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TILAPIA, *OREOCHROMIS* SP. (L.): SEX REVERSAL AND PERFORMANCE ON ALTERNATIVE SOURCES OF PROTEIN

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

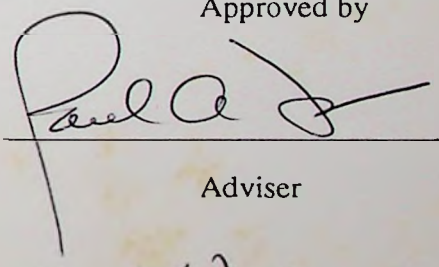
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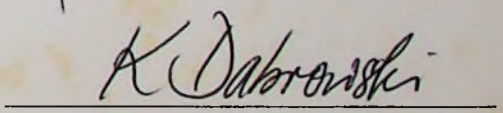
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ABSTRACT

Tilapias, (*Oreochromis* spp) have become increasingly important as cultured fish species worldwide. However, the problem of overpopulation in ponds, caused by prolific breeding, is still a major constraint in the development of tilapia aquaculture in developing countries. The potential for improved yields through the use of controlled reproduction has provided an impetus for studies in monosex tilapia production. The current strategy is to emphasize production of male-only groups because they have faster growth than females. Production of monosex tilapia has been achieved through the use of androgens to reverse the sex, hybridization between specific tilapia species, chromosomal manipulation and, more recently, through the heat treatment of embryos and larvae.

Experiments on breeding and nutrition were conducted in a recirculating, closed system. Water was pumped through a UV unit, a biofilter and a reservoir where it was heated before flowing into 32 L glass aquaria. The water temperature at 25±1.0°C was regulated by an automatic thermostat control.

Fish diet is the largest single expenditure in fish culture operations. Since the world fish meal production is declining, alternative sources of proteins need to be identified. We evaluated performance of tilapia on sources of protein which are alternative to high-cost fish meal. Evaluations were performed on growth rate, feed utilization, digestibility, survival and general condition of the fish fed graded levels of animal- and plant-based products.

To evaluate the success of heat treatment in producing sex reversal, one group of tilapia embryos was kept at 36±1.0°C and control group was maintained at 23±1°C. Both

groups were fed diets which had the fish meal component gradually replaced by animal by-products meal for 5 months. The heat treatment produced 74% males in comparison to the control group which had 51% males. With respect to growth, all the heat treated tilapia performed better than the control during the first two months even at 0% fish meal content in their diets. Males of each group grew faster than females. Chemical body composition of the two groups did not differ significantly. We concluded that heat treatment produced predominantly male offspring and enhanced utilization of animal by-product meal-based diet.

A set of experiments was performed to evaluate the use of cottonseed meal as a replacement for fish meal. Good growth performance was obtained by feeding tilapia up to 50% cottonseed meal inclusion. At higher percentages the fish became stressed, had retarded growth, demonstrated mineral deficiency and showed 11% mortality. These problems were associated with the presence of gossypol, a toxic compound which complexed with some of the minerals and accumulated in fish tissues. Reproductive parameters were not affected by the level of gossypol found in the formulated diets, although final conclusion can only be made after carrying out histological studies.

Progeny test results performed on the heat-treated males showed that 15% of the males could have been sexually reversed from genotypic females to phenotypic males. However, none of those males gave 100 % female progeny, suggesting that the sex in this strain of tilapia is not determined by simple XY factors. The sex determining system could have been compromised if the tilapia strain used in the present study was a hybrid species. Although molecular markers suggested that it is closely related to Nile tilapia (*Oreochromis niloticus*), it is possible that in the past the Piketon tilapia strain was back-crossed to species with a homogametic male system, such as *Oreochromis aureus.*

In loving memory of my late parents

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FIELDS OF STUDY

Major Field: Zoology

Specific Area: Aquaculture Program

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INTRODUCTION

About ten species of tilapia, (Perciformes: Cichlidae), have been successfully cultured. As a group, tilapia represents one of the most widely produced food fishes in the world. The ancient Egyptians and the Chinese cultured tilapia and the carp respectively long before the Christian era (Trewavas, 1982). Tilapia, being warm water fish, were originally farmed in Africa, then spread to Asia and Latin America. Tilapia include species of three major genera, *Oreochromis* (maternal mouthbrooders), *Sarotherodon* (paternal or biparental mouthbrooders) and *Tilapia* (substrate spawners) (Trewavas, 1982). All the species of the three genera are usually lumped together and commonly referred to as tilapias or cichlids.

Tilapias are among the most known hardy fishes. They are produced in nearly every environment, in waters of various qualities and salinities, from geothermal waters of the temperate to lowland tropical ponds and raceways. They are tolerant to poor water quality conditions, relatively resistant to disease, easy to reproduce and the fact that they can utilize plant proteins makes them excellent candidates for aquaculture. They grow fast and, importantly, their desirable flesh qualities make them a highly marketable food fish (Purdom, 1993). About 4 to 6 months are required to grow tilapia from fingerling to market size in a closed, recirculating system (Pullin, 1991). Some tilapia species can grow to a large size. For instance, *Oreochromis niloticus* can grow 2-3 kg. In addition, tilapias are easily bred in captivity without complex hatchery equipment or hormonal induction for spawning. They breed more or less a year-round in the tropics and have a short generation time of about 4 to 6 months with fecundities in the order of a few hundred to a few

thousand swim-up larvae per individual spawning for the mouth brooders and substrate spawners, respectively (Keenleyside, 1991). Females of *Oreochromis* species carry fertilized eggs in their mouth where the embryos are incubated and periodically churned. The eggs hatch within the mouth and the sac-fry remain in the mouth for protection. When the larvae become free-swimming, they leave the female's mouth in search of food but remain under the protection of the mother until the juvenile stage.

Tilapias have become increasingly important as cultured species not only in the tropics but also in the developed countries. Aquaculture is one of the fastest growing sectors of agriculture in many American nations, and tilapia aquaculture is one of the most rapidly growing sectors of aquaculture (Aquaculture Outlook, 1999). Tilapia are produced in many states of the US in a wide array of environments: ponds, raceways, tanks and cages. With the continuing development of recirculation system technologies, a new opportunity for tilapia production continues to expand. The use of solar greenhouse structures and insulated buildings which can capitalize on inexpensive sources of heat such as geothermal energy, is allowing tilapia culture in increasingly colder locations. Tilapia aquaculture production was estimated at nearly 5,910 tons in 1994 and by 1997, domestic production of tilapia was estimated to reach about 7,725 tons, according to the American Tilapia Association (ATA) (Stickney, 1997).

Tilapia aquaculture is also growing fast in many countries of the Americas due to increased market acceptance by domestic consumers and lucrative export markets. Commercial tilapia farms and polyculture of tilapia with shrimp are developing rapidly in Central and South America to meet a growing demand for tilapia in the United States where imports have risen. The US imported an estimated 28,180 tons of tilapia in 1998, an increase of 14% over the previous year (Aquaculture Outlook, 1999). Tilapia was the third largest imported seafood product behind shrimp and Atlantic salmon (Costa-Pierce, 1997).

ATA predicts further growth in tilapia imports, especially imports of tilapia fillets from the rapidly developing industries in Central and South America.

The problem of overpopulation of tilapia in ponds, caused by prolific breeding, has been recognized as a major constraint in the development of tilapia aquaculture. Pond-reared tilapia mature as early as at two months of age and are capable of breeding once every month thereafter. Overcrowding in ponds results in low yields of harvest-size fish. Culture practices of tilapia in the developing countries are still based on mixed-sex culture. The problem of stunting and overcrowding in production ponds are either tolerated or at best addressed by selective harvesting of larger fish. This practice has serious genetic consequences. Culling the best growers results in retaining the early maturing runts for breeding The potential for improved yields in culture of tilapia through the use of controlled reproduction to eliminate the problem of precocious maturity and undesirable reproduction has provided an impetus for studies in unisex tilapia production. The strategy adapted currently by the intensive and extensive tilapia farmers is to grow monosex tilapia particularly males because they grow faster than females. Females invest more food resources in production of gametes at the expense of somatic growth. By using only males, the tilapia breeder not only avoids undesirable natural reproduction but also raises bigger fish. This practice would particularly be an advantage for African and Asian countries where tilapia are primarily farmed for local consumption.

A number of techniques are used to control natural reproduction in tilapia (Guerrero, 1982). Four of these methods have been most frequently used:

a) Manual sexing, which is not very efficient, as half of the seeds are discarded and errors allow some females to enter the production stock and breed. The technique is labor intensive. It can be improved by stocking a predator to consume any recruits;

b) Artificial sex reversal to produce monosex populations (Guerrero and Abella, 1976; Guerrero, 1979; Tave, 1995). The technique involves providing feed laced with a

synthetic male hormone (androgenic steroid) such as 17 α-ethynyl- or methyl-testosterone to sexually undifferentiated tilapia larvae at an effective dosage and duration;

c) Production of monosex male F1 hybrid progeny from certain interspecific crosses. The most commonly produced hybrid in the US and Israel is that of the *O. niloticus* and *O. aureus* cross (Tave, 1995). But the crosses are not always 100% successful and success depends on maintaining pure genetic lines of the parental stocks. The technique is, in addition, management intensive;

d) Chromosomal manipulation, aimed at producing all sterile triploid fishes (Dunham, 1990; Riley et al., 1993).

The most recently developed technique to produce a male population of tilapia is the use of supermale broodstock (Mair et al., 1997). Supermales (males with a YY genotype) can only produce male offspring in female homogametic species such as Nile tilapia. Although this technique has great promise, it requires great managerial skill and extensive progeny testing.

In the recent past, however, another technique to produce mostly male tilapia, has been developed (Baroiller et al., 1995). It involves raising embryos and early stages of fish larvae at higher but sublethal temperatures. Sullivan and Schultz (1986), had earlier reported on the interaction of temperature and genotype in determining variable sex ratios in strains of *Poeciliopsis lucida*. Conover et al. (1992) reviewed the effects of thermal environment on primary sex differentiation of Atlantic silverside (Atherinidae), *Menidia menidia*, another thermosensitive fish species. Results of Conover et al. (1992) suggest that temperature sex determination (TSD) also exists in other fish species, including several species of tilapia with strong genotype-temperature interactions. The technique was also used by Baroiller et al. (1995) to raise tilapia embryos and larvae at high temperature for several days. They found that high temperatures (34-36°C) significantly increased the proportion of males (69-91% being an extreme percentage) but low temperatures (19-23°C)

did not affect the sex ratio. This study demonstrated that higher temperatures can have a strong effect on the sex ratio of *O. niloticus*, and the significance of external factors on sex determination in gonochoristic fish species. This technique has the advantage that no steroid hormones are used. The use of hormones raises ethical and health issues and consumer resistance.

Although some success in producing a high proportion of male tilapia can be achieved using heat treatment, little is known about what possible effect heat could have on gonads, reproductive development (for example, gonadogenesis), sex ratio of subsequent generations, growth rates, and the nutritional response of both males and females of F1 and other generations. Thermally sex-reversed fish are presumed to have normal gonads and to be sexually functional. This may not be completely true. The sensitivity of the germ cells to heat has not yet been fully confirmed although Strüssmann and Patino (1995) observed occasional occurrence of individuals of *Odontesthes bonariensis* and *Patagonia hatcheri* whose gonads were undeveloped or completely devoid of germ cells in groups reared at high temperatures. Temperature-induced sterilization is likely to occur in fish subjected to heat treatment at an early stage of development. High maintenance and periodic replenishment of sex-reversed broodstock would make this technique laborious. The impact of heat treatment on reproduction could be determined through a set of progeny testing experiments, and used to evaluate the effectiveness of the technique relative to reversing of the phenotypic sex of fish. All progeny of sexually-reversed males crossed with normal females in female homogametic *O. niloticus* would be expected to be females.

Most of the tilapia species brought into the United States came from stocks obtained in Israel and Egypt (Legner and Fisher, 1980). They were initially introduced in the southern states where water temperature conditions were favorable. Today, tilapia are produced in nearly every state, including those in the more temperate, northern and central states of the United States. The most cultured tilapia species in the United States are *O.*

mossambicus, *O. niloticus*, *O. aureus*, *O. urolepis hornorum* and *Tilapia zillii* or likely hybrids among those species (Costa-Pierce and Rakocy, 1997). Barrett (1983) reported that *O. mossambicus* and. *O. u. hornorum* had undergone so much hybridization that they were essentially one reproducing population. He also reported that *O. aureus* was the dominant cichlid in Arizona and that recent evidence had indicated that *O. niloticus* genes were also present, implying that hybridization between the two species had occurred in the wild. There is always a chance that hybridization will occur between closely related tilapia species that may be genetically distinct but reproductively compatible due to their relatively recent divergence. Given those circumstances, it was desirable to determine the true identity of the tilapia species we used in the present studies.

To be able to interpret and understand the results of sex reversal and progeny testing, we would need to correctly identify the type of tilapia species that we were dealing with. We used a tilapia species which had been reared at Piketon Research and Extension Center, Piketon, Ohio, for a few years. The fish were originally obtained from Purdue University, West Lafayette, Indiana. Although the fish were referred to as the Nile tilapia (*O. niloticus*), their identity was questionable on the basis of morphological characteristics. The appearance of the fish does not quite match with the morphological features of *O. niloticus* as described by Trewavas (1983).

The most recent classification of tilapiine species was based on breeding behavior, morphological characteristics and biogeography (Trewavas, 1983). However, such characters are of limited value for species identification because they showed considerable interspecies overlap or seasonality. Genetic markers based on DNA methods were therefore chosen to identify genetic variability among tilapia species. In recent years, several genetic studies using molecular markers to characterize various tilapia strains or species have been reported (Macaranas, et al., 1986; Harris et al., 1991; Seyoum and Kornfield, 1992). Among the DNA-based methods available for strain identification is the

use of randomly amplified polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR). The method allows the examination of genomic variation without prior knowledge of DNA sequences (Williams et al., 1990, and 1993, Hadrys et al., 1992). The technique was successfully used to generate genetic markers by Bardakci and Skibinski (1994) for distinguishing among tilapiine species. The authors provided evidence that RAPD markers were useful for systematic investigation at the level of species and, to a certain extent, for subspecies' identification. In our study, RAPDs were used to determine the taxonomic position of our experimental tilapia strain in comparison with other known species of *Oreochromis* genus.

Apart from investigating the impact of heat treatment on the reproductive biology of the tilapia, we were also interested in the performance of our experimental strain on fish meal-free diets. Currently, intensive research on nutritional responses of cultured fish is going on to develop alternative sources of high quality proteins to replace high-cost fish meal. Fish diet is normally the single largest expenditure in semi-intensive and intensive fish culture operations, representing 40-60% of total costs (Rodriguez-serna et al., 1996). This is due to extensive reliance on marine animal protein sources such as fish meal, shrimp meal and squid meal to meet the high dietary protein requirements of cultured fish. These feedstuffs have high nutritional value and palatability but are expensive and not always readily available (Lim and Dominay, 1990). Rumsey (1994) noted that the world supply of fish meal increased by 27% during the past 20 years and that the output by major fish meal producing countries actually declined. The world fish meal production was predicted to decline by about 5% between 1990 and year 2000 (Rumsey, 1993 and 1994). Therefore, in order to develop economical aquaculture systems, there is need to identify alternative sources of cheap but high quality proteins. In our study, we used both animal-based and plant-based sources of protein components, in varying proportions and evaluated

the performance of tilapia on the basis of growth rate, feed utilization, digestibility, steroid hormones, gonadal development, survival and general health of the fish.

Most of the experiments on breeding and nutrition of tilapia were conducted in a recirculating closed system. Dechlorinated water supplied from the Municipality of Columbus, Ohio, U.S.A. flowed to a bottom of 64 L reservoir from which it was pumped through a UV unit and a biofilter to an upper 86 L reservoir before it was distributed to individual glass aquaria. The temperature of the recirculating water was regulated by an automatic thermostat at a desired level.

OBJECTIVES

In this study, we sought to:

1. investigate the effect of heat treatment on sex ratio and growth of a strain of tilapia, *Oreochromis* sp. which was being raised at Piketon Research and Extension Center;
2. evaluate the effectiveness of a heat treatment technique in inducing sex reversal in tilapia through progeny testing;
3. identify genetically the tilapia strain which was being used to masculinize progeny using heat treatment and in the nutritional studies;
4. evaluate the performance of tilapia which were fed diets with protein component (fish meal) replaced by animal by-products or plant sources, and
5. investigate the effect of cottonseed meal-based diets on the reproductive parameters of tilapia.

In the following dissertation, a summary of our findings are presented in each Chapter :

Chapter 1: **Effects of heat treatment on sex ratio of tilapia and performance on animal by-products**

Embryos and larvae of tilapia which were raised at high temperatures of 36±1°C for 60 days of development after hatching, produced 74% males compared to 51% males for the control. The control fish had been raised at a lower temperature of 23±1°C. The conclusion was that some of the females had their sex reversed into males, accounting for the extra males above the theoretical 1:1 sex ratio. The chapter also reports on the effect of heat treatment on growth rates of tilapia which were fed different proportions of animal by-products as the major source of protein in the diet. Growth rates, size distribution and gonadosomatic indices of the heat-treated groups were compared to those of the control group. There were no significant differences in the performance of fish among the fish fed different diets within the same group (treated or control). Differences in growth rates and size distribution were significant between groups. Heat-treated fish grew faster than the control group during the first two months of feeding experiments, and males in each group grew faster than the females. Chemical body composition of the two groups was comparable.

Chapter 2: **Evaluation of heat treatment technique**

The Chapter reports on the success of heat treatment in masculinizing tilapia by performing back crosses with untreated females. Three out of 16 heat-treated males tested produced significantly higher than 50:50 proportions of female offspring. It was presumed that among these males, some of them had their sex reversed from females into phenotypic males by heat treatment. Theoretically, we expected the sexually-reversed males to produce 100% female populations as a genetic proof of sex reversal. This did not happen, suggesting that the process of sex reversal using heat treatment may not have been 100% successful. The results suggest that the heat-treated males were reproductively active and

able to produce viable offspring but final conclusion of the viability of gametes can only be made after the histology of gonads has been carried out. Mortalities of the tilapia larvae were high but probably expected at those early delicate stages of development, later declined as they developed to swim-up stage.

Chapter 3: **Genetic identification of the experimental tilapia strain**

Using the random amplified polymorphic DNA markers, revealed that the Piketon *Oreochromis* strain was more closely related to the *O. niloticus* samples than any of the other species used in the study. Although the Piketon strain appeared to be primarily *O. niloticus* in origin its morphological characteristics indicated that the strain could have some genetic contribution from other closely related *Oreochromis* species, and therefore might be a genetic hybrid. The morphological features of the Piketon tilapia strain matched the description of *O. aureus* (Trewavas, 1983) and the species is highly suspected to have hybridized with *O. niloticus* in the production of American aquacultural tilapia. Final identity of the strain will have to await more genetic screening using DNA samples from *O. aureus* along with other known tilapia species.

Chapter 4: **Performance of tilapia on cottonseed meal-based diets**

The chapter presents results of the performance of the tilapia on cottonseed meal (CSM)-based diets and assessment of its nutritional value for tilapia. Fish meal protein was gradually replaced with CSM from 0% to 100%. We determined growth rate of the fish, feed utilization, feed intake, its digestibility and chemical composition of the fish body. Less than 50 % CSM protein inclusion was found to promote tilapia growth. Greater than 50% CSM protein depressed fish growth; the diet was poorly digested and utilized. At 100% CSM protein some mortalities of tilapia were experienced and the fish suffered from mineral deficiencies, especially iron. Some of the minerals were not

biologically available to the fish due to complexing of the elements with phytate compounds found in cottonseed meal. Furthermore, iron in particular was bound by gossypol, a yellow, toxic and phenolic compound which occurs in free and bound form in cottonseed products. Levels of gossypol in liver increased with increasing CSM component in the diet. Fish meal protein replacement with CSM protein could therefore safely be incorporated at less than 50% protein replacement due to gossypol effects on tilapia.

Chapter 5: **Effects of cottonseed meal-based diets on reproductive parameters**

Investigations on possible effects of CSM on growth of different sexes and reproductive system of tilapia are presented in Chapter 5. Gossypol, a toxic component of CSM was reported to interfere with some of the physiological processes of reproduction in many vertebrates (Hadley et al., 19810). In particular, it was shown *in vitro* to alter steroidogenesis in both male and female bovines (Gu et al., 1991). Earlier studies were designed mainly to investigate the effect of CSM on fish growth (Fowler, 1980; Robinson and Brent, 1989; El-Sayed, 1990). In our study, males gained more weight than females among the dietary treatments. But the growth rates of both sexes were affected by 75% and above substitution of fish meal protein with CSM protein . Plasma steroid hormones (testosterone, estradiol-17β, 11-ketotestosterone and 17,20β-dihydroxy-4-pregnen-3-one were not significantly affected by the dietary treatments, nor did gonadosomatic index of either sex show significant differences among dietary treatments. However, plasma levels of testosterone and 11-ketotestosterone were significantly correlated with the gonadosomatic indices of both sexes. Low levels of progesterone were observed in both sexes. Because of low concentrations of free gossypol in the test diets, it did not induce significant changes in plasma sex steroids in tilapia. High concentrations of gossypol have been shown to inhibit steroidogenesis in domestic animals (Gu et al., 1990). However,

gossypol could still modify directly or indirectly other reproductive parameters such as motility of sperm and quality of gametes. Further studies need to be carried out in this area.

CHAPTER I

EFFECTS OF HEAT TREATMENT ON SEX RATIO AND GROWTH OF TILAPIA, *OREOCHROMIS* SP. (L.) FED ANIMAL BY-PRODUCTS IN A RECIRCULATING SYSTEM

INTRODUCTION

Tilapia, *Oreochromis* species have become increasingly important as cultured species not only in the tropics but also in the developed countries. Farmed tilapia today account for an ever-increasing proportion of the United States fish market. Tilapia imports increased to about 28,000 metric tons of product weight in 1998, 14% higher than the previous year (Aquaculture Outlook,1999). The potential for improved yields in culture of tilapia through the use of controlled reproduction to eliminate the problem of precocious maturity and undesirable reproduction has provided an impetus for studies in unisex tilapia production. Therefore, the strategy adapted currently by the intensive and extensive tilapia farmers is to grow monosex tilapia species. The females breed prolifically leading to overcrowding and consequently to stunted population. This can be detrimental to the fish farmer. By using only males, the tilapia breeder not only avoids undesirable natural reproduction but also raises bigger fish. Furthermore, male tilapia have a faster growth rate than females. This would particularly be an advantage for African countries where tilapia are essentially farmed for local consumption.

