

Genetic manipulation of sex ratio for the large-scale production of all-male tilapia *Oreochromis niloticus* L.

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Abstract

We describe the results of a breeding programme for Nile tilapia *Oreochromis niloticus*, incorporating estrogen induced sex reversal of male to female and progeny testing, to generate novel 'YY' male genotypes. 'YY' male genotypes proved to be as viable and fertile as normal XY males, and to sire a mean progeny sex ratio of 95.6 % male. While these results confirm the hypothesis of predominantly monofactorial sex determination, there appears to be a genetic basis for the occurrence of occasional females in the progeny of 'YY' males. It is likely that these arise through the action of several autosomal sex modifying genes. We report the first instance of feminization of YY genotypes in tilapia and the mass production of 'YY' males through YY x YY matings, obviating the need for time consuming progeny testing to discriminate XY and YY male genotypes. This enables the production of 'YY' males and their all male progeny, known as genetically male tilapia (GMT), to be mass produced on a commercial scale. We conclude that the "YY male technology" provides a robust and reliable solution to the serious and widespread problem of early sexual maturation, unwanted reproduction and overpopulation in tilapia culture.

Introduction

The Nile tilapia, *Oreochromis niloticus* is widely used in tropical aquaculture and is becoming an increasingly popular species for culture in environmentally controlled conditions in temperate countries. Aquaculture statistics for 1993 place annual world production for this species at > 365,000 metric tonnes (FAO 1995) which is likely to be an underestimate. Production is presently expanding rapidly, especially in Southeast Asia, and the Nile tilapia is now the principal species of choice in freshwater aquaculture in many tropical countries.

This species has been the focus of considerable biological research with genetics receiving much emphasis (Pullin and Maclean 1992). Many studies have concentrated on the genetic basis of sex determination in *O. niloticus* and other *Oreochromis* species, as reviewed by Mair et al. (1991a and b), and Trombka and Avtalion (1993). The interest in the genetics of sex determination in tilapia is motivated by the practical and commercial implications of the production of monosex progeny for use in aquaculture in addition to fundamental scientific curiosity.

Culture of monosex progeny, preferably males, which, in tilapia species, grow faster and to a larger size than females, has long been recognised as the most effective solution to the widespread problem of early sexual maturation and uncontrolled reproduction in tilapia culture. To date, this has been achieved either through manual sexing, direct hormonal sex reversal or hybridization, the relative merits of which are reviewed by Mair and Little (1991) and Wohlfarth (1994). All of these techniques have significant disadvantages in their application and none has become widely used in aquaculture, especially in developing countries. This is in contrast to the widespread culture of monosex female salmonids produced through genetic manipulation of sex determination (Bye and Lincoln 1986).

Although much studied, the precise mechanisms by which sex is determined in tilapia, specifically *O. niloticus*, are not fully understood. Early hypotheses were based on the sex ratios observed in hybrid crosses of different species, the most comprehensive of these hypotheses being that based on a theory of autosomal influence (Hammerman and Avtalion 1979). However, so far, no theory based on hybrid sex ratios successfully explains all observed sex ratios, which may be highly variable in some crosses such as those between *O. niloticus* and *O. aureus*. Recent research has concentrated on intra-specific sex ratios. Several authors have presented evidence which indicates that *O. niloticus* has a predominantly monofactorial mechanism of sex determination with heterogametic XY males and homogametic XX females (Mair et al. 1991a; Trombka and Avtalion 1993). However, this simple monofactorial hypothesis fails to explain some deviations from predicted sex ratios based on studies involving sex reversal and chromosome set manipulation. Hussain et al. (1994) hypothesised the existence of an autosomal sex modifying locus (with alleles *SR* and *sr*) epistatic to the gonosomal locus and which induces female to male sex reversal when *sr* is homozygous. This hypothesis was developed to explain the occurrence of varying proportions of males in heterozygous and homozygous meiotic and mitotic gynogenetic progeny (Mair et al 1991a and Hussain et al., 1994). However, this hypothesis still fails to explain some of the aberrant sex ratios observed in crosses of hormonally sex reversed fish, and it has been concluded that these arise through additional autosomal influences and/or environmental influences (Mair et al. 1990; Trombka and Avtalion 1993). Baroiller et al. (1995) presented evidence for a temperature effect on sex differentiation. In putative all-female progeny from androgen sex reversed males (XX) crossed with normal females, they observed significantly higher proportions of aberrant males in progeny reared at high temperatures (36°C) during the period of sex differentiation. Similar results have been obtained in our laboratory using different strains of *O. niloticus* (J.S. Abucay, FAC, CLSU, N. Ecija, unpubl. data). The hypothesis of a temperature effect on sex determination still does not explain the occurrence of a small percentage of males in the putative monosex female progeny reared at ambient temperatures. Thus, currently available data indicate that sex determination in *O. niloticus*, while influenced by several factors, is best described as “predominantly monofactorial”, with an underlying mechanism of male heterogamety playing the major role.

