & 364 (2)
CTU
(h immersed)
EtOH
TA
MT

Figure 19

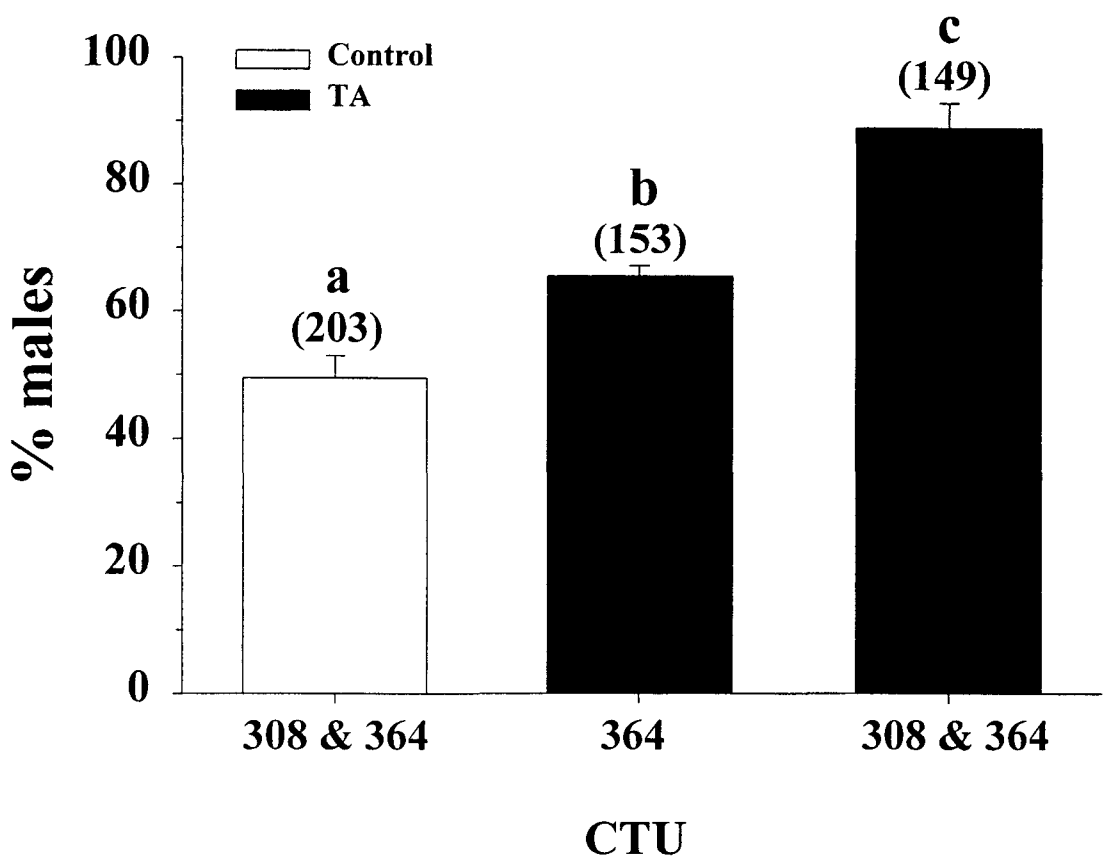


Figure 20. Comparison of the effects of single and double immersions in steroid on masculinization of Nile tilapia. Graph depicts the percentage of males (mean + SE) from fish immersed once in TA for 3 h at 364 CTU and fish immersed twice at 308 and 364 CTU. Each treatment was triplicated. Numbers of fish sampled are provided in parentheses. Common letters indicate treatment values that are not significantly different from each other.

Effects of Steroid Dosage (*Experiment 6*). Because of the size of the brood, no replication was performed in this experiment. The control group was composed of 45% males (Fig. 21). Significant masculinization was observed in all groups immersed in TA ($P < 0.001$), but no differences were found between the fry immersed in 500 and 750 µg/l (70 and 80% males, respectively). The fry immersed in 1000 µg/l had a higher percentage of males (94%) than the other two dosages ($P = 0.008$). Fry immersed in 500 µg/l of MDHT did not differ in percentage of males (47%) from the control group; however, the group immersed in 1000 µg/l showed significant masculinization with as many males as the groups immersed in 500 and 750 µg/l of TA.

Effects of EtOH and DMSO as administration vehicle (*Experiment 7*). Fry in the control groups immersed in either EtOH or DMSO had a female-biased sex ratio (29.3 and 26.4% males, respectively; Fig. 22). Significant masculinization was achieved when fry were immersed in 500 µg/l of MDHT using EtOH as vehicle (40.9%; $P < 0.01$); in contrast no effect of MDHT was observed when DMSO was used as vehicle (30.2% males). TA immersions with EtOH and DMSO as vehicle resulted in significantly higher percentage of males (69.2 and 62.0%, respectively; $P < 0.0001$) than their respective control group, no statistically significant differences were observed between the two vehicles.

Mortality and Growth. Table 2 summarizes mortality and final weights in all experiments. Within most experiments mortality and final weight data were not significantly different among treatment groups. Comparisons between experiments were not made because sampling was not standardized for dpf over experiments. In those instances where final weight was found to be significantly different, mortality played a significant role in accounting for the differences (Experiments 3 and 4b). In

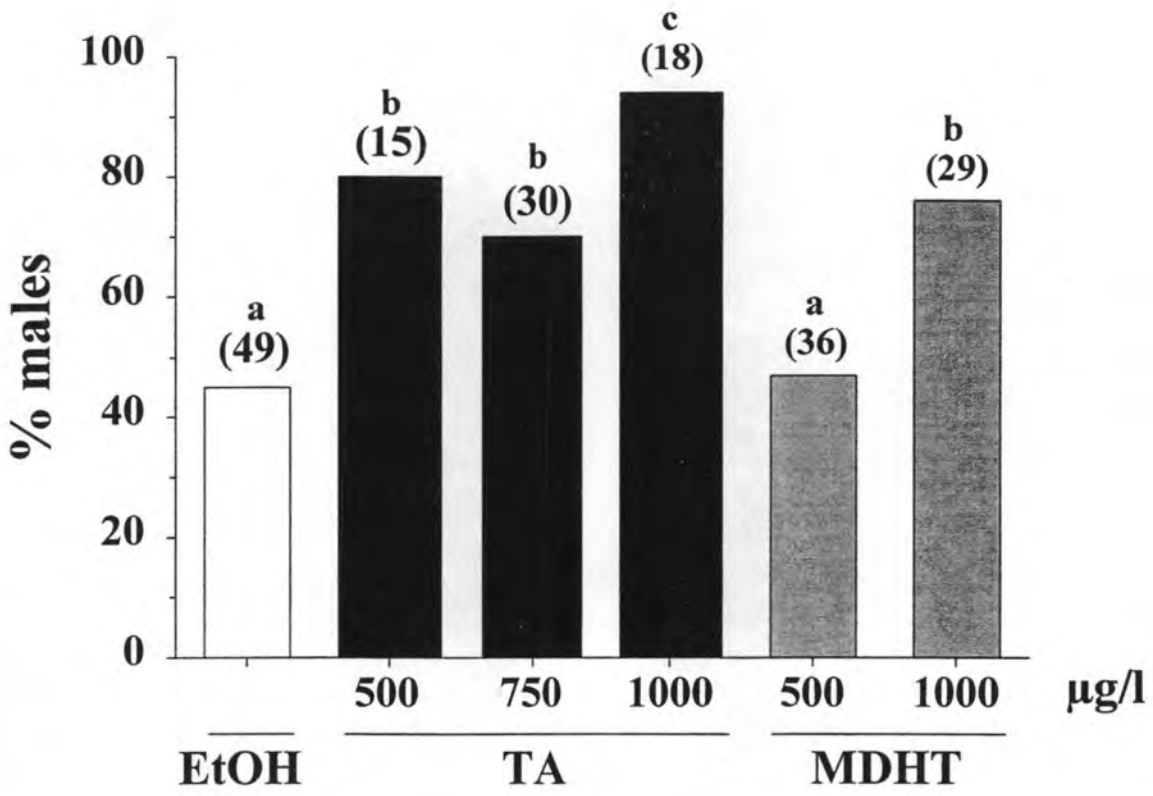


Figure 21. Effects of hormone dosage in Nile tilapia fry immersed in TA or MDHT. Graph depicts the percentage of males produced by each treatment (each experiment consisted of one tank). Control fish were immersed in 0.5 ml of EtOH vehicle. Numbers of fish sampled are provided in parentheses.

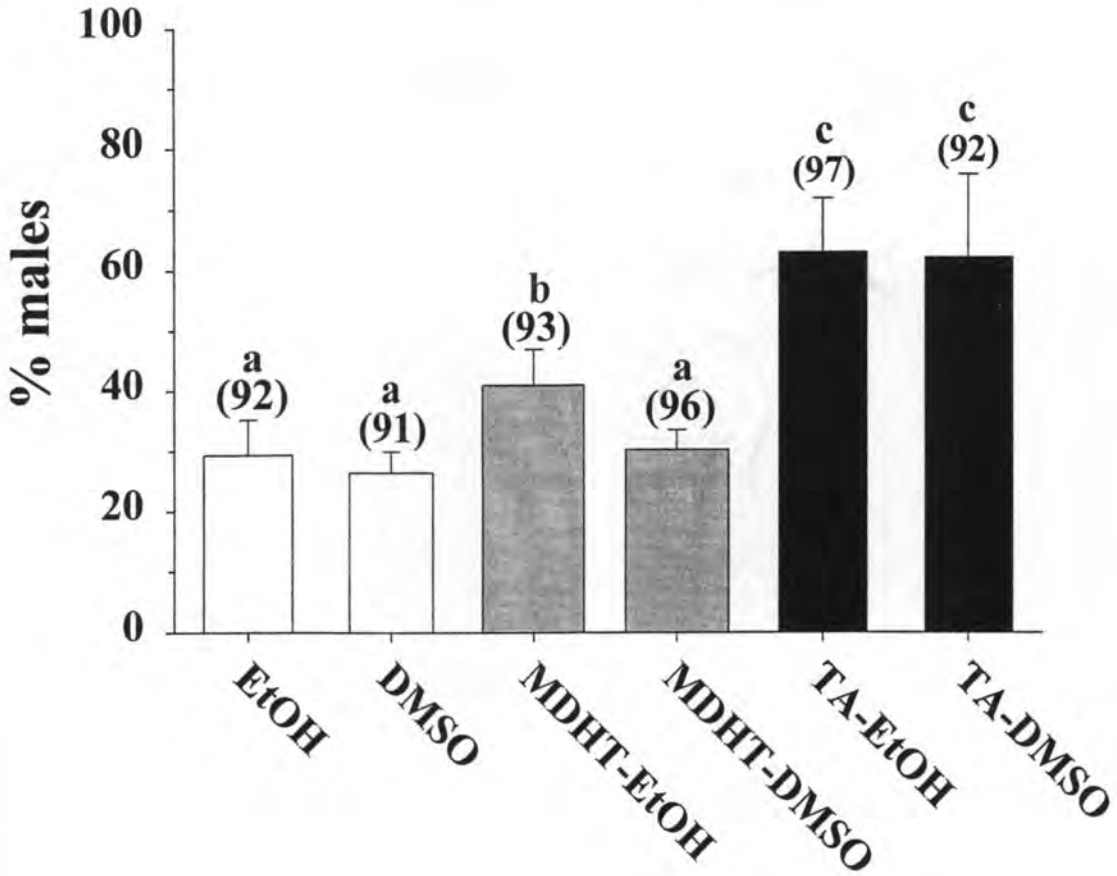


Figure 22. Effects of solvent used as hormone vehicle on Nile tilapia fry masculinization. Graph depicts the percentage of males (mean ± SE) produced after immersion in 500 μg/l of TA or MDHT for 3h at both 308 and 364 CTU. Control fish were immersed in 0.5 ml of either EtOH or DMSO vehicle. Number of fish sampled are provided in parentheses. Each treatment was triplicated. Data from replicates were pooled in each treatment. Common letters indicate treatment values that are not significantly different from each other.

Table 2. Mean final weight (in grams) and final percent mortality (± SE) of fish immersed in steroid or vehicle. The days post-fertilization when final weights were measured (dpf), the number of fish sampled (n) and statistical significance for overall ANOVA or Chi square (N.S. = not significant $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) are provided for each treatment. C = Control, TA = trenbolone acetate, MDHT = 17 α-methyldihydrotestosterone, NG = norgestrel, n.a. = statistical analysis not performed.

Experiment	dpf	Treatment	weight (g) ± SE	n.	S. S.	Mortality (%) ± SE	S. S.
1a	72	C 336 CTU	3.98 ± 0.1	92	N.S.	7.0 ± 2.7	**
		TA 336 CTU	3.39 ± 0.1	81		18.0 ± 6.0	
		TA 364 CTU	3.81 ± 0.1	94		9.0 ± 7.6	
		TA 392 CTU	4.07 ± 0.1	98		2.0 ± 2.0	
		TA 420 CTU	4.07 ± 0.1	89		10.0 ± 3.6	
		C 420 CTU	4.31 ± 0.2	89		10.0 ± 5.6	
1b	68	C 336 CTU	0.92 ± 0.1	99	N.S.	1.01 ± 1.0	N.S.
		TA 336 CTU	0.80 ± 0.1	101		0.00 ± 0.0	
		TA 364 CTU	0.75 ± 0.1	100		0.00 ± 0.0	
		TA 392 CTU	0.88 ± 0.1	101		0.00 ± 0.0	
1c	94	C 350 CTU	4.03 ± 0.1	89	N.S.	11.0 ± 0.8	**
		TA 350 CTU	4.30 ± 0.2	55		23.0 ± 8.9	
		C 364 CTU	3.84 ± 0.1	90		10.0 ± 3.3	
		TA 364 CTU	3.31 ± 0.1	73		7.0 ± 5.7	
		C 378 CTU	4.13 ± 0.1	84		16.0 ± 9.8	
		TA 378 CTU	3.49 ± 0.1	69		5.0 ± 0.8	
		C 392 CTU	3.74 ± 0.1	67		7.0 ± 0.8	
		TA 392 CTU	3.28 ± 0.	71		0.0 ± 0.0	
2	58	C (33 f/l)	2.47 ± 0.1	60	n.a.	0.0 ± 0.0	N.S.
		TA (33 f/l)	2.88 ± 0.1	61		0.0 ± 0.0	
		TA (66 f/l)	1.98 ± 0.1	60		3.5 ± 1.8	
		TA (100 f/l)	1.69 ± 0.1	60		1.3 ± 0.7	
		TA (200 f/l)	1.63 ± 0.2	31		1.0 ± 0.0	

Table 2

Experiment	dpf	Treatment	weight (g) ± SE	n	S. S.	Mortality (%) ± SE	S. S.
3	52	C 280	2.23 ± 0.1	90	N.S.	26.3 ± 9.1	***
		TA 280	1.86 ± 0.1	91		9.0 ± 0.9	
		TA 310	1.64 ± 0.1	95		10.5 ± 1.7	
		TA 280 & 310	1.73 ± 0.1	96		6.3 ± 2.3	
		TA 364	1.69 ± 0.1	93		3.8 ± 2.3	
4a	72	C 2h	5.19 ± 0.2	102	***	1.0 ± 1.0	N.S.
		TA 2h	4.76 ± 0.2	103		0.0 ± 0.0	
		TA 3h	4.46 ± 0.2	100		0.0 ± 0.0	
		MDHT 2h	4.86 ± 0.3	104		2.0 ± 2.0	
		MDHT 3h	4.46 ± 0.2	98		2.0 ± 2.0	
		NG 2h	4.90 ± 0.2	101		5.0 ± 2.6	
		NG 3h	4.59 ± 0.2	99		3.0 ± 3.0	
		MT 2h	5.78 ± 0.3	97		2.0 ± 2.0	
4b	81	C 280 2h	2.63 ± 0.1	61	N.S.	31.0 ± 5.0	***
		TA 308 2h	1.68 ± 0.1	60		4.5 ± 4.5	
		TA 280 48h	1.76 ± 0.1	62		0.0 ± 0.0	
		TA 308 4h	2.26 ± 0.1	63		30.5 ± 5.5	
		TA 280 & 308 2h	1.58 ± 0.1	63		0.0 ± 0.0	
		MT 280 48h	1.72 ± 0.1	60		3.5 ± 3.5	
		MT 280 & 308 2h	2.08 ± 0.1	30		0.0 ± 0.0	
5	102	C 308 & 364 CTU	5.02 ± 0.1	203	N.S.	2.2 ± 0.1	N.S.
		TA 364 CTU	4.81 ± 0.1	153		1.9 ± 0.1	
		TA 308 & 364 CTU	5.12 ± 0.2	149		1.6 ± 0.1	

Table 2 continued

Experiment	dpf	Treatment	weight (g) ± SE	n.	S. S.	Mortality (%) ± SE	S. S.
6	63	C	1.33 ± 0.1	49	**	11.0	n.a.
		TA 500 µg	2.77 ± 0.3	15		65.0	
		TA 750 µg	1.73 ± 0.1	30		40.0	
		TA 1000 µg	3.40 ± 0.4	18		52.0	
		MDHT 500 µg	1.56 ± 0.1	29		51.0	
		MDHT 1000 µg	1.73 ± 0.1	36		24.0	
7	92	C-ETOH	6.07 ± 0.2	92	N.S.	3.98 ± 0.1	N.S.
		C-DMSO	5.96 ± 0.2	91		3.39 ± 0.1	
		MDHT-ETOH	5.78 ± 0.2	93		3.81 ± 0.1	
		MDHT-DMSO	5.62 ± 0.2	96		4.07 ± 0.1	
		TA-ETOH	6.26 ± 0.2	97		3.81 ± 0.1	
		TA-DMSO	5.89 ± 0.2	92		4.31 ± 0.2	

Table 2 continued

Experiment 2, no statistical comparisons on final weight were made because the fish were raised in different-sized tanks.