Monosex production in tilapia can be achieved through: (1) treatment with steroid hormones, (2) hybridization, (3) chromosomal manipulation and more recently, (4) using heat treatment. The most frequently used procedure to masculinize tilapia, has been the use of methyltestosterone, a synthetic hormone. However, hormonal treatment has a restricted application in some countries like the United States. The use of hormones raises ethical, health issues and consumer resistance. With heat treatment, however, modified gonad development of embryos of tilapia results in offspring which are predominantly males. A success of sex reversal as high as 62 to 90% male tilapia production, was recorded using heat treatment method in *Oreochromis niloticus*. (Baroiller *et al.*, 1995). Among the males produced, some of them are sexually-reversed males, i.e. they are genotypically females but phenotypically males with a male reproductive system. Our study was designed to investigate how heat treatment, applied at early stages of development, would affect the sex ratio of tilapia strain available in midwest United States aquaculture facilities.

Related to the successful and profitable aquaculture is the reduction of the cost of production. Fish feeds represent the single most expensive item in fish production. They account for as much as 30-50% of the total production costs in some cases (Steffens,1994). Protein, and in particular, fish meal is the most expensive dietary component in fish food. The goal of the study was therefore to determine the utility of low-cost sources of protein to replace fish meal without compromising the performance of the cultured fish in a recirculation system. This is particularly an important consideration for fish farming in developing countries where the expense of fish meal-based feeds is a particular problem.

MATERIALS AND METHODS

Breeding experiments

The offspring for these experiments were collected from naturally spawning group of females and males of tilapia at Piketon Research and Extension Center, Piketon, Ohio,

USA. The fish were originally obtained from Purdue University, West Lafayette, Indiana, USA and had been described as Nile tilapia (*Oreochromis niloticus*). However, the fish will be referred to as *Oreochromis* sp. or simply tilapia throughout this study since the exact determination of the species was not completely resolved. The fishes were suspected to be of hybrid origin or a backcross hybrid of Nile tilapia and some other tilapia species.

Hatched larvae of about six to eight days old were obtained from two mouth brooding and divided into two groups. The first group was raised in a 32 L aquarium at a water temperature of 35±1° C and heat-treated for 60 days. The second group was raised in a similar tank but at a lower temperature of 22±1° C to serve as control group. The two groups of larvae were raised in a recirculating system and fed trout feeds (Zeigler Brothers Co. Ltd, PA) *ad libidum*.

The recirculating system consisted of dechlorinated water supplied from the Municipality of Columbus, Ohio, USA in a 64-L bottom reservoir from which it was pumped through a UV unit and a biofilter to an upper 86-L reservoir before it was distributed to individual glass aquaria. Water left the aquaria through an outlet behind the aquarium into a collecting pipe and back to the bottom reservoir. The same water recirculated through the system again. About 20 liters of water in the bottom reservoir were replenished daily to compensate for loss through evaporation and occasional leakage. The water pipes were made of PVC material. The water was heated by an element (200 Watts, model WCS-2215AR19, Cleveland Process Corp., FL, USA and the temperature of the recirculating water was regulated by an automatic thermostat (CN 76000, Microprocessor-Based Temperature Controller, Omega Technologies Co., Stamford, CT., USA).

Diets and feeding experiments

Feeding trials for the heat-treated group of fish with test diets started immediately after heat treatment in March, 1996. The fish meal was gradually replaced by a fish meal

Ingredients	Diets				
	1	2	3	4	5
FM menhaden	20.00	15.00	10.00	5.00	0.00
FM herring	20.00	15.00	10.00	5.00	0.00
FM Analog**	0.00	9.50	19.01	28.51	38.01
Yeast	9.00	9.00	9.00	9.00	9.00
Corn Gluten	15.00	15.00	15.00	15.00	15.00
Soybean extract	13.00	13.00	13.00	13.00	13.00
Whey	7.00	7.00	7.00	7.00	7.00
Methionine	0.00	0.10	0.21	0.31	0.41
Lysine	0.00	1.20	0.25	0.37	0.49
Vitamin Mixture*	0.50	0.50	0.50	0.50	0.50
Mineral Mixture*	1.50	1.50	1.50	1.50	1.50
Alphacel	3.50	2.73	2.13	1.46	0.78
Vit. C-MP	0.05	0.05	0.05	0.05	0.05
Choline Chloride	0.10	0.10	0.10	0.10	0.10
Cod Oil	10.35	11.30	12.25	13.20	14.16
Total	100.00	100.00	100.00	100.00	100.00
Calculated values (%) based on nutrient tables or nitrogen analysis					
Protein	46.50	46.70	47.00	47.20	47.40
Energy (kcal/Kg)	4117	4117	4117	4117	4117
Methionine	1.20	1.20	1.20	1.20	1.20
Lysine	2.96	2.96	2.96	2.96	2.96

*Source: Dabrowski, (1990).

**FM analog: mixture of feather meal, blood and meat meal, bone meal, poultry by-product meal.

Table 1.1: Diet composition (%) for tilapia species nutrition experiment (FM-- fish meal).

analog composed of animal by-products in the proportions of 0% (diet 1) to 100% (diet 5)(Table 1.1). The fish were divided into fifteen groups to correspond with triplicate groups per diet. The stocking density was eight to ten fish, an average of about 100g per aquarium. The fish were initially fed their respective diets at a rate of 3% of their body weight per aquarium per day, later diminished to 1.5% towards the end of the experiment. Growth of fish was monitored by recording fresh body weight biweekly and the feed ration adjusted accordingly. Feeding experiments for the control group with the same test diets did not start until after two months after the start of the feeding experiment for the heat-treated fish. This allowed the control fish to grow to the initial size of the heat treated group when feeding experiments started. The control group of fish were then treated in a similar manner as the heat treated group in the feeding experiments. Specific growth ratios for each diet and for both the heat-treated and control fish were calculated to adjust for the age differences. All aquaria were maintained at 25±1° C throughout the experimental period. The feeding trials were conducted for 160 days for both groups of fish.

Determination of sex ratio

At the end of the feeding experiments, a random sample of 50 and 60 fish from the heat-treated and control groups, respectively, were sacrificed to determine sex ratios. By this time, the gonads were mature. The gonads were removed and weighed. The ratio of gonad weight to the fish weight was used to calculate the gonadosomatic index (% GSI) of each group of fish. Chi-square test ($p < 0.05$) was used to determine significant differences in the sex ratios.

Chemical composition of diets and fish body

Six fish samples of the sacrificed fish per diet (two per tank) were randomly selected, freeze-dried and ground into powder for proximate chemical analysis at The Ohio State University Research and Extension Analytical Laboratory, Wooster, Ohio, USA.

Samples were prepared for analysis using a dry-wet ashing procedure (AOAC, 1984). Samples in dry porcelain crucibles were dried in a freeze-dryer and later placed in an ashing oven (600 C) for two hours, removed and allowed to cool. Samples were moistened with distilled water, then nitric acid (9 N) was added, and samples gently heated on an electric hotplate to evaporate the liquid until only residual moisture remained. Ashed samples were solubilized in 6 N HCl and brought to volume with distilled water to obtain a ratio of 1:5 acid:water. Appropriate dilutions were prepared to bring mineral concentrations within standard reading ranges. Ionic composition of fish whole-body ash was measured by the inductively coupled plasma (ICP) emission spectrophotometry method with the use of an ARI-3560 Spectrometer (Applied Research Labs, Valencie, CA) according to Watson and Issac (1990).

RESULTS

Growth and size distribution

Heat-treated tilapia consistently performed better than the control group in terms of somatic growth whether or not they were fed different diets whose fish meal content had been gradually replaced by animal by-product, particularly during the first 8 months (Fig. 1.1 and Table 1.2). For example, mean body weight gain by heat treated fish exceeded that of control group fed five different diets at the beginning of the the feeding experiments. Evidently, control fish were slow at utilizing the diets during the first 8 weeks of feeding. The growth rates of heat-treated fish fed 100 % fish meal (diet 1) and 100% fish meal analog (diet 5) were higher than those for the control group during the first 8 weeks of feeding. However, the growth rates for the control fish improved after the 10th week of feeding (Table 1.2). Survival for all treatments was 100% right from larval stage to the time of sex determination.

Size differences between heat-treated and control fish were observed in both male and female tilapia. Females among the heat-treated group did not grow as much as the males. Most of them were confined to a size 100g or less while few males were as small as 80g by the end of the experiment, and most males weighed between 120 and 160g (Fig.1.2a). A similar trend was observed among the control group with most of the females weighing less than 80g, and few exceeded a weight of 100g (Fig. 1.2b). Most of the males weighed 100g or more and a few of them, in some cases represented by one fish, weighed up to 200g. In both groups, no female weighed more than 120g after five months of growth.

Sex ratio

The heat-treated group of tilapia had 74% males, while the control group had 51% males. The gonadosomatic index (GSI) also reflected the effects of heat treatment and diets. The GSI for heat-treated males was of particular interest. It was about half or less that for the control group and tended also to drop with the reduction of the fish meal content. Among the females, the GSI was notably low for diets 2 and 5 where it was less than half of the control fish group. There were no females to represent diet 4 among the heat-treated fish. GSI of female fish for diets 1 and 3 were not affected by heat treatment.

Chemical Composition of the body

There were no significant differences observed between the two groups of fish in the nitrogen and phosphorus levels among all the diets used (Fig. 1.4). Copper and iron concentrations were found to be higher in heat-treated than the control group fed diet 1. With the exception of control fish fed diets 4 and 5, which showed a lower concentration of iron, other treatments did not differ significantly (Fig. 1.5a and 1.5b). Concentration of zinc did not show differences among the different diets or between the two groups of fish

	100% Fish Meal		100% Fish Meal Analog	
Period (weeks)	Heat-treated	Control	Heat-treated	Control
2-4	2.5	0.8	2.3	0.5
6-8	2.1	1.2	1.8	1.0
10-12	1.2	1.5	1.2	1.8
14-16	1.3	1.2	1.2	1.2

Table 1.2: Specific growth ratios (%) of heat-treated and control fish at various time periods adjusted for age difference. The fish were fed 100% fish meal and 100% fish meal analog.

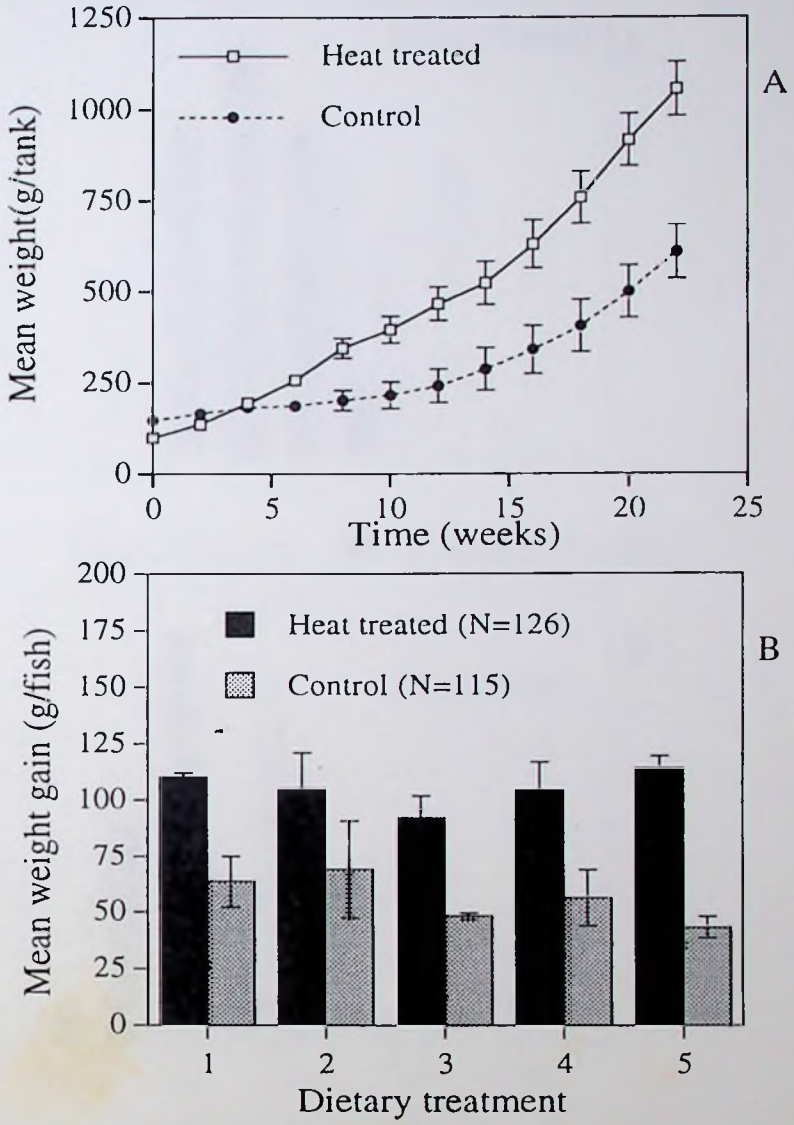


Figure 1.1: Growth of heat-treated and control tilapia after feeding on 100% fish meal (A)and weight gain after feeding on graded levels of animal by-products meal(B). Error bars stand for standard deviation.

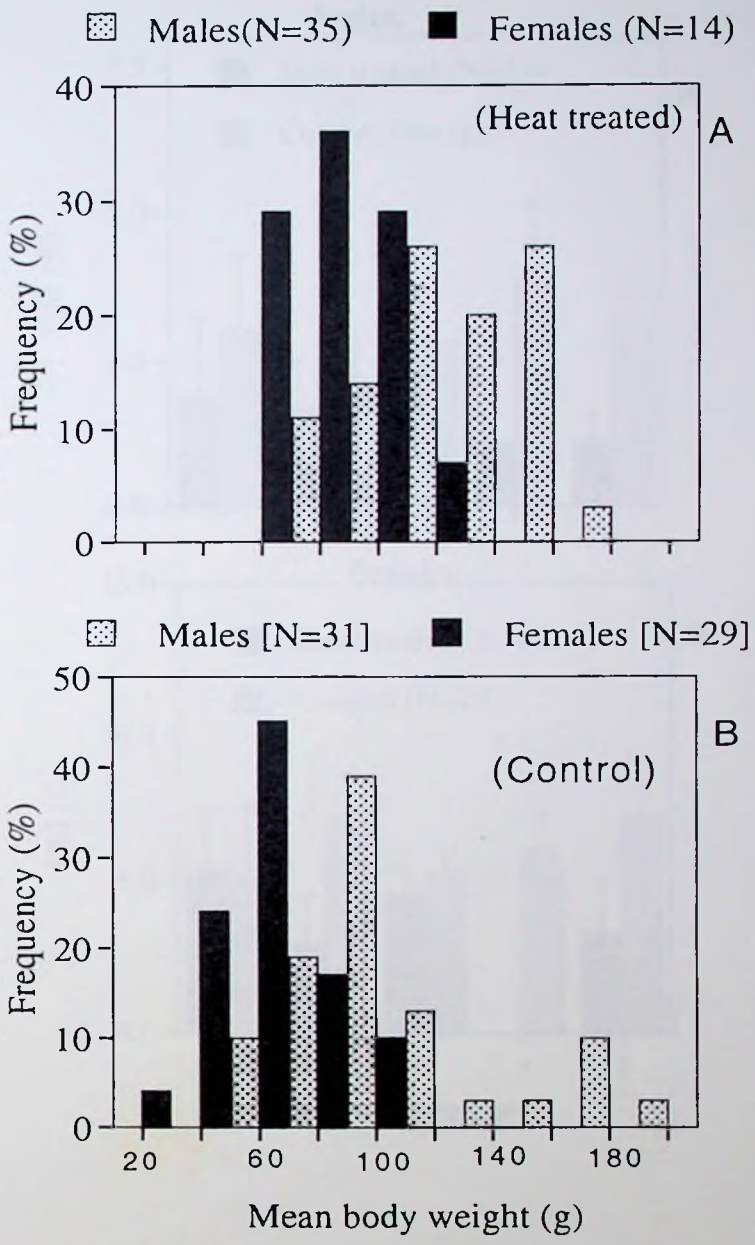


Figure 1.2: Size distribution of heat-treated (A) and control (B) tilapia fed animal by-product meal-based diets.

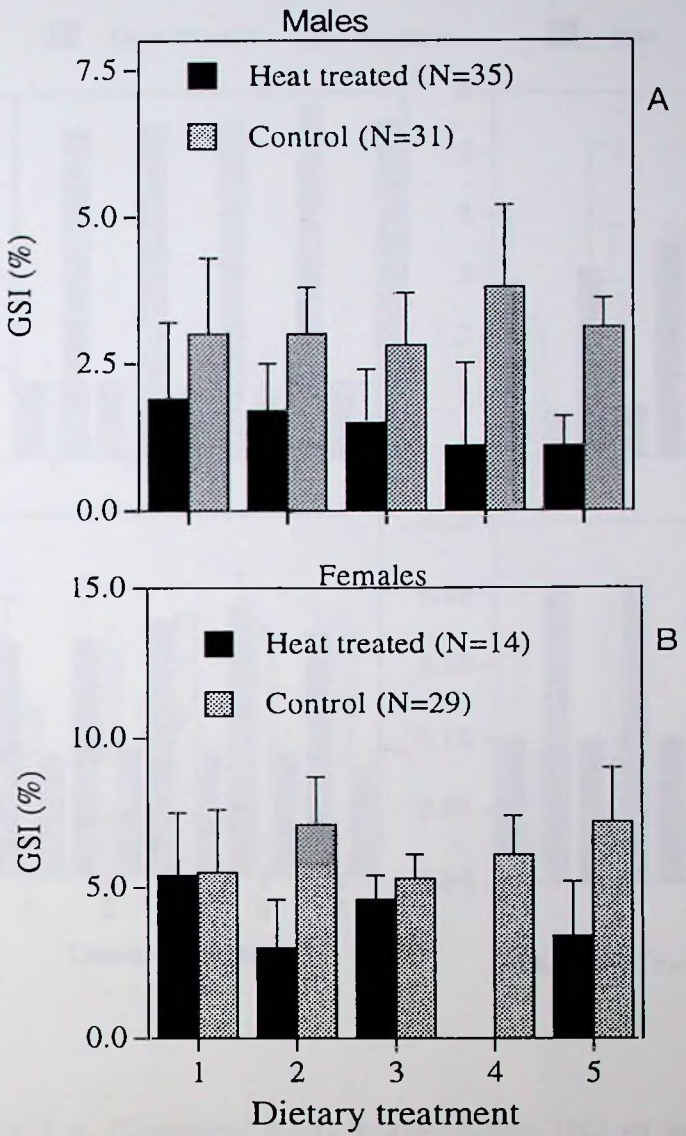


Figure 1.3: Gonadosomatic indices of heat-treated and control tilapia fed diets containing graded levels of animal by-products meal. Error bars stand for standard deviation.

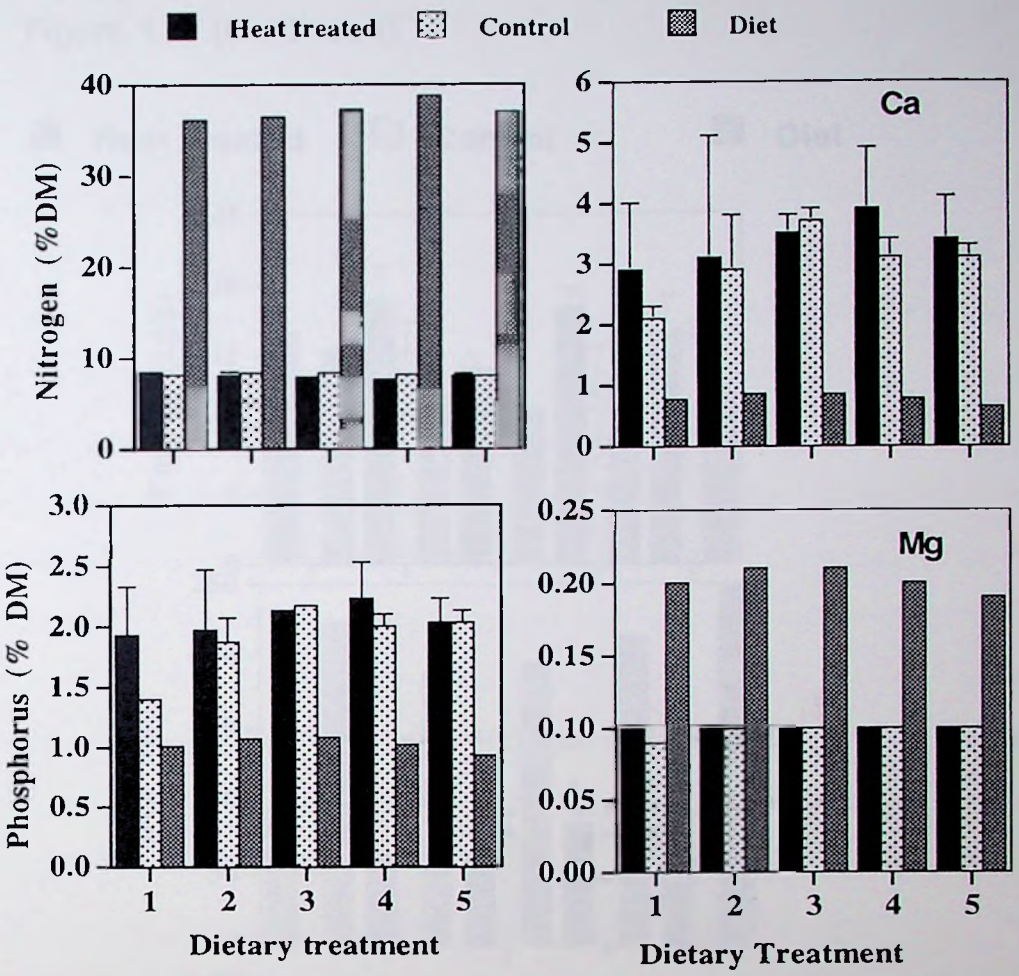


Figure 1.4: Chemical body composition (%) of tilapia fed animal by-products meal-based diets. The asterisk shows minerals which are significantly different compared to other diets. Error bars stand for standard deviation. Sample size used was 6 fish per dietary treatment.