Based on this hypothesis of predominantly monofactorial sex determination, several authors have proposed a breeding programme through which novel ‘YY’ males could be generated in *O. mossambicus* (Yang et al. 1980; Varadaraj and Pandian 1989) and in *O. niloticus* (Mair 1988; Baroiller and Jalabert 1989; Scott et al. 1989). These authors demonstrated the viability of very small numbers of ‘YY’ males, but none demonstrated that these could be produced and spawned on the large scale that would be necessary for the technology, termed the “YY male technology”, to be applied to aquacultural practice. Our objectives in this study were to further investigate sex determination in *O. niloticus*, to determine the viability of, and the progeny sex ratios produced by, ‘YY’ males, and to test the feasibility of large scale production of all-male tilapia through genetic manipulation of sexual phenotype.

Materials and Methods

Genetic origins of stocks and genetic nomenclature

The strain of *O. niloticus* used in these investigations, known in the Philippines as “Egypt-Swansea”, originates from L. Manzala, Egypt, where it was collected and transferred to the University of Stirling, Scotland in 1979 (Hussain 1992). Several introductions of this strain were made to the University of Wales Swansea in the 1980s. Several hundred fish, including a number of estrogen treated phenotypic females, representing more than ten families, were transferred in 1989 to the Philippines where our experiments were carried out.

In this paper the genetic nomenclature for the description of hormone treated fish described by Mair et al. (1987) is used: the sex symbol refers to the functional reproductive phenotype, with a delta (Δ) prefix denoting functional sex reversal. Thus, a genetic male reversed to female by estrogen treatment is denoted as $\Delta \text{♀}$.

The breeding programme

The breeding programme adopted (Fig. 1) has a number of distinct steps:

1. Feminization of sexually undifferentiated progeny from normal crosses: this has been successfully achieved in this strain (Mair 1988 and Scott et al. 1989) and by several authors in a number of other *Oreochromis* species (Scott et al. 1989; Varadaraj 1989; Rosenstein and Hulata 1994).
2. Identification of sex reversed females ($\Delta \text{♀♀}$ - XY) by progeny testing: the morphology, behaviour and karyotypes of sex reversed females is indistinguishable from that of their genetically female siblings and thus they can only be identified by progeny testing them in crosses with normal XY males. Progeny sex ratios approximating 3:1 are indicative of a maternal XY genotype. Identification by progeny testing of hormonally induced and one naturally occurring $\Delta \text{♀♀}$ (XY) was reported by Mair et al. (1991a). Five further estrogen treated $\Delta \text{♀♀}$ were identified from among fish transferred to the Philippines.
3. Crossing of the $\Delta \text{♀♀}$ (XY) identified in step 2, with normal males (XY): this should generate progeny including approximately 25% ‘YY’ males. Each of the five identified $\Delta \text{♀♀}$ (XY) was crossed to normal males and the progeny reared for use in progeny testing.
4. Progeny testing of males to identify YY genotypes: a total of 54 males from these five crosses were progeny tested with normal females. It was hypothesised that ‘YY’ males would sire only male offspring. Mair et al. (1991a) previously reported the identification of four ‘YY’ males in the progeny of a single naturally occurring $\Delta \text{♀}$. Although it is possible to identify ‘YY’ males at this stage investment of time and resources in progeny testing would be impractical on a large scale. It is for this reason that it was necessary to complete the succeeding stages of the programme.
5. Crossing of identified ‘YY’ males with previously identified $\Delta \text{♀♀}$: six $\Delta \text{♀♀}$ (five were second generation $\Delta \text{♀♀}$ produced by estrogen treatment of progeny from the initial XY x XY crosses, progeny tested as in step 2) and six ‘YY’ males were used in nine crosses to generate families with a 1:1 ratio of YY and XY genotypes. The progeny from these

crosses were each divided into two batches, one of which was treated with estrogens for feminization.