Water quality. Water quality in rearing tanks was maintained close to the optimal values for tilapia culture. Temperature was maintained at 28 ± 1.0 °C; dissolved oxygen averaged 6.4 ± 0.2 mg/l; pH averaged 7.4 ± 0.2; nitrites (NO_2) ranged between non-detectable values to 0.14 ± 0.02 mg/l; and ammonia (NH_3) values were always below 0.01 mg/l.

Histology. According to the recent classification of testicular developmental stages proposed by Grier and Lo Nostro (2000), the histological analysis of all testes sampled indicated that males at 52 dpf were in early to mid-maturation class characterized by a discontinuous germinal epithelium near the ducts, while a continuous germinal epithelium was near the testis periphery (Fig. 23). All samples analyzed seemed to indicate normal development. In few instances, isolated oocytes were observed interspersed in gonads composed mainly of testicular tissue (Fig. 24).

Discussion

We found that short-term immersions of Nile tilapia fry in synthetic steroids cause significant masculinization of the fry. The efficacy of this process is governed by a combination of factors, with time of immersion playing a significant role on the level of masculinization achieved. In systems maintained at 28 °C, the period of gonadal sensitivity to exogenous steroids is limited to five to six days, and as in many biological systems, the timing of gonad sensitivity to steroids resembles a bell-shaped curve (typical of a normal distribution). In our tilapia, sensitivity to masculinization starts at 11 dpf, increases at 12 dpf, reaches a maximum at days 13 and 14 and declines

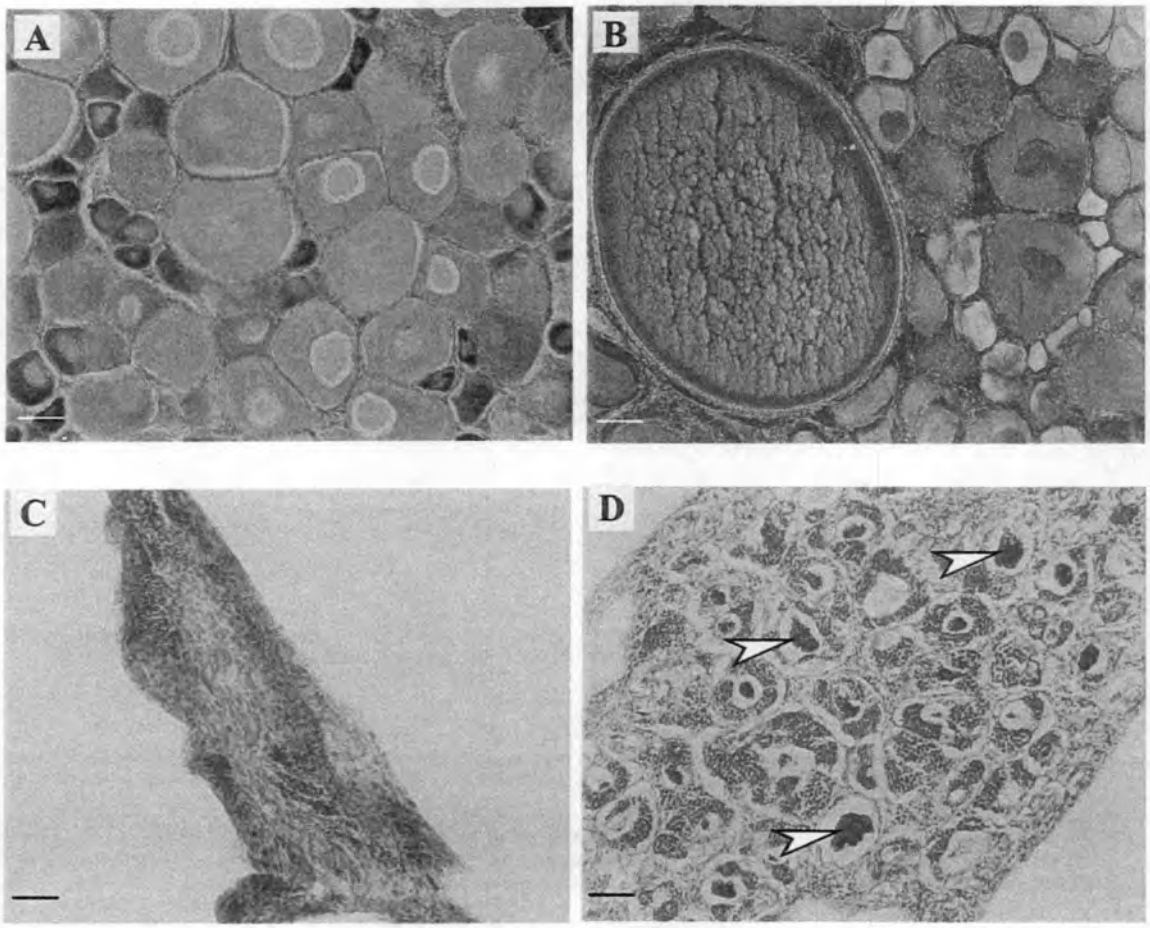


Figure 23. Gonadal histology of Nile tilapia fry at 52 days post-fertilization (dpf) from control females (A and B) and MT-treated males (C and D). All sections are sagittal. (A) Ovary during early stages of maturation showing vitellogenic oocytes. (B) Ovary initiating vitellogenesis showing a few enlarging oocytes. (C) Testis from a juvenile male; the germinal epithelium displays homogeneous staining and no sperm conglomerates. (D) Testicular tissue from a maturing male showing large amounts of sperm concentrated in the center of tubules (indicated by arrows); Bar, 100 μm.

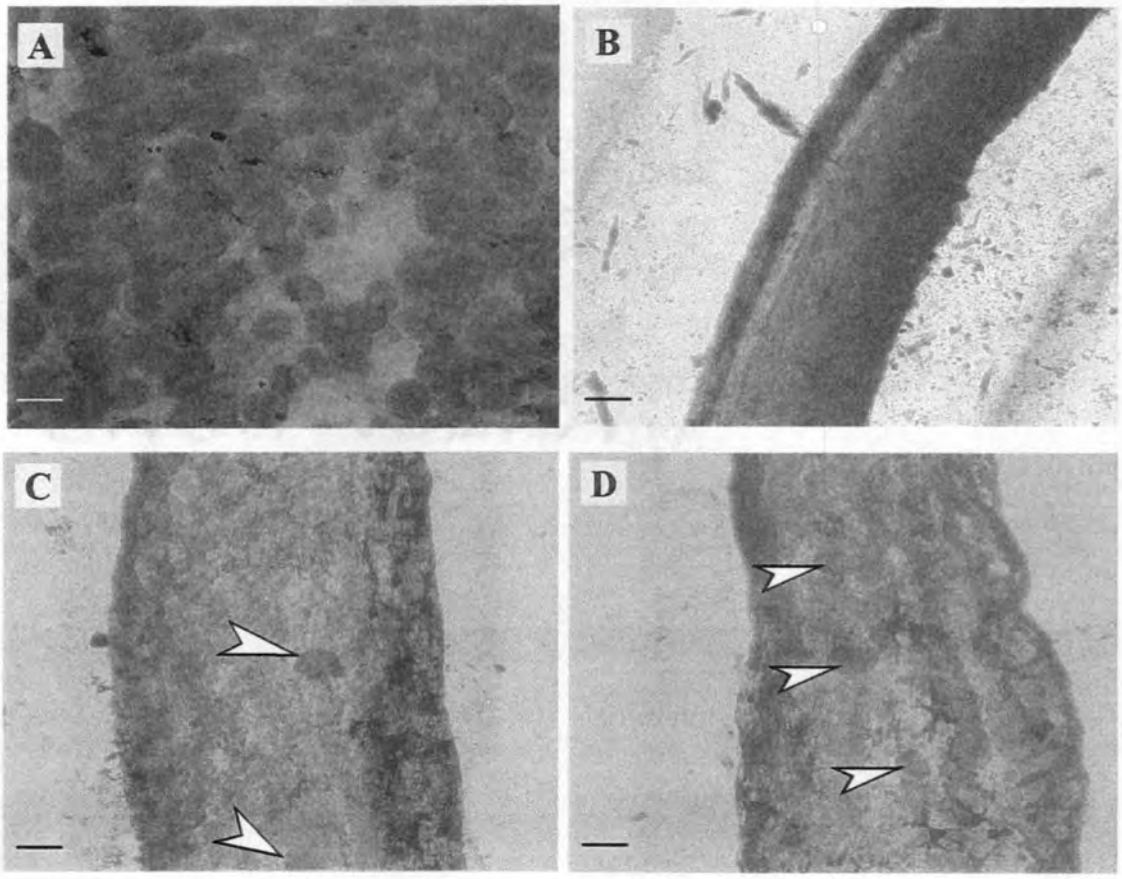


Figure 24. Gonads from fish immersed in either EtOH-vehicle (control) or TA. (A) Ovary from a control female showing normal development and (B) testis from a control male showing normal development. Gonads from TA treated fish showing a large majority of testicular tissue with a few isolated oocytes (C and D). Photographs were taken after squash method using Wright's stain. Arrows indicate scattered oocytes surrounded by testicular tissue; Bar, 100 μm.

by 15 dpf. Piferrer and Donaldson (1993) illustrated a similar pattern in response to both estrogen and androgen treatments in coho salmon, *Oncorhynchus kisutch*, fry. This suggests that variation exists among individuals even from a similar brood and may be the result of developmental differences between the fry.

In chinook salmon, *O. tshawytscha*, Baker et al. (1988) indicated that the labile period for sex reversal was near the hatching time. Later, Piferrer and Donaldson (1989) specified that sensitivity to estrogenic compounds occurs before hatching while sensitivity to androgens appears after hatching. In that paper, the sensitivity of coho salmon fry to estrogens was highest one day before 50% of the fry had hatched, and sensitivity to androgens peaked at 6 days post-hatch. In a study with rainbow trout, *O. mykiss*, Feist et al. (1995) also found that efficacy for androgen treatment peaked one week post-hatching.

A difference between our results and those obtained with salmonid species is that single immersions of coho and chinook salmon fry in MT can produce 90-100% males (Baker et al., 1988; Piferrer and Donaldson, 1989). In contrast, our experiments showed that the maximum number of males obtained with single immersions in TA were around 80%, with lower numbers of males produced when MT was used. Feist et al. (1995) obtained 76% males from a 100% genotypic female rainbow trout progeny with a single immersion. These results suggest that different species have different susceptibility to the androgen treatment, a theory strongly supported by recent results presented by Galbreath and Stocks (1999) who reported the ineffectiveness of MDHT, MT and TA immersion treatments to masculinize brook trout, *Salvelinus fontinalis*. Furthermore, our data also indicate that the response to the androgen treatments varied between broods, indicating differential sensitivity between broods of the same species.

Another factor to consider regarding sensitivity to androgenic steroids is that the development of salmonid fry is notably slower than that of Nile tilapia. As suggested by the studies on salmonids, the labile period at which the gonad can be affected by androgen treatment lasts 21 to 28 days (Piferrer and Donaldson, 1989; Feist et al., 1995). In Nile tilapia, the length of this period lasts only five days. The window of opportunity for masculinizing any individual Nile tilapia may last just a few hours, thus decreasing the overall effectiveness of masculinizing a group of tilapia fry by short-term immersions.

We found that the number of fry per liter of treatment solution influenced the efficacy of the immersion treatment as expected. However, changes in the proportion of males obtained did not show a strong density-dependent pattern. Our results indicate that 33 fish per liter provides the highest number of males. This stocking density is nearly 5-times that reported by Torrans *et al.* (1988) in a study in which *O. aureus* were masculinized by immersion for 5 weeks in mibolerone. The effectiveness of our treatment decreased by 9% when either 66 or 100 fry were treated, and no differences were found between these two stocking rates. In essence, density trials are similar to dosage trials; therefore, the number of males produced using a dose of 5.0 μg of steroid per fish did not differ from a dose of 7.6 μg per fish, while a dose of 15.2 μg of steroid per fish produced 9% more males.

In experiments designed to optimize the immersion protocol for Nile tilapia, we found that the procedure for immersion on 10 and 13 dpf proposed by Gale et al. (1999) could be improved. Instead of using dpf for defining the initiation of treatment, we standardized the protocol by using CTU, compensating for temperature-dependent developmental changes. Our results indicate that a single immersion on 364 CTU (13 dpf) resulted in similar masculinization as two immersions, one at 280 followed by

another at 364 CTU (corresponding 10 and 13 dpf at 28 °C). However, in another experiment, we found that the number of males produced by a double immersion on 308 and 364 CTU provides higher masculinization than a single immersion on 364 CTU. Based on these results, we recommend the use of two 3-h immersions, one at 308 and a second at 364 CTU.

The length of the immersion played a significant role in masculinization of tilapia. Independently of the hormone used, three and four-hour immersions resulted in more efficient masculinization than two-hour immersions. In contrast, in some species of salmonids studied, single 2 h immersions in MT were sufficient to produce near total masculinization (Baker et al., 1988; Piferrer and Donaldson, 1989). However, in most species it has been proposed that double immersions may ensure high masculinization rates (Piferrer and Donaldson, 1993)

Several experiments demonstrated that the synthetic steroid TA was a more potent masculinizing agent than MDHT, NG, and MT. When different dosages of both TA and MDHT were compared, TA produced consistently more males than MDHT. In some instances, TA provided almost twice as many males than MDHT. Small differences were observed between dosages of 500, 750 and 1000 μg/l of TA, and due to the lack of replication in that particular experiment, the results could be explained by variability inherent to the sampling and the small sample size. These results indicate that above 500 μg/l of TA, masculinization does not seem to increase substantially. A different pattern was observed when 1000 μg/l of MDHT where used, since this dosage significantly improved masculinization when compared against 500 μg/l of the same hormone. Gale et al. (1999) found that a dose of 500 μg/l of MDHT produced consistently more males than 100 μg/l.

We performed histological analysis of gonads obtained from an experiment that resulted in significant masculinization (Experiment 3) and found that all the testes analyzed looked normal. In individuals older than 100 dpf, milt production was evident and histological sections indicated significant sperm production. It has been reported that TA administered in the food successfully masculinized channel catfish (Galvez et al., 1995) and blue tilapia (Galvez et al., 1996). However, Davis et al. (2000) clarified that after raising the catfish treated with TA to reproductive age (three years), the fish were not functional males, but infertile organisms. The experiment involving blue tilapia did not report spawning of the treated fish. In our study, we found no indication of sterility in a series of progeny testing trials using fish from TA immersions (data not published), and sex ratios of the progeny produced did not provide evidence that verified that the males bred were genotypic sex reversed females. Furthermore, in tilapia culture the goal of masculinization treatments is to impede undesired reproduction in the grow-out systems; therefore, regardless of whether the treatment results in infertile or non-functional males, the treatment can still be considered as a success.