(Fig. 1.5c). Figure 1.5 shows that the diets contained and supplied adequate amounts of minerals except calcium and phosphorus.

DISCUSSION

Temperature has for a long time been recognized as one of the important environmental factors in influencing primary sex differentiation (Conover and Fleisher, 1986; Conover and Heins, 1987b; Conover and Kynard, 1981; Francis, 1992; Mair et al., 1990; Strüssmann and Patino, 1995). The present study further demonstrates that heat treatment applied to sexually undifferentiated tilapia embryos and juveniles can influence the sex ratio of the offspring. Sublethal temperatures (34-36° C) significantly increased the proportions of males (74%) but lower temperatures (20-25° C) did not affect the sex ratio. Baroiller et al. (1995) while studying the temperature sex determination in *O. niloticus* and the Red Tilapia (Red Florida strain), found that high temperatures (34-36°C) significantly increased the proportion of males (69-91% for the former and 60-97% for most progenies of the latter strains), but lower temperatures (19-23°C) did not affect the sex ratio. They concluded that temperature sex determination could exist in several species of tilapia, with strong genotype-temperature interactions. The same authors observed variations in thermosensitivity among different fish species and a strong parental influence on the response to high temperature treatment. Although production of 74% males by the tilapia strain found in Central Ohio is relatively moderate compared to the above findings, these findings further confirm that the heat treatment technique is promising and could revolutionize aquaculture production of tilapia in the future. The technique would have the further advantage of being able to effectively replace the traditional method of administering sex hormones to fish, a practice resented by many fish consumers, as one of the major ways of masculinizing tilapia.

Not only did heat treatment alter the sex ratio of the offspring but it also enhanced the rate of feeding of the fish and enhanced their growth rate compared to fish that were not heat-treated. Significant differences in average weight gain were observed between the two groups that were given five different diets with different levels of fish meal replacement, at least during the first 8 weeks. This suggests that fish which were heat-treated had a higher metabolic rate right at the very start of the feeding experiments and therefore consumed more food which resulted in higher average weight gain and greater somatic growth. It could also be related to the presence of higher proportions of males among the heat treated group. However, each diet with different levels of fish meal replacement did not affect growth of either group; whether 100% or 0% replacement. This showed that the fish meal analog (FMA) was as good a source of protein as the fish meal itself and did not affect the growth performance of the fish. This means that if fish diets are formulated in such a way that they meet the quality of fish meal-based diets, i.e.provide the available amino acids and other essential nutrients, fish meal could be effectively replaced. Size differences observed in the two groups of fish were sex-related as it occurs in nature, bimodal in heat-treated and unimodal in the control fish, (Fig.1.2). In both groups, females exhibited a slower growth rate than males and their gonads were larger than males (Fig 1.3).

It should be pointed out, however, that gonads of heat-treated males were regressed as indicated by their low GSI values. The regression of the gonads was made worse by the deprivation of fish meal in their diet. The possible consequence of this effect would be to render most of the males sterile and incapable of producing viable gametes. If this were the case, then it would confirm the report of Nakamura and Nagahama (1995) that there was nearly complete elimination of germ cells in the gonads of *O. niloticus* exposed to 36-37° C between 7-50 days after hatching. They also showed that these effects were permanent and probably resulted in sterile adults.

Faced with the high costs of feeds due to increasing demand and diminishing supply of fish meal, several feedstuffs, both plant and animal, have been tested and proposed as substitutes for fish meal. The formulation of fish meal analog was aimed at meeting such needs. This new product should normally contain no less than 35% protein, no measurable amount of antiproteinase activity, match amino acid profiles of fish meal, contain less than 15% ash and no less than 0.5% of available phosphorus (Dabrowski and Hardy, 1994). Further specifications for FMA formulation requires the consideration of essential fatty acids and preventing degradation of the feeds during storage.

The present study showed that fish meal analog that was composed of blood meal, meat and bone meal, feather meal and poultry by-product meal, can completely replace fish meal in practical diets for tilapia without impairing its performance. There is increasing evidence that the nutritional value of several alternative nutrient sources are not that inferior to that of fish meal, and their value can be increased significantly by adding other ingredients and limiting nutrients. Fowler (1990) found that in Chinook salmon diet, 38% of the fish meal was replaced successfully by feather meal. For rainbow trout, poultry by-product meal with feather meal appeared to be an excellent replacement of fish meal in the diet formulation. It is also known that different fish species have different nutrient requirements which in turn determines how diets are formulated. For example, Tacon and Jackson (1985) found that a mixture of meat and bone meal (4:1) successfully replaced up to 50% of fish meal in rainbow trout diet formulation. However, meat and bone meal alone was a poor substitute for herring meal in Chinook salmon diet (Fowler and Banks, 1976). In common carp, diet formulations with rapeseed meal were inferior to a control diet that was fish meal-based (Dabrowski and Kozlowska, 1981). Viola and Zohar (1984) reported that up to 50% of fish meal could be replaced by poultry by-products in the diets of tilapia hybrids. Otubusin (1987) reports that blood meal levels exceeding 50% of fish meal protein in diets for tilapia fingerlings, reared in cages for 120 days resulted in a significant

reduction in fish growth, while a 10% level of replacement was found the most effective. Therefore, we conclude that a fish-meal free diet (diet 5) in the present study is a significant achievement in low cost-diets formulation for this species.

Heat-treated fish, on the whole, showed higher concentrations of mineral elements like iron, copper and zinc in their body composition compared to the control group. With the exception of diet 3, heat treated fish raised on the other four diets clearly showed a significantly higher content of iron, a very important prosthetic element in hemoglobin. This seems to suggest that heat treatment promoted the absorption and concentration of iron in the body. Nitrogen and phosphorus concentrations were about the same in treated and control and among dietary groups of fish. This observation provided further evidence that the animal by-product supplied an equivalent amount of nutrients to the tilapia just as did fish meal.

Fish absorb some minerals not only from their diets but also from their external aquatic environment. The latter include calcium, sodium, potassium, whereas magnesium, iron, zinc, and copper are essential dietary components. Phosphate and sulfate, however, are more effectively obtained from feed sources (Lall, 1989). Feeds are considered the major source of iron for fish because natural waters usually contain low amounts of soluble iron. Fish accumulate zinc from both water and dietary sources; however, dietary zinc is more efficiently absorbed than waterborne zinc. It is also known that bioavailability of zinc to fish can be reduced by high dietary concentration of calcium and phosphorus which complex with it and this affects its absorption (Satoh et al., 1989). These and other trace metals, although required in very small quantities (usually less than 100 mg/kg dry diet), are absolutely essential for normal growth of fish. Dietary phosphorus requirements for most fish range between 0.5-1.0%, for instance, carp, rainbow trout and salmon (Ogino and Takeda, 1978; Watanabe et. al., 1980). The availability of phosphorus in fish meal for tilapia was reported to be lower compared to that for rainbow trout and chum salmon

(Watanabe et al., 1980). However, despite a low dietary content of calcium and phosphorus, the fish were able to accumulate enough minerals in their body. Based on the performance of the fish in our experiments, the animal by-product-based diets supplied most of these nutrients in an adequate amount, and no particular mineral deficiency was detected among the fish.

In conclusion, a combination of successful heat treatment and a preponderance of males in the recirculating systems made it possible for the tilapia to utilize the feeds whose protein source was solely contributed by the fish meal analog, which was composed of animal by-products. The experiment demonstrates first that a predominantly male progeny can be achieved through heat treatment and secondly that tilapia can be raised relatively cheaply on diets whose protein source can be solely contributed by animal by-product in recirculation systems. In general, feed utilization and growth functions were elevated by the high temperature treatment at an early stage of fish development.

CHAPTER 2

EVALUATION OF HEAT TREATMENT TECHNIQUE IN MASCULINIZATION OF TILAPIA, *OREOCHROMIS* SP.(L.)

INTRODUCTION

The production of the predominantly male tilapia offspring using heat treatment was presented in Chapter 1. Although some success was achieved in producing a higher proportion of males than in controls, it was not clear what impact the treatment might have on the reproductive physiology of the fish produced, including modifying early gonadogenesis or what proportion of the males was sexually reversed by heat treatment. Thermally sex-reversed fish have been presumed to have normal gonadal histology and sexual function (Strüssmann and Patino, 1995). The sensitivity of the male germ cells to heat is uncertain. For instance, Strüssmann and Patino (1995) quoted Nakamura and Nagahama who reported the complete elimination of germ cells of the gonads of *Oreochromis niloticus* exposed to 36-37°C between 7-50 days after hatching, leading to possible sterilization. Strüssmann et al. (1996, 1997) reported the occurrence of germ cell-deficient atherinid fish (*Odontesthes argentinensis* and *Patagonina hatcheri*) among groups exposed as larval stages to high temperatures during gonadal sex differentiation. My hypothesis is that the water temperature used to raise the tilapia larvae and juveniles during the sex differentiation period could induce sterility among the fish requiring periodic replenishment of the sex-reversed-broodstock. However, as demonstrated earlier, thermal

treatment of tilapia had a positive effect on fish growth and this may prove to be a practical approach to culturing this species.

A series of breeding experiments was therefore carried out to determine whether heat treatment affected the primordial germ cells of gonads and therefore the ability of males to produce viable gametes. The work would also demonstrate, for the first time since the introduction of the technique, its effectiveness to generate viable offspring that were predominantly male. The offspring of the sexually reversed males were expected to be females if the siring tilapia males were genotypically female but phenotypically males. This assumed that the sex determining system in *Oreochromis* sp. strain kept in Piketon was XY-XX; in which case the offspring of sexually reversed XX phenotypic male, crossed with normal XX female was expected to produce XX female offspring (Tave, 1992). This sex determination system would be true of *O. niloticus* but would not hold if the strain was a hybrid between *O.niloticus* and *O.aureus* which has a ZZ-WZ sex determining system (Tave, 1992).

MATERIALS AND METHODS

Broodstock

Mature male tilapia (n=49) that had been raised earlier from the heat-treated group were used as donors of milt for the breeding experiments. They were kept in a 400 L circular tank at 23±1°C. The males ranged from 641.6 g to 1, 084 g in weight and were about 2 years old at the time of study. Untreated female tilapia (200 - 300 g body weight) were selected from an existing stock at the aquaculture facility at Piketon Research and Extension Center, Piketon, Ohio, USA. They too, had been kept at 23±1°C in 400 L tanks. The tanks were continually aerated. The broodstock fish were fed trout commercial diet (Zeigler Brothers, Inc., PA) at a daily ration of 2% of their body weight. Illumination

was provided by electrical lighting and the photoperiod regulated to maintain a daily minimum of 14 hours light and 10 hours dark.

Spawning

Initially natural pair spawning was attempted but the approach had to be changed because of the aggressive nature of males towards females. Each male and female was kept in separate 32 L aquaria filled with a 3-5 cm-layer of sand to induce spawning behavior. A female tilapia was identified as ripe and ready to spawn when the genital papilla was red and distended. However, in order to speed up the process of spawning most males and females were induced to spawn by administering leutinizing hormone releasing hormone (LHRH) at the rate of 40-50 μg/kg body weight for females and 30 μg/kg body weight for males. Females for hormone injection were selected based on the intraovarian biopsy results following the procedure developed by Garcia-Abiado (1995). A soft silastic tubing (2 mm internal diameter) was inserted into the ovipore and the eggs gently aspirated. If the eggs were found large and fully yolked, it was a sign that the female was about to spawn. The fish were given a dose of hormone by injecting them intraperitonally and stripped after 24 hours, usually 6-8 hours of light. Milt from the males was collected by applying pressure along the belly. The first few drops of milt were usually contaminated with urine and were discarded. Milt emerging from the genital pore was collected with a pasteur pipet, transferred into eppendorf tubes and refrigerated at 4°C until eggs were ready for fertilization. Ripe females were stripped of eggs into a plastic cup. The eggs were discarded if found attretic or contaminated with blood or connective tissue. Good eggs were subdivided into smaller batches (about 200-300 eggs) together with the ovarian fluid in separate plastic cups. A few drops of the milt were placed over the eggs, swirled gently for 5 minutes to allow fertilization to take place before they were incubated at 26±1°C.

Egg Incubation

About 100-200 fertilized eggs were placed in individual incubation glass chambers, each 17cm long, connected by plastic tubing that continually renewed the water flow. The main incubation tank (40 x 22.5 x 15 cm, internal dimensions) was made of perspex material and water left through an overflow tube back to the water reservoir. A total of 10 glass chambers, was surrounded by a water level of about 9 cm high. One or two glass beads (2 mm diameter) were placed at the bottom of each glass chamber to prevent loss of eggs in case of backflow of water. Each glass chamber was clearly labeled with the tank numbers of the broodstock and date of fertilization. A small plastic basket whose bottom was net-meshed (about 4 cm diameter x 6 cm high) was connected to an overflow tube of each glass chamber to trap any overflowing eggs from the incubation chamber. The continuous flow of water through the glass chambers ensured the churning action of the fertilized eggs and constant water renewal. The chambers were checked daily to remove and record any dead eggs. Dead eggs were more buoyant than the live eggs and were suspended higher up in the incubators where they were siphoned off. The percentage fertilization success was recorded 3 hours after fertilization. Egg survival was assessed using a binocular dissecting microscope by taking the same number of eggs or embryos in duplicate, counting and recording the number alive. Embryos were observed daily to monitor their development and survival until they hatched and further into swim-up stage. To determine embryo survival the following criteria for a living embryo were used: (i) the presence of a well defined germ ring, prior to the heart beat; (ii) in older embryos, the presence of heart beat.

Rearing of larvae and fingerlings

Ten days after hatching, the fish larvae, still with incompletely absorbed yolk, were counted and transferred to glass aquaria that were supplied with the same water going through the egg incubation chambers and maintained at the same temperature of 26±1°C. The larvae were given a a diet formulated in this laboratory (Kolkovski et al, 1999) or

larval fish Kyowa (Kyowa Hakko Kogyo Co.Ltd., Tokyo, Japan) for several days. The diet was changed to trout commercial feeds (Zeigler Brothers Inc., PA) at varying feed particle sizes (0.1cm -0.5cm diameter) depending on the size of the fingerlings. The fish were raised to juvenile stage for a period of 4 months and over, after which they were ready for sexing.

Sexing and sex ratio determination

All progeny of all male parents tested were sexed by dissection except where broods exceeded 70 fish. In these cases, a more representative sample of progeny was randomly selected for sex determination. When the gonads were not fully developed and difficult to sex, they were stained in aceto-carmine solution as described by Guerrero and Shelton (1974) and examined under the binocular dissecting microscope using magnifications of x25 to x100. The significance of the differences in the percentages of males and females of each individual male offspring were analyzed by the chi-squared test and binomial distribution statistic. Statistical significance was defined as $P < 0.05$.

RESULTS

The average fertilization success was 75.2±15.6% and ranged between 43 and 95% 2 hours after fertilization (Table 2.1). Table 2.1 also shows how the relative survival of the offspring gradually changed at different stages of development. Highest losses of offspring occurred between pigmentation (72 hours after fertilization) and, between 5 days at hatching stage and 10 days after fertilization with a relative survival drop of about 20%. After the tenth day, when the fish larvae were transferred from the incubation chambers to aquaria, proportions of larvae surviving improved and stabilized. There were two extremes of survival after hatching: a group of larvae from one brood which did not survive for long after hatching, designated as 0% survival and those which survived at a high proportion, designated as 73% of the survival (Table 2.1).

Activity/Dev. Stage	Time	Mean Success/ Survival (%)	Range (%)	Relative Survival (%)
Fertilization	2 Hrs	75.2±16	43-95	-----
Embryo	24 Hrs	62.6±24	6-91	83.2
Embryo	48 Hrs	54.2±24	3-88	86.6
Pigmentation	72 Hrs	46.0±22	2-76	84.9
Hatching	5 Days	35.5±19	2-76	77.2
Larvae	10 Days	21.2±18	0-73	59.7
Larvae	30 Days	16.2±19	0-73	76.4
Juveniles	120 Days	15.7±19	0-73	96.9

Table 2.1. Success and survival of progeny sired by heat-treated male tilapia at various stages of development.

Fish (M) ID.	Fish Total	Fish sexed	Males (%)	Females (%)	Age (days)
1	24	24	29	71[a]	143
2	72	72	58	42	146
3	51	51	43	57	152
4	27	27	33	67[a]	125
5	22	22	41	59	132
6	22	22	59	41	133
7	78	48	44	56	158
8	86	56	46	54	154
9	47	47	38	62	158
10	59	59	44	56	159
11	272	161	24	76[a]	176
12	190	102	47	53	180
13	37	37	51	49	153
14	153	76	43	57	154
15	185	146	47	53	162
16	20	20	60	40	153

[a]: proportion of females is significantly higher than the normal sex ratio of 1:1 at at $P < 0.05$.

M : male tilapia

Table 2.2: Proportions of male and female offsprings sired by heat-treated tilapia males. Last column shows the age of the progeny at the time the sex was determined.

	Males			Females		
Siring Male #	Mean Wt	N	GSI	Mean Wt	N	GSI
1*	31.60 ± 3.7	7	0.71 ± 0.5	29.60 ± 2.6	17	1.27 ± 0.3
2	21.80 ± 1.0	42	0.40+ ± 0.1	19.11 ± 0.9	30	0.56 ± 0.1
3	25.90 ± 2.1	22	0.78+ ± 0.2	22.20 ± 1.3	29	3.00 ± 0.3
4*	36.90 ± 3.3	9	0.18 ± 0.0	33.50 ± 1.6	18	0.16 ± 0.0
5	47.64 ± 2.1	14	0.60 ± 0.1	36.42 ± 2.0	16	1.33 ± 0.2
6	43.20 ± 4.1	9	0.13 ± 0.0	34.93 ± 2.4	13	0.19 ± 0.0
7	52.50 ± 1.9	13	0.19+ ± 0.0	40.30 ± 2.1	9	0.43 ± 0.3
8	35.13 ± 1.3	21	0.21+ ± 0.1	30.91 ± 1.1	27	0.35 ± 0.1
9	32.92 ± 1.8	26	0.17+ ± 0.0	31.30 ± 1.4	30	0.28 ± 0.1
10	41.30 ± 1.5	18	0.36+ ± 0.0	31.90 ± 1.4	13	0.88 ± 0.2
11*	38.72 ± 3.9	8	0.23+ ± 0.1	33.30 ± 1.3	22	0.59 ± 0.1
12	37.00 ± 1.5	16	0.40+ ± 0.2	27.65 ± 0.9	15	0.82 ± 0.1
13	58.10 ± 1.9	19	0.31+ ± 0.1	46.60 ± 2.1	18	0.75 ± 0.2
Control	92.15 ± 4.4	8	0.37 ± 0.1	71.70 ± 4.1	12	0.67 ± 0.1

*: Male tilapia suspected to have been sexually reversed. N= Size of the progeny examined.
+ Mean GSI values of male and female progeny of the same sire which are significantly different from one one another at $p < 0.05$.

Table 2.3: Mean gonadosomatic indices (±S.E.) of the progenies of heat-treated male tilapia with their corresponding mean weights (±S.E.).

Table 2.2. shows the sex ratio of the progeny sired by 16 out of 49 heat-treated male tilapia tested. The progenies of the following three tilapia males (about 20% of the tested males), ID numbers 1, 4 and 11 (Table 2.2) showed a sex-ratio where female proportion was significantly higher ($p > 0.05$) than the theoretical sex ratio of 1:1. This implies that about 20% of the originally heat-treated males could have been sexually reversed by the heat treatment at the early sexually undifferentiated stage. There were a few more progenies whose female number was higher than males, above 55% and therefore skewed but those ratios were not statistically significant ($p > 0.05$).

Gonadosomatic index (GSI) values of the progeny of heat-treated males suspected to have been sexually reversed, were not significantly different from the GSI of progeny of the rest of the treated male tilapia. Both of the two groups shared the low and the high values of GSI. About 70% of the GSI for male progenies were significantly different from those of female progenies (Table 2.3). The GSI of the progenies ranged between the following means±S.E.: 0.17 ± 0.0% and 0.78% ± 0.2% for males and, 0.16 ± 0.1 and 3.00 ± 0.3% for females (Table 2.3). These values were much lower compared to those recorded in earlier studies for normal (control) tilapia (shown in Chapter 1).

DISCUSSION

The primary objective of this study was to verify whether heat treatment of early stages of *Oreochromis* sp. is effective as a technique for masculinizing of presumptive females to functional males. Three of the 16 heat-treated tested males (20%) produced significantly higher proportions of female offspring. The assumption was that among the original heat treated males, 24%, i.e. above the theoretical 50:50 sex ratio would have had their sex reversed from females into phenotypic males by heat shock. In Chapter 1, we reported having obtained 74% masculinization success. This meant that 30% of the total population of heat-treated females were sexually reversed in which case we would have

expected at least 5 out of 16 tested males to produce predominantly female progeny. Therefore, although a figure of 3 sexually-reversed females is small, it may not differ significantly from the expected value of 5.

Theoretically, we should have expected the sexually-reversed "males" to produce 100% female populations as a genetic proof of sex reversal just like in similar studies of progeny testing on sex reversals in Mosambiquan tilapia (Clemens and Inslee, 1968). However, this was not confirmed in the present study. This would suggest that the process of sex reversal using heat treatment may not have been 100% but only achieved partially. Some of the germ line cells at primordial stage could have retained their potential to form Y sex chromosomes, hence the appearance of males in the offspring, rather than having XX sex chromosomes only for female offspring. We also suspect that some portions of the Y sex chromosomes were not completely deactivated by heat treatment. Tsumura et al. (1991), while studying sex reversal in rainbow trout, also found a small percentage of female offspring produced by XX phenotypic males.

In a study using methyltestosterone to masculinize female *Tilapia mossambica* (*O. mossambicus*), Clemens and Inslee (1968) achieved 29% success where, among those fish treated early in life, sired all-female offspring when crossed with normal females. This indicated that successful sex reversal had been achieved and that the females were homogametic for sex inheritance; that is, the sex chromosomes of normal females are XX (Tave, 1992).