6. Progeny testing of estrogen treated females ($\Delta^{\text{♀}}$ - XY and $\Delta^{\text{♀}}$ - YY) and non-treated males for identification of YY genotypes: it was hypothesised that 50% of all sex reversed females would be of the YY-genotype, assuming no differential feminization of XY and YY genotypes. In the progeny testing of these sex reversed females with normal XY males, XY females would be expected to produce 3:1 sex ratios, whilst 'YY' females would give all- or nearly all-male progeny. In practice it is difficult to discriminate between these two progeny sex ratios statistically for YY x XY crosses which produce some female offspring. Thus females from all but one of these families were progeny tested using previously identified androgen sex reversed males ($\text{XX}\Delta^{\text{♂}}$). Using these males, XY and 'YY' females would produce 1:1 and 1:0 sex ratios respectively, which are easily separated statistically. A total of 113 sex reversed females were progeny tested in this way. A total of 158, non-treated males were progeny tested, as a control to test the hypothesis that 50% of genotypes would be YY.
7. Crossing of identified 'YY' males with identified 'YY' females ($\Delta^{\text{♀}}$): in order to produce 'YY' males in large numbers without the need for each to be progeny tested, it was necessary to demonstrate that 'YY' males and 'YY' females could be bred together and would generate sufficient numbers of viable YY male progeny.
8. Progeny testing of males from YY x YY matings to verify their YY genotype: a total of 61 males from several YY x YY crosses were reared and progeny tested. In addition some progeny from several YY x YY crosses were feminized to produce future YY male producing broodstock. Twelve of these females were progeny tested using $\text{XX}\Delta^{\text{♂}}$.

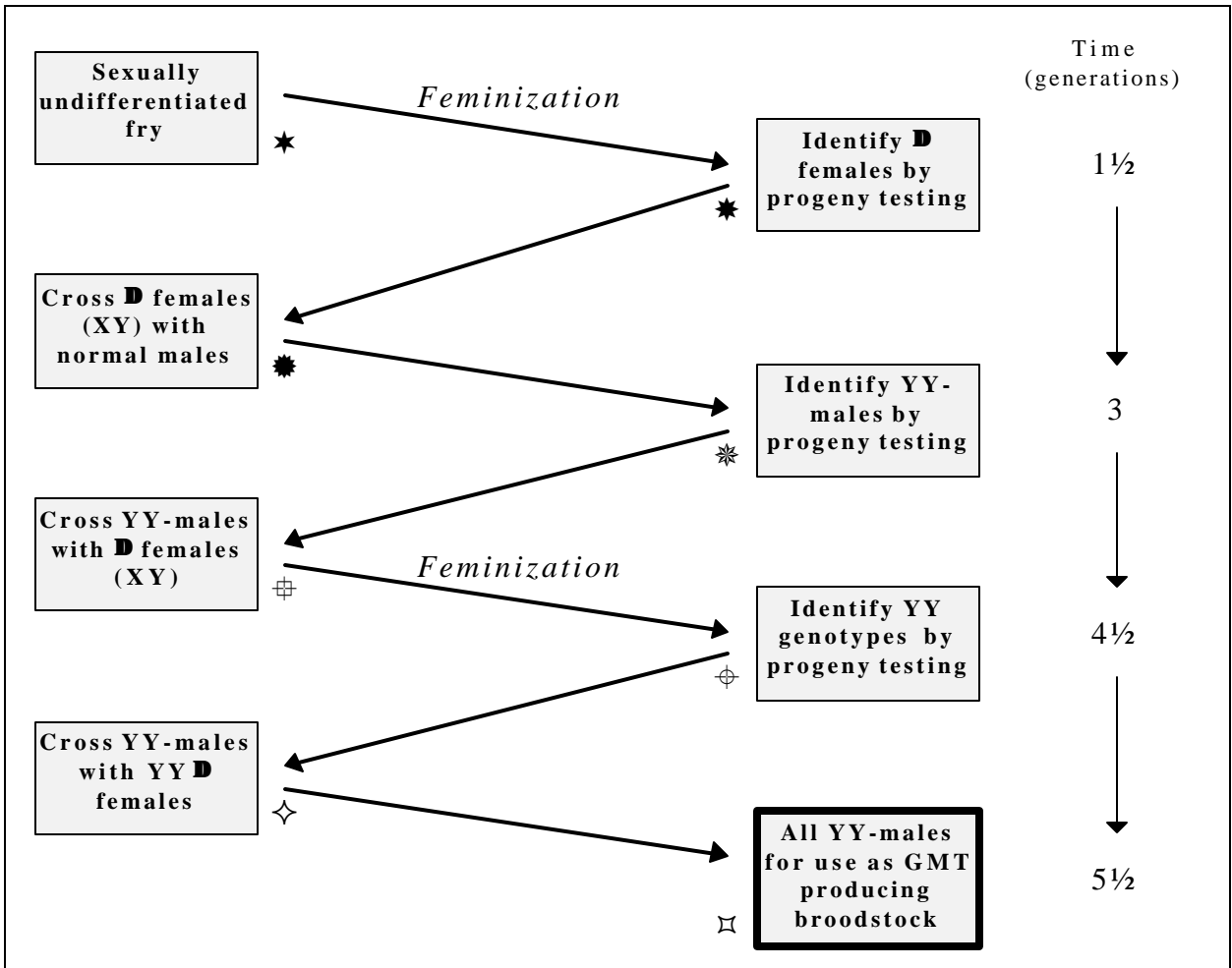
In anticipation of the future production of large numbers of 'YY' males and for the provision of sex reversed males ($\Delta^{\text{♂♂}}$ - XX) to be used in progeny testing, we also developed all-female lines in the same strain of *O. niloticus*. In this programme, progeny from normal crosses were masculinised by oral application of the androgen 17 α -methyltestosterone. These males were progeny tested with normal females and sex reversed $\Delta^{\text{♂♂}}$ (XX), and identified based on the hypothesis that they should produce only female progeny. A second generation of masculinization enabled the production of large numbers of $\Delta^{\text{♂♂}}$ (XX) obviating the need for time consuming progeny testing. The $\Delta^{\text{♂♂}}$ (XX) were viable and produced mean sex ratios of approximately 95% female (G.C. Mair, University of Wales Swansea, unpubl. data).