DMSO and EtOH are commonly used as solvent vehicles for the administration of compounds that do not dissolve readily in water (Castro et al., 1995). DMSO has been used as vehicle of suspected endocrine disrupters in many studies involving fish. It is considered a good vehicle because it is readily absorbed through the skin of many organisms. EtOH, on the other hand, is a good solvent, easy to obtain, and economically affordable. In our study, we found that TA exerts its masculinizing effects with the same efficiency in either solvent vehicle, while MDHT showed no masculinization if administered with DMSO. These results suggest that EtOH is a better option for delivering steroids to fish via immersions. However, more research is

needed to determine if other solvents or dosages may influence the efficacy of the steroid used.

The results of this study confirm that significant masculinization of Nile tilapia fry can be achieved by short-term immersion in masculinizing agents. However, in contrast to the results obtained with various species of salmonids, immersions of Nile tilapia fry in steroids does not result in a consistent pattern of high masculinization rates. In chapter IV, we will investigate multiple immersions in steroids as a potential option for increasing masculinization success and decreasing the variability of masculinizing trials.

Acknowledgments

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Chapter 5

Masculinization of Nile Tilapia, *Oreochromis niloticus,* by Immersion in Synthetic Steroids: II. Effect of Multiple Immersions, Exposure Time, Dose and Fish Density[1]

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Short title: Factors involved in masculinization of tilapia by hormonal immersion

Keywords: Masculinization, Immersion, Methyldihydrotestosterone, Trenbolone Acetate, Nile tilapia, *Oreochromis niloticus.*

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Abstract

We investigated the masculinizing effects of multiple short-term immersions in trenbolone acetate (TA) and 17α-methyldihhydrotestosterone (MDHT) on Nile tilapia fry. Short-term immersions of fry on two different days provided statistically significant masculinization. The combination of immersions at 11 and 13 days post-fertilization (dpf) resulted in more males than those at 13 and 14 dpf. The highest percentage of males obtained under this immersion regime was 93.4. Multiple immersions in TA were as effective as double immersions at providing significant masculinization, with the highest percentage of males obtained in an experiment being 93.7. We explored the influence of density, duration of immersion, steroid dosage, and their interactions using a fractional factorial design. Despite significant masculinization results, the fractional factorial experiment also indicated that the masculinization rates are highly variable and the combination of dosage, duration and density did not show a consistent masculinization pattern. Our results suggest that the window of sensitivity for masculinizing Nile tilapia at the individual level is very short and may vary between broods, which in turn, supports the idea that there are other major factors influencing susceptibility to androgen-induced masculinization. Heterogeneity of developmental stages, developmental rate, and sensitivity of progeny to steroids may play important roles in the efficacy of immersion treatments.

Introduction

Masculinization of tilapia (*Oreochromis* spp.) for the production of male-biased populations continues to be an important tool for aquaculturists to prevent unwanted reproduction and to produce the sex with the larger growth potential. Previous work in our laboratory has shown that short-term immersion in androgenic steroids can result in masculinization of Nile tilapia, *O. niloticus* (Gale et al. 1999; Contreras-

Sánchez et al., Chapter IV). These studies show that immersion in androgen has the potential to be an alternative to dietary treatment with steroids for the masculinization of tilapia. A variety of androgens--especially synthetic androgens--are effective masculinizing agents (Hunter and Donaldson 1983); however, there may be differences in their potency. In the first part of this study (Chapter IV), we showed that single immersions in the non-aromatizable synthetic androgens 17α-methyldihydrotestosterone (MDHT) and trenbolone acetate (TA) were effective in masculinizing Nile tilapia fry. Either treatment resulted in significantly more males being produced than that in the controls. While these results demonstrate the potential for immersion as a mean of administering the compounds, they did not determine optimal treatment conditions. We have had variable success masculinizing tilapia by immersion in MDHT and TA and our results indicate that TA is more potent and more consistent than MDHT in masculinizing Nile tilapia.

In order to determine the best treatment conditions for masculinization by immersion, studies must be conducted on those factors that are believed to play a critical role in determining efficacy. For masculinization by immersion, the major factors are type of hormone, timing of treatment (relative to fish development), hormone dosage, duration of exposure, and density of fish during immersion. Our previous study showed that significant masculinization could be achieved by exposure of fry between 11 and 15 days post-fertilization (dpf) and provided evidence that double immersions within these days may produce the best results.

Because fish density, hormone dosage and length of exposure are factors that may interact, a factorial design must be employed to determine the minimum dosage, the highest density, and the shortest exposure that in combination yield an acceptable rate of masculinization. Because there are many factors that may influence treatment

efficacy, conducting a single experiment in which all factors are examined simultaneously at different levels would require unreasonably large numbers of tanks and more tilapia fry than can be produced from a single spawning, the latter being necessary to eliminate variation attributable to differences in susceptibility between different broods (Contreras-Sánchez et al., Chapter IV). Heretofore, our approach was to examine one factor at a time while holding all others constant. However, this approach limits the amount of information that can be gained on the interactions among the various factors. Therefore, we designed an experiment that was carried out using a fractional factorial design (Kuehl 1994) to examine multiple factors (hormone dosage, exposure duration, and fish density) simultaneously with the TA. This design allows information to be obtained on factors of interest in the early stages of experimentation when the number of treatments exceeds the resources (Kuehl 1994).

We investigated the feasibility of increasing masculinization of Nile tilapia using multiple immersions. We also tested the fractional factorial experimental design combining multiple immersions during the labile period and different levels of density of fish rearing, dosage of hormone and duration of the immersion time.

Materials and Methods.

Fish rearing and breeding. Studies were conducted at the Oregon State University's Fish Performance and Genetics Laboratory, Corvallis, OR. Adult Nile tilapia were separated by sex in 1,100 l tanks supplied with a constant flow of recirculating water. Adult fish were fed three times a day with floating pellets (Silver Cup Fish Feed; Nelson and Sons, Inc). Breeding families were placed in 200 l aquaria (one male to three females), and checked every 2 hours for spawning activity. Once breeding

occurred, the other fish were removed and the brooding female was left to incubate the progeny. Water temperature in all systems was maintained at 28 ± 1°C.

Experimental design. Because efficacy of masculinizing treatments is dependent on developmental stage (Hunter and Donaldson, 1983), only fry from individual broods were used in each experiment to ensure as much between-fry similarity as possible. Temperature in the spawning tank was monitored every 30 minutes with an Optic Stowaway® data recorder (Onset Computer Corp.), and the average temperature was estimated between time of spawning to time of fry capture. Celsius Temperature Units (CTU) were estimated by multiplying the mean water temperature by the number of days or estimated hourly when needed (i.e. 10 days at 28 °C = 280 CTU). At 280 CTU post-fertilization, fry were collected from the tank (if the female released the fry from her mouth) or from the female and randomly assigned to experimental units. Immersions were carried out in 3.8 l glass jars and the number of replicates per treatment was based on the total number of fry in each brood. Treatments consisted of immersions in either steroid or ethanol (EtOH) vehicle, which was mixed with dechlorinated water 1 min before addition of fry. Steroids were obtained from Sigma Chemical Company (St. Louis, Missouri) and stored at 4 °C in stock solutions of vehicle (1 mg/ml). For control and hormone treatments, 500 µl of EtOH vehicle or hormone stock solution per liter of treatment water were used. The final EtOH concentration in the immersion water was 0.05%.

Effects of Double Immersions (Experiments 1a-1c). Results from the previous study (Contreras-Sánchez et al., Chapter IV) indicated that double immersions in synthetic steroids can provide significant masculinization; here we identified the specific timing of immersions necessary to yield the highest rate of masculinization. Based on

previous results, hormone dosage was maintained at 500 µg/l, fish density at 33 fry/l, and duration of the immersion at three hours.

Experiment 1a. Fry immersed in TA at 308 and 364 CTU (11 and 13 dpf at 28 °C). This experiment was run four times. Each trial consisted of two to four replicates (each trial used a different brood). Control groups consisted of EtOH vehicle immersions at 308 and 364 CTU.

Experiment 1b. Fry immersed in TA at 364 and 392 CTU (13 and 14 dpf at 28 °C). This experiment was conducted three times. Each trial consisted of three replicates. Control groups consisted of EtOH vehicle immersions at 364 and 392 CTU.

Experiment 1c. Fry immersed at 308, 308 and 364, 308 and 392, or 392 CTU (11 dpf, 11 and 13 dpf, 11 and 14 dpf, or 14 dpf, respectively). This experiment was run once using three replicates per treatment. The control group consisted of EtOH vehicle immersions at both 308 and 364 CTU.

Effects of Multiple Immersions (Experiments 2a-2c). These experiments were designed to test if more than two immersions were able to improve masculinization rates. Hormone dosage was maintained at 500 µg/l, fish density at 33 fry/l; duration of the immersion was three hours, except when the brood size allowed for more treatments.

Experiment 2a. Treatments consisted of fry immersed at both 308 and 364 CTU (11 and 14 dpf) for either three, six or 24 hours; fry immersed at 336, 364 and 392 CTU (12, 13 and 14 dpf) for three hours; fry immersed at 336, 364, 392, and 420 CTU (12, 13, 14, and 15 dpf) for three or six hours. The control group was immersed at the same times as the last treatment in 0.5 ml/l of EtOH vehicle. All treatments were triplicated.

Experiment 2b. Treatments consisted of fry immersed at 308, 336, 364, 392, and 420 CTU (11, 12, 13, 14, and 15 dpf) for three hours in either TA or MDHT; and fry

immersed at 336, 364, 392, and 420 CTU (12, 13, 14, and 15 dpf) for three hours in TA. The control group was immersed in EtOH vehicle at the same times as the first treatment. This experiment was conducted twice using different broods.

Experiment 2c. Treatments consisted of fry immersed in either TA or EtOH vehicle at 308, 336, 364, and 392 (11, 12, 13, and 14 dpf) for three hours. This experiment was conducted twice using different broods.

Fractional Factorial (Experiment 3). We used a fractional factorial design to examine the effects of fish density, hormone dosage, and exposure duration simultaneously. Fry were immersed at 308, 336, 364, 392, and 420 CTU (11, 12, 13, 14, and 15 dpf) in either TA or EtOH. Fry densities were either 12, 25, 50, 100, or 200 fish/l; hormone dosages were either 62.5, 125, 250, 500, or 1000 μg/l; exposure duration was either 0.75, 1.5, 3, 6, or 12 hours. Because we decided to use a fractional factorial design, only certain combinations of treatment conditions were used (see table in the Results section for combinations used). These conditions were chosen by generating a model using Statistical Analysis Systems for Windows, release 6.10 (SAS Institute Inc., Cary, NC). Under this model, only replication around the middle treatment level for each factor is recommended. The fractional factorial design is effective in screening studies to check on numerous factors, under the assumption that only a few effects are important. The fractional factorial design carries the caveat that follow-up experiments must be conducted using suitable replication once the levels for the various factors are chosen.

For each experiment, fry were collected after each immersion, jars were thoroughly cleaned, and then fish were reallocated in fresh dechlorinated tap water. After 5-7 days, fry were transferred to 75 l fiberglass tanks in a recirculating system. Water temperature in the grow-out system was maintained at 28 ± 2°C. Fish were fed to

satiation 4 times a day with Natureboy Basic Food Flakes (Rexotic Products, Portland, OR, USA). At 60-70 dpf, fish were killed with an overdose of the anesthetic tricaine methanesulfonate (MS-222) and sex ratios were determined by examination of gonads using squash (100X) preparations after Wright's staining (Humason, 1972).

Statistical analysis. Data were checked for evidence of tank effects within treatments (Fisher's test or ANOVA). Because no significant differences between replicates were found, data were pooled. Sex ratio and mortality data were analyzed using Chi square tests. When significant differences between all treatments were found, pairwise comparisons were performed using Fisher's exact test, with exact p-values (a more conservative test than the chi-square test for small sample sizes) estimated in GraphPad Prism™ (GraphPad Software Inc.). For Experiment 3, a response surface regression analysis was performed using the RSREG procedure in SAS. In this analysis, linear and quadratic equations primarily form contours that may show how the response increases or decreases based on the interactions of the factors tested, as well as the trends along levels of the factors. The mean final weights of fish were analyzed in Statgraphics Plus, release 3.0 (Statistical Graphics Corp.) for differences between groups using multivariate analysis of variance, including mortality and sex as possible confounding variables. For all analyses, differences were considered statistically significant when the p-value (P) was less than 0.05.

Results

Effects of Double Immersions (Experiments 1a-1c)

Experiment 1a. All four trials resulted in statistically significant differences between the control and the TA immersed groups (Fig. 25a-25d). Mean (± SE) percentage of males from the control groups were 47.8 ± 6.0; 55.6 ± 11.1; 50.5 ± 3.8; and 54.9 ± 2.5,

Figure 25. Effects of double immersions on masculinization of Nile tilapia fry. Graph shows mean percentage of males (± SE) obtained after immersions for 3h in 500 µg/l of TA or EtOH vehicle (control). Fish were immersed at 308 and 364 CTU (11 and 13 dpf; a, b, c, and d) or at 364 and 392 CTU (13 and 14 dpf; e and f). The numbers in parentheses indicate the numbers of fish sampled for each treatment. Asterisks indicate significant differences between control and treatment groups.

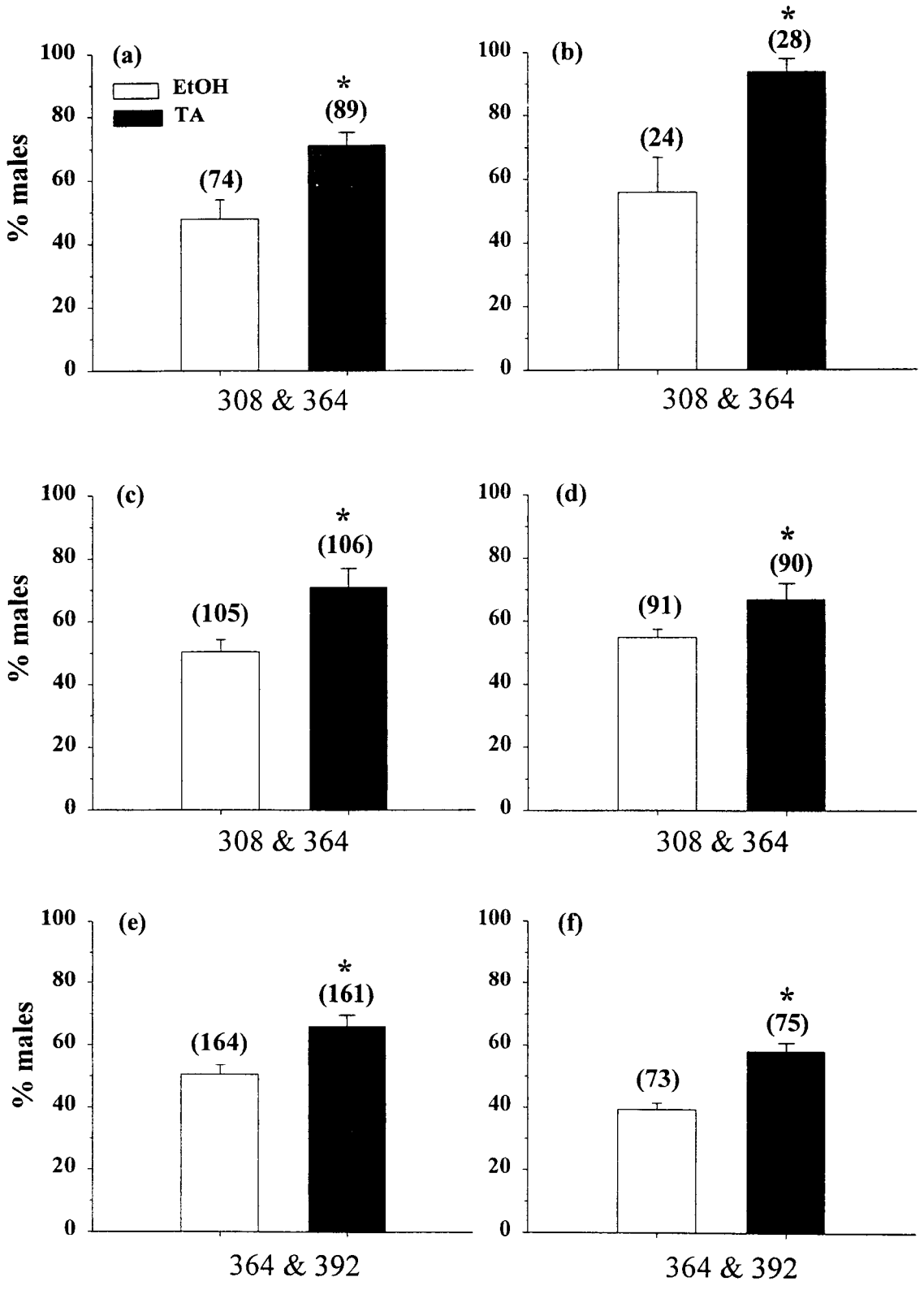

(a)
EtOH
TA
(74)
*
(89)
(b)
(24)
*
(28)
(c)
(105)
*
(106)
(d)
(91)
*
(90)
(e)
(164)
*
(161)
(f)
(73)
*
(75)
% males
100
80
60
40
20
0
308 & 364
364 & 392
CTU post-fertilization

Figure 25

respectively. TA treated groups had 70.8 ± 4.1; 93.4 ± 4.2; 70.8 ± 6.1; and 66.7 ± 5.1 percent males, respectively. All comparisons resulted in P values < 0.01.

Experiment 1b. Two out of three trials resulted in significant differences between the control group and the fish immersed in TA at 364 and 392 CTU (Fig. 25e-25f). Control groups had averages of 50.5 ± 3.1 and 39.4 ± 2.0 percent males, while TA treated groups had 65.9 ± 3.8 and 58.0 ± 2.8 percent males, respectively. The trial that resulted in no masculinization had an average of 53.4 ± 1.0% males in the control group and 50.5 ± 3.2% males in the TA treatment (graph not shown).

Experiment 1c. No significant differences were found when all treatments were compared. Mean percentage of males in the control group was 54.0 ± 4.6 (Fig. 26).

Effects of Multiple Immersions (Experiments 2a-2c).

Experiment 2a. Every treatment that involved TA resulted in significantly more males than the control group ($P < 0.001$; Fig. 27a). Mean percentage of males in the control group was 48.8 ± 8.8. The percentage of males in the TA immersed groups ranged between 81.3 and 93.7. When comparisons were made between TA treatments, significantly more males ($P < 0.001$) were produced in those groups that included immersions at 336 CTU (12 dpf) compared with those that started at 364 CTU (13 dpf). No significant differences were found among those treatments that started on day 12 nor among those that started on day 13.

Experiment 2b. Only the fry immersed in TA at 308, 336, 364, 392, and 420 CTU resulted in significantly more males than the control group (Fig. 27b). Mean values for the control and TA treated group were 40.4 ± 4.8 and 50.7 ± 1.3 % males, respectively. All other comparisons resulted in no significant differences when

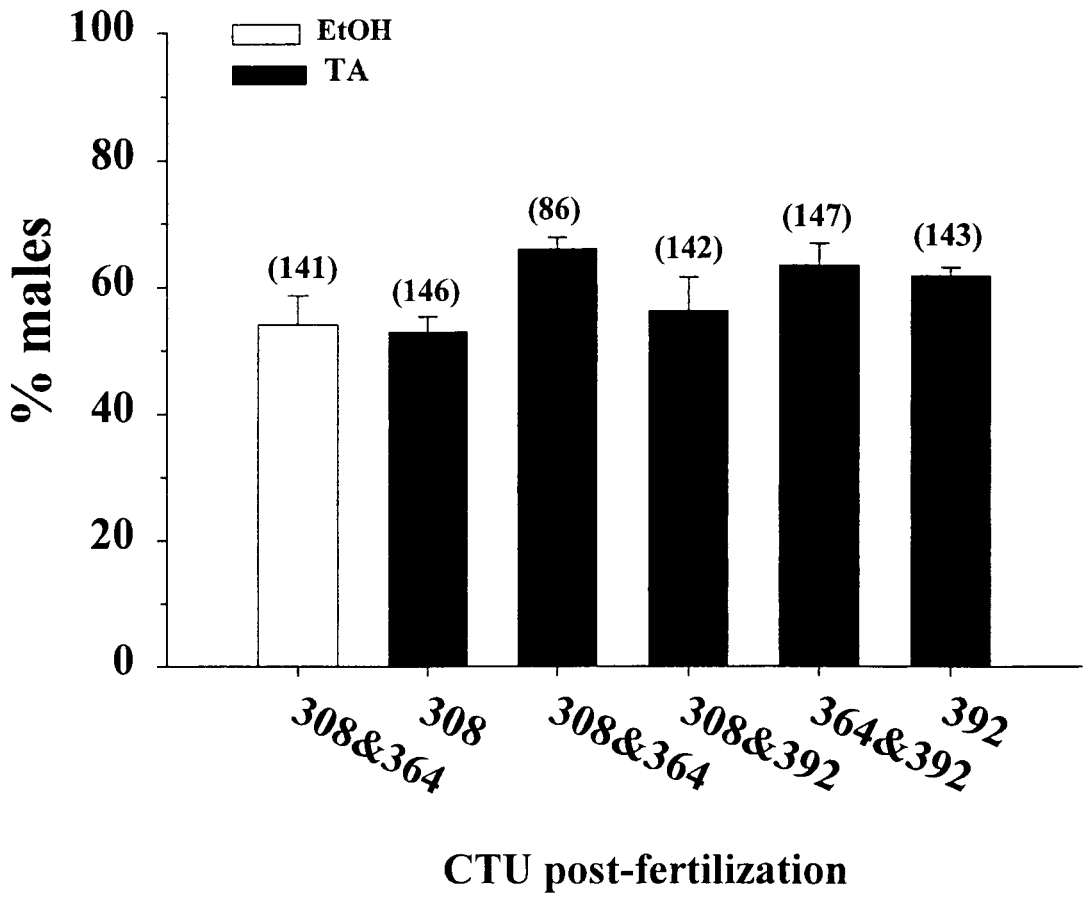


Figure 26. Mean percentage of males (± SE) obtained after single and double immersions of Nile tilapia fry in 500 µg/l of TA. Treatments consisted of fry immersed at 308, 308 and 364, 308 and 392, or 392 CTU (11, 13, and 14 dpf). The control group was immersed in EtOH vehicle at 308 and 364 CTU. The number of fish sampled in each treatment is provided in parentheses. Each treatment had three replicates.

Figure 27. Masculinizing effects of multiple immersions in 500 μg/l of TA or MDHT on Nile tilapia fry. Each treatment was triplicated and data from replicates were pooled. (3a) Fry immersed at 308 and 364 CTU (11 and 14 dpf) for either three, six or 24 hours (shown in parentheses below the bars); fry immersed at 336, 364 and 392 CTU (336-392) for three hours; fry immersed at 336, 364, 392, and 420 CTU (336-420) for either three or six hours; the control group was immersed in EtOH vehicle at the same times as the last treatment. (3b) Fry immersed at 308, 336, 364, 392, and 420 CTU (308-420) for three hours in either TA or MDHT; fry immersed at 336, 364, 392, and 420 CTU (336-420) for three hours; and the control group was immersed in EtOH vehicle at the same times as the first treatment. (3c) Fry immersed in either TA or EtOH vehicle at 308, 336, 364, and 392 (308-392) for three hours. The numbers of fish sampled are provided in parentheses (above the bars). Common letters indicate treatment values that are not significantly different from each other.

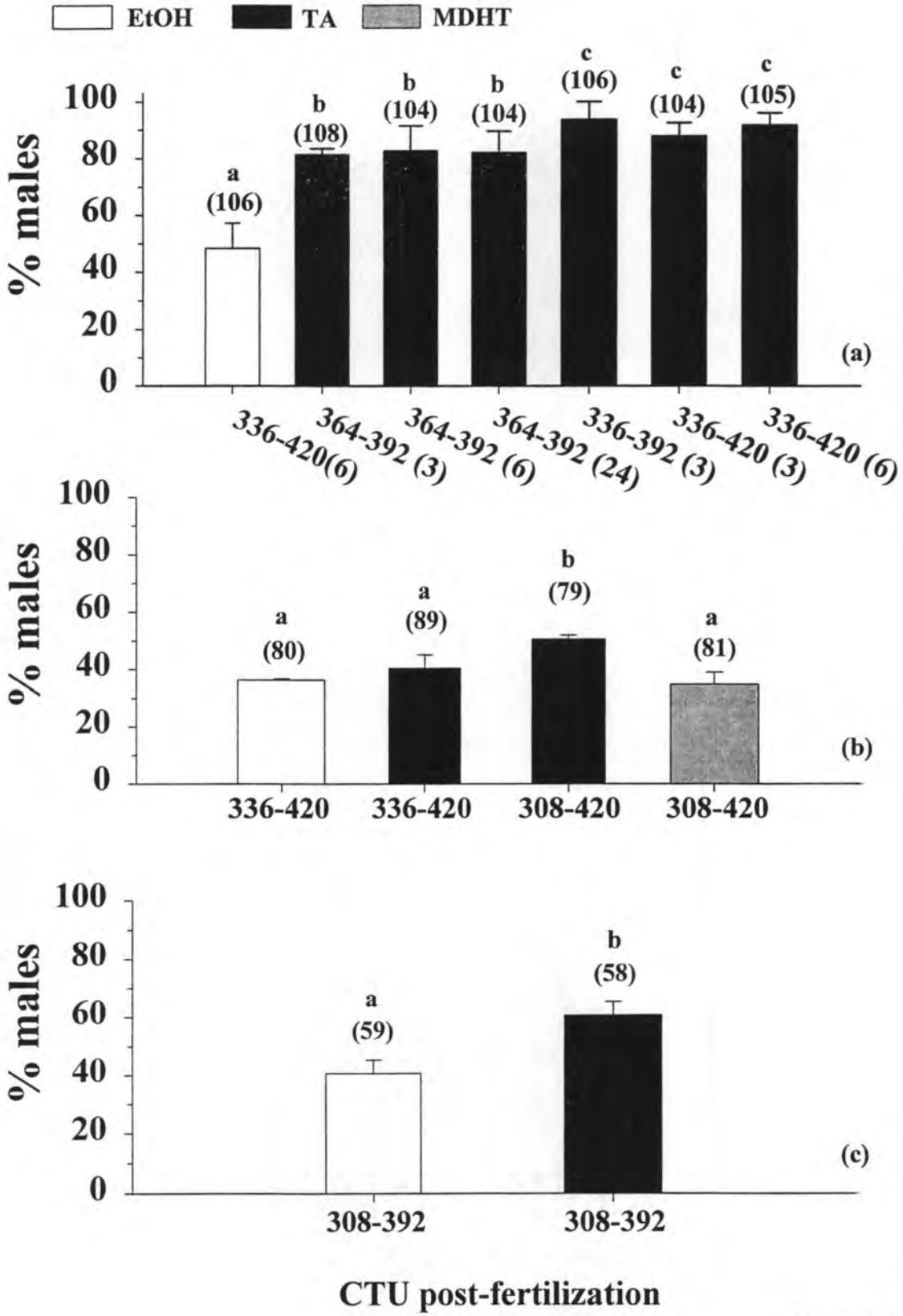

EtOH
TA
MDHT
% males
100
80
60
40
20
0
a (106)
b (108)
b (104)
b (104)
c (106)
c (104)
c (105)
(a)
336-420(6)
364-392 (3)
364-392 (6)
364-392 (24)
336-392 (3)
336-420 (3)
336-420 (6)
a (80)
a (89)
b (79)
a (81)
(b)
336-420
336-420
308-420
308-420
a (59)
b (58)
(c)
308-392
308-392
CTU post-fertilization

Figure 27

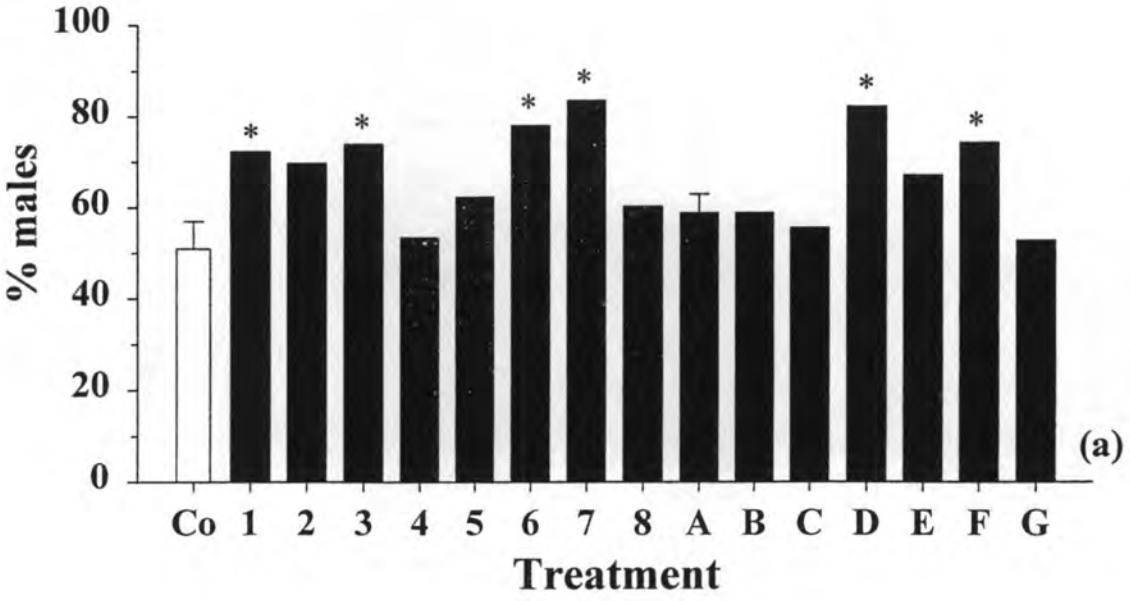
compared against the control. The second brood treated with TA showed no significant differences in males produced between treated and control groups. Mean percentage of males in the control group was 57.1 ± 7.4.

Experiment 2c. Fish immersed in TA had significantly more males than the control group (Fig. 27c). Mean percentage of males for the control and TA groups were 40.7 ± 0.7 and 60.7 ± 0.4, respectively. No significant masculinization was obtained from fish immersed in TA in the second trial (not shown). Mean percentage of males in the control group for this brood was 53.4 ± 2.4.

Fractional Factorial (Experiment 3). Significant masculinization was obtained with different combinations of the three factors used (Fig. 28a); however, the surface response regression analysis indicated that none of the factors or combinations were able to produce a significant trend in masculinization ($P > 0.05$), indicating that significant masculinization was obtained at either high or low levels of the factors studied. Mean percentage of males in the control group was 50.9 ± 0.5, while highest masculinization rates of treated fish ranged between 72.1 and 83.3% males. Figure 28b shows that the dispersion of the values with significantly higher percentage of males had no clear pattern of interaction between the three factors tested.

Mortality and Growth. Table 3 summarizes mortality and final weights in all experiments. Significant differences in final weights were found between treatment groups in Experiments 1c, 1e, 3a and 3b. However, these results did not indicate a consistent treatment effect. In some instances, the controls grew more than the hormone-immersed treatments (Experiments 1c and 3a), while in other experiments the reverse was observed (Experiments 1e and 3b). Significant differences in

Figure 28. Percentage of males in the treatment groups generated by the fractional factorial experimental design (a). Only the control (EtOH immersed) group and "A" treatments (38 fish/l; 250 μg/L of TA; 1.5 h immersions) were replicated (3 and 6 replicates, respectively) and their standard errors are indicative of the expected variability throughout the entire experiment. Asterisks indicate significant differences in comparison to the control group. Figure 28b depicts the data generated by the fractional factorial experiment indicating immersion length, density and dosage at which each treatment was assigned. Letters and numbers in parentheses indicate the treatment "coordinates" assigned by the fractional factorial design. Shaded boxes represent those treatments that were significantly different from the controls. Note the lack of pattern in the arrangement of treatments with significant differences.



Immersion Length (h)	Density (fish/l)	Dosage µg/l				
		62.5	125	250	500	1000
0.375	10 19 38 75 150			(G) 52.4		
0.75	10 19 38 75 150		(3) 73.7 (1) 72.1		(4) 53.3 (2) 69.6	
1.5	10 19 38 75 150	(B) 58.6		(D) 81.8 (A) 58.7 ± 4.1 (E) 66.7		(C) 55.2
3	10 19 38 75 150		(7) 83.3 (5) 62.2		(8) 60.0 (6) 77.8	
6	10 19 38 75 150			(F) 73.7		

(b)

Figure 28

Table 3. Mean final weight (in grams) and final percent mortality (± SE) of fish immersed in steroid or vehicle. The days post-fertilization when final weights were measured (dpf), the number of fish sampled (n) and statistical significance for overall ANOVA or Chi square (N.S. = not significant $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) are provided for each treatment. C = Control, TA = trenbolone acetate, MDHT = 17 α-methyldihydrotestosterone, n.a. = statistical analysis not performed.