Heat treatment as a technique to masculinize fishes is relatively new compared to other methods, the classical one being the use of steroid sex hormones. Therefore studies of this kind are still limited. Considering that the tilapia species used in the progeny test experiments could not be precisely identified (findings are presented in Chapter 3), this may partly explain the type of results obtained in this study. There was evidence to show that the tilapia species used in this experiment was a hybrid between *Oreochromis niloticus*

and some other closely related species of *Oreochromis*, most likely *O. aureus*, since the two species can be easily hybridized. It was also shown that *O. niloticus* contributed a larger component in the genetic constitution of the species. Because of the possible genetic mixing, the species would not be expected to produce progenies' sex ratio according to the rule and obtain 100% all-female offspring following heat treatment. It is probable that heat treatment may not have succeeded in complete deactivation of the sex chromosome set due to the presence of other unclear genetic factors that may have a role in determining sex in tilapia. For example, it was suggested that a polygenic system or a multifactorial mechanism of sex determination exists in tilapia (Majumdar and McAndrew, 1983).

Contrary to the predicted effect of heat treatment, like heat-induced germ cell deficiency and possible cause of sterility in fish, most males, in this study, were found reproductively active and able to produce viable sperm cells. If there was any damage at all, it could only have been partial. Strussmann et al.(1998) found that while most individuals of *Patagonina hatcheri* subjected to 27 ± 1.5°C and of *Odontesthes bonariensis* subjected to 29°C had sterile gonads, a few individuals had only partial loss of germ cells. These gonads with partial germ cell deficiency were characterized by isolated cysts in which the germ cells seemed to proliferate normally, interspersed with large gonadal regions completely devoid of germ cells. This suggested that there was a variation in the heat-sensitivity of individual cells (Strüssmann et al., 1997). However, the lower mean GSI of the tilapia offspring of heat-treated males in the present study in comparison to controls seem to suggests that gonadal development was suppressed to a certain extent (Table 2.3). This is in great contrast with GSI values obtained for the untreated control group of fish of similar size and age; their GSI values were above 2.5% for males and 5.0% for females (Fig. 1.2) presented in Chapter 1.

Losses due to mortality of the fish embryos and larvae were high at the early stages of development and stabilized later towards the swim-up stage. There was no difference in

the mortalities of progeny of heat-treated and untreated males (data not presented). The incubation temperature for the developing embryos fell within the recommended range of 25 and 30°C for *O. niloticus* (Rana, 1990) and would not have caused the high mortalities observed among the fish during the study. Many abnormalities including missing fins, twisted tails, deformed jaws, vertebral column and various abnormally shaped gonads were observed in the early sets of the offspring populations. Some of these defects seemed to depend on the quality of water in recirculating system because some of them disappeared when the water quality was improved by allowing a slow flow of water through the system. Mean survival of hatched embryos was low (35.5 ± 19.2%, Table 2.1) compared to findings of other investigators who carried out artificial fertilization, incubation and hatching of *O. niloticus*. Myers and Hershberger (1991) reported a hatching success of 68.6±3.6% for *O. niloticus* whose eggs and sperms were also strip-spawned.

Using semen from heat treated phenotypic males did not seem to greatly affect the hatching time recorded for the tilapia species. Rana (1985), obtained hatching times of 4 days for *O. mossambicus* in round-bottom containers at 28°C. This timing is very close to our finding. It took about 120 hours (5 days) after fertilization for the eggs to hatch. Rana (1986), however, warns that data on hatching times for the artificially reared eggs of mouth-brooders should be compared cautiously. In addition to factors such as oxygen and pH levels (Braum, 1978), mechanical stress on the chorions of tilapia eggs during artificial incubation may also influence hatching times (Rana, 1986).

Our evaluation of the effectiveness of heat treatment as a technique to masculinize genotypic female tilapia to phenotypic males is one of the few studies to be undertaken. Further investigations are warranted to address progeny sex ratio in pure, genetically verified cichlids. The results confirm that environmental sex determination occurs in the family of cichlids as reported by Baroiller et al. (1995) and that the response of differentiating gonads to heat can vary. The experiments also raise some questions on the

effectiveness of heat treatment technique with respect to masculinization of tilapia and whether the phenotypic males produced are reproductively functional. Histological studies on the gonads of heat-treated males to assess the extent of possible damage relative to untreated male gonads would determine whether the gonads of heat-treated males are fully or partly functional. This will also help in decision making about whether or not heat treatment as a new technique for masculinizing tilapia can be recommended for application in aquaculture practice. If adapted, production of all or predominantly male progenies in tilapia culture using heat treatment would be received as significant progress in aquaculture of this tilapia species.

CHAPTER 3

GENETIC CHARACTERIZATION OF TILAPIA (*OREOCHROMIS* Sp.) REARED AT PIKETON RESEARCH AND EXTENSION CENTER, OHIO, USA

INTRODUCTION

Tilapia form a group of warmwater fish which belongs to the family Cichlidae. They were originally native to the lakes and rivers of Africa before they were introduced into various waters of the world. Tilapia are classified into three major genera, namely, *Oreochromis* (the maternal mouthbrooders), *Sarotherodon* (the paternal or biparental mouthbrooders) and *Tilapia* (the substrate spawners) (Trewavas, 1982). All the species of the three genera are usually lumped together and commonly referred to as tilapia. Tilapia had been introduced from Africa into Asia and the Far East before the First World War (Schuster, 1952). However, from the 1930s to mid 1970s, tilapia were widely exported from their native ranges in sub-Saharan Africa. Introductions were made into Asia and over 50 countries throughout the tropical world for aquaculture development, aquatic weed and insect control (Costa-Pierce and Rakocy, 1997). This widespread seeding of tilapia in the world's warm water ecosystems is referred to as the "tilapia craze," similar to the carp "craze" in the 19th century.

Most of the tilapia species brought into the United States came from stocks

obtained in Israel and Egypt (Legner and Fisher, 1980). They were initially introduced in the southern states where water temperature conditions were favorable. Today, tilapia are produced in nearly every state in the United States, using thermal structures, greenhouses, waste heat and geothermal waters in the more temperate northern and central states. The most frequently cultured tilapia species in the United States are *O. mossambicus, O. niloticus*, *O. aureus* (blue tilapia), *O. urolepis hornorum* and *Tilapia zillii* or, likely, hybrids among those species. Barrett (1983) reported that *O. mossambicus and O. u. hornorum* had undergone so much hybridization that they were essentially one reproducing population. He also reported that *O. aureus* was the dominant cichlid in Arizona and that recent evidence had indicated that *O. niloticus* genes were also present, implying hybridization between the two species. Evidently, hybridization may occur between closely related tilapia species that may be genetically distinct but reproductively compatible because of their relatively recent divergence.

Our earlier studies on sexual reversal in tilapia prompted the need to characterize the tilapia at the Piketon Research and Extension Center, Ohio, USA. Their identification as Nile tilapia (*Oreochromis niloticus*), was questionable because they did not fit the morphological features of *O. niloticus* as described by Trewavas (1983). The morphology of the tilapia strain at Piketon could easily be confused with other described species of tilapia which shared its external features. The upper margin of the dorsal fin and the caudal fin of the Piketon strain are characteristically red-orange as breeding colors. The same appearance can be found among several other tropical tilapia species such as *O. variabilis*, *O. mossambicus* and *O. aureus*. As mentioned above, the last two species now

occur in the United States. The Piketon strain is certainly an *Oreochromis* species because only the females of the Piketon strain brooded eggs.

Earlier results with the Piketon strain (unpublished) of sex reversal using high sublethal temperature (35±1°C) produced a sex ratio of 74% males compared to a normal 50:50% sex ratio for embryos raised at a lower temperature (25±1°C). Boroiller et al. (1995) reported that high temperatures (34-36°C) significantly increased the proportion of males, with sex ratios ranging from 69-91% males in *Oreochromis niloticus* and between 60 and 97% males for red tilapia of the Red Florida strain. It is documented that different species of *Oreochromis* may have different chromosomal mechanisms of sex determination under normal temperature conditions (Wohlfarth and Wedekind, 1991) so we needed to identify the species of the Piketon strain and determine whether it was a hybrid between tilapia species or a pure line of one *Oreochromis* species

The most recent classification of tilapiine species was based on breeding behavior, morphological characteristics and biogeography (Trewavas, 1983). However, such characters are of limited value for species identification because they show considerable interspecies overlap (such as brooding behavior) or may be seasonal (such as sexual coloration), hence we turned to molecular markers.

In recent years, several genetic studies using molecular markers to characterize various tilapia strains or species have been reported (Macaranas et al.,1986; Harris et al., 1991; and Seyoum and Kornfield, 1992). These genetic markers can be used to identify genetic variability among species. The methods include isozymes (Second, 1982; Tanksley and Orton, 1983), which are limited by the relatively low levels of polymorphism

detectable. DNA based methods include restriction fragment length polymorphisms (RFLP), a technique which has the disadvantage of having to use radioactive material and requiring large quantities of relatively pure DNA (Tanksley et al., 1989; Wang et al., 1992), mitochondrial DNA (Capili, 1990; Seyoum and Kornfield, 1992), which has maternal inheritance and may give misleading results for hybridization studies, and very recently, DNA microsatellites (Harris et al., 1991), which are only beginning to be applied to tilapia. Among the DNA-based methods available for strain identification is the use of Randomly Amplified Polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR). This method allows the examination of genomic variation without prior knowledge of DNA sequences (Williams et al., 1990, 1993; Hadrys et al., 1992). The number and the size of amplified fragments depend on occurrence in the genome of sequences complementary to short, single and arbitrary PCR primers. Priming sites are assumed to be randomly distributed throughout a genome and polymorphisms (presence versus absence, where absence indicates a change in the sequence of the complementary site) in such sites result in differing amplification products, detected electrophoretically by the presence or absence of fragments. These polymorphisms have been shown to be inherited in a Mendelian fashion and can be used as genetic markers (Williams et al., 1990). The technique was successfully used by Bardakci and Skibinski (1994) in identifying markers which distinguished tilapiine species. The authors provided evidence that RAPD markers were useful for systematic investigation at the level of species and, to a certain extent, for subspecies identification. In this study, we used RAPDs to determine the taxonomic position of the Piketon *Oreochromis* strain in comparison with other

known species of *Oreochromis*. This molecular technique has a big advantage in that it requires only small amounts of DNA which can be extracted from very small samples of fish tissue. This makes it possible to collect tissue samples from distant locations, and preserve and transport them to the laboratory for molecular analysis.

MATERIALS AND METHODS

Collection of fish specimens

Five population groups of known tilapia species were used to compare with the Piketon tilapia strain which was being investigated in the related reproductive and feeding studies. The groups consisted of fish samples of *Oreochromis niloticus* from Lake Albert, Lake Victoria in East Africa and Boston University in the United States; *Tilapia zillii*, also from Lake Albert, and a sample of *O. mossambicus* obtained from Pomona, California. Tissue samples from Africa were collected from fish caught in their respective lakes by nets and preserved in 95% ethanol before the samples were transported to the laboratory at Ohio State University. With the exception of samples of *O. aureus*, whose tissues we could not obtain, the other species represented all species which were suspected to have a possible relationship with the uncharacterized Piketon *Oreochromis* strain.

DNA Extraction

A small piece of muscle tissue (about 1 cm^3) was minced with a fresh razor blade on a glass plate and transferred to an eppendorf tube in 0.5 ml ABI lysis buffer (Applied

Biosystems Inc., 0.1 M Tris. 4M Urea, 0.2M NaCl, 0.01 M CDTA, and 0.5% n-laurelsarcosine). The formed slurry was transferred into a 1.5 ml microfuge tube. A fresh blade was used for each sample and the glass plate was cleaned with soap and water and, wiped with alcohol between samples. A solution of 25 µl of proteinase K (20mg/ml) was added to the tube to degrade the proteins. Samples were incubated overnight at 45-50°C. The following day the samples were divided equally into two 1.5 ml eppendorf tubes. DNA was extracted by two phenol/chloroform series followed by one chloroform extraction as follows: 250 ul of Tris saturated phenol, pH 8.0 or higher and 250 ul chloroform/isoamyl alcohol were added to each tube for the first two extractions, followed by 500 ul of chloroform/isomyl alcohol (24:1). The extraction was performed until the solid phase no longer formed. Chloroform was added during the first extraction because of the high concentration of salt in the ABI lysis buffer and because it facilitated the separation of the layers.

Samples were mixed by inversion for two minutes and then spun in a microcentrifuge for five minutes after each step resulting in two layers separated by a solid phase. The top layer was removed and put in a fresh tube. DNA was precipitated by adding 2 volumes (1 ml) of 95% ethanol, followed by a 70% ethanol wash. Excess ethanol was removed and precipitated DNA in the form of pellets left to dry overnight. The pellets were resuspended in 250-400 ul TE, pH 7.5 and incubated at 65°C for two hours. DNA was quantified by spectrophotometry and quantifications were confirmed by electrophoresis on a 1% agarose gel. The genomic DNA stock concentration of each sample was brought to 25g/ul with TE buffer for analysis by the RAPD's method.

Primer codes	Sequences (5'---> 3')
OPM 02	ACAACGCCTC
OPM 03	GGGGGATGAG
OPM 07	CCGTGACTCA
OPM 11	GTCCACTGTG
OPM 12	GGGACGTTGG
OPM 13	GGTGGTCAAG
OPM 15	GACCTACCAC
OPM 19	CCTTCAGGCA

Table 3.1: Sequence and operon codes of the RAPD primers used in the comparison study of tilapia species.

RAPD Reaction

Preliminary screening of a subset of the DNA samples used in this study with the Operon RAPD primers of the M set showed that most primers of the twenty in the set, were potentially useful, producing 8-10 bands which were polymorphic in the series of test samples. For the purposes of the experiment, a total of eight primers were identified based on the number of bands generated and level of variation revealed. The primers used to screen the DNA samples were used to perform duplicate amplifications in the series of samples from the experiments (Table 1).

RAPD reaction conditions were established and optimized in preliminary studies and subsequently strictly controlled in all the experiments that followed, since the generation of RAPD bands is very sensitive to reaction conditions. Reactions were carried out in duplicate to ensure reproducibility. Eppendorf tubes (0.75ml), tips and distilled water were autoclaved for 20 minutes and exposed to ultra-violet light for 15 minutes before use. 10X RAPD buffer was also sterilized in UV light to minimize contamination. A total of thirty one reaction tubes were set up in one set of experiment. These reactions represented 10 reactions for tilapia samples from Piketon, five for *T. zillii* from L. Albert, five for *O. niloticus* from L. Albert, five for *O. niloticus* from L. Victoria, three for *O. mossambicus* and two for *O. niloticus* from Boston. One tube was set up as the negative control. An aliquot of 2 µl (50ng) of each genomic DNA sample was placed in labeled tubes, excepting the last tube for the negative control. Tubes were closed and placed on ice. An adequate amount of a stock solution containing the remaining reaction reagents was prepared and added to the DNA template in each of the reaction tubes, using a

separate tip for each reaction tube. Each reaction tube contained 2.5 µl 10X RAPD buffer (100mM Tris-Cl, pH 8.8. 500mM KCl, and 15mM $MgCl_2$), 2.5 µl dNTP's (1.25 mM final concentration of each of the four nucleotides), 3.0 µl of a specific RAPD primer, 0.1 µl (1 Unit) *Taq* Polymerase (Sigma Chemical Co.) and 14.9µl dH_2O (HPLC Grade). *Taq* polymerase was added last to the stock on ice. Each of the above reagent volumes was multiplied by thirty-two reactions, mixed thoroughly and dispensed 23.0 µl into each tube. The negative control tube got 25.0 µl of the stock solution. This brought the final volume to 25.0 µl. The template and the stock solutions were mixed well followed by an addition of two drops of sterile mineral oil to each tube. The lids of the tubes were tightly closed before they were placed into the PCR machine wells to start the reactions.

RAPD PCR Reactions

The following cycle conditions were used to amplify the RAPD bands in the thermal cycler:

Step 1 : 30 seconds at 94°C,

Step 2 : 1 minute at 35°C

Step 3 : 2 minutes at 72°C,

Number of cycles : 45,

Soak cycles : 5 minutes at 72°C after the final cycle followed by a step to a 4°C soak.

When the thermal cycler reached 93°C on the first cycle, the PCR was paused for 3 minutes (hot start reaction), then the cycling reaction was started. The PCR reaction took about 4-5 hours to complete. The reaction tubes were kept at 4°C for a few hours until electrophoresis was set up.

Electrophoresis

The products of the RAPD reactions were separated into bands on an Agarose/Synergel gel matrix with two lanes of 123bp standard marker (Sigma) per gel. The gel matrix was made up of Agarose/Synergel mixture, equivalent to 1.6% agarose using 0.5 X TBE buffer. Ethidium bromide staining solution was added to the agarose solution at about 56°C before it was poured gently onto a gel mold (20 cm wide by 21cm long). The mold had already been taped at the open ends and set flat with two sets of combs 15 cm apart. The combs were gently removed from the gel after 13-15 minutes setting and solidifying. The gel was cut into two portions and the two separated to individual electrophoretic tanks which were filled with 0.5X TBE buffer, enough to cover the gels.

To each of the tubes containing the PCR products, 3 µl of "blue juice" (Bromphenol Blue, Xylene Cyanol, and Ficoll) was added and mixed. The ladder (marker) was also prepared by mixing thoroughly 1.0 µl of the marker, 3.0 µl of blue juice and 21.0 µl of 0.5X TBE buffer for each lane in each gel. A total volume of 25.0 µl of each PCR products was loaded into each well. Fifteen samples and two separate lanes of the marker were loaded on each gel. Both gels were electrophoresed at 150 volts for two hours after which the gels were removed and polaroid photographs taken under UV illumination.

Analysis

The bands shown on the photographs were scored relative to the positions of the

standard 123 bp DNA ladder. The bands were matched with the corresponding standard band positions of migration up to the 10th band (123-1230bp), and were scored as "1" if present at that position or 0 if absent for each individual lane. Each band position scored in any amplification for each primer was assumed to represent a single locus. The RAPD patterns of individuals were compared within and between different tilapia groups. Genetic distances between groups were estimated using the computer software RAPDistance programs, version 1.04 for analysis of RAPD pattern (Nei & Li, 1979; Amstrong et al., 1994). The proportion of band sharing and index of similarity between individuals was calculated using the formula (Bardakci and Skibinski, 1994):

$S_{xy} = 2n_{xy}/ (n_x+n_y)$,

where n_{xy} is the number of bands shared by individuals x and y and n_x and n_y are the number of bands scored for each individual (Lynch, 1990). Within group similarity (S) was calculated as the average of S_{xy} across all possible comparisons between individuals within a group. Genetic distance values were used to construct a dendrogram using the neighbor joining method.

RESULTS

The eight RAPD primers generated a total of 127 reproducible RAPD bands. The number of bands generated per primer varied between nine and 22. The average total number of bands observed in each strain sample varied between 26 and 39, with L.Victoria strain giving the lowest and Boston strain giving the highest number of bands. Primer OPM 15 gave the best discrimination between the two genera *Oreochromis* and *Tilapia*.

Primer	Pike	O.n.(V)	O.n.(B)		O.mos	T.zil	Total
OPM 2	2	1	0	0	0	1	4
OPM 3	0	0	4	0	0	2	6
OPM 7	1	2	0	1	0	0	4
OPM 11	1	0	1	1	0	2	5
OPM 12	1	0	1	1	1	0	4
OPM 13	2	0	0	0	0	0	2
OPM 15	0	0	1	0	0	1	2
OPM 19	0	0	0	2	0	5	7
Total	7	3	7	5	1	11	34

Table 3.2: Overal number of bands and unique bands (alleles) at different loci for various tilapia. species analysed by RAPD method.

	Pike	O.n(V)	O.n(A)	O.n(B)	O.mos	T.zil
Piketon	0.81	0.40	0.30	0.37	0.26	0.24
O.n.(V)		0.81	0.31	0.39	0.44	0.24
O.n.(A)			0.62	0.29	0.26	0.29
O.n.(B)				0.82	0.46	0.29
O.mos					0.97	0.28
T.zil(A)						0.74

Table 3.3: Proportion of band sharing among samples of tilapia species characterized by RAPD analysis.

Pike/Piketon : *Oreochromis* strain from Piketon, OSU Aquaculture Center, USA,

O.n(V) : *Oreochromis niloticus* from Lake Victoria, East Africa,

O.n(A) : *Oreochromis niloticus* from Lake Albert, East Africa,

O.n(B) : *Oreochromis niloticus* from Boston University, USA,

O.mos : *Oreochromis mossambicus* from South Africa,

T.zil(A) : *Tilapia zillii* from Lake Albert, East Africa

Primer	Pike	O.n.(V)	O.n.(A)	O.n.(B)	O.mos	T.zil
OPM2	2.8	2.4	2.0	2.0	2.0	3.8
OPM3	4.0	3.0	4.6	6.5	2.3	4.2
OPM7	4.5	5.6	2.6	6.0	5.0	3.6
OPM11	3.6	3.4	3.2	5.0	2.7	4.2
OPM12	7.1	3.0	3.6	2.5	4.0	2.4
OPM13	5.3	3.8	5.6	9.0	5.0	4.8
OPM15	2.1	1.8	2.0	4.0	2.7	1.4
OPM19	4.5	2.6	5.0	4.0	2.0	8.6
Total	33.9	25.6	28.6	39	25.7	33

Table 3.4: Total number of bands per individual tilapia strain per RAPD primer.