Sex reversal by oral application of estrogens and androgens.

Functional sex reversal is most easily achieved through oral application of estrogens and androgens incorporated into the feed and administered during the period of sex differentiation which is known to be fixed by between 14 (Srisakultiew 1993) and 30 (Alvencia-Casauay and Carino 1988) days post-hatch.

First feeding fry were collected from incubators following artificial incubation. Fry were transferred at a density of approximately 1,000 per m², to fine mesh net cages (1 x 1 x 1m) suspended in fertilized earthen ponds and fed with a hormone treated feed,

Figure 1. Schematic diagram of the breeding programme for the large scale production of YY-male broodstock.



ad libitum, four times daily. Adaptations of protocols for feminization optimized by Rosenstein and Hulata (1994) and Mair and Santiago (1994) were applied as follows: fry were fed with a powdered feed containing $1,000 \text{ mg.kg}^{-1}$ of Diethylstilbestrol (DES) for 20 days. Adaptations of commercial protocols were applied for masculinization (Guerrero III and Guerrero 1988 and Vera Cruz and Mair 1994). Fry were fed a feed containing 17α -methyltestosterone at 40 mg.kg^{-1} for 25 days. Following hormone treatment, fry were transferred to net cages for rearing to sexual maturity.

Progeny testing

For progeny testing sexually mature fish (150 - 200 days) were stocked in 1m^2 fine mesh cages immersed in earthen ponds to a depth of 60cm, for spawning, either as single pairs or with one male to three females. Cages were checked every seven days and eggs or fry removed from incubating females. Spawners were then individually tagged using Passive Integrated Transponder (PIT) electronic tags and stocked communally in concrete tanks. Eggs and fry were incubated artificially up to the completion of yolk sac absorption whereafter they were stocked as sib groups in fine mesh cages, for rearing. Fry were reared for approximately three months until they attained a mean mass of 3g, before being killed and sexed using the gonad squash technique of Guerrero and Shelton (1974). In larger families where it was not deemed necessary to sacrifice the entire family, a minimum sub-sample of 100 fish was taken at random, avoiding size bias, which may have favoured the selection of larger males.

All sex ratios were tested against a 1:1 sex ratio (or 3:1 when testing for XY Δ ♀ crossed with normal XY males) using the chi-squared test. A stringent statistical criterion was adopted for the designation of parental genotypes when progeny testing for 'YY' males or females, hypothesised to produce all- or nearly all-male progeny. A 5% level of probability was not deemed sufficiently stringent to permit the confident designation of genotypes. Thus potential YY genotypes producing sex ratios, in crosses with XX genotypes, that were not significantly different from 1:1 or only significant at the 5% level ($0.01 < P < 0.05$) were designated as XY. Only those producing male-skewed sex ratios different from 1:1 at a probability level of 0.1% ($P < 0.001$) were designated YY. This procedure also helped to minimize the chance of making a type I error in the identification of 'YY' males. No genotype was designated for parents of families falling between these two criteria ($0.05 > P > 0.01$).

Proportions of 'YY' males identified in progeny of XY x XY and XY x YY crosses were tested against the 1:1 expectation using the chi-squared test.

Results

Five sex reversed females (Δ ♀ ♀) had previously been identified among DES treated fish and were used in seven crosses with normal males (XY Δ ♀ x XY ♂) to produce families including 25% YY genotypes for progeny testing (Mair et al. 1991a and unpublished data).