Experiment	dpf	Treatment	Weight (g) ± SE	n	S. S.	Mortality (%) ± SE	S. S.
1a	113	C 308 & 364 CTU	11.31 ± 0.60	74	N.S.	18.6 ± 2.3	N.S.
		TA 308 & 364 CTU	12.07 ± 0.79	89		19.3 ± 3.5	
1b	68	C 308 & 364 CTU	3.19 ± 0.46	24	N.S.	73.0 ± 18.2	N.S.
		TA 308 & 364 CTU	2.92 ± 0.25	28		68.0 ± 12.8	
1c	59	C 308 & 364 CTU	4.16 ± 0.26	105	***	1.3 ± 0.8	N.S.
		TA 308 & 364 CTU	1.93 ± 0.08	106		2.0 ± 0.6	
1d	60	C 308 & 364 CTU	4.34 ± 0.15	91	N.S.	7.0 ± 2.7	N.S.
		TA 308 & 364 CTU	3.98 ± 0.14	90		9.0 ± 1.6	
1e	70	C 308 & 364 CTU	5.61 ± 0.14	164	***	17.3 ± 1.3	N.S.
		TA 308 & 364 CTU	6.66 ± 0.12	161		18.7 ± 0.7	
1f	87	C 308 & 364 CTU	5.58 ± 0.24	73	N.S.	10.0 ± 1.6	N.S.
		TA 308 & 364 CTU	5.59 ± 0.33	75		12.0 ± 0.0	
2	82	C 308 & 364 CTU	3.97 ± 0.14	140	N.S.	6.0 ± 2.0	***
		TA 308 CTU	5.26 ± 0.12	146		2.7 ± 1.3	
		TA 308 & 364 CTU	4.95 ± 0.30	86		42.7 ± 12.2	
		TA 308 & 392 CTU	5.25 ± 0.15	142		8.0 ± 0.0	
		TA 364 & 392 CTU	5.04 ± 0.14	147		2.0 ± 1.2	
		TA 392 CTU	4.50 ± 0.14	143		11.3 ± 2.9	
3a	86	C 336-420 CTU	2.03 ± 0.07	106	***	18.2 ± 8.3	N.S.
		TA 364-392 (3) CTU	1.40 ± 0.05	108		14.1 ± 5.3	
		TA 364-392 (6) CTU	1.74 ± 0.05	104		13.1 ± 3.1	
		TA 364-392 (24) CTU	1.65 ± 0.05	104		22.2 ± 12.4	
		TA 336-392 (3) CTU	2.24 ± 0.09	104		18.2 ± 6.1	
		TA 336-420 (3) CTU	1.78 ± 0.06	106		22.7 ± 13.6	
		TA 336-420 (6) CTU	1.90 ± 0.06	105		15.2 ± 7.6	

Table 3

Experiment	dpf	Treatment	Weight (g) ± SE	n	S. S.	Mortality (%) ± SE	S. S.
3b	50	C 336-420 CTU	2.01 ± 0.10	80	***	19.9 ± 2.7	N.S.
		TA 336-420 CTU	1.02 ± 0.03	89		10.1 ± 1.0	
		TA 308-420 CTU	1.87 ± 0.11	79		20.2 ± 2.0	
		MDHT 308-420 CTU	2.85 ± 0.05	81		18.2 ± 0.0	
3c	58	C 336-420 CTU	4.20 ± 0.11	N.S.	59	21.3 ± 1.3	N.S.
		TA 336-420 CTU	4.09 ±0.10		58	22.7 ± 2.7	
4	60	C	1.93 ± 0.09	n.a.	94	C = 16.7	n.a.
		1	1.37 ± 0.09		43	1 = 26.7	
		2	1.08 ± 0.07		46	2 = 28.0	
		3	1.78 ± 0.17		19	3 = 0.0	
		4	2.25 ± 0.31		15	4 = 21.1	
		5	1.20 ± 0.10		45	5 = 25.3	
		6	1.94 ± 0.18		36	6 = 52.0	
		7	2.29 ± 0.15		18	7 = 5.3	
		8	2.30 ± 0.42		10	8 = 47.4	
		A	1.75 ± 0.09		133	A = 39.9	
		B	1.43 ± 0.11		29	B = 23.7	
		C	1.53 ± 0.16		29	C = 23.7	
		D	2.71 ± 0.26		11	D = 0.0	
		E	2.71 ± 0.18		27	E = 46.0	
		F	1.68 ± 0.22		19	F = 50.0	
		G	1.72 ± 0.14		21	G = 44.0	

Table 3 continued

mortality were observed in Experiment 2, when fish immersed in TA at 11 and 13 dpf had significantly more fish dying (42.7%) than the rest of the treatments (mean = 6.0%). In Experiment 4, no statistical comparisons on final weight or mortality were made because the design of the fractional factorial included different densities in treatment tanks.

Water quality. Water quality in rearing tanks was maintained close to the optimal values for tilapia culture. Temperature was maintained at 28 ± 1.0 °C, dissolved oxygen averaged 6.4 ± 0.6 mg/l, pH averaged 7.1 ± 0.5; nitrites (NO_2) ranged between 0.10 to 0.8 ± 0.1 mg/l; and ammonia (NH_3) concentrations were always below 0.5 mg/l.

Discussion

Short-term immersions of Nile tilapia fry twice, each one on different days, provided statistically significant masculinization. The highest percentage of males obtained was 93.4, but this extreme result occurred only in one out of eight trials. Most experiments produced between 60-80 % males and two trials resulted in no masculinization. The combination of immersions at 11 and 13 dpf resulted in slightly more males than those at 13 and 14 dpf.

Multiple immersions in TA were as effective as double immersions for providing statistically significant masculinization. The highest percentage of males obtained in any experiment was 93.7, but two broods produced only 50-60% males. Despite the statistical significance of these results when compared with their respective controls, the number of males obtained does not reach the recommended values for successful aquacultural practices (95% or more males; Popma and Green, 1990). Anderson and

Smitherman (1978) cautioned that a population with as low as 1-5% females can result in high reproduction and overcrowding of ponds.

TA seems to be more potent than MDHT, supporting the results presented in Chapter IV. TA was found to be very effective in masculinizing channel catfish, *Ictalurus punctatus* (Galvez et al., 1995) and blue tilapia, *O. aureus* (Galvez et al., 1996) by oral administration. However, despite promising results, the administration of TA via short immersions produces highly variable rates of masculinization. TA presents the advantage of being approved as a growth promoter by the US Food and Drug Administration for its use in the cattle industry, and several studies have been conducted regarding its efficacy, residency in the animals treated, and elimination pathways (e.g. Galbraith and Watson, 1978; Hermesmeyer et al., 2000; Yoshioka et al., 2000). Therefore, efforts to maximize its masculinizing efficacy could have a significant impact on aquacultural practices.

The fractional factorial design presented an opportunity to explore the influence of several major factors and their interactions while using multiple immersions. However, despite significant masculinization, the fractional factorial experiment also indicated that the masculinization rates are highly variable and the combination of dosage, duration and density did not show a consistent pattern in masculinization rates. This may indicate that other factors could be involved in controlling gonadal differentiation in Nile tilapia.

The results suggest that the window of sensitivity for masculinizing Nile tilapia at the individual level is very short and may vary between broods, which in turn, supports the idea that there are other major factors influencing susceptibility to androgen-induced masculinization. The rate of development for tilapia is relatively fast if

compared with species that have been shown to have high susceptibility to immersion treatment with steroids (e.g. salmonids). Several species of salmonids have been successfully masculinized by short immersions (Piferrer and Donaldson, 1993; Pandian and Sheela, 1995), and the susceptibility to androgen treatment seems to last between 21 to 28 days (Piferrer and Donaldson, 1989; Feist et al., 1995). We have demonstrated that the labile period for tilapia masculinization lasts only 5 to 6 days at 28 °C. The fast developmental rate of tilapias may restrict the period of gonadal sensitivity to exogenous steroids delivered by immersions to a few hours.

It is common knowledge that tilapia have a very heterogeneous distribution in size present at all life stages. In our laboratory, Rik Hornick (unpublished data) found that tilapia fry size at 10 dpf (range of average lengths from broods = 7.84 - 8.37 mm, range of average weights from broods = 7.37 - 8.31 mg) varies between broods independently of the size of the mother. Hornick also found that the length of the fry at 10 dpf within a brood ranged between 6.8 to 8.5 mm with a coefficient of variation of 4.9, and that by 15 dpf the variability in the data remained constant. Little is known about the variability in developmental stages of tilapias, but the heterogeneity in size may serve as a good indicator of heterogeneity in development. Therefore, if the labile period of gonadal sensitivity of Nile tilapia fry lasts a few hours and developmental stages are variable among the fry, the chances of successful masculinization are limited. Perhaps longer immersion periods may be required to obtain all male populations; however, immersions as long as 24 h were tested in the current study and found to provide no significant improvement in masculinization. Other factors that can potentially influence the variability of the results are the sensitivity of broods to the steroid used and inbreeding. Strain sensitivity to steroids was mentioned by Varadaraj et al. (1994) as a potential factor influencing masculinization efficiency in tilapias. However this hypothesis has not been tested.

Despite significant masculinization in several experiments, short-term immersions in androgenic steroids also resulted in low rates of masculinization in other experiments. Independently of the number of short immersions used during the labile period, the percentage of males produced never reached 95% or higher. This poses a significant disadvantage for the immersion technique in terms of its feasibility for aquacultural purposes if 99% or more male populations are required. Early studies on immersions of tilapia fry in androgens reported 100% masculinization; however, these studies involved protocols that required 1 to 5 weeks of treatment (Varadaraj and Pandian 1987; Torrans *et al.* 1988). Such a long term protocol defeats the purpose of the immersion treatment (i.e. short-term usage of steroids, small amounts of hormone used, little manipulation of the fry).

We have defined that the critical stage of development when tilapia are susceptible to masculinization by short-term immersion occurs between 11 and 15 dpf. This may provide important clues as to when tilapia are susceptible to masculinization by treatments that do not involve steroids (e.g. temperature, pH). More research is needed to investigate if the immersion protocol can be improved by a combination of such factors and hormonal treatment (see Appendix). The development of this technology for masculinization of tilapia may enable farmers to masculinize tilapia with androgens while minimizing the risk of contamination of ponds with MT. Currently, we are investigating the effects of steroid solubility and temperature on the susceptibility to steroids during the labile period.

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Chapter 6

Identification of Unique Genes Expressed During Sex Inversion of Nile Tilapia, *Oreochromis niloticus* by cDNA Subtractive Hybridization[1]

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Short title: Gene expression during sex inversion of tilapia

Keywords: cDNA library, Masculinization, Immersion, Trenbolone Acetate, Nile tilapia, *Oreochromis niloticus.*

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Abstract

The genetic control of sex differentiation in mammals has been linked to the expression of several specific genes, including SRY, the purported testis-determining factor; however in fish, these genes have either not been identified or are present in both sexes. Therefore, to identify the genes involved in sex differentiation in fishes, we have utilized induction of sex inversion in Nile tilapia (*Oreochromis niloticus*) to produce phenotypic males and combined this with subtraction hybridization techniques to investigate the mechanism of sex determination and gonadal differentiation. Fry were immersed for brief periods in the masculinizing synthetic steroid, trenbolone acetate, during early development; shortly thereafter, RNA from treated and untreated fish was isolated and used to identify the expression of unique mRNAs during the process of sex inversion through the use of suppression subtractive hybridization. mRNA collected 16 hrs after treatment suggests that short-term immersion of tilapia fry in trenbolone acetate induces the expression of unique genes. We have sequenced 165 clones from which 61 proteins have been identified. A significant number of these genes seem related to the anabolic effects of trenbolone acetate. We have also identified 12 genes related to reproductive tissues; seven of which have unique or enriched expression in the testes. Some of the genes and protein products that have been identified are linked to gonadal development and testicular protein synthesis in other species.

Introduction

Among vertebrates, fishes have the highest variability of sex determining mechanisms, ranging from polygenic systems to socially and environmentally controlled (or at least influenced) mechanisms (Yamamoto, 1969; Bull, 1983; Conover and Heins, 1986). Different models have been proposed to explain sex determination in fishes, including the traditional homogametic-heterogametic system and multifactorial autosomal systems (Trombka and Avtalion, 1993). However, the specific genetic elements that control the initial events in sex determination and the components of the processes downstream of genetic sex determination remain unknown. The search for distinctive genetic material that has been found in other vertebrates (specifically in mammals) have met with little success, for example, *Sry*-like sequences occur in both male and female fish (Tiersch *et al.*, 1992). We suggest that looking for the specific gene products that are expressed during the course of sex differentiation in both sexes might be a fruitful approach to examine the process of sex differentiation in fish.

The plasticity observed in sex development in fish allows for manipulation of sex differentiation. The fact that sex differentiation in several species can be controlled by exogenous treatment with steroids led Yamamoto (1969) to hypothesize that steroids may be the natural inducers of gonadal differentiation. The sensitivity to steroid-induced sex inversion has been the basis for the development of techniques for the production of single sex populations in several fish species. This potency does not prove that steroids are the natural inducers of gonadal differentiation; nevertheless, it provides a powerful tool for exploring the physiological process of sex differentiation.

By resolving the mechanisms by which steroids induce differentiation to one sex or the other, insight may be gained in understanding the molecular events surrounding sex determination in fishes. The results of recent experiments support the presence of an androgen receptor in the ovaries of juvenile coho salmon, *Oncorhynchus kisutch*, (Fitzpatrick, *et al.*, 1994). Furthermore, Gale (1996) reported the existence of an androgen receptor in the gonadal cytosol of adult and juvenile Nile tilapia *Oreochromis niloticus*. The presence of androgen receptors in the gonads suggests that this tissue is the target of exogenous steroid treatment, but the presence of androgen receptors and their possible role in undifferentiated gonads is still unknown.

Induction of sex inversion has been extensively used in the search for mechanisms of sex determination and gonadal differentiation in fishes (Yamamoto, 1969; Pandian and Sheela, 1995). At the same time, it has become an important tool for enhancing aquacultural practices, since monosex populations are desired for increased production in many systems (Green *et al.*, 1997). Short term immersions in synthetic steroids are efficient for masculinizing several species of salmonids (*Oncorhynchus,* spp; Hunter and Donaldson, 1983) and recent studies have focused on applying this technique to warm-water species, such as tilapia (Gale et al., 1999). One important feature of the short term immersions technique is that it provides additional information regarding the labile period during which the administered steroid can stimulate (or inhibit) the mechanisms that leads to sex differentiation. Determining when developing fish are specifically susceptible to steroid-induced sex inversion should identify those periods when genes responsible for sex differentiation are expressed. Identifying the unique

mRNAs expressed during this sex determining period of development might provide the necessary probes needed to examine the mechanism for sex differentiation in fishes.

Until now, the conventional approach for examining sex differentiation in fish has focused on searching for sexually-distinct genetic material that has been found in mammals or other vertebrates. These efforts have met with little success. The development of new techniques in molecular biology, such as suppression subtractive hybridization which is used to identify unique mRNAs (Diatchenko *et al.*, 1996) and the production of single sex populations through steroid treatment, create a unique opportunity to describe some of the major steps of sex differentiation in fish. The subtractive hybridization technique compares two populations of mRNA and identifies differentially expressed mRNA transcripts, and it has been emphasized that this technique can be used to isolate genes that are expressed as a result of differences in developmental stages or tissues responding to exogenous stimulation (Jin *et al.*, 1997). Applying this technique to androgen-immersed tilapia fry, the genes turned on during sex inversion may be identified. Furthermore, once these gene products are identified, molecular probes can be developed to determine if sex-specific and tissue-specific expression occurs during natural sex differentiation.

The combination of innovative techniques that allow the production of monosex populations by short-term immersions and suppression subtractive hybridization to identify unique mRNAs, create an opportunity to describe some of the major steps of

sex differentiation in fish. We investigated the mechanism involved in steroid-induced sex differentiation of Nile tilapia by creating a subtracted library that contained specific genes that were actively transcribed during the process of sex inversion by a synthetic androgen.