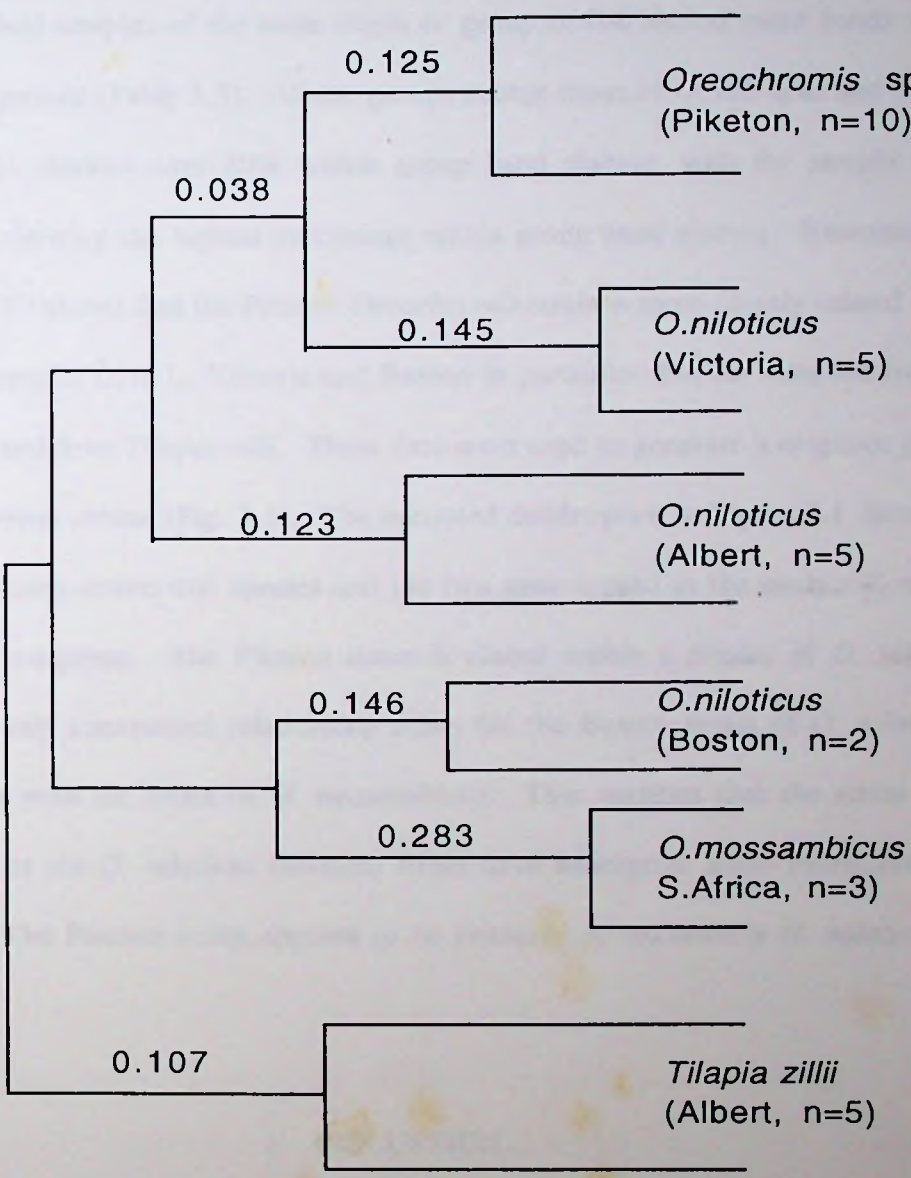


Figure 3.1: A dendrogram of examined strains of tilapia in relation to Piketon *Oreochromis* sp. strain using neighbor-joining method. Numbers indicate genetic distances calculated from data for all RAPD primers.

Tilapia zillii had the highest number of unique alleles followed by *Oreochromis* strain of Piketon and *O. niloticus* of L. Albert (Table 3.2). RAPD primer OPM 19 generated the highest number of unique alleles.

Individual samples of the same origin or group of fish shared more bands within than between groups (Table 3.3). All the groups except those of *O. niloticus* and *T. zillii* from L. Albert showed over 80% within group band sharing, with the sample of *O. mossambicus* showing the highest percentage within group band sharing. Examining the data in Table 3.3 shows that the Piketon *Oreochromis* strain is more closely related to the *O. niloticus* samples from L. Victoria and Boston in particular, but far removed from *O. mossambicus* and from *Tilapia zilli*. These data were used to generate a neighbor joining tree of the various strains (Fig. 3.1). The unrooted dendrogram in Figure 3.1 shows the relationship among strains and species and the two genera used in the study. *T. zillii* is shown as the outgroup. The Piketon strain is placed within a cluster of *O. niloticus* strains. The only unexpected relationship exists for the Boston strain of *O. niloticus,* which clusters with the strain of *O. massambicus*. This suggests that the strain of *O. massambicus* or the *O. niloticus* (Boston) strain have undergone some hybridization in their origin. The Piketon strain appears to be primarily or exclusively *O. noloticus* in origin.

DISCUSSION

Comparison of strains using the RAPD markers, suggests that the strain of *Oreochromis* at Piketon is closer to the Nile tilapia (*O. niloticus*) strain than any other of

the species studied. Because the strain shares many alleles of *O. niloticus*, it would appear that it originated from the *niloticus* group of species.

The problem of taxonomy of the tilapiine species is usually complicated by the fact several species of the genus *Oreochromis* are capable of hybridization. Hybridization among these species in the wild has been found to occur especially in ecologically disturbed habitats and in cases where closely related species have been brought together through introductions and transplants. For instance, hybridization among tilapiine species in Lake Victoria is reported to have occurred between *O. niloticus*, an introduced species and the endemic species of *Oreochromis* such as the *O. esculentus* (Fryer and Iles, 1972; Trewavas, 1983; Ogutu-Ohwayo, 1990). The same situation could occur in the development of domesticated strains of tilapia. In this case, species might be brought together under conditions in which normal species isolating mechanisms would be demphasized, and hybridization could occur. Whether this hybridization was planned by the breeder or accidental would be irrelevant to the outcome, a hybrid strain.

Although our results suggest that the Piketon strain is likely to be a Nile tilapia, since it is placed firmly within the *niloticus* cluster of comparisons, its true identity will be resolved when DNA samples from *O.aureus* are analyzed with DNA from other samples of *Oreochromis* species. Because we did not have all the material from other tilapia species for comparison, it is possible that the strain could have originated as a hybrid between *O. niloticus* and its close relatives such as the blue tilapia (*O. aureus*) or *O. hornorum*, whose contribution in genetic material, in any case is likely to be minor. The two latter species of tilapia occur in the United States and have been found to readily

hybridize with *O. niloticus* (Costa-Pierce and Rakocy, 1997). The morphology of the Piketon strain closely resembles the description of the blue tilapia especially in coloration and sexual dimorphism (Trewavas, 1983). Trewavas (1983) describes the blue tilapia as having vermilion or orange upper margin of the dorsal and a broad pink to red margin of the caudal fin, the rest of which is without markings or with irregular spots or a dark reticulum with light interstices over part or nearly the entire fin as the breeding colors for males of blue tilapia. On the other hand, the Nile tilapia is characteristically distinguished by the regular dark vertical stripes on the caudal fin for both male and female fish. The male does not develop any color in the dorsal fin during breeding. The only visible difference between males and females of the same age in *O. niloticus* during breeding is size; males tend to be larger than females. It is probable that the Piketon strain which fits the above description could be of hybrid origin between Nile tilapia and blue tilapia. This would support the belief that *O. aureus* was originally distributed from Egypt to America under the name of Nile tilapia and most of the supposed 'niloticus' in America have been proven to be the 'blue' tilapia, though not yet scientifically verified. It is also known that more recent exports in the Middle East have been from ponds where *O. niloticus* have been hybridized with *O. aureus* (Mires, 1977).

The study has shown that genetic markers can be of great value and relevant to the management of cultured fish. The genetic identification and discrimination of aquaculture stocks is clearly a fundamental requirement in any culture program. For instance, genetic markers could be used to detect varying degrees of similarities among stocks and guide fish breeders to minimize the deleterious effects of inbreeding (Gjedrem, 1992) by crossing

fish from genetically different strains. Further, informed decisions on donor strains can be made only with knowledge of genetic similarity because strains differ to varying degrees and, therefore are not equally suitable for crossbreeding. Such information is especially required when detailed pedigree information is lacking (Ferguson et al., 1993).

CHAPTER 4

GROWTH, FEED UTILIZATION AND BODY COMPOSITION OF TILAPIA, *OREOCHROMIS* SP. FED COTTONSEED MEAL-BASED DIETS IN A RECIRCULATING SYSTEM

INTRODUCTION

Fish diet is normally the single largest expenditure in semi-intensive and intensive fish culture operations. This is due to extensive reliance on marine animal protein sources such as fish meal, shrimp meal and squid meal to meet the high dietary protein requirements for fish. These feedstuffs have high nutritional value and palatability but are expensive and not always readily available (Lim and Dominay, 1990). Rumsey (1994) noted that the world output by major fish meal producing countries actually declined. The world fish meal production was predicted to decline by about 5% between 1990 and year 2000 (Rumsey, 1993, 1994). Therefore, in order to develop economical aquaculture systems, alternate sources of high quality proteins will have to be identified to replace high-cost fish meal. Currently a great deal of research is going on to address the use of plant protein concentrate feeds for fish.

Several sources of plant proteins to partially or completely replace fish meal have been experimented on varous finfish. The plant proteins examined include soybean meal (Quartararo et al., 1998), cacoa husks (Pouomogne et al., 1997), various cereals (Al-Ogaily et al., 1996), brewery draff (Pouomogne et al., 1992), napier grass (Chikafumbwa,

1996) and cottonseed meal (Robinson et al., 1984; El-sayed, 1990). For tilapia diets,typical plant protein alternatives have included soybean meal (Brandt, 1979; Jackson et al., 1982), and sunflower seed meal (Jackson et al., 1982) among others. Results ranged from high to poor performance in terms of growth and survival of tilapia species.

Cottonseed meal is widely used as a protein supplement for ruminant livestock because of its high protein content and ample availability. Cottonseed meal which ranks third in the world in tonnage among the vegetable protein concentrates produced(Swick and Tan, 1995), and is available at a much lower cost than animal proteins. Furthermore, cottonseed is a by-product of cotton, a cash crop widely grown in the tropics where extensive tilapia culture is practiced. The nutritional value of cottonseed has been evaluated for several species of fish such as chinook and coho salmon (Fowler, 1980), tilapia (Jackson et al., 1984; Viola and Zohar, 1984; El-Sayed, 1990), and channel catfish (Robinson,1991; Robinson and Li, 1994; Robinson and Tiersch, 1995). However, the use of cottonseed meal in fish diets is limited by the presence of gossypol, a toxic component for monogastric animals and by its low content of lysine, an essential amino acid. Generally, it has been reported that the amount of cottonseed meal that can be included in feeds depends on the fish species, and levels of free gossypol, dietary protein and available lysine (Martin, 1990).

The researchers mentioned above did not agree about the effects of feeding cottonseed meal to tilapia. Robinson et al. (1984a) have reported that tilapia can tolerate gossypol and so respond favorably to diets based on cottonseed meal as a major source of protein. Other investigators have found that the growth of tilapia is indeed adversely affected by cottonseed meal as an ingredient in the feeds. Jackson et al. (1982) obtained good growth of tilapia (*Sarotherodon mossambicus*) when 35.2% prepressed, solvent-extracted cottonseed meal (0.03% free gossypol) was used as a substitute for 50% of fish meal protein. The growth rate was essentially the same as in the control group at 100%

substitution level. Likewise, El-Sayed (1990) demonstrated that cottonseed meal could be used as a main dietary protein source for *O. niloticus*. Viola and Zohar (1984) found that low-gossypol cottonseed meal could be included in feeds for hybrid tilapia *O. niloticus* x *O. aureus* to the same extent as soybean meal. In contrast, Robinson et al. (1984a) reported that neither glanded nor glandless cottonseed meals appeared to be as high in nutritive value for *Tilapia aurea* as soybean and peanut meals. Ofojekwu and Ejike (1984) also reported a much lower weight gain and feed efficiency of *O. niloticus* fed a cottonseed cake diet as compared to tilapia fed the fish meal control diet.

The present study was conducted in view of the above arguments and to evaluate the effect of inclusion of graded levels of cottonseed meal has on growth, feeding efficiency, protein digestibility, concentration of gossypol in fish liver and survival of the tilapia strain being raised in Ohio. Feed digestibility was studied by incorporating chromic oxide in the feeds as a marker, and feed intake was estimated by incorporating glass beads in the dietary treatments and x-raying the fish soon after feeding. At the end of the experiment, proximate analysis was conducted on the fish body to estimate their chemical composition. The study was unique in that the fish investigated were sired by males that had been heat-treated at an early stage of larval development. Experiments were set up to test the hypothesis that fish fed a high protein cottonseed-based diet would perform as well as those given a fish meal-based diet in a recirculating system.

MATERIALS AND METHODS

Growth and Feed Utilization

Individuals of tilapia juveniles (ranging from 13 to 16 in number and from 10.2 to 13.4g in average weight per fish) were randomly distributed among 15 glass aquaria (32 L capacity each) and water temperature maintained at 27±1°C. Each tank was well aerated.

Ingredients	Diet No. 1 (0% Cot)	2 (25% Cot)	3 (50%Cot)	4 (75% Cot)	5 (100%Cot)
FM menhaden	20.00	15.00	10.00	5.00	0.00
FM herring	20.00	15.00	10.00	5.00	0.00
Cottonseed Meal	0.00	14.70	29.42	44.11	58.80
Krill hydrolysates	5.00	5.00	5.00	5.00	5.00
Wheat middlings	28.00	21.20	14.40	7.50	0.60
Corn gluten meal	11.60	12.60	13.60	14.60	15.60
Yeast	6.00	6.00	6.00	6.00	6.00
Methionine	0.00	0.10	0.20	0.30	0.40
Lysine	0.00	0.20	0.40	0.60	0.80
Vitamin Mixture[a]	0.50	0.50	0.50	0.50	0.50
Mineral Mixture[a]	0.50	0.50	0.50	0.50	0.50
Vitamin C-MP[b]	0.05	0.05	0.05	0.05	0.05
Choline	0.10	0.10	0.10	0.10	0.10
Cod Oil	8.00	8.90	9.80	10.70	11.60
Cellulose	0.25	0.15	0.03	0.04	0.05
Total	100	100	100	100	100
Proximate analysis					
Protein	40.94	43.06	42.88	42.44	42.94
Lipid	14.38	14.48	14.59	14.69	14.79
Methionine	1.18	1.17	1.17	1.16	1.16
Lysine	2.78	2.71	2.63	2.55	2.48
Energy (Kcal/kg)	4180	4177	4176	4170	4163
Analyzed gossypol (%)					
Free	0.000	0.026	0.052	0.079	0.099
Total	0.000	0.112	0.251	0.351	0.439

[a] Dabrowski *et al.* (1995)
[b] Mg-L-ascorbyl-2-phosphate (Showa Denko K.K., Tokyo, Japan)

Table 4.1: Diet composition (%) for tilapia feeding experiments (FM= fish meal).

The water was constantly replaced by continuous flow at a rate of 1L/hour to provide oxygen and remove excess nitrogenous wastes.

Five diets, each containing a different proportion of cottonseed meal (Table 4.1) were prepared for feeding the fish and stored at -10°C. Each diet was assigned randomly to three tanks, i.e. three replicates per diet. The fish were fed diets for two weeks to acclimate them to the feed before taking growth parameters periodically at an interval of two weeks. Initially the fish were fed their respective diets at a rate of 3% of their body weights per day, and later diminished to 1.5% towards the end of the experiment. An equal amount of diets was given 3 times a day at 11:00, 14:00 and 17:00 hours for 13 days and the fish were then starved for 24 hours before recording their weights. Feeding was then recommenced immediately after weighing. Each fish in each tank was weighed and the average weight gain per tank calculated. The feeding experiments were conducted for 16 weeks (114 days) from May 21 to September 11, 1998.

Estimation of Feed Intake

The experiment was carried out to estimate feed intake at the end of the growth study using radiographic methods (Jobling et al., 1989; Arnesen et al., 1993).

Diet Preparation

About 100 g of dry feed pellets of each reference diet were ground in a mortar into powder to which 4% (w/w) of glass beads (0.4 - 0.6mm diam.) were added as a marker and mixed thoroughly. Enough cold water was added to the mixture and stirred thoroughly to form a thick paste. This was then re-pelletted by extruding the paste through a mincer, resulting in "spaghetti-like" strings. They were placed on aluminium foil and freeze-dried. The feeds were later broken up and sieved into convenient pellet sizes for the fish and stored at -20°C until used.

Fish feeding and X-raying

Before the fish were given the diet containing the marker, they were starved for 24 hours to allow evacuation of most of the gut contents. The fish were then fed their respective diets labelled with glass beads (supplied by D.F. Houlihan of the University of Aberdeen, Scotland, UK) until satiation (within about 15 minutes) early in the morning. Two hours after feeding, four fish were randomly picked out of each tank. Each fish was marked for later identification by fin clipping. Each group of fish from each tank was anesthetized with MS-222 (Tricaine Methanesulfonate) at the concentration of 100 mg/L. They were laid out one by one on a flat plate for x-raying. Radiographic images were obtained using an x-ray machine (Picker VTX 1050, Picker International, Cleveland, Ohio) operating at 44 kVp, 250 mA, 1.9 mAS; and a high detail radiographic film and screen combination (ultra-vision L film/ultra-vision detail screens, Sterling Diagnostic Imaging, Inc., Newark, DE). The fish were returned to their original buckets where they recovered from anesthesia within 5 minutes. They were weighed and returned to their respective tanks. The x-raying was carried out at the Ohio State University Veterinary Hospital, Radiology Department. The exercise took two days.

Exposed X-ray plates were developed and the numbers of glass beads revealed in the gut of individual fish counted. A standard curve describing the relationship between the amount of labelled feed (x) and the number of glass beads (y) was prepared by x-raying weighed samples of labelled food. The number of glass beads in the gut of each fish was converted into grams of feed by the following formula:

$y = 271.20x - 8.40$, ($r^2 = 0.965$, $n = 20$).

The results were reported as the mean ± standard deviation. The mean values were compared using Student's t-test, and the differences at $p < 0.05$ were considered significant.

Feed Digestibility

Digestibility experiments commenced at 11th week of the feeding study. The fish were starved for 24 hours before feeding them. Early each morning, for three days, the fish were fed their normal respective diets to satiation. Each diet contained 0.5% chromic oxide marker which gave a green appearance to the feed pellets. Two hours after feeding, the excess feed was siphoned off and the fish transferred to circular tanks with a steep conical bottom, an apparatus designed to collect fish feces. Three conical tanks each holding about 50 L of water were used. Water maintained at 28°C continually flowed in at the top and out near the bottom of the tanks at a rate of 1L/min. Opening the tap at the end of the tank, allowed feces to sink and settle through a 5-cm drain into a 2-cm diameter, 10-cm long, settlement chamber which tapered into a 2-cm diameter, 8-cm length of pvc-tubing. The tubing into which the feces settled was packed in ice and kept at less than 4°C to reduce bacterial action. The feces were collected three times a day at an interval of three hours (over 9 hours) by detaching the collecting tube. Fish were prevented from stirring the feces by a circular net mesh placed at the base of the tank. Any feces held on the walls of the tank were siphoned in a container and filtered together with the feces obtained in the collecting tubes to remove excess water, transferred into petri dishes and stored at -20°C. Collection of feces took 14 days to complete.

Dietary and fecal samples were freeze-dried and analyzed in triplicate for total nitrogen and other elements at the Research-Extension Analytical Laboratory, Wooster. Chromic oxide was analysed using the method described by Scott (1978) in the same Laboratory. The fecal matter was digested with perchloric acid. The apparent digestibility (AD) of a given nutrient was calculated using the following formula (Spyridakis et al.,1989):

AD (%)= 100 - {100 (% Cr_2O_3 diet/% Cr_2O_3 feces)(% nutrient feces/% nutrient diet)}.

One way Anova was performed after transformation of the data into arcsin square root in order to demonstrate the significance of differences among treatments. Means were compared with the Newman-Keuls multiple range test (Sokal and Rohlf, 1981) at the 0.05 probability level.

Analysis of Gossypol in Liver

Gossypol concentration in liver tissue was determined according to the method described by Kim and Calhoun (1995) with some modifications. Wet liver tissue was used for (+)- and (-)-isomers of gossypol, because the detection of gossypol in freeze-dried liver sample was lower than in wet sample from the preliminary analysis which was done to compare the two processing methods. The wet liver was weighed, and 10 volumes of complexing reagent was added to obtain the 2-amino-1-propanol derivatives of (+)- and (-) isomers of gossypol. The complexing reagent consisted of 2% 2-amino-1-propanol and 10% glacial acetic acid in N, N-dimethylformamide. The liver sample in complexing reagent was homogenized on ice for 10 sec, heated at 95^{0}C for 30 min, cooled on ice, and then centrifuged at 5500 rpm for 5 min. After centrifugation, the aliquot of the supernatant was taken, diluted with mobile phase to make desirable concentrations, and centrifuged again to prevent the syringe filter (0.45μm) from clogging, and then the supernatant was filtered with the syringe filter before injection to HPLC. The sample volume used for injection was 20 μl. The HPLC system consisted of Beckman 506A with a 4.6 mm × 150 mm C-18 reverse phase column (Shodex, Japan) packed with octadecyl-bonded totally porous silica gel (5 μm), and an UV detector (Programmable detector module 166, Beckman, USA). The mobile phase was made of 80% acetonitrile and 20% 10mM KH_2PO_4 adjusted to pH 3.0 with H_3PO_4. The retention time for (+)- and (-)-isomer of gossypol were 2.1 and 3.4 min, respectively, with flow rate of 1.8 ml/min at 254 nm. The

standard gossypol (+)- and (-)-isomers were provided by Dr. Cass from Brazil. Recovery rate for extraction of gossypol enantiomers in liver was 92.4%, and detection level was 1.0 ng gossypol / 20 µl of injection volume with a S/N of 3.

Chemical Body Composition of Fish

Three fish samples of the sacrificed fish per tank (i.e.nine per diet) were randomly selected, freeze-dried and ground into powder for proximate chemical analysis at the Ohio State University Research-Extension Analytical Laboratory, Wooster. The minerals were determined in the whole-body dry matter. Samples were prepared for analysis using a dry-wet ashing procedure (AOAC, 1984). Samples in dry porcelain crucibles were dried in a freeze-dryer and later placed in an ashing oven at 600°C for two hours, removed and allowed to cool. Samples were moistened with distilled water, then nitric acid (9 N), and gently heated on an electric hotplate to evaporate the liqud until only residual moisture remained. Ashed samples were solubilized in 6 N HCl and brought to volume with distilled water to obtain a ratio of 1:5 acid:water. Appropriate dilutions were prepared to bring mineral concentrations within standard reading ranges. Ionic composition of fish whole-body ash was measured by the inductively coupled plasma (ICP) emission spectrophotometry method with the use of ARI-3560 Spectrometer (Applied Research Labs, Valencie, CA) according to Watson and Issac (1990).

RESULTS

Growth and Feed Utilization

Tilapia growth did not differ ($p > 0.05$) with up to 50% substitution with cottonseed meal in the diet given over a period of 16 weeks (Fig. 4.1a). Above 50% fish meal replacement, growth response started to decline with time after the 12th week so that by the end of the feeding trial period, there was hardly any noticeable weight gain. Fish

which had 100% cottonseed meal in their diet had the lowest average weight gain, less than half of the weight gained by fish given diets 1 to 3 (Figure 4.1b).