Progeny testing of males from XYΔ♀ x XY♂ crosses

A total of 54 males from seven XY x XY matings were progeny tested in crosses to randomly selected normal females. More than 3,500 fry were sexed with an average of 69.5 fry per family (Table 1). This was deemed an adequate sample size to gain an accurate estimate of family sex ratio. Using pre-defined criteria for allocation of genotypes, 50% of these males were classed as XY, 38.9% as YY and 11.1% were not classified because their progeny sex ratio was different from 1:1 at $P < 0.01$ but not at $P < 0.001$.

The frequency distribution of family sex ratio is clearly bimodal, those from XY males being normally distributed around a 1:1 sex ratio and a peak at 100% male representing the modal sex ratio for progeny of designated 'YY' males (Fig. 2a).

The heterogeneity chi-squared (based on the difference from 1:1 ratios) for the sex ratios of designated XY males was not significant ($\chi^2_{[26]} = 36.33$). However, the sex ratios produced by designated 'YY' males were significantly heterogeneous ($\chi^2_{[20]} = 50.21$) due to one outlying sex ratio of 67.0% male. If this outlier is removed the χ^2 value becomes non-significant ($\chi^2_{[19]} = 15.80$). When all sex ratios are considered together, they are highly heterogeneous ($\chi^2_{[53]} = 775.64$; $P < 0.001$).

Progeny testing of males from XYΔ♀ x YY♂ crosses

The results from the progeny testing of males from the later crosses of XYΔ♀ x YY♂ follow a similar trend. Of 158 males tested in crosses with normal females, 41.8% were designated as XY, 54.4% as YY and only 3.8% were unclassified (Table 1). The proportion of 'YY' males was not significantly greater than the predicted 1:1 ratio. Similar range (71.7 - 100%) and mean (96.4%) proportions of males were observed as with the previously identified 'YY' males. Designated XY males produced sex ratios slightly skewed to male (56.6% male) in this set of progeny tests. The family sex ratio frequency distributions (Fig. 2b) shows a pattern similar to that from testing progeny of XY x XY crosses although, as hypothesised, there was a relatively higher proportion of 'YY' males.

Chi-squared heterogeneity values for sex ratios from males designated as XY and as YY were not significant ($\chi^2_{[65]} = 90.89$ and $\chi^2_{[78]} = 103.90$ respectively). All sex ratios considered together were highly heterogeneous ($\chi^2_{[157]} = 2,168.58$; $P < 0.001$).

Progeny testing of DES treated females from XYΔ♀ x YY♂ crosses

Females were selected for progeny testing at random from nine DES treated families of XYΔ♀ x YY♂ crosses (see Table 2). A total of 113 females were tested, the majority (105) in crosses with sex reversed XXΔ♂ to facilitate statistical discrimination of XY and YY genotypes. No 'YY' males were designated from the eight progeny tests of females from family 1, which were tested with XY males. This is lower ($P < 0.01$) than the expected proportion and is possibly a consequence of the difficulty in designating genotypes associated with the 3:1 sex ratios expected from tests of XYΔ♀. Overall 50.4% of the 113 females were classed as XY, 45.1% as YY, and only 4.5% were unclassified. Other than family 1, no families had proportions of 'YY' females differing ($P > 0.05$) from the expected 1:1 although it should be noted that only a small number of females were tested in each family. The overall

range (74.0 - 100.0% male) and mean (97.3% male) of the sex ratios produced by 'YY' females was in accordance with those produced by 'YY' males.

The sex ratio frequency distribution for the progeny testing of DES treated females (Fig. 2c) closely resembles that for the males (Fig. 2b).

Progeny testing of males and females from YYΔ♀ x YY♂ crosses

Following successful identification of both 'YY' males and 'YY' females, a number of YY x YY crosses were attempted. Several spawnings were obtained and progeny were divided into two, with one group receiving DES treatment. Table 3 shows the summary of the results from the progeny testing of 61 males (with normal XX♀♀) from four such crosses and 12 sex reversed females (with sex reversed XXΔ♂♂) from three crosses. Following the sexing of over 4,300 progeny, all tested males and females were designated as YY genotypes. All 12 females produced 100% male progeny (Fig. 2e) and the 'YY' males produced a mean sex ratio of 98.9% male (Fig. 2d). Sex ratios from these 'YY' males were highly homogeneous ($\chi^2_{[60]} = 11.27$; $P > 0.99$).

Summary of sex ratios produced by XY and YY genotypes

In the progeny testing of males from XY x XY and XY x YY crosses, designