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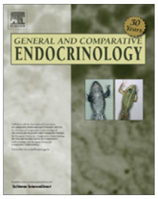

Pubertal effects of 17α-methyltestosterone on GH–IGF-related genes of the hypothalamic–pituitary–liver–gonadal axis and other biological parameters in male, female and sex-reversed Nile tilapia

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ABSTRACT

The influence of 17α-methyltestosterone (MT) on growth responses, biological parameters and the expression of genes involved in the GH–IGF pathway of the hypothalamic–pituitary–liver–gonadal axis were investigated in female, male, and sex-reversed Nile tilapia to evaluate the relationship between sex and MT-induced changes in these parameters. Female fish had a lower growth rate than male and sex-reversed fish, and MT increased growth performance and duodenal villi in females. Most but not all biological parameters of sex-reversed fish were similar to those of male fish. Male fish had higher red blood cell counts and hemoglobin levels than female and sex-reversed fish, suggesting that these hematological indices reflect a higher metabolic rate in male fish. Greater blood triglyceride levels indicated the vitellogenin process in female fish. MT increased the alternative complement activity in female fish ($P < 0.05$). Sex and MT had no significant effects on the hypothalamic mRNAs of GHRH and PACAP. Although not statistically significant, females tended to have higher GH mRNA levels than male and sex-reversed fish. Additionally, MT tended to decrease and increase GH mRNA levels in female and male fish, respectively. There were significant differences among sexes in the expression of GHR, and IGF mRNAs at the peripheral level in the liver and gonads. Females had lower hepatic GHRs and higher ovarian GHRs than male and sex-reversed fish. While the mRNA levels of IGF-1 were lower in the ovary, the levels of IGF-2 were higher compared with those in testes. A significant correlation between GHRs and IGFs was demonstrated in the liver and gonad (except for IGF-1). Multiple regression analysis showed a significant relationship between GH mRNA and both GHRs and IGFs in the liver and gonad. MT exerted androgenic and, to some extent, estrogenic effects on several physiological parameters and GH–IGF action.

1. Introduction

Fish generally exhibit a variety of sexual dimorphisms, including sexual dichromatism as well as differences in body parts and growth and behavior. These sex-related characteristics are regulated by sex hormones, which are influenced by both genetics and the environment, and are thereby displayed after sex differentiation [32]. Sexual dimorphism with respect to growth is of considerable interest for farmed fish production and varies among fish species. Among Cichlids, the Nile tilapia (*Oreochromis niloticus*) is one of the most economically important farmed species. The Nile tilapia shows strong sex growth rate dimorphism, with the growth rate of males being greater than that of females [68]. Thus, the sex-reversal of Nile tilapia via the production of all male fish is now used in commercial tilapia production. The synthetic hormone 17α-methyltestosterone (MT) is widely used to produce all male populations of Nile tilapia. Although sex-reversed Nile tilapia exhibited phenotypic characteristics similar to genotypic male fish, several differences in secondary sexual characteristics are distinguishable (Supplemental Fig.). MT has been used in several different applications in teleost fish because of its anabolic and androgenic effects. Nevertheless, how MT affects physiological and immunological parameters in fish remains to be determined. In addition, the effect of administering MT to fish on the action of the growth hormone (GH)-insulin-like growth factor (IGF) axis remains unclear. Moreover, the immunocompetence handicap hypothesis [14], in which males are more immunosuppressive

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than females due to androgen immunosuppression and/or behavior [29], was proposed to explain the observed gender dimorphism for immunity. Therefore, a comparative study on sex differences (male, female and sex-reversed) in biological parameters and the expression of the GH–IGF-axis would provide useful information not only for principal information on the long-lasting effects of MT but also for all-male tilapia aquaculture. In addition, the effects of long exposure to MT in tilapia during juvenile growth on the expression of the GH–IGF axis would provide the molecular basis of hormonal cross-talk between sex steroids and the GH–IGF axis.

Sex growth performance differences may be attributed to sex hormones directly and/or indirectly through sex-related behavior, physiological factors, and/or the activity of the GH–IGF axis. Nile tilapia spawns throughout the year. Females collect fertilized eggs and brood them; therefore, females undergo fasting frequently. The inferior growth rate of females is likely caused by their mouthbrooding behavior and/or the higher metabolic energy output required for egg production. Sex-related compensatory growth performance has been evaluated in the Nile tilapia. After a fasting period, male Nile tilapia were found to exhibit greater hyperphagia, resulting in higher compensatory growth performance compared with that of females [2]. The expression IGF-1 was demonstrated to be distinctly involved in compensatory growth in sea bream [39]. Growth hormone receptor (GHR) was related to egg size in cichlid (*Simochromis pleurospilus*). Additionally, the expression of GHR accompanied compensatory growth during the juvenile stage [59].