Growth rates, feed conversion ratio (FCR) and feed efficiency ratio (FE) for tilapia varied depending on diet given (Table 4.2). Fish fed the control diet (100% fish meal) and diets including 50% cottonseed meal had significantly better daily weight gain, daily feed intake, SGR and feed efficiency ratio ($P < 0.05$) than those fed 100% cottonseed meal. Furthermore, fish fed 100% cottonseed meal experienced some low levels of mortality (Table 4.2). Although fish fed diets 2 and 3 had higher daily weight gains than the control (diet 1), the differences in weight gain were not significant ($P > 0.05$).

Gossypol levels increased with higher proportions of cottonseed meal inclusion in the diets (Table 4.1).

Feed Intake

Figure 4.2 shows radiographic picture of two examples of x-rays used to calculate the number of ingested glass beads by the fish. The feed intake values were calculated from a calibration curve showing the relationship between the number of glass beads and feed pellet weights (Fig. 4.3a). The average feed intake by the fish given varying levels of cottonseed meal-based diets is shown in Figure 4.3b. Fish fed diet 1 consumed more compared to fish of the rest of the diets, least being fish fed diets 4 and 5. Fish fed diet 3 consumed more than those fed diet 2, however, the difference was not significant ($P > 0.05$). It was also observed that diets 4 and 5 were frequently spitted after tasting.

Digestibility of Feeds

Apparent digestibility of protein and other nutrients are presented in Figure 4.4 and Table 4.3. Apparent digestibility of N-protein was high (above 80%) and at about the same level for diets 1 to 4 while it was significantly lower in diet 5 (100% cottonseed meal).

Mean Values	Diet				
	1	2	3	4	5
W_i	15.4	16.8	16.1	14.1	14.2
W_f	77.5[a]	84.1[a]	82.5[a]	55.4[b]	42.1[c]
Wt.Gain (g/day)	0.54[a]	0.59[a]	0.58[a]	0.36[b]	0.25[c]
Feed intake (g/day)	8.6[a]	8.6[a]	8.6[a]	7.4[a]	5.7[b]
SGR[1]	1.4	1.4	1.4	1.2	1.0
FCR[2]	1.3[a]	1.3[a]	1.3[a]	1.7[b]	1.9[b]
FE[3]	403[a]	397[a]	412[a]	294[b]	201[c]
Survival	100	100	100	100	89

Table 4.2: Performance of tilapia species fed diets containing graded levels of cottonseed meal. Values in the same row with different superscripts a, b or c are significantly different at P = 0.05.

W_i = initial body weight (g/fish)
W_f = final body weight (g/fish)
[1]Specific growth rate (%)
[2]Feed conversion ratio (g dry diet fed/ g fish weight gain)
[3]Feed efficiency ratio {weight gain (g)/initial weight (g)}100.

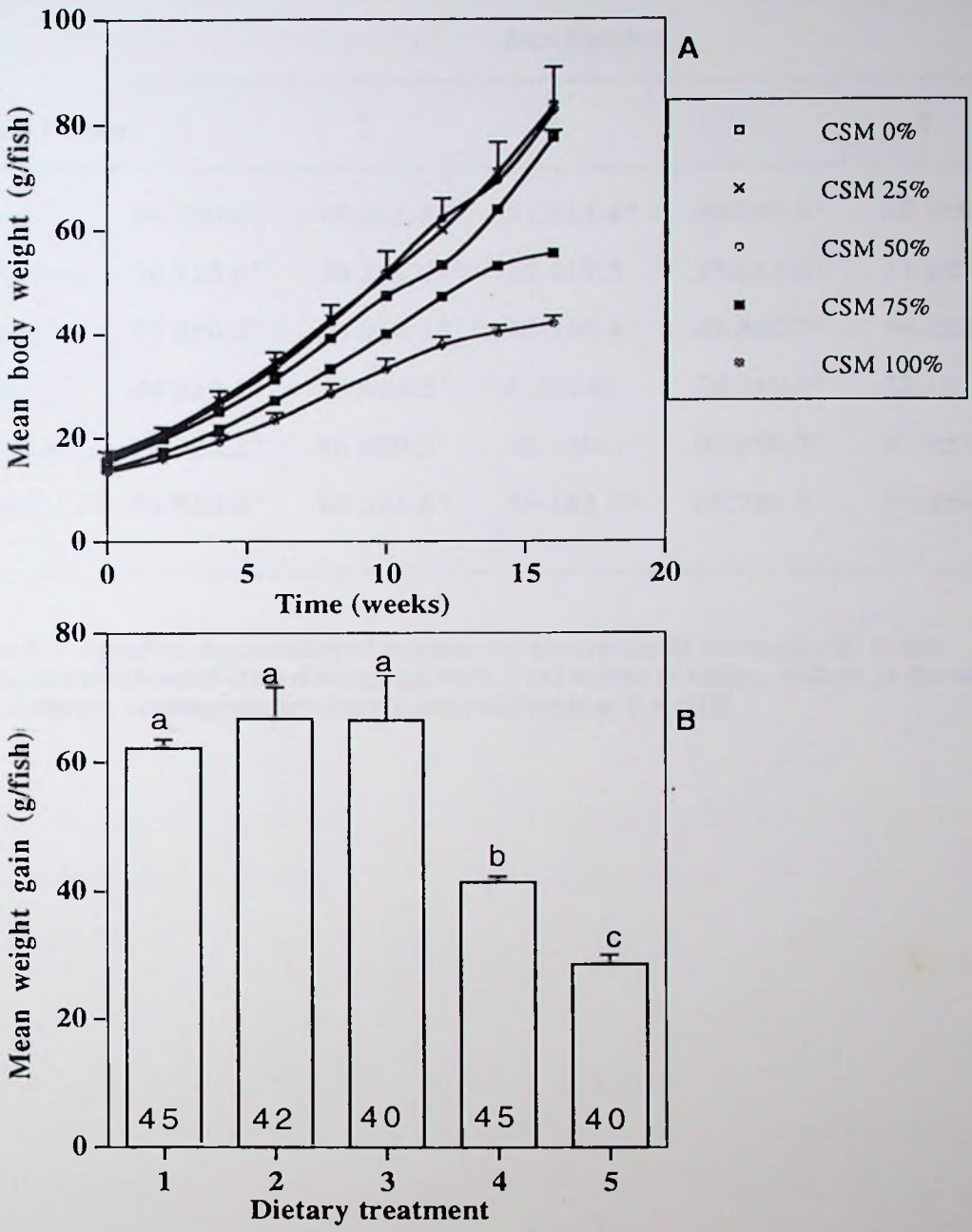


Figure 4.1: Growth (A) and weight gain (B) of tilapia after 16 weeks of feeding graded levels of cottonseed meal. Fish sample size is shown on each histogram. Different letters indicate the means are significantly different from each other ($p < 0.05$).

Dietary Nutrient	Diet Number 1	2	3	4	5
Protein	88.7±0.8[a]	86.6±1.8[a]	81.3±1.4[a]	82.2±0.8[a]	69.8±0.6[b]
Phosphorus	36.7±0.6[a]	30.2±13.3[a]	22.4±7.5	27.6±1.6[b]	11.2±5.4[c]
Magnesium	61.9±0.2[a]	53.9±8.1[a]	43.7±6.4[b]	49.8±2.7[b]	44.0±8.7[b]
Copper	24.8±9.1[a]	10.6±9.5[b]	6.2±3.0[c]	28.1±9.9[a]	33.1±1.9[a]
Potassium	98.1±0.2[a]	96.8±0.5[a]	95.1±0.6[a]	95.1±0.5[a]	93.9±1.7[a]
Sodium	93.9±0.4[a]	86.2±1.6[a]	86.1±3.6[a]	88.7±1.8[a]	86.9±4.5[a]

Table 4.3: Apparent digestibility of protein and absorption of minerals (%) in dry cottonseed meal-based diets determined from fecal matter of tilapia. Values in the same row with different superscripts are significantly different at P = 0.05.

The water was constantly replaced by continuous flow at a rate of 1L/hour to provide oxygen and remove excess nitrogenous wastes.

Five diets, each containing a different proportion of cottonseed meal (Table 4.1) were prepared for feeding the fish and stored at -10°C. Each diet was assigned randomly to three tanks, i.e. three replicates per diet. The fish were fed diets for two weeks to acclimate them to the feed before taking growth parameters periodically at an interval of two weeks. Initially the fish were fed their respective diets at a rate of 3% of their body weights per day, and later diminished to 1.5% towards the end of the experiment. An equal amount of diets was given 3 times a day at 11:00, 14:00 and 17:00 hours for 13 days and the fish were then starved for 24 hours before recording their weights. Feeding was then recommenced immediately after weighing. Each fish in each tank was weighed and the average weight gain per tank calculated. The feeding experiments were conducted for 16 weeks (114 days) from May 21 to September 11, 1998.

Estimation of Feed Intake

The experiment was carried out to estimate feed intake at the end of the growth study using radiographic methods (Jobling et al., 1989; Arnesen et al., 1993).

Diet Preparation

About 100 g of dry feed pellets of each reference diet were ground in a mortar into powder to which 4% (w/w) of glass beads (0.4 - 0.6mm diam.) were added as a marker and mixed thoroughly. Enough cold water was added to the mixture and stirred thoroughly to form a thick paste. This was then re-pelletted by extruding the paste through a mincer, resulting in "spaghetti-like" strings. They were placed on aluminium foil and freeze-dried. The feeds were later broken up and sieved into convenient pellet sizes for the fish and stored at -20°C until used.

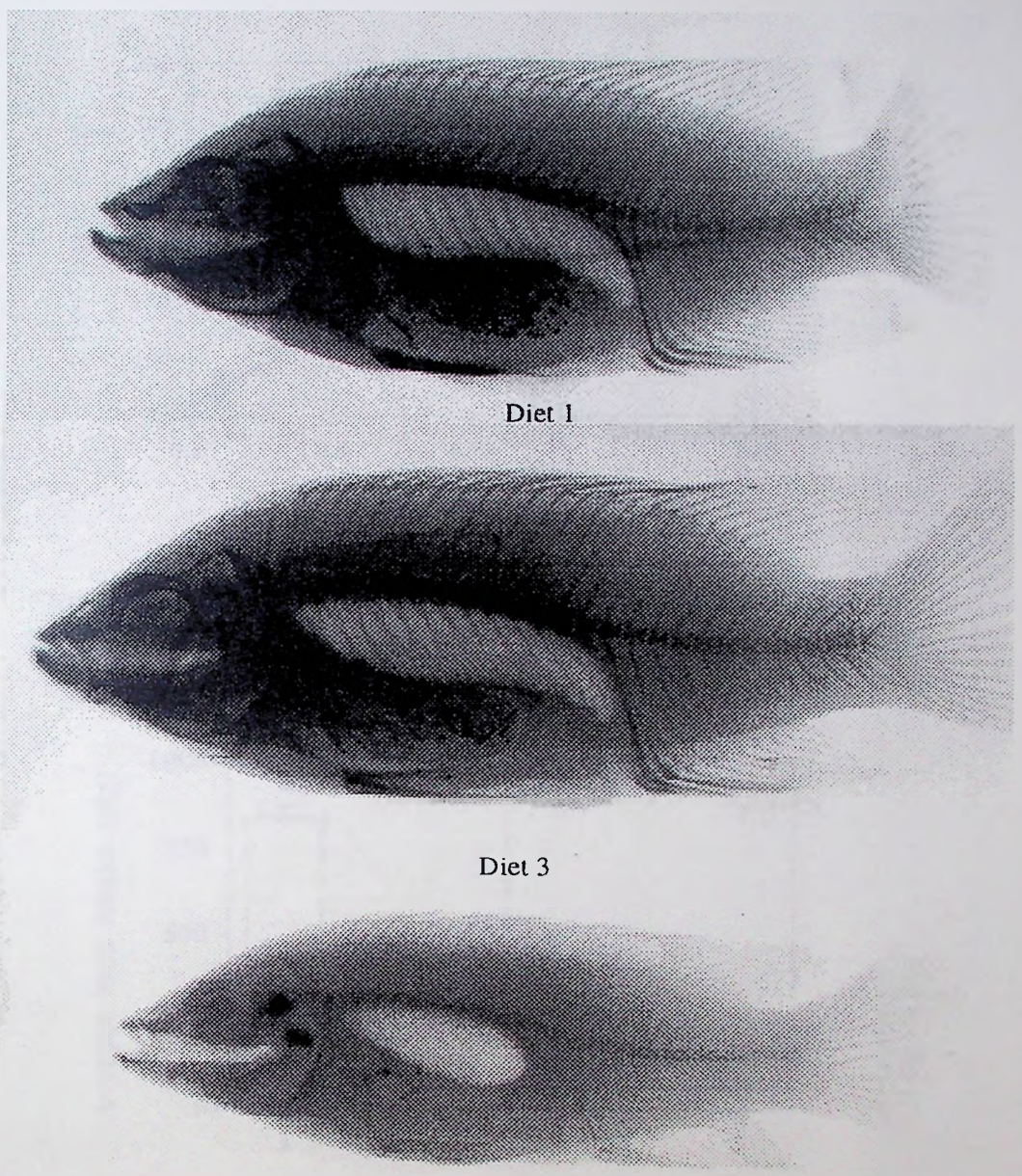


Figure 4.2: Radiographic pictures showing feed intake of diets 1, 3 and 5 indicated by the amount of glass beads in the gut.

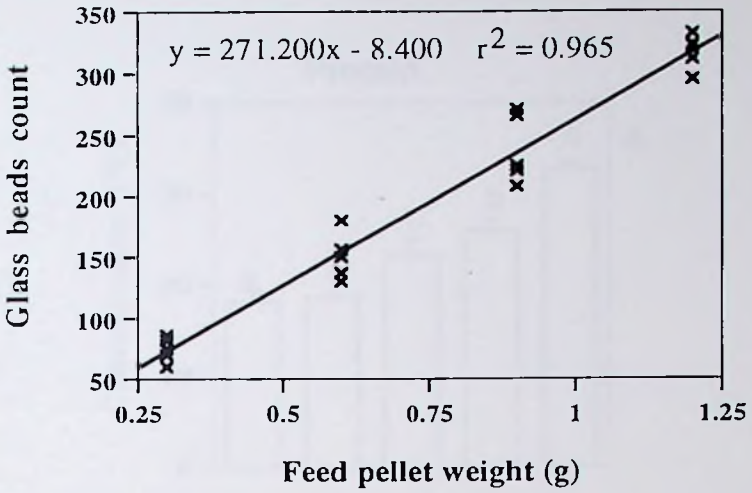


Figure 4.3a: Calibration curve showing the relationship between number of glass beads and feed pellet weight.

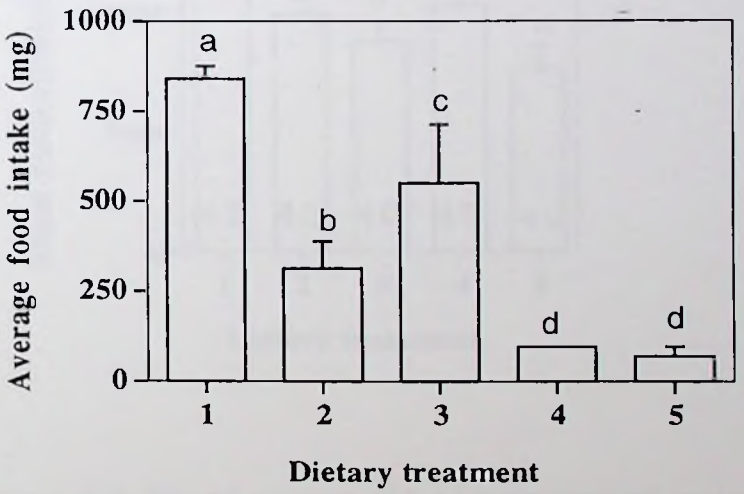


Figure 4.3b: Mean food intake by tilapia (calculated from the glass beads counted in their guts, N=12 fish/diet). Different letters indicate the means are significantly different from each other ($p < 0.05$).

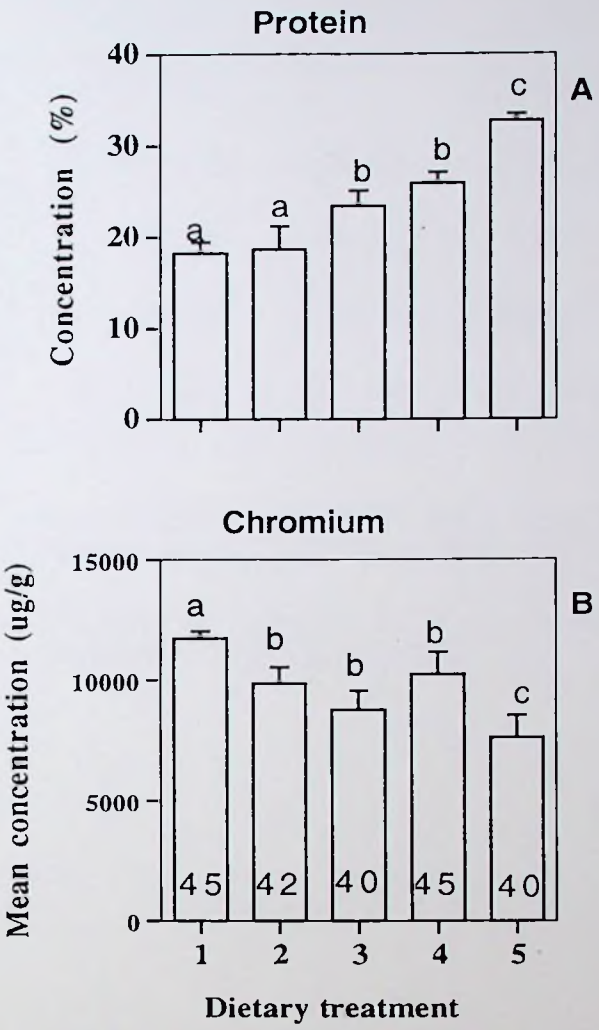


Figure 4.4: Fecal protein (A) and chromium concentration (B) determined from fecal matter after feeding tilapia diets1 to 5. Sample size of the fish used is shown on each histogram. Different letters indicate the means are significantly different from each other ($p < 0.05$).

Diets	Gossypol			(-)-isomer/total ratio
	(+)-isomer	(-)-isomer	Total	
Control (1)	1.32±0.64[e]	-	1.32±0.64[e]	0
CSM25 (2)	22.90±5.98[d]	9.39±4.33[d]	32.29±10.3[d]	28.3±4.00
CSM50 (3)	51.3±8.31[c]	21.00±3.36[c]	72.30±11.6[c]	29.1±0.90
CSM75 (4)	72.3±5.80[b]	27.1±1.95[b]	99.4±5..93[b]	27.3±2.17
CSM100 (5)	95.9±14.2[a]	36.2±1.51[a]	132.1±15.7[a]	27.6±2.16

Table 4.4. Average gossypol concentration (ppm) in liver of tilapia fed five experimental diets having different levels of cottonseed meal for 16 weeks [1].

[1] Means of six individual fish; Values ± Std. dev. in the same column with different superscript are significantly different ($P < 0.05$).