Materials and Methods

Rationale. In previous studies, we demonstrated that short-term immersion in steroids can effectively masculinize tilapia fry (Gale et al., 1999; Contreras-Sánchez et al., Chapters IV and V). Steroid hormones induce transcription of specific genes and also increase the stability of several of the mRNA encoded by those genes (Alberts et al., 1994). We found that a combination of two immersions--one during the period of early responsiveness to masculinizing agents (11 days post-fertilization; dpf) and one during the period of peak responsiveness (13 dpf)--can produce populations composed of 90% or more males. If the mechanisms leading to sex inversion are activated by this protocol, the expression of the transcribed messages might peak within hours after the steroid immersion on the day of peak responsiveness. Therefore, sampling mRNA at 12-16 hrs might capture those expressed genes.

Fish rearing and breeding. Studies were conducted at the Oregon State University's Fish Performance and Genetics Laboratory, Corvallis, OR. Adult Nile tilapia were separated by sex in 1,100 l tanks supplied with a constant flow of recirculating water. Breeding families were placed in 200 l aquaria (one male to three females), and checked every 2 hours for spawning activity. Once breeding occurred, the other fish

were removed and the brooding female was left to incubate the progeny. At 10 dpf, the female was forced to release the fry from her mouth. Fry were counted and randomly assigned to experimental units. Each replicate consisted of 100 fry housed in a 3.8 l glass jar with 3 l of dechlorinated tap water. Each treatment consisted of three replicates and all the fry used in an experiment were the product of a single brood. The water in all treatments was maintained at 28 ± 1°C under constant aeration.

Immersions. Fry were immersed for three hours in either 500 µg/l of trenbolone acetate (TA) in EtOH or EtOH vehicle alone at 11 and 13 dpf (308 and 364 Celsius Temperature Units; CTU) at a density of 33 fish/l. After each immersion, fry were collected and jars were thoroughly cleaned. Then, the fish were placed in jars with fresh dechlorinated tap water. At 14 dpf, 50 fry per replicate were sampled for total RNA isolation (see below), the rest of the fish were reared in 75 l fiberglass tanks all connected to a recirculating system at 28 ± 2°C. Fish were fed to satiation four times a day for 68 days. At this time, fish were killed by overdose of anesthetic (tricaine methanesulfonate, MS-222, 600 mg/L) and sex ratios were determined by examination of gonads using squash (100X) preparations with Wright's stain (Humason, 1972). Once the success of the masculinization treatment was confirmed, we processed the tissues for RNA extraction.

Total RNA sampling and isolation. At 14 dpf (16 hours post immersion), 50 fry per replicate were killed by overdose in MS-222. Fry from replicates were pooled in each

treatment; excess water was removed by drying on tissue paper, and the total weight of the sample was measured. Total RNA from all 150 fry (~1.25 g) from ethanol immersed (Control) and TA immersed (TA) treatments were isolated by single-step method using the RNA STAT-60™ isolation reagent (Tel-Test, Inc.).

mRNA and cDNA synthesis. Polyadenylated RNA was isolated from the total RNA pools using the Oligotex™ mRNA purification kit (Qiagen Inc.), according to the manufacturer's instructions. Double-stranded cDNA was synthesized from 1.5 μg of poly(A)$^+$ RNA from the control or TA treatments using the SMART™ PCR cDNA synthesis kit (CLONTECH Laboratories, Inc.).

Suppressive subtractive hybridization. A cDNA subtracted library was constructed using the CLONTECH PCR-Select™ cDNA subtraction kit (CLONTECH Laboratories, Inc.) following procedures described by the manufacturer with minor modifications. We performed a forward subtraction to detect expression of up-regulated genes following TA treatment. Because of the limited amount of poly A$^+$ RNA obtained, we were not able to perform a reverse subtraction to identify down-regulated genes. Briefly, tester and driver cDNAs were prepared from the two populations of genetic material. The cDNA from the TA-treated group was used as tester and the ethanol-immersed group as driver. Digestion of the cDNA with a four-base-cutting restriction enzyme (Rsa I) yielded cDNAs with blunt ends. The tester cDNA was subdivided in two portions, and each portion was ligated with a different cDNA adaptor. After two hybridizations, the formation of ds-tester molecules with

two different adaptors on either end allowed for the preferential amplification of the subtracted and normalized fractions using PCR. A secondary PCR amplification using nested primers was performed to reduce the background from PCR products and enrich the differentially expressed sequences. Conditions for thermal cycling were slightly modified from the manufacturer's recommended conditions. The PCR mixture was heated for 30 s at 94 °C, followed by 15 cycles of PCR amplification at 94 °C, 30 s; 66°C, 30 s; 72 °C, 1.5 min. An aliquot of eight µl of the PCR reaction was analyzed in a 2% agarose/Ethidium Bromide (EtBr) gel run in 1X TAE buffer (Sambrook et al., 1989). All PCR and hybridization steps were performed on an Amplitron II (Thermolyne) thermal cycler.

Cloning and sequencing of subtracted cDNA products. Subtracted cDNA products were cloned directly into pCRII.1 (TA Cloning kit, Invitrogen Inc.). Plasmid DNAs were prepared using QIAprep Spin Miniprep kit (Qiagen Inc.) following the manufacturer's protocol. Transformed bacteria were plated onto ampicillin–containing agar plates and colonies were grown at 37 °C until visible. The bacterial colonies were maintained at 4 °C until blue/white staining was clearly distinguishable. The inserts in pCRII were amplified by PCR using M13 forward and reverse primers (which flank the multiple cloning site of pCRII). The amplified inserts were analyzed in a 2% agarose/EtBr gel run in 1X TAE buffer (Sambrook et al., 1989) and purified using Qiaquick Spin PCR purification kit (Qiagen Inc.).

cDNA sequencing and analysis. Sequencing was performed at the Oregon State University's Center for Gene Research and Biotechnology on a 373 DNA sequencer (Applied Biosystems, Inc.). The Taq Dideoxy terminator cycle sequencing kit with Amplitaq (Perkin Elmer Inc.) and the M13 reverse primer were also used. Resulting sequences were compared against the GenBank/EMBL database using the BLAST queuing system. Nucleic acid and amino acid homologies searches were conducted using the BLASTn and BLASTx programs respectively.

Northern blot analysis. Validation of the efficacy of the subtractive hybridization was performed by Northern blot analysis. 1.6 μg of Poly A^+ RNA were electrophoresed on a 1% agarose-formaldehyde denaturing gel in 1X MOPS (Sambrook et al., 1989) and then transferred onto a nylon membrane (GeneScreen Plus®; Pall Corporation). The mRNA was crosslinked to the membrane with a UV Stratalinker 1800 (Stratagene, Inc.). Six different clones were selected for analysis. Probes were generated to the individual cDNA inserts by taking 100 ng of plasmid DNA and labeling the inserted DNA with the North2South® Direct HRP labeling and detecting kit (PIERCE Inc.) according to the manufacturer's protocol. Membranes were hybridized with the probes at 55 °C for four hours according to the manufacturer's protocol. Membranes were then exposed to autoradiography film at room temperature for 15-90 s. To determine intensity of the expression, quantification of Northern blots was performed using the image analysis program Image QuaNT™ (Molecular Dynamics Inc.). Normalization against β-actin was not possible because several actin genes were induced by the treatment (see Results).

Results and Discussion

Masculinization of Nile tilapia. Microscopic analysis of gonads at 72 dpf indicated that immersion of Nile tilapia fry in TA at 11 and 13 dpf resulted in significant masculinization (Fig. 29). Fish in the control group had an average of 49.5 % males (± 3.5) while the TA immersed group resulted in a mean of 88.6 % males (± 4.0). Since these results confirmed successful masculinization, we proceeded to construct the subtracted cDNA library.

Construction of subtracted libraries. A subtracted cDNA library was generated with secondary PCR products from Nile tilapia cDNAs (Figure 30). The size of the products obtained in the subtracted library ranged from 0.1 to 1.3 Kb. One hundred sixty-five transformed colonies were selected for screening; 161 had cDNA inserts and 4 had no inserts. The analysis of the 161 cDNAs sequenced using the BLAST algorithm indicated that 93 clones (57.8 %) had significant nucleotide or amino acid matches in the GenBank/EMBL database and 68 (42.2 %) had very low or no matches and were likely novel genes. The analysis of the 93 clones with significant matches led to the identification of 61 genes (32 were repeats). Forty of these cloned sequences were identified by matching both the nucleic acid and amino acid sequences (Table 4) with highly significant probability (E) values. Nineteen of the clone sequences matched previously reported amino acid sequences of the GenBank/EMBL database with high probability, but no matches to their corresponding nucleic acid sequences were found (Table 5A). Two of the cloned sequences matched the nucleic acid sequences of the GenBank/EMBL database with high probability values, but the

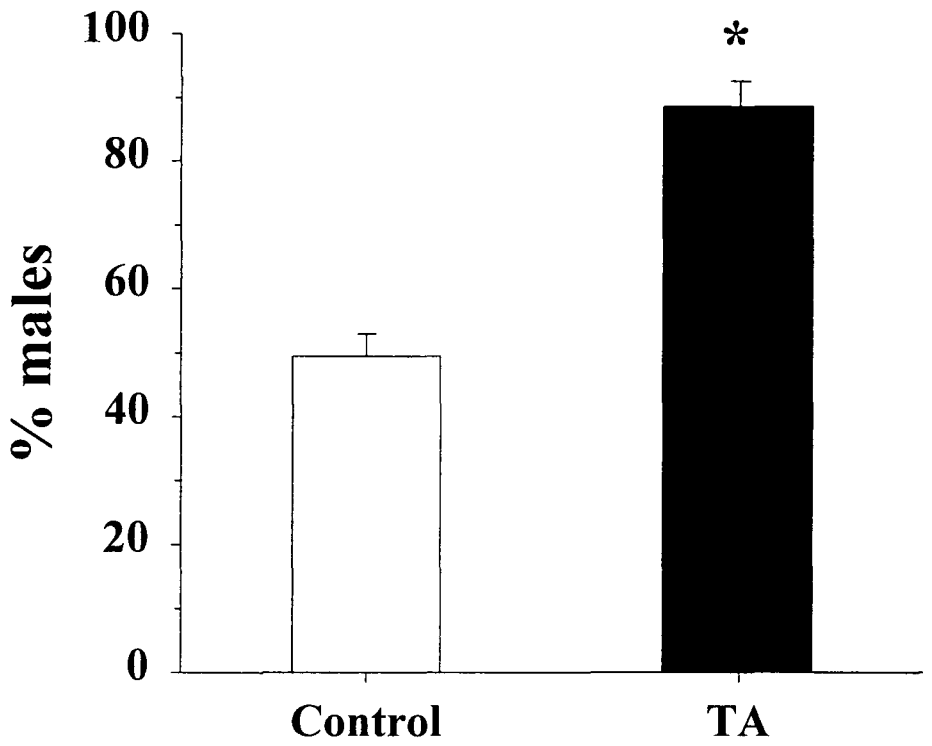


Figure 29. Masculinizing effects of short-term immersions of Nile tilapia fry in 500 μg/l of the synthetic steroid trenbolone acetate (TA). Graph depicts the percentage of males (mean ± SE) from fish immersed in TA for 3 h at 11 and 13 dpf (308 and 364 Celsius Temperature Units). Each treatment was run in triplicate. The number of fish sampled was 150 for each treatment. Asterisk indicates statistically significant difference from the control.

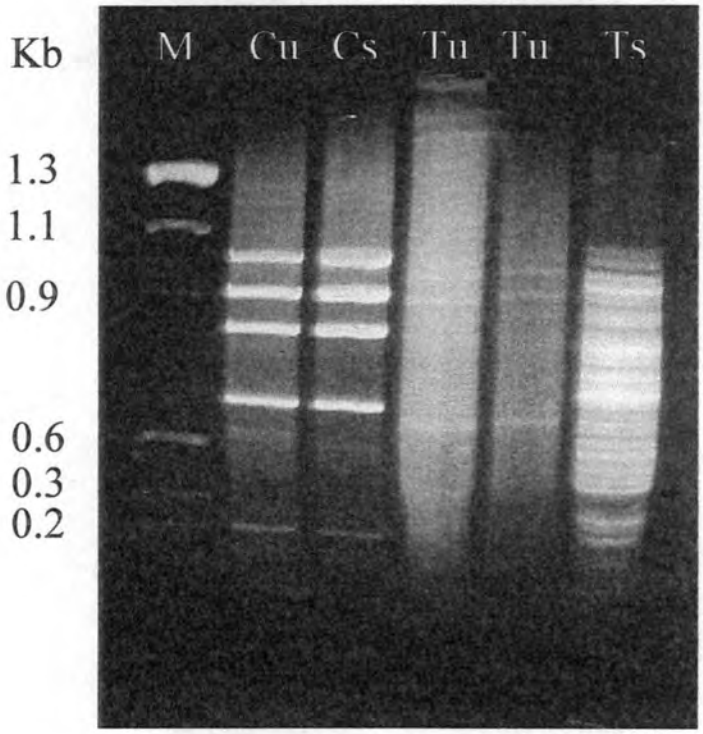


Figure 30. Secondary PCR products after subtraction hybridization analyzed by 2% agarose gel. (M) DNA markers; (Cu) Skeletal muscle cDNA control unsubtracted; (Cs) Skeletal muscle cDNA subtracted; (Tu) Tilapia cDNA tester unsubtracted; (Ts) Tilapia cDNA tester subtracted. The two lanes with unsubtracted tester are products of two different PCR reactions that resulted in different amounts of cDNA. The large amount of staining in the subtracted lane compared with the unsubtracted lane indicates successful subtraction of sequences, leaving an enriched fraction of sequences resulting from TA treatment.

Table 4. Genes identified using the BLAST algorithm that matched both nucleotide and amino acid sequences from the GenBank/EMBL database. The name and species cited correspond to those matches with highest scores in the results. [a]Number of base pairs or amino acids reported for the species listed. [b]Location of the alignment obtained, in some instances matches were not continuous; therefore, only the first section of the match is shown (* indicates example). [c]Percent identity for the match. [d]Percent homology for the match. [e]Number of clones analyzed containing the sequence.