Sexual differences in the action of the GH–IGF axis might occur at centrally hypothalamic–pituitary levels and/or at a peripherally responsiveness level. Testosterone-mediated GH secretion act at the hypothalamic level by promoting GHRH function [4]. A number of studies have demonstrated the effects of sex hormones *in vitro*. For instance, sex steroid exerted an estrogenic effect on the increasing the secretion of GH, and decreased transcription of the GHRs and IGFs mRNAs [43,69]. Tilapia treated with estrogen during early development had long-lasting effects that impaired the GH–IGF-1 axis [61]. Androgenic effects were shown to stimulate the expression of GHRs and IGFs [35,43]. However, the effects of sex steroid on IGF-1 expression were different between male and female tilapia [52,61]. In fish, variable sex steroid effects have been reported with respect to sex differences in the GH–IGF axis at the hypothalamic–pituitary–hepatic and gonadal levels due to variation in the patterns and degree of sexual size dimorphism. Thus, the investigation of sex differences in the expression of a gene involved in the GH–IGF of the hypothalamic–pituitary–liver and gonadal axes would provide valuable information on the action of the GH–IGF axis on sexual size dimorphism, especially in Nile tilapia, which has strong sex differences in sizes even at an early puberty phase.

Sexual differences are present in a number of physiological processes, including hematological indices, blood chemicals, nutritional status, and immunity, indicating that sex steroids play a role in a variety of biological processes. For example, testosterone stimulates erythropoiesis in mammals, and estrogen reduces the hematocrit and hemoglobin levels in triploid fish [15,58]. Variable androgen effects on blood glucose, plasma protein, and cholesterol were demonstrated [42,56,72]. Intestinal morphology affects physiology and nutrient utilization. Increases in gut are attributed to increased ingestion, absorption, and dietary utilization. The oral administration of sex steroid in fish has been widely performed in a number of studies on sex steroids and the production of sex-reversed fish for culture. Therefore, whether sex steroids affect intestinal integrity remains to be investigated. Although gender differences in immunity have focused on paratism [27,44], little information is available on sexual dimorphism in innate immune parameters. Compared with other vertebrates, the innate immune system is more important in fish because it is the first line of defense against nonspecific pathogens. Nevertheless, sexual dimorphism related to the immune system has been demonstrated in a few teleost species [44,50,70].

Therefore, the objectives of this study were to comparatively investigate differences by sex (female, male, sex-reversed) in genes involved in the GH–IGF axis at the hypothalamic–pituitary–liver and gonad levels in Nile tilapia. The effects of long-term exposure to MT on the sex-related gene expression of the GH–IGF axis were also examined. Because sex steroids are involved in the physiological network, we provide valid information on the effects of MT on sex-related biological parameters, including hematological indices, blood metabolites, gut morphology and immune responses.

2. Materials and methods

2.1. Fish

The experimental Nile tilapia (*O. niloticus*, Chitralada 3) used in this study were male, female, and sex-reversed. Fry (swim-up stage) were collected from the breeding pond of Suranaree University of Technology (SUT Farm) and randomly divided into two groups: one included normal male and female fish that had been fed with feed (powdered fish meal; 58% crude protein), and the other included sex-reversed fish that had been produced by feeding the swim-up fry with 50 mg kg^{-1} MT-treated feed for four weeks. To evaluate the sex-reversal efficiency, one hundred randomly sampled fish from each group were sacrificed to determine the sex ratio. The gonads were removed and prepared using the squash method with a microscopic examination. The efficacy of sex reversal was considered acceptable at 98%. Then, each group of Nile tilapia was fed with a diet that consisted of 350 g kg^{-1} crude protein until the experiment began.

2.2. Experimental design, diet and fish culture

A 3 × 2 factorial design was employed to study the effects of sex-related differences among female, male, and sex-reversed Nile tilapia and MT (0 and 10 mg kg^{-1}-feed) on growth, the expression of genes involved in the GH–IGF axis and biological parameters. Two hapas ($2 \times 2.5 \times 2$ m^3) were randomly assigned to each combination of treatment factors and maintained in the SUT Farm reservoir. Prior to the initiation of the experiments, female, male and sex-reversed Nile tilapia of 79 ± 12 g (means ± SD) were hand-sexed, and forty fish of each sex were randomly distributed in the experimental hapas. The basal diet (32% protein and 300 kcal g^{-1}) was formulated to consist of a mixture of fish meal (30.0%), soybean meal (27.0%), rice bran (15.0%), corn meal (14.5%), cassava chips (12.0%) and vitamins and minerals (1.5%). The two experimental diets were the basal diet (0 mg kg^{-1}) and the basal diet supplemented with MT (10 mg kg^{-1}). The fish were fed the basal diet to acclimatize them to the experimental environment for 2 weeks. During the experimental period, fish were hand fed *ad libitum* twice daily for 10 weeks, and growth performance parameters were evaluated. The air and water temperatures were measured daily. The dissolved oxygen (DO) and pH were measured weekly using a DO meter and pH meter, respectively. The air and water temperatures (means ± S.D.) were 30–34 °C and 28–30 °C, respectively. The pH and DO were within acceptable ranges, which were a pH of 8.0–8.5 and a DO of 6.0–9.0 mg L^{-1}. Any dead fish were removed daily and recorded.

2.3. Fish sampling, blood collection and hematological examination

At the end of the experimental period (10 weeks), the fish were not fed for 18 h before fish sampling. Five fish from each replication of each combined treatment were sampled and anesthetized with 2-phenoxethanol (0.2%). Blood samples were collected from