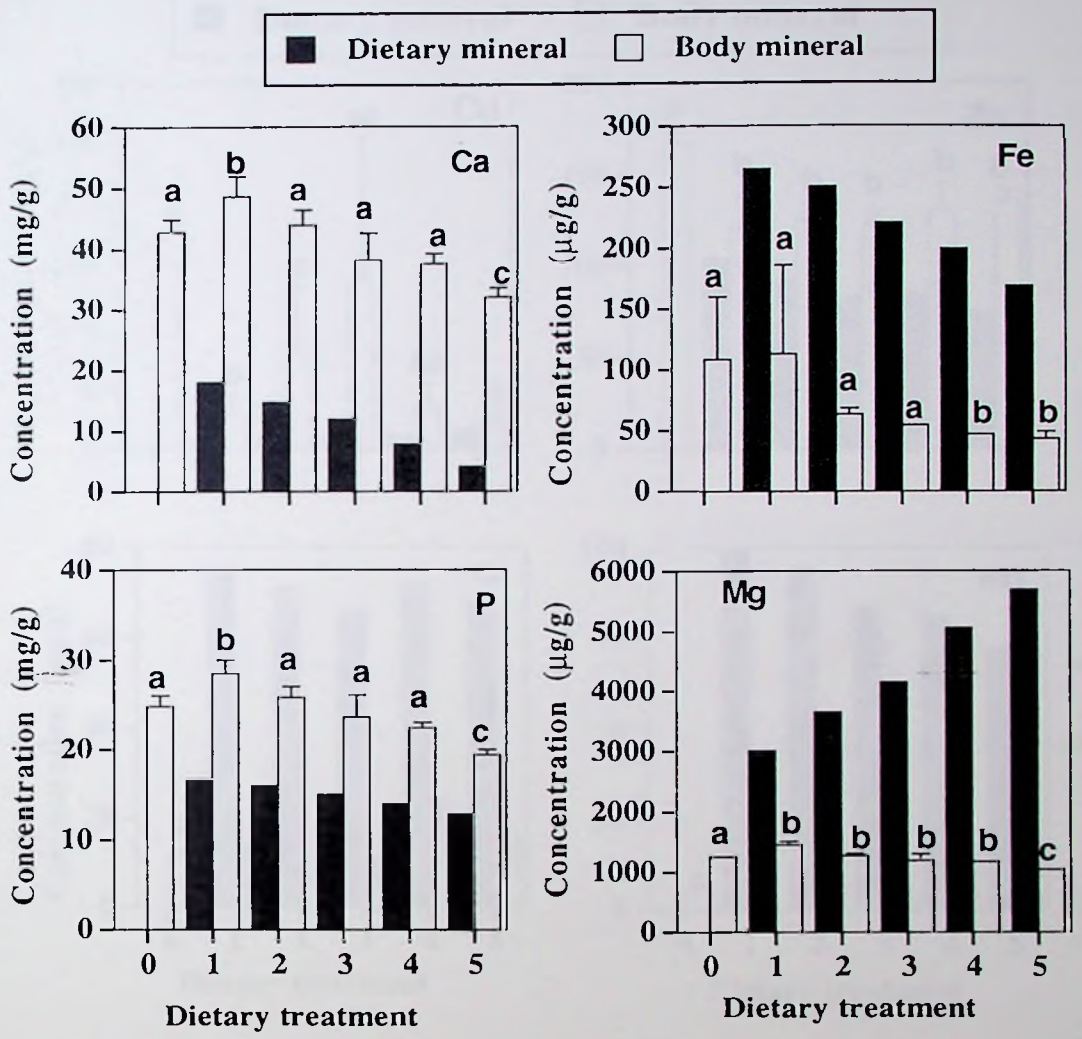
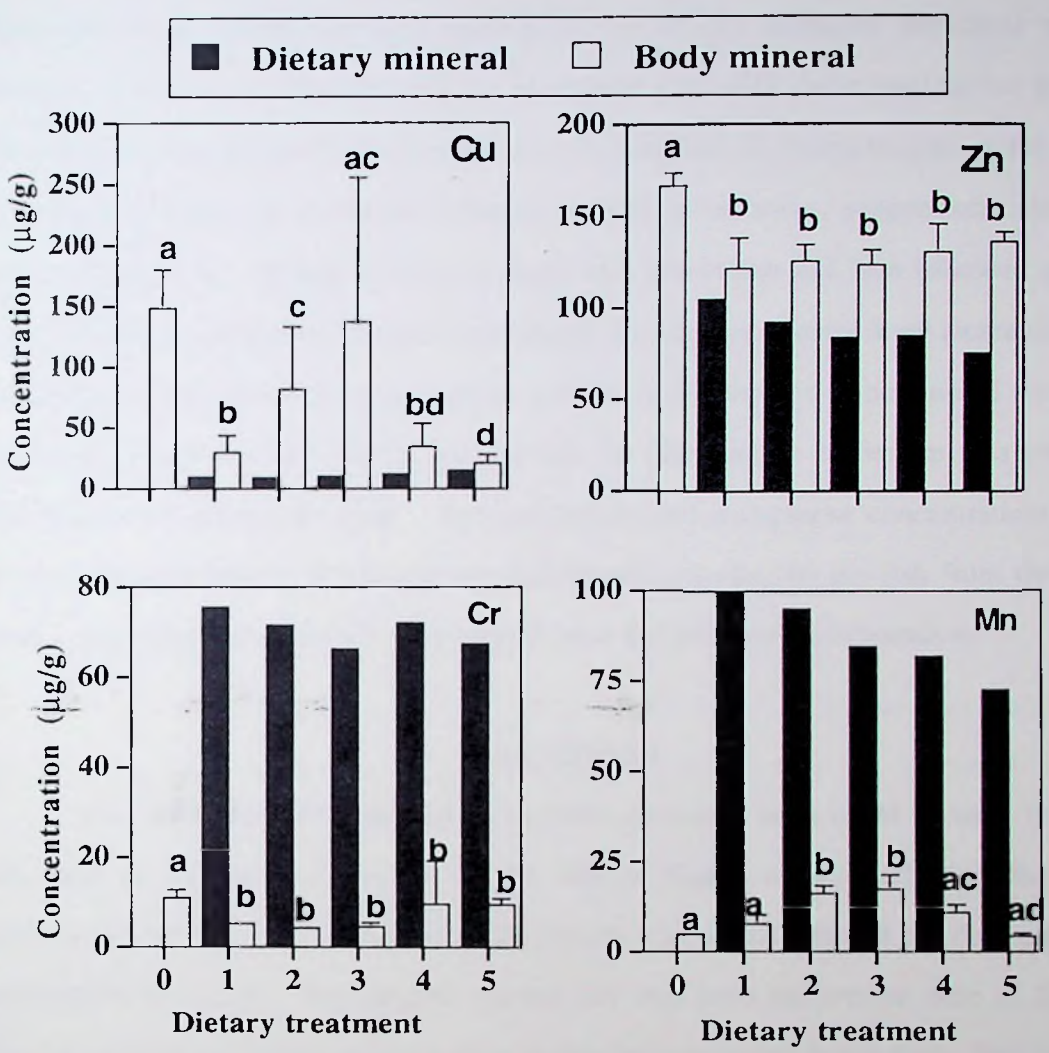


Figure 4.5: Mineral concentrations of Ca, P, Fe, Mg, Cu, Cr, Zn and Mn in test diets and fish body before (0) and after feeding tilapia for 16 weeks. The sample size was 9 fish per diet. Different letters indicate the means are significantly different from each other ($p < 0.05$).

Figure 4.5: (Continued).



affected significantly by the difference in the composition of feeds. The average protein in fish fed cottonseed meal-free diet (diet 1) was not significantly different from fish fed the other four diets. While the concentration of zinc in fish gradually decreased with the increase of cottonseed meal proportion, its content was at the same level across the diets. The mineral elements in fish body which showed a decline in concentrations as the amount of cottonseed meal was increased included calcium, phosphorus, magnesium, aluminium and iron (Fig.4.5). Dietary calcium, phosphorus, aluminium and iron followed a similar trend. However, dietary magnesium inceased as the cottonseed meal level increased. Only chromium content in fish body showed an increase as the amount of cottonseed component increased from 0 to 100% and in spite of the fact that dietary chromium was uniformly distributed throughout the diets. Average copper and manganese concentrations in fish showed a peak in diet 3. While little manganese was absorbed by the fish from their diets, more copper was accumulated in the body from a diet with low concentrations.

DISCUSSION

This study demonstrates that up to 50% cottonseed meal could be used to replace fish meal as a source of protein in the diet of tilapia without affecting the overall performance of fish. Beyond that level, growth was depressed and the fish population experienced mortality. The specific growth rate and food conversion ratio of fish was directly affected by dietary protein source in fish fed cottonseed-based diets. The results of the present experiments also indicate that tilapia cannot be raised successfully by feeding diets formulated on cottonseed meal alone as a source of protein. The findings are consistent with those reported by other investigators. Ofojekwu and Ejike (1984) reported that cottonseed meal could not be used as a sole protein source for *O. niloticus* because they exhibited poor growth, food conversion and specific growth rate. Robinson et al. (1984b) also observed that glanded cottonseed meal had limited utilization in tilapia and resulted in

poor growth and decreased feed conversion. Similar results were reported by Robinson et al. (1984b) for channel catfish fed glanded cottonseed meal. These findings contradict the earlier held views that tilapia grows well on cottonseed meal-based protein, even at 100% level of inclusion (Jackson et al., 1982 and 1990). Fowler (1980) found that cottonseed meal was efficiently utilized as a potential replacement for fish meal protein in diets for chinook and coho salmon.

The results of the decreased digestibility confirmed earlier observations that diets 4 and 5 were less utilized for growth in the second phase of the study, after the 12th week. At the end of the 16-week trial, feed intake was low in both diets and the least eaten was diet 5 where cottonseed meal was 100%. The level of cottonseed meal in diets 4 and 5 rendered them so unpalatable that it supressed the appetite of the fish. The effect of poor appetite became worse after the twelth week of feeding when retarded growth was clearly noticed in fish fed these two diets. The other two diets (2 and 3) gave values of digestibility and feed intake comparable to the those of the control diet 1. These studies are a further proof that the glass bead-radiographic bioassay is a useful method for assessing total feed intake by individual fish.

The values for digestibility of proteins and absorption of mineral elements brought out several important observations. Whereas most of the protein and a few minerals are available in the cottonseed meal-based diets, a number of other minerals like calcium, phosphorus, copper, iron and zinc were not as biologically available to the fish, especially in fish that had a higher dietary inclusion of cottonseed meal. Most of these minerals in the diets were lost in excreted feces. Yet minerals are required by fish for normal life processes. The minerals are responsible for skeleton formation, maintanance of colloidal systems, regulation of acid-base equilibrium and as cofactors for biologically important compounds such as hormones and enzymes (Watanabe et al., 1997). Mineral deficiencies can cause biochemical, structural and functional pathologies like those found in tilapia

which were subjected to the test diets. According to Watanabe et al. (1997), the biological availability of a mineral varies depending on the feedstuffs and the composition of the diet. Several factors influencing bioavailability include the level and form of the mineral, particle size and digestibility of the diet, nutrient interactions which may be either synergistic or antagonistic, physiological and pathological conditions of the fish and the species concerned. In the present study, the concentrations of minerals in the test diets met the requirement levels for tilapia (NRC,1993).

The distribution of data for chemical composition of the fish body in response to dietary levels could be divided into four groups. The first group refers to elements for which fish body composition did not show any marked change with the graded level of cottonseed meal(e.g. protein and zinc). Sun and Jeng (1998) reported that zinc was uniformally distributed throughout the body tissues and organs of tilapia (*O. mossambicus*) but more concentrated in the gut tissue of the common carp (*Cyprinus carpio*). The authors did not explain why zinc was concentrated and retained in the fish body in comparison to other minerals. They suggested that zinc might be associated with the membrane proteins, a property which could make the element more stable in the fish body. It is well established in some monogastric animals that zinc bioavailability can be reduced by dietary calcium and phytate (Lo et al., 1981) which is present in several feedstuffs of plant origin. Phytic acid present in cottonseed meal was expected to strongly chelate divalent minerals, including zinc, to form insoluble phytates in the intestinal lumen, thereby lowering zinc availability. McClain and Gatlin (1988) found that bioavailability of dietary zinc to blue tilapia (*O. aureus*) was significantly reduced by dietary phytate and recomended that to satisfy the dietary zinc requirement of tilapia, relatively high levels of supplemental zinc would be required in practical diets to overcome the binding capacity of phytate.

The second group was composed of minerals such as calcium, phosphorus, magnesium, aluminium and iron whose concentrations in fish body fell progressively as

the the level of cottonseed meal increased (Fig. 4.5). The low content of these minerals could be related in part to their low amount absorbed by the fish from the diets as noted earlier. It is therefore unclear whether it was the amount of mineral concentration in the diets or the elevation of cottonseed meal that influenced the final concentration of the minerals in the fish body. However, Skonberg et al. (1997) noted that whole body calcium, phosphorus and magnesium concentrations in salmonid fish were responsive to graded levels of their dietary concentrations. This is probably also true for tilapia, given the fact that fish derived these elements mostly from the diet. Phosphorus is required in rather high levels by fish in comparison with other mineral elements (Robinson et al., 1987). Fish can absorb dissolved calcium from the surrounding water mostly through their gills. This was the reason why the mineral was by far more concentrated in the body tissues of the tilapia compared to what was available in their diets (Fig.4.5).

Unlike calcium, however, the primary source of phosphorus for fish is of dietary origin and its requirements range from 0.5 to 0.8% of dry diet for various aquatic species (Wilson et al., 1982). While imbalances in the dietary Ca:P ratio can lead to malabsorption of one of the elements in terrestrial vertebrates (Maynard et al., 1979), the dietary Ca:P ratio does not impair growth or tissue mineral concentrations in fish reared in freshwater as long as dietary phosphorus is adequate and calcium is present in the rearing water (Wilson et al., 1982). This observation was supported by the direct relationship that existed between the amount of phosphorus found in the fish body and the amount of the mineral available in the fish diets. Watanabe et al.(1980) found that the availability of phosphorus in fish meal for tilapia was low compared to that for rainbow trout and chum salmon. The differences in the availability of phosphorus to salmonids and tilapia was attributed to the limited secretion of gastric juices by tilapia. Feedstuffs that originate from plant seeds contain phosphorus primarily as the calcium-magnesium salt of phytic acid known as phytin. Phytin phosphorus is unavailable to animals with simple stomachs because they

lack the enzyme phytase in the gastrointestinal tract (NRC, 1993). Phytic acid also forms insoluble salts with many minerals in the digestive tract. Hence, the availability of phosphorus in most plant products is low, for example, that of soybean meal in catfish is between 29 and 54% (Wilson et al., 1982)

Iron concentration in tilapia fed 100% cottonseed meal diet (diet 5) declined to only half of that in fish fed fish-meal-based diet (diet 1) (Fig.4.5). Pellett et al (1990) reported the conversion of iron from soy diet to hemoglobin iron in rats to lie between 40 and 47% and even lower (29-34%) from the cottonseed source. An antagonistic relationship between dietary iron and whole body manganese, but not body zinc, was observed in Atlantic salmon by Andersen et al. (1996). This relationship seem to hold true for tilapia fed diets 1-3. Therefore, we can conclude that the decreasing dietary iron could improve the absorption of manganese through competition for the same binding sites (Fig.4.5). In contrast, chromium was the only mineral which showed gradual increase from diets 1 to 5 and yet diet 5 contained relatively lower amount of chromium compared to diet 1. This would suggest that the bioavailability of the mineral increased as the level of cottonseed meal went up. The importance of chromium essential in fish nutrition has been recently recognized (Watanabe et al., 1997), although the effects of its deficiencies have not been reported. A supplemental dietary chromium was found to increase the weight gain, energy deposition and liver glycogen content in tilapia fed glucose diets (Shiau and Lin, 1993).

Copper and manganese which constituted the fourth group, showed peak concentrations in fish fed diet 3 (50% cottonseed meal) and lower levels in fish fed the rest of the diets. It was not clear why this level of cottonseed content favored fairly high concentrations of copper and manganese in the bodies of fish fed diet 3. It was, however, noted that whereas the quantity of dietary copper increased with the increase of the proportion of cottonseed meal, dietary manganese correspondingly decreased. The requirement for copper depends to a great extent on the physiological state of the fish, the

copper concentration in the water, and the levels of zinc, iron, cadmium and molybdenum, which are metabolic antagonists of the mineral. Copper and zinc may compete for binding sites on proteins responsible for mineral absorption and / or synthesis of metalloenzymes (Watanabe et al., 1997). Berntssen et al. (1999), showed that dietary copper concentrations of 500 mg Cu / kg and above caused toxic responses in Atlantic salmon fry as concluded from the antagonistic interaction with selenium. In the present study with tilapia, dietary levels of copper were well below that level. The highest concentration was about 15 µg/g dry diet in diet 5. The concentration of manganese in the diets ranged between 72 mg/kg for diet 5 and 100 mg/kg for diet 1, far above the normal fish meal range of 4 to 38 mg/kg, depending on the fish species (Watanabe et al., 1997). In general, the diets contained most of the required mineral nutrients in adequate, though in varied amounts.

One of the problems which limit the use of cottonseed meal as a fish feed is its free gossypol content (El-Sayed, 1990). Besides being toxic to some fishes, gossypol may render lysine, an essential amino acid, unavailable (Jauncey and Ross, 1982). However, this action was diminished as the diets with cottonseed were supplemented with the recommended amount of synthetic lysine (Table 4.1). The response of fish to gossypol is said to be species specific. For instance, rainbow trout fed a cottonseed-based diet containing 0.03% free gossypol, exhibited poor growth and high mortality (Herman, 1970). Tilapia and channel catfish tolerated higher levels of free gossypol without adverse effects on their growth rates (Robinson et al., 1984a; Dorsa et al., 1982; Robinson et al., 1984b). The values of free gossypol determined in the test diets for tilapia (Table 4.1) were high enough to affect the health and growth rates of tilapia which were investigated. Free gossypol is reported to be a membrane-active agent with cytocidal properties and the ability to inhibit membrane-bound enzymes, causing hemolytic anemia at high concentrations (Makinde et al., 1997). Indeed in the accompanying studies on tilapia

hematology, we observed the levels of hematocrit and hemoglobin to be between 36±0.5% for diet 1 and 9.3±1.2% for diet 5 and, 8.7±0.2 g/dl for diet 1 and 1.7±0.1 g/dl for diet 5 respectively (unpublished). Analysis of gossypol in liver of tilapia revealed its high concentration in that organ (Table 4.4). Roehm et al. (1967) reported that the amount of total and free gossypol were highest in liver than any other tissues determined in rainbow trout. Skutches et al.(1974) while studying the effect of dietary free gossypol on blood components in swine and rats suggested that free gossypol bound the iron from the liver of those animals, thus reducing the liver iron concentration. After this complexing action of free gossypol and iron, the levels of hemoglobin and hematocrit in the animals went down. The authors postulated further that there was a possibility that gossypol could interfere with the intestinal absorption of iron. Skutches et al. (1974) also found that cottonseed-based diets containing 0.02 to 0.03% free gossypol caused a high incidence of death in growing pigs. Some of the clinical signs of chronic gossypol toxicity seen in a variety of animals as compiled by Berardi and Goldblatt (1980) include loss of appetite, weakness, emaciation and weight loss, among others. The present study showed that these signs were observed among tilapia fish fed diets containing high level of gossypol derived from cottonseed meal.

In conclusion, cottonseed meal can replace fish meal only partially as a main source of protein in compounded feeds at a limited amount of no more than 50% for tilapia raised in recirculation systems. Plant protein sources as alternatives to fish meal are comparatively inexpensive and abundant, but the use may be limited by the presence of a number of antinutritional factors. These include various protease inhibitors, phytic acid, tannins, a high fiber content and natural toxicants such as gossypol, erucic acid, glucosinolates and isothiocyanates (Davies et al., 1990). In fact, besides gossypol, cottonseed also contains cyclopropenoid fatty acids, namely, malvalic and sterculic acids, which have been shown to have detrimental effects on salmonid fish (Hendericks et al.,

1980). The authors also reported that the fatty acids greatly increasd the incidence and severity of liver cancer in rainbow trout. Until ways of removing or degrading these antinutritional components have been found, cottonseed meal might be recommended at maximum of 50% fish meal replacement level in diet formulations for tilapia. This is in contrast with the animal by-products which were shown to completely and effectively replace fish meal as protein sources and other nutrients in tilapia diets as reported in the first Chapter.

CHAPTER 5

FISH MEAL REPLACEMENT WITH COTTONSEED MEAL IN DIETS FOR TILAPIA: EFFECTS ON REPRODUCTIVE PARAMETERS

INTRODUCTION

Due to the increasing demand, the decreasing supply and the high cost of fish meal, fish nutritionists concentrated their effort to find an alternative source of proteins to substitute fish meal in the diet of fish. Thus, cottonseed meal (CSM), a by-product of the cotton fiber and cottonseed oil industries, has been used to replace partially or totally fish meal in fish diets (Fowler, 1980; Robinson et al., 1984a and b; Robinson and Brent, 1989; El-Sayed, 1990; Robinson and Li, 1994; Robinson and Tiersch, 1995; Dabrowski et al., 1999). However, the major problem associated with CSM use is the presence of the gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxaldehyde].

Gossypol interferes with numerous physiological processes of the reproduction. In numerous species including invertebrates, sea urchin (Burgos et al., 1980), birds, Japanese quail (Lin et al., 1988), domestic cock (Mohan et al., 1989), and mammals, rat (Hadley et al., 1981; Baccetti et al., 1986), hamster (Chang et al., 1980; Waller et al., 1981), boar (Tso and Lee, 1981), monkey (Shandilya et al., 1982; Stephens et al., 1983) and man (Coutinho et al., 1985; Waites et al., 1998), gossypol has been reported as a male antifertility agent. In female, this yellow pigment inhibited embryo implantation (Lin et al., 1985) and development (Brocas et al., 1997). Finally, *in vivo* (Lin et al.,1980; Oko and

Hrudka, 1984; Ahluwalia et al., 1986) and *in vitro* (Gu et al., 1990 a and b, 1991) experiments, revealed that gossypol can also alter steroidogenesis in both sexes. In fish, most of investigations were designed to study the effect of CSM on growth (Fowler, 1980; Jackson et al., 1982; Dorsa et al., 1982; Ofojekwu and Ejike, 1984; Robinson et al., 1984a and b; Robinson and Brent, 1989; El-Sayed, 1990). Nevertheless, Robinson and Tiersch (1995) reported that reproductive capacity in channel catfish *Ictalarus punctatus* in terms of relative testis weight and sperm motility was not significantly affected by high levels of CSM. The reproductive functions (gonad histology and anatomy, level of plasma testosterone) were also similar between Nile tilapia *Oreochromis niloticus* fed a formulated diet containing 0.1% gossypol and a control diet (Huiguang et al., 1995).

The objective of this study was to evaluate if CSM can be used as a partial or total replacement of fish meal without affecting reproduction of tilapia. Growth, gonadal development and plasma levels of sex steroids were monitored as indicator of gossypol effect.

MATERIALS AND METHODS

Experimental diets

Five experimental diets were formulated to contain increasing percentages of cotton seed meal (0, 25, 50, 75 and 100%). Fish meal proteins (menhaden and herring in equal proportion) were systematically decreased to maintain the crude protein level at approximately 42% in all diets (isonitrogenous). All diets were also designed to be isocalorific. Cotton seed meal was obtained from Southern Cotton Oil Co. (Memphis, TN). The diets were mixed and steam pelleted at The Ohio State University feed meal facility (Wooster, OH). The composition of the experimental diets are given in Table 1. The concentration of gossypol in diets was determined according to the method described by Hron et al. (1990) with some modifications (Dabrowski et al., 1999). The results of this

analysis revealed an increase of free and total gossypol associated the increase of CSM in the diet (Table 2).

Experimental system and sampling procedures

The experiment was conducted in a flow-through water recirculating system including fifteen 32-L glass aquaria. Each aquarium was equipped with continuous aeration and was continuously supplied with water at a rate of 1L/min and thermoregulated at 27 ± 1°C. The photoperiod was maintained constant (12h L/12h D). Aquaria were cleaned and the left over feed siphoned out daily.

Two hundred and nineteen juvenile tilapia (11.3 ± 3.9 g) were randomly distributed into all aquaria at a rate of 13 to 16 fish per aquarium, with three replications per treatment. Fish were acclimated for two weeks to the laboratory conditions and to the diets. Then, fish were anaesthetized in a MS-222 solution (50 ppm) and individually weighed (initial weight). The experiment started in May and lasted for 16 weeks. Fish were fed three times a day (11h00, 14h00 and 17h00) by hand at a rate of 3% of their body weight until early June. Then, the feeding rate was gradually decreased to reach 1.5% in late July. All fish were weighed every two weeks and rations were adjusted. At the end of the experiment, blood was withdrawn using heparinized syringe from the caudal vein of 2 anaesthetized fish per tank (6 fish/diet). Blood samples were centrifuged at 1,500 g for 15 min at 4°C, and the plasma was stored at -20°C until radioimmunoassays. Then all fish were individually weighed and sacrificed. Fish were sexed and gonad were weighed to calculate the gonadosomatic index (GSI = gonad weight x 100)/ total weight). The gonads of the fish blood sampled were fixed in Bouin's solution for histological examination. After a week in fixative, gonads transferred to 70% ethyl alcohol. Then tissues were dehydrated in a series of ethyl alcohol and xylene baths and embedded in paraffin. Thin sections (5 µm) were cut and mounted on albumin-coated slides. The slides were stained with trichrome:

hemalun, phloxine and green light (Langeron, 1942).