#	Code	Name	Species	[a]Length	[b]Match	[c]Id (%)	[d]+ (%)	[e]# Isol
1.	T66	Actin	Mouse, *Mus musculus*	1770 bp	879-1378	80		1
			Urchin, *Heliocidaris tuberculata*	376 aa	241-332	39	57	
2.	T59	Alpha-actin	Tilapia, *Oreochromis mossambicus*	1555 bp	518-957	100		2
			Carp, *Cyprinus carpio*	377 aa	151-296	100	100	
3.	T36x	Amyloid A4 Protein Precursor	Rat, *Rattus norvegicus*	2170 bp	516-553	89		1
			Rat	770 aa	90-203	49	62	
4.	T32x	Apolipoprotein AI Precursor	Sea-bream, *Sparus aurata*	1020 bp	*803-848	95		1
			Sea-bream	260 aa	169-260	44	61	
5.	T56	Apolipoprotein E	Turbot, *Scophthalmus maximus*	698 bp	2-206	82		1
			Zebrafish, *Danio rerio*	281 aa	42-175	57	76	
6.	T44	Autoantigen NGP-1	Human, *Homo sapiens*	2331 bp	674-1053	77		1
			Human	731 aa	192-349	91	96	
7.	T61	Band 3 anion exchange protein	Trout, *Oncorhynchus mykiss*	3096 bp	2141-2545	83		3
			Trout	918 aa	636-837	82	89	
8.	Tilm39	Beta-actin	Tilapia, *Oreochromis mossambicus*	1881 bp	521-960	99		1
			Atlantic salmon, *Salmo salar*	375 aa	144-293	98	98	
9.	Til10y	Ca transporting ATPase End. Ret.	Blue marlin, *Makaira nigricans*	4092 bp	1437-2028	90		1
			Chicken, *Gallus gallus*	994 aa	354-552	64	75	
10.	Til2y	Calmodulin	Yellow Perch, *Perca flavescens*	1644 bp	339-599	90		1
			Human, *Homo sapiens*	149 aa	101-149	100	100	
11.	T55	Carboxylesterase	Atlantic salmon, *Salmo salar*	1795 bp	619-709	82		2
			Rat, *Rattus norvegicus*	597 aa	196-341	65	80	
12.	T27	Clathrin coat assembly protein AP50	Human, *Homo sapiens*	1828 bp	939-1075	88		1
			Human	435 aa	299-343	100	100	
13.	Tilh66	Collagen Alpha 1	Mouse, *Mus musculus*	4437 bp	2422-2450	96		2
			Human, *Homo sapiens*	1669 aa	900-916	82	87	
14.	T58	Creatine kinase	Carp, *Cyprinus carpio*	1498 bp	458-960	86		4
			Mouse, *Mus musculus*	381 aa	134-313	74	80	

Table 4

#	Code	Name	Species	[a]Length	[b]Match	[c]Id (%)	[d]+ (%)	[e]# Isol
15.	Til9y	Elastase 1 precursor	Flounder, *Paralichthys olivaceous*	960 bp	392-738	81		3
			Cow, *Bos taurus*	266 aa	48-226	59	69	
16.	TILH9	Elongation Factor 1-Alpha	Yellowfin, *Seriola quinqueradiata*	1386 bp	740-1176	88		2
			Zebrafish, *Danio rerio*	462 aa	240-416	87	92	
17.	TILM8	Elongation Factor 2	Chicken, *Gallus gallus*	3144 bp	2383-2489	89		2
			Chicken	858 aa	719-858	82	91	
18.	T33	Eukaryotic initiation factor	Human, *Homo sapiens*	1536 bp	1022-1300	81		4
			Human	411 aa	348-410	96	97	
19.	T49x	Glucose 6-Phosphate Isomerase	Human, *Homo sapiens*	1987 bp	227-518	79		1
			Human	558 aa	37-173	79	90	
20.	Tilh53	Growth factor-B1-	Human, *Homo sapiens*	2023 bp	264-538	82		1
			Human	144 aa	1-102	63	74	
21.	T39x	GTP binding protein (rac2)	Human, *Homo sapiens*	579 bp	112-474	83		2
			Human	192 aa	33-177	94	96	
22.	Tilm32	Keratin	Human, *Homo sapiens*	1360 bp	630-681	91		4
			Goldfish, *Carassius auratus*	467 aa	236-421	58	77	
23.	Tilh51	Lim domain protein	Rat, *Rattus norvegicus*	1696 bp	558-637	86		1
			Rat	327 aa	6-83	53	67	
24.	T37	Maltase glucoamilase	Human, *Homo sapiens*	6513 bp	3989-4034	92		1
			Human	1857 aa	1265-1449	60	73	
25.	Tilm34	Matrix Metalloproteinase 16	Human, *Homo sapiens*	2116 bp	286-344	87		1
			Human	607 aa	31-119	65	77	
26.	T69x	Myosin heavy chain	Pollock, *Theragra chalcogramma*	3441 bp	804-1303	90		1
			Chicken, *Gallus gallus*	1938 aa	1100-1251	67	70	
27.	Tilh64	NA/K transporting ATPase A-chain	Tilapia, *Oreochtomis mossambicus*	3390 bp	2200-2886	97		3
			European eel, *Anguilla anguilla*	1022 aa	738-914	94	97	
28.	T63x	Neurofilament subunit	Human, *Homo sapiens*	2944 bp	208-240	90		2
			Human	544 aa	193-265	41	57	

Table 4 continued

#	Code	Name	Species	[a]Length	[b]Match	[c]Id (%)	[d]+ (%)	[e]# Isol
29.	T65x	O-GlcNAc transferase	Human, *Homo sapiens*	4256 bp	2979-3136	86		2
			Human	920 aa	721-885	90	94	
30.	T62x	P4 receptor-associated immunophilin	Human, *Homo sapiens*	1694 bp	396-431	91		1
			Human	457 aa	47-290	56	72	
31.	Tilm37	Parvalbumin Beta	Atlantic salmon, *Salmo salar*	781 bp	86-372	85		1
			Carp, *Cyprinus carpio*	108 aa	1-108	74	76	
32.	T42x	Polymerase II subunit hsRPB4	Human, *Homo sapiens*	1902 bp	288-430	81		1
			Human	142 aa	44-141	81	85	
33.	Tilh68	Putative TSC-22	Human, *Homo sapiens*	2023 bp	264-568	83		1
			Human	144 aa	1-116	64	77	
34.	T9	Ribosomal protein L36	Rat, *Rattus norvegicus*	364 bp	90-292	84		1
			Rat	105 aa	1-92	90	95	
35.	T24	Ribosomal protein S15	Atlantic salmon, *Salmo salar*	473 bp	117-297	88		1
			Platyfish, *Xiphophorus maculatus*	145 aa	78-100	86	94	
36.	Tilh63	Ribosomal protein S3	Mouse, *Mus musculus*	818 bp	165-719	84		1
			Mouse	243 aa	50-241	92	93	
37.	T38x	Voltage dependent anion channel	Human, *Homo sapiens*	1806 bp	766-824	83		1
			Human	283 aa	212-282	69	73	
38.	T43x	X -Chromosome linked phosphoglycerate	Rat, *Rattus norvegicus*	1675 bp	1136-1263	86		1
			Chicken, *Gallus gallus*	417 aa	272-416	80	84	
39.	Til26y	60S Acidic ribosomal protein P1	Chicken, *Gallus gallus*	459 bp	76-281	83		2
			Chicken	114 aa	1-62	91	95	
40.	T32	60S Ribosomal protein L21	Human, *Homo sapiens*	521 bp	120-430	80		3
			Human	160 aa	14-160	87	94	

Table 4 continued

Table 5. Genes identified using the BLAST algorithm that matched only amino acid sequences, but no match was found to the corresponding nucleotide sequence (A); and genes identified by matching only nucleotide sequences, but no match was found to the corresponding amino acid sequence in the GenBank/EMBL database (B). Legends are similar to those described in Table 4.

(A)

#	Code	Name	Species	[a]Length	[b]match	[c]Id	[d]+ (%)	[e]# Isol
41.	Til23y	Aminopeptidase N	Human, *Homo sapiens*	967 aa	586-689	52	68	1
42.	Til19y	ATP syntase alpha chain	Fruit fly, *Drosophila melanogaster*	552 aa	432-477	97	99	1
43.	Til6y	Carboxypeptidase A2 precursor	Rat, *Rattus norvegicus*	417 aa	201-312	70	83	1
44.	Tilm46	Collagen Alpha 1 Precursor	Chicken, *Gallus gallus*	1019 aa	900-1018	52	73	1
45.	Til25y	Cytochrome C Oxydase Polypeptide II	Goldfish, *Carassius auratus*	230 aa	34-153	51	59	1
46.	Til14y	Epithelial zink-finger protein	Human, *Homo sapiens*	355 aa	240-299	38	49	1
47.	T1	Fibrinogen Beta chain precursor	Rat, *Rattus norvegicus*	479 aa	19-83	46	60	3
48.	Til22y	Insulin receptor substrate	Rat, *Rattus norvegicus*	1235 aa	352-418	45	57	1
49.	Till23	Lipoprotein Lipase Precursor	Chicken, *Gallus gallus*	490 aa	329-388	55	60	1
50.	Til4y	Myosin-binding protein C	Chicken, *Gallus gallus*	1132 aa	593-639	46	63	2
51.	Til3y	Oviduct specific glycoprotein precursor	Mouse, *Mus musculus*	383 aa	35-138	58	75	2
52.	Til18y	Serotransferin precursor	Medaka, *Oryzias latipes*	690 aa	522-648	79	88	1
53.	Tilm33	Ubiquitin Carboxyl-Terminal Hydrolase	Human, *Homo sapiens*	1087 aa	105-300	55	71	1
54.	T39	ADP, ATP Carrier protein	Human, *Homo sapiens*	298 aa	1-50	90	94	2
55.	T4	Proto/oncogene serine/threonine Kinase	Mouse, *Mus musculus*	313 aa	216-291	84	91	2
56.	T41x	Pre-RNA Splicing factor ATP-Dependent	Human, *Homo sapiens*	1227 aa	987-1125	31	53	1
57.	T45	Myomesin (M protein)	Chicken, *Gallus gallus*	1450 aa	1115-1287	46	65	1
58.	T46x	Very long Acyl-COA Synthase	Rat, *Rattus norvegicus*	620 aa	551-620	47	68	1
59.	T51	Coagulation factor X precursor	Human, *Homo sapiens*	490 aa	198-290	50	71	1

(B)

#	Code	Name	Species	[a]Length	[b]match	[c]Id	[e]# Isol
60.	Tilh67	E2 Receptor	Tilapia, *Orechromis aureus*	4337 bp	1888-2110	95	1
61.	Tilm48	28S ribosomal protein	Puffer, *Tetraodon nigroviridis*	420 bp	238-410	93	1

Table 5

corresponding amino acid sequences did not identify any protein sequence with the BLASTx program (Table 5B).

The analysis of all the genes identified indicates that 20 (32.8 %) of the sequences matched genes or proteins from fish submitted to the GenBank/EMBL database, while 41 (67.2 %) have not been reported to the database from fish. Of the genes reported in fish, only four (6.6 %) have been sequenced in tilapias (genus *Oreochromis*) and one has been reported in the species used for our study (Nile tilapia). In this regard, our study becomes a significant contribution to the tilapia gene database, and we have submitted the sequences of the clones identified to the GenBank database.

Genes linked to testis development. We found that 12 of the identified sequences are related to genes expressed in reproductive tissues of other species (Table 6). Eleven of these genes have been linked to different stages of testicular development and one has been reported only in the female reproductive tract of higher vertebrates (oviduct specific glycoprotein).

Because the fish were so small (mean = 0.009 g) at the time of sampling, it was impossible for us to test individual body regions or organs. Hence, the subtracted library was obtained from whole-body extracts, and we can only speculate about the potential role of these genes in governing the development of testicular tissue.

Table 6. Summary of genes that have been linked to gonadal development. The references shown are provided as examples and are not indicative of all published documents that link these sequences to gonadal development.

	Gene	Function/Event	Species	References
1.	Elongation Factor 1-Alpha	Support protein synthesis	mouse, medaka	Furuchi et al., 1996; Kinoshita et al., 2000.
2.	Autoantigen NGP-1 (testicular antigen)	Signal transduction? meiosis and/or early spermiogenesis	rat	Racevskis, et al., 1996.
3.	Creatine Kinase M chain	Provides energy for metabolism	trout	Garber et al., 1990; Saudrais et al., 1996.
4.	Phosphoglycerate kinase testis specific	Provision of nutrients to the germ cells; signal for spermatocyte differentiation	human	McCarrey et al., 1996; Tascou et al., 2000.
5.	Protooncogene serine/threonine kinase	Cell proliferation	mouse, rat	Sorrentino et al., 1988; Wingett et al., 1992.
6.	Growth factor TSC-22	Transcription regulating factor	mouse	Hamil & Hall, 1994.
7.	Glucose 6-Phosphate Isomerase	Differentiation mediator	hamster, human	Kalla et al., 1990; Yakirevich & Naot, 2000.
8.	Estrogen receptor	Several functions; normal development of testis	mouse	Couse et al., 1999; Jefferson et al., 2000.
9.	Collagen Alpha 1	Cell to cell communication; testis development	mouse, human	Kallury & Cosgrove, 2000; Frodjman et al., 1998.
10.	Calmodulin	Associated with mitotic divisions and/or spermatogenesis	mouse, rat	Slaughter, et al., 1989; Trejo & Delhumeau, 1997.
11.	Aminopeptidase N	Protein processing mechanisms	mouse, human	Huang, et al., 1997; Agrawal et al., 1989.
12.	Oviduct specific glycoprotein	Early embryonic development	mouse, hamster	Lee, et al., 2000; Merlen & Bleau, 2000.

Table 6

In our search for sex determining genes in tilapia, we considered the possibility of finding genes similar or at lest related to those present during the process of sex differentiation in mammals. The model proposed for mammalian sex differentiation considers the *Sry* gene to be responsible for sex determination and gonadal differentiation in mammals. The *Sry* gene gives rise to the testis-determining factor (TDF) which triggers a cascade of events that leads to the emergence of Sertoli cells surrounding germ cells and later formation of the seminiferous cords, as well as the appearance of Leydig cells responsible for the secretion of testosterone (Wachtel and Tiersch, 1994). In a recent study, Schmahl et al. (2000) suggested that *Sry* induces a rapid increase in proliferation in the mouse XY gonad, this proliferation being initially observed in Sertoli cell precursors. They also concluded that this proliferation of cells is responsible, at least in part, for the increase in testicular mass observed at the outset of testis differentiation. The Sertoli cells also play an important role in sex differentiation by secreting the anti-Müllerian hormone, which causes regression of Müllerian ducts (Nagai, 1992). In addition to this, one of the members of the *Sox* family, the *Sox-9* gene, has been linked to testis determination in vertebrates (Da-Silva *et al.*, 1996; Kent *et al.*, 1996). Da-Silva *et al.* (1996) proposed that *Sox-9* plays an essential role in sex determination, probably immediately downstream of *Sry* in mammals, and acts as a critical factor during differentiation of Sertoli cells in all vertebrates. Kent *et al.* (1996) proposed that this gene might play a broad role in the development of the urogenital system. Despite these advances in the understanding of the mechanism that controls sex determination and gonadal differentiation in

mammals, little is known about these mechanisms in birds, reptiles, amphibians, and fishes.

We found no evidence for the activity of a genetic switch directing the fate of the gonads (i.e. *Sry* related sequences, *Sox* family genes). But this may be due to the potential bias caused by the sampling time. It is possible that sex inversion via steroid administration may not require the presence of a sex determining factor. Hormonal treatment may override female differentiation by inducing cells in the primordial gonad to undergo male differentiation. This process may act through the activation of a mechanism that induces differentiation of the gonadal somatic cells into Sertoli and Leydig cells, with the Sertoli cells potentially directing the fate of the germ cells into spermatogonia (a process similar to that suggested by Schmahl et al., 2000). In regards to this, Hashimoto et al., (1990) did demonstrate that testicular differentiation in the mouse is independent of germ cells. They demonstrated that the transplantation of aggregates of female somatic cells into ovariectomized, nude mice resulted in the formation of testis cords and differentiation into Sertoli cells. These results indicated that female gonadal somatic cells have the potential to form primordial testicular tissue. Once the somatic cells are differentiated, the proliferation and differentiation of testicular stem cells into highly specialized germ cells (spermatozoa) are largely controlled by the adjacent supporting Sertoli cells (Hellani et al., 2000) and the fate of the gonad is directed to a functional testis.

Irrespective of the mechanism, it has been suggested that the differential growth of the testis determines the sex of an organism (Mittwoch, 1986). If this hypothesis is correct, then the masculinizing properties of TA may be linked to initiating testicular cell proliferation before the cells in the primordial gonad are programmed to develop as ovarian tissue.

The induction of differential expression of the estrogen receptor and the receptor-associated immunophilin in the TA treated group, suggest that masculinization of Nile tilapia might be associated with the expression of the estrogen receptor gene and molecular chaperones involved with the subcellular trafficking of steroid receptors (i.e. HSP binding immunophilin). This may indicate an active role of the estrogen receptor in early testis differentiation. In recent studies, it has been demonstrated that estrogen receptors are essential for the development of functional testes (Couse et al., 1999; Dupont et al., 2000). These studies showed that mice lacking the alpha form of the estrogen receptor still developed grossly normal reproductive structures and exhibited various stages of spermatogenesis; however, the number of germ cells (Dupont et al., 2000) and the number and motility of sperm (Couse et al., 1999) were significantly reduced. Despite this evidence, little is known regarding the role of estrogenic compounds and estrogen receptors during early testicular differentiation.

Anabolic effects of TA. A large proportion (80 %) of the genes identified in the cDNA subtraction library were related to cell proliferation, growth, movement and early differentiation. It is well known that TA is a potent anabolic steroid and for this

reason, has been used to enhance growth in the cattle industry with great success (Mader, 1998). Having such a large proportion of anabolic and housekeeping genes expressed in the subtracted library may have reduced the efficiency of the technique to isolate unique genes related to sex differentiation. On the other hand, several of these genes can be implicated in the development of testicular tissue. For example, actins, collagen and connexins, have been found in testicular tissue at different developmental stages (Kim et al., 1989; Fröjdman et al., 1998; Bobo et al., 1999).

Northern blots. We validated the efficacy of the subtractive hybridization process to identify specific genes expressed after TA treatment by Northern blot analysis with six genes obtained in the library (Figure 31). The differential expression of testis specific phosphoglycerate kinase, estrogen receptor, protooncogene serine/threonine kinase, progesterone receptor-associated immunophilin, elongation factor 1-alpha, and the autoantigen NGP-1 genes was 92.9, 54.2, 168.2, 240.9, 11.3, and 10.1 % higher in the TA treated group than in the control group, respectively. These results demonstrate that the subtracted library contained cDNA fragments representative of mRNA that are expressed during early development in tilapia, and that there is an effect of steroid treatment on the expression of several genes. Differences in the amount of overexpression are evident and this may be due to the relation between sampling time and the time at which a particular gene transcription was induced.

Future research. We have identified unique gene expression during the process of sex inversion in Nile tilapia. Understanding the contribution of the genes found in our

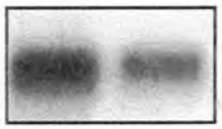
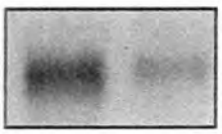
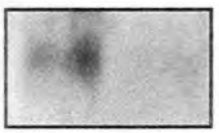
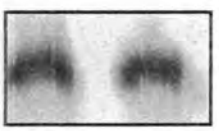
Northern Blot TA EtOH	Clone	Gene	mRNA (Kb)	Differential Expression (TA:EtOH)
	T43x	Phosphoglycerate kinase testis specific	1.8	1.92
	Tilh67	Estrogen receptor	4.2	1.54
	T49	Protooncogene Serine/Threonine kinase	2.6	2.68
	T62x	P4 receptor-associated Immunophilin	2.2	3.41
	T68	Elongation Factor 1-Alpha	1.7	1.18
	T44	Autoantigen NGP-1	2.6	1.14

Figure 31. Northern blots of Nile tilapia fry genes differentially expressed after trenbolone acetate (TA) or ethanol vehicle (EtOH) immersions. The differential expression was estimated as the ratio of the TA expression to that measured in the control. The size of the mRNA was estimated by electrophoresis in 1% agarose-formaldehyde denaturing gel.

study to the early differentiation of testicular tissue in fish can be accomplished by in situ hybridization of the clones sequenced, their correlation with gonadal cell proliferation, and their Sertoli cell differentiation. Further research should be oriented towards eliminating the background caused by the anabolic effects of the steroid employed for sex inversion, thereby significantly increasing the efficiency of the subtraction to unmask genes that are underrepresented in the subtracted library. Serial samplings will also help to identify specific patterns of gene expression involved with gonadal differentiation during development after the TA treatment and specifically determine if the expression of the genes identified in our study is closely related to the appearance of Sertoli cells. We now have some of the probes to initiate this study.

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Chapter 7

Conclusion

The main objective of this thesis was to identify the mechanism of sex differentiation in Nile tilapia by using androgen-immersed fry and identifying the differential expression of genes turned on during sex inversion. We also wanted to document if methyltestosterone (MT) persists in the environment after oral administration of tilapia fry for masculinization purposes and determine if short term immersions in synthetic steroids can offer significant masculinization of fry, providing an alternative to oral administration of steroids.

The results presented in this thesis support the concept that sex differentiation in tilapia is a very labile process. Short term immersions in steroids and high temperatures significantly biased sex ratios toward males providing evidence for a sex determination system that is regulated by interactions between genetic elements and environmental factors (at least temperature).

We found no evidence for the activity of a genetic switch directing the fate of the gonads (i.e. *Sry* related sequences, *Sox* family genes). But this may be due to the potential bias caused by the sampling time. However, it is also possible that sex inversion via steroid administration may not require the presence of a sex determining factor. The genes isolated in the subtracted library may be indicative of the activation of gonadal cell proliferation. Steroid treatment may override female differentiation by activating cell proliferation and inducing cells in the primordial gonad to undergo

male differentiation. This process may act through the activation of a mechanism that induces differentiation of the gonadal somatic cells into Sertoli and Leydig cells, with the Sertoli cells potentially directing the fate of the germ cells into spermatogonia--a process that has been suggested by Schmahl et al. (2000)--under the regulation of the testis determining gene *Sry*. Schmahl et al. (2000) suggested that *Sry* induces a rapid increase in proliferation in the mouse XY gonad with this proliferation being initially observed in Sertoli cell precursors. They also concluded that this proliferation of cells is responsible, at least in part, for the increase in testicular mass observed at the outset of testis differentiation. The Sertoli cells also play an important role in sex differentiation by secreting the anti-Müllerian hormone, which causes regression of Müllerian ducts (Nagai, 1992).

Masculinization via exposure of Nile tilapia fry to elevated temperatures apparently follows a different pathway. In the absence of a testis determining factor, female differentiation requires the activation of estradiol synthesis. This process involves the conversion of testosterone into estradiol by the enzymatic action of aromatase. In Nile tilapia, female differentiation is inhibited by elevated temperatures during a sensitive period. We have found that this thermosensitive period may comprise the same days at which masculinization can be induced by the exposure of fry to synthetic steroids and significant masculinization can be achieved by exposures to 34 °C for as short as 3.5 days. Previous studies masculinized tilapia fry using long-term exposures (30-40 days) to elevated temperatures (Baroiller *et al.*, 1995; D'Cotta et al., 1999; Wang and Tsai, 2000), but apparently such a long treatment may not be needed.

In mammals, sex differentiation results from a cascade of events triggered by a genetic "switch." In the presence of the testis determining factor, the organism develops as a male; otherwise, ovaries form. Consequently, sexual differentiation at the brain and pituitary levels is determined by the differentiation of the gonads (Wachtel, 1994). However, Francis (1992) hypothesized that teleosts may follow a different pattern in which the brain determines gonadal development. According to this hypothesis, the sex determining gene initiates events in the brain that lead to gonadal differentiation. Francis concluded that hormonal manipulation, and social and environmental interactions mediate sex differentiation acting via brain-pituitary axis.

Our results support the idea that steroid treatment may induce gonadal cell differentiation directly in the gonad. The presence of isolated oocyte-like cells surrounded by testicular tissue in several experiments may be indicative of localized steroid action in the gonad. It is possible to speculate that synthetic steroids interact with steroid receptors present in the primordial gonad in order to exert its masculinizing effects. Due to the short duration of our treatments, some germ cells may not be reached by the steroid, either because treatment occurred too early and not all germ cells have steroid binding capabilities, or because treatment occurred too late and some germ cells have already committed differentiation towards oogonia. If sex inversion was to be controlled by the brain, the entire gonad could be regulated by such a mechanism without missing individual cells.

In Chapter III, we presented evidence for a significant effect of environmental conditions on the masculinizing efficacy of oral administration of steroids. First, we

found a significant leakage of MT to the rearing water and a subsequent accumulation of the steroid in the sediments. The low levels of masculinization obtained when tilapia fry were fed twice the recommended dose of MT, or when MT was allowed to remain in the system indicate that masculinization is being inhibited or that paradoxical feminization is occurring. Other studies have suggested that paradoxical feminization is the most likely process. Even though the mechanism has not been demonstrated, overexposure of fish to MT could result in paradoxical feminization due to a hypothesized conversion of MT into estrogen by the enzymatic action of aromatase. Paradoxical feminization has been reported in several species of fish (Goudie et al., 1983; Solar et al., 1984; Davis et al., 1990; Piferrer and Donaldson, 1990; Piferrer et al., 1993; Varadaraj et al., 1994; Rinchard, *et al.*, 1999; Eding *et al*, 1999) and has been reported to occur even when fish are immersed for only two hours in concentrations of 6.4 or 10.0 mg/l of MT (Piferrer and Donaldson, 1990; Piferrer et al., 1993). The evidence suggests that MT is being actively converted to an estrogenic compound, and that the amount required to cause paradoxical feminization will vary according to the species and the conditions under which the organisms are treated.

This is the first study that has demonstrated that reducing MT from the water significantly increases MT masculinizing efficiency. We speculate that the recommended dose of MT for masculinizing tilapia is higher than needed and a significant portion of it separates from the food and remains in suspension in the water. We recommend the use of activated charcoal filtration systems to eliminate

excess MT and to potentially increase masculinization and reduce the risk of environmental and health impacts.

Results from Chapters IV and V suggest that the window of sensitivity for masculinizing Nile tilapia at the individual level is very short and may vary between broods, which in turn, supports the idea that there are other major factors influencing susceptibility to androgen-induced masculinization. We found that the period at which Nile tilapia is susceptible to steroid immersion treatment for masculinization starts at 11 dpf, increases at 12 dpf, reaches a maximum at days 13 and 14 and declines by 15 dpf.

Despite significant masculinization in several experiments, short-term immersions in androgenic steroids also resulted in low rates of masculinization in other experiments. Independently of the number of short immersions used during the labile period, the percentage of males produced reached more that 95% only once. This poses a significant disadvantage for the immersion technique in terms of its feasibility for aquacultural purposes if 99% or more male populations are required. We are currently initiating a series of studies to determine the potential synergistic effects of steroid immersions and elevated temperatures. We feel confident that the promising results presented in Appendix 1 are opening new alternatives to improve the immersion protocol.

It is evident that steroids play a very important role in sex differentiation of fish and differences between males and females might be related to differential expression of genes regulating the enzymes responsible for the conversion of androgens into estrogens. The induction of differential expression of the estrogen receptor and the receptor-associated immunophilin in the TA treated group, suggest that masculinization of Nile tilapia might be associated with the expression of the estrogen receptor gene and molecular chaperones involved with the subcellular trafficking of steroid receptors. This may indicate an active role of the estrogen receptor in early testis differentiation and support Yamamoto's (1969) hypothesis that steroids play a significant role in sex differentiation in fishes. We have identified unique gene expression during the process of sex inversion in Nile tilapia and have generated molecular probes that can support new research for understanding sex determination and sex differentiation in Nile tilapia.

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APPENDICES

Appendix 1

Effects of Temperature and Double Immersion in TA. Previous studies have demonstrated that long-term exposure of Nile tilapia fry to elevated temperatures (35 °C) result in masculinization of Nile tilapia fry. Results from this thesis (Contreras-Sánchez et al., Chapters IV and V) indicated that double immersions in synthetic steroids can provide significant masculinization, here we identified if the combination of elevated temperatures and immersions in TA at 11 and 13 dpf can yield significant masculinization. Fry were immersed in TA or EtOH vehicle at three different temperatures: 28, 34 and 38 °C. Hormone dosage was maintained at 500 µg/l, fish density at 33 fry/l, and duration of the immersion at three hours.

Fry were collected after each immersion, jars were thoroughly cleaned, and then fish were reallocated in fresh dechlorinated tap water. After 5-7 days, fry were transferred to 75 l fiberglass tanks in a recirculating system. Water temperature in the grow-out system was maintained at 28 ± 2°C. Fish were fed to satiation 4 times a day with Natureboy Basic Food Flakes (Rexotic Products, Portland, OR, USA). At 60-70 dpf, fish were killed with an overdose of the anesthetic tricaine methanesulfonate (MS-222) and sex ratios were determined by examination of gonads using squash (100X) preparations after Wright's staining.

Results. Significant masculinization was achieved by rearing fry at 34 °C for 3.5 days and by immersing Nile tilapia fry in TA at all temperatures tested (Fig. 1). Immersing Nile tilapia fry in EtOH vehicle at 34 °C resulted in significantly more males (68.3%

males ± 5.8) than the control group (52.5% males ± 4.2). Despite a slight increase in males, the immersions in EtOH vehicle at 38 °C (62.0% males ± 2.8) did not yield significantly more males than those obtained in the controls. Immersions in TA at 11 and 13 dpf at 28, 34, and 38 °C resulted in 99.0, 99.0, and 100% males, respectively. Only one female was found in each of the groups treated with TA at 28 and 34 °C. These fish were the largest fish of their respective group, suggesting that they may not have been masculinized because they have already passed the period of gonadal sensitivity to the steroid when the steroid treatment was applied. These results support our hypothesis that once the gonads have committed to ovarian development, masculinization by short-term immersion in the steroid is not possible. Groups immersed at 28 and 34°C had 3.5 and 11% mortalities, respectively. Immersions in either EtOH or TA at 38 °C resulted in higher mortalities than the groups immersed at 34 or 38 (21 and 30%, respectively). This may have caused differential mortality of males in the 38 °C group resulting in the lack of significance in male percentage between this group and the controls.

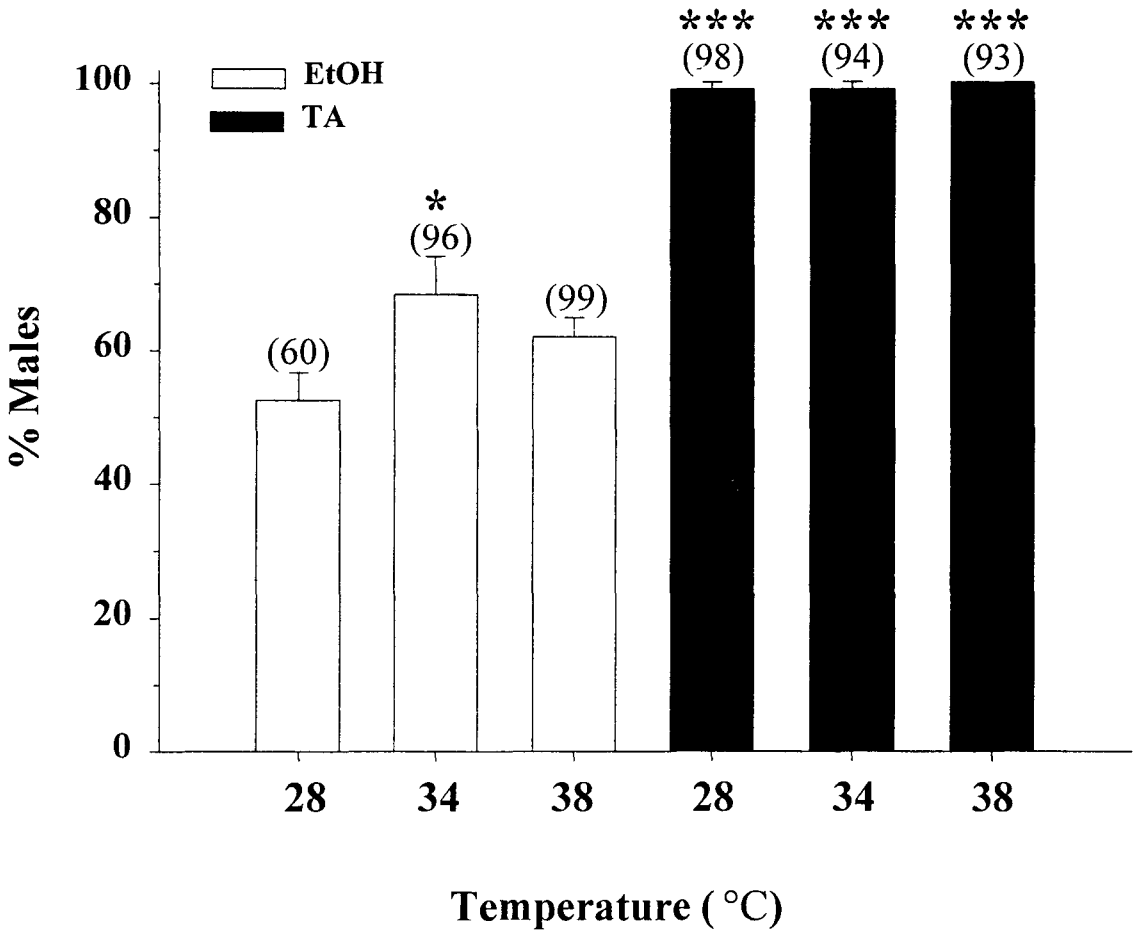

Figure 1. Masculinizing effects of temperature and short-term immersion of Nile tilapia fry in 500 μg/L of the synthetic steroid trenbolone acetate (TA). Graph depicts the percentage of males (mean ± SE) from fish immersed in TA for 3hr at 11 and 13 dpf. Each treatment was run in triplicate, with the exception of EtOH-vehicle immersion at 28 °C which was run in duplicate. Asterisks indicate statistically significant differences from the control group maintained at 28 °C. The numbers of fish sampled for each treatment are presented in parentheses.

Appendix 2

Figure 2. Schematic representation of the sex ratio from different crosses, indicating a high degree of variability. Numbers in boxes indicate the male identification number. The sex ratio is shown as the percentage of females in individual broods and the female that was used for each cross is represented by different symbols. Note the variability in the percentage of females in the progenies produced by the female represented by a dark triangle and the female represented with an oval symbol with a cross inside when they spawned with different males.

Progeny sex ratios