Radioimmunoassays

Plasma steroid hormones (testosterone, estradiol-17β, 11-ketotestosterone and 17,20β-dihydroxy-4-pregnen-3-one) were determined by radioimmunoassay according to the methods similar to those of Ottobre et al. (1989). The steroids were previously extracted with ethyl ether. [1,2,6,7-^{3}H]testosterone (96.5 Ci mmol^{-1}) and [2,4,6,7,16,17-^{3}H]estradiol (141 Ci mmol^{-1}) were purchased from NEN Life Science Products (Boston, MA, USA). [^{3}H]11-ketotestosterone and [^{3}H]17,20β-dihydroxy-4-pregnen-3-one were a gift from Dr. C.B. Schreck (Oregon State University, OR) and Dr. A. Fostier (INRA, Rennes, France). Unlabelled steroids were purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA), Sigma (St. Louis, MO, USA) and Steraloids (Wilton, NH, USA). The testosterone (T) antiserum was provided by Dr. R.E. Ciereszko (Institute of Animal Physiology, University of Agriculture and Technology, Olsztyn, Poland), the estradiol-17β (E2) antiserum by Dr. R.L. Butcher (West Virginia University, WV, USA), the 11-ketotestosterone (11-kT) antiserum by Dr. D.E. Kime (University of Sheffield, United Kingdom), and the 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) antiserum by Dr. A. Fostier. The characteristics of these antisera have been reported previously (Dabrowski et al., 1995, Butcher et al., 1974, Kime and Manning, 1982 and Fostier and Jalabert, 1986, respectively). The assays were validated for use with tilapia plasma (Table 3).

	T	11-kT	E2	17,20βP
Within-assay CV (%) (n=6)	2.9		1.6	1.7
Between-assay CV (%) (n=2)	4.4		1.7	5.1
Accuracy (coeff. of determination)	0.985		0.998	0.976
Sensitivity (pg /ml)	1		2	1
Recovery of extraction (%)	96		89	72
Parallelism: Serial dilutions of plasma samples showed parallelism with the standard curve between 20 and 200 µl.				

Table 5.1: Radioimmunoassay characteristics of steroid hormones in tilapia.

Statistical analysis

Data were subjected to analysis of variance (ANOVA), and subsequent comparison of means by Scheffe's test (Dagnelie, 1975). Prior to analysis, homogeneity of variance was confirmed for all data using Bartlett's test and all percentages were transformed using an arc sin transformation. The relationship between particular parameters was estimated by calculation of the correlation coefficient (r) and its statistical significance was tested (Dagnelie, 1975).

RESULTS

On the basis of final body weight, no significant ($P>0.05$) difference existed between males or females fed fish meal-based diet (diet 1) and diets where 25 and 50% of fish meal proteins were replaced by CSM. However, the substitution of 75 and 100% of fish meal proteins induced a significant ($P<0.01$) decrease of the body weight in both sexes (Figure 1). At the end of the experiment, male body weight was also significantly ($P<0.01$) higher than female one among the different dietary treatments (Figure 1).

In both sexes, GSI were not significantly ($P>0.05$) different among dietary treatments (Figure 2-A). Moreover, due to the different stage of maturity in the ovarian development, relative gonad weight in females presented large variation ranging from 0.04 to 6.05 % among treatments. To detect any changes in GSI, females were divided in two groups: GSI below or upper 1% (Figure 2-B). In this case, higher GSI were observed in females fed diet 3 in which fish meal proteins were substituted at 50% by CSM, however, no significant differences ($P>0.05$) were observed among the different dietary treatments.

Although plasma levels of T and 11-kT were higher in males fed diet 3 at the end of the experiment, they were not significantly ($P>0.05$) different from those found in males fed other dietary treatments (Figure 3-A). Moreover, the concentrations of these two androgens were lower in males fed fish meal-based diet (diet 1) than the ones fed other dietary treatments. Plasma levels of T were significantly correlated with GSI (Figure 4-A) and plasma levels of 11-kT (Figure 4-B). Concentrations of plasma E2 were detected in males ranging from 0.3 to 0.6 ng/ml but no significant ($P>0.05$) differences was observed among the dietary treatments. In females, no significant effect ($P>0.05$) of dietary treatment on the plasma levels of T and E2 was observed at the end of the experiment (Figure 5). Like in males, GSI was inversely correlated with plasma levels of T (Figure 4-C). In both sexes, 17,20βP was detected in fish plasma fed different dietary treatments but the levels of this progestogen were low (Figure 6).

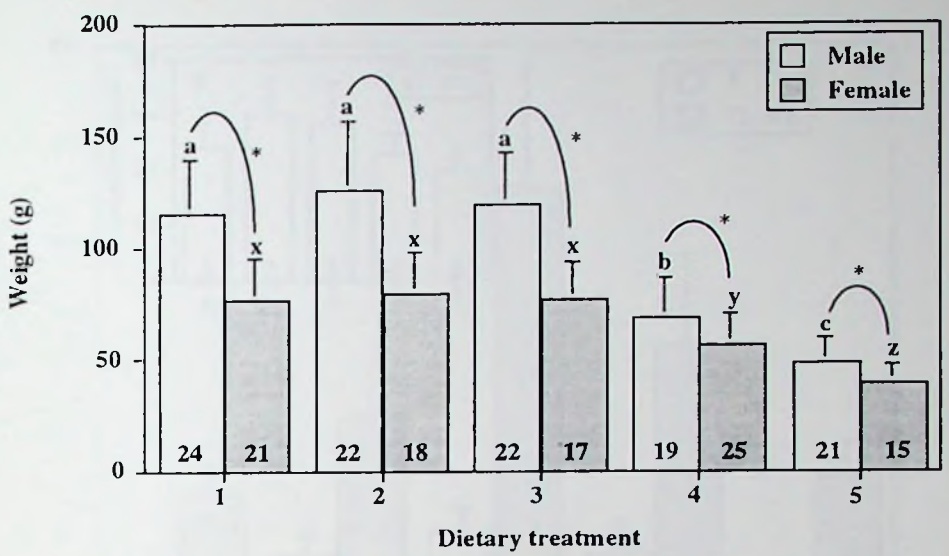


Fig. 5.1: Final weight of male and female tilapia fed different levels of CSM for 16 weeks. Data are means ± SEM. For each sex, means with the same letter are not significantly different ($p>0.05$). Asterik denotes significant difference between male and female within a dietary treatment. Number of fish sampled are given on bottom of bars.

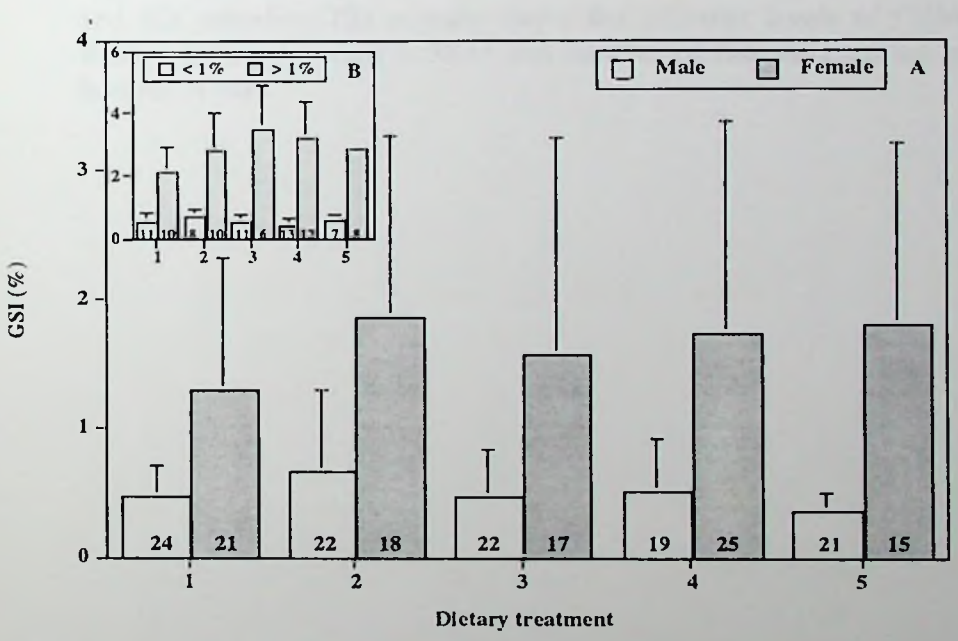


Fig. 5.2.A: Gonadosomatic index (GSI) in male and female tilapia fed different levels of CSM for 16 weeks. Data are means ± SEM and number of fish sampled are given on bottom of bars.

B: GSI in female tilapia fed different levels of CSM for 16 weeks. Data are means ± SEM and number of fish sampled are given on bottom of bars.

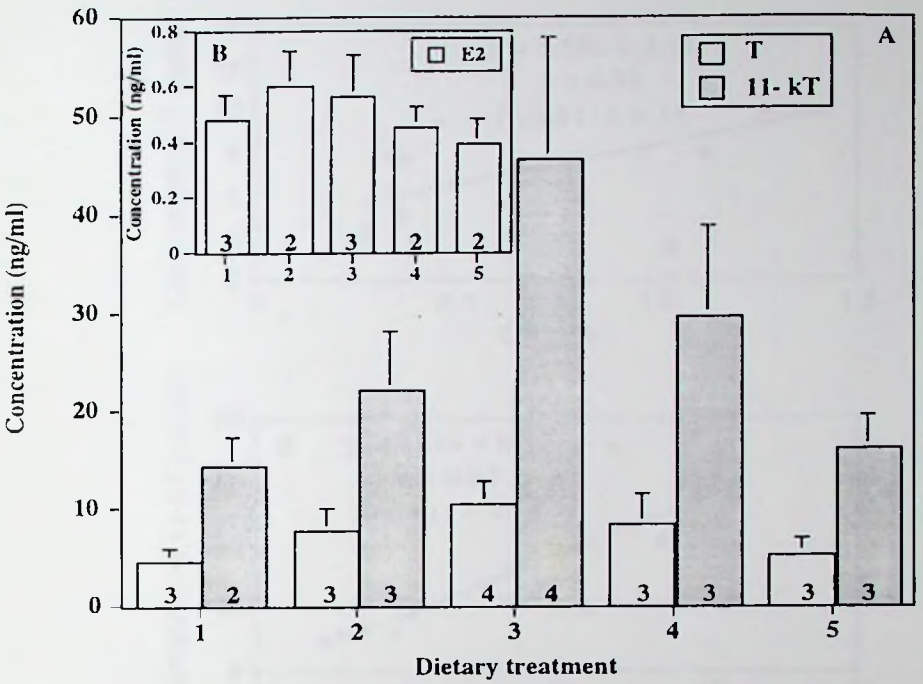


Fig. 5.3: Plasma sex steroids (T: testosterone, 11-kT: 11-ketotestosterone and E2: estradio-17β) in male tilapia fed different levels of CSM for 16 weeks. Data are means ± SEM and number of fish sampled are given on bottom of bars.

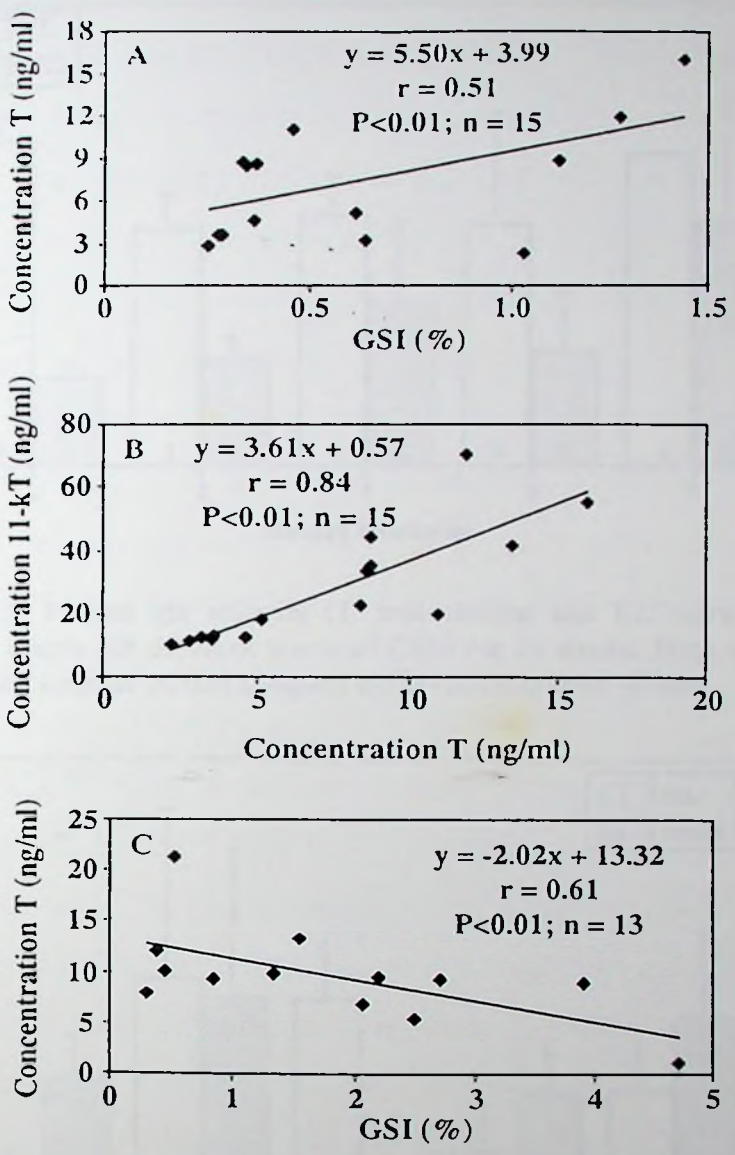


Fig. 5.4: Relationship between (A) gonadosomatic index (GSI) and plasma levels of testosterone (T) in male tilapia fed different levels of CSM for 16 weeks: (B) plasma levels of T and 11-ketotestosterone (11-kT) in male tilapia fed different levels of CSM for 16 weeks: (C) gonadosomatic index (GSI) and plasma levels of testosterone (T) in female tilapia fed different levels of CSM for 16 weeks.

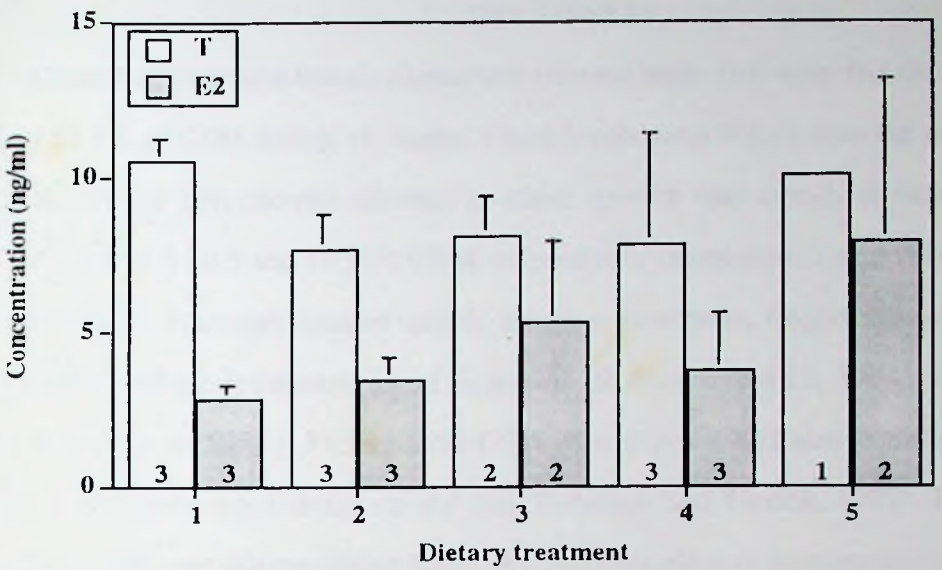


Fig. 5.5: Plasma sex steroids (T: testosterone and E2: estradio-17β) in female tilapia fed different levels of CSM for 16 weeks. Data are means ± SEM and number of fish sampled are given on bottom of bars.

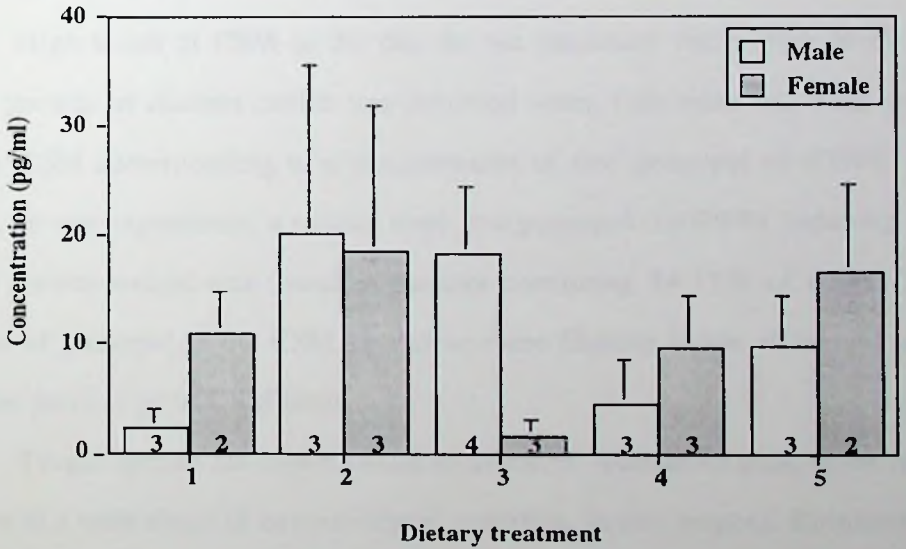


Fig. 5.6: Plasma levels of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) in male and female tilapia fed different levels of CSM for 16 weeks. Data are means ± SEM and number of fish sampled are given on bottom of bars.

DISCUSSION

Growth of male and female tilapia was affected when fish were fed diets containing 44.11 or 58.8% of CSM during 16 weeks. These levels were higher than the ones reported in *Tilapia aurea* or *Oreochormis niloticus* in which growth was already reduced when fish received diets with 26.5 and 18.51% CSM, respectively (Robinson et al., 1984; Ofojekwu and Ejike, 1984). Although channel catfish, *Ictalarus punctatus*, fingerlings tolerated up to 50% of CSM without detrimental effect on growth (Robinson and Li, 1994), males of this species fed diets containing 37.5 or 52% CSM over 2 years had also significantly lower weights in comparison to fish fed control diet (Robinson and Tiersch, 1995). Those results indicated that fish can tolerate higher levels of CSM in feeds than monogastric animals. One factor responsible of the growth depression can be associated with the decrease of the levels of available lysine in diets containing CSM. However, in our experiment, the requirement for this essential amino acid was satisfied in all diets.

High levels of CSM in the diet do not necessary mean high levels of gossypol. Thus, growth of channel catfish was inhibited when fish were fed diets with more than 17.4% CSM corresponding to a concentration of free gossypol of 0.09% (Dorsa et al., 1982). In our experiment, a similar level free gossypol (0.079%) inducing a decrease of tilapia growth weight was found in the diet containing 44.11% of CSM. Therefore, the content of gossypol in the CSM should be more limiting factor of the use of CSM rather than the percentage of CSM itself.

Tilapia species are high resistant to diseases, tolerant to poor water quality and can survive in a wide range of environmental condition. In this respect, Robinson et al. (1984) found that *Tilapia aurea* can tolerate high dietary levels of free gossypol (up to 0.18%) without significant effect on growth. In contrast, the growth of the fish used in the present study was already affected by low levels of free gossypol. Using the same dietary treatments, Dabrowski et al. (1999) reported that growth of rainbow trout, *Oncorhynchus*

mykiss, fed up to 58.8% CSM over a period of 200 days was not reduced. Thus, it appears that in fish as in mammals the tolerance to gossypol is species specific.

In most of tropical fish, such as tilapia, females spawn several times a year (Baroiller and Jalabert, 1989; Brummett, 1995). The ovarian development in a given population is not synchronized and consequently females in a given time are not at the same stage of maturity. Therefore, large variations in the reproductive parameters can be expected.

In the present study, the reproductive parameters (GSI and concentration of plasma sex steroids) in male and female tilapia fed CSM diets did not differ significantly from fish fed meal-based diet. GSI and plasma sex steroids in all treatments were similar to those reported in other tilapiine fish (Yaron et al., 1983; Rocha and Reis-Henriques, 1996) indicating a well-development of the gonads. Gossypol has been shown to possess inhibitory effects on steroidogenesis. A decrease of plasma T levels has been reported in rats fed or implanted with gossypol (Lin et al., 1980; Hadley et al., 1981; Oko and Hrudka, 1984; Ahluwalia et al., 1986). Moreover, gossypol also inhibits biosynthesis of progesterone in cultured bovine luteal cells (Gu et al., 1990). However, this antisteroidogenic effect appears at high concentrations of gossypol. Thus, the low concentration of free gossypol contains in the CSM diets used in this experiment (Table 2) did not induce any changes in plasma sex steroids. Huiguang et al. (1995) also reported that plasma T concentrations of Nile tilapia *Oreochromis niloticus* fed a formulated diet containing 0.1% gossypol acetate for 4 months did not differ significantly from these fed control diet. Surprisingly, it appeared that male and female tilapia fed diet in which CSM was substituted at 50% presented the highest concentrations of plasma androgens and estrogen, respectively. These findings were also found in rainbow trout (Dabrowski et al., 1999).

Although the substitution of fish meal by CSM did not affect the plasma sex steroids, we can not exclude that CSM and more specifically gossypol can modify directly or indirectly other reproductive parameters. Thus further studies are needed to confirm that gossypol did not affect the concentration, the motility and the fertilizing ability of the spermatozoa in males or quality of the eggs in females. Stress has been also reported to affect some parameters of the reproductive system in fish. Female tilapia *Oreochromis mossambicus* treated with cortisol showed a retardation of oocyte growth associated with a decrease of plasma T and E2 (Foo and Lam, 1993). Huiguang et al. (1995) reported that plasma cortisol increased in tilapia *O. niloticus* fed diet containing 0.1% gossypol acetate.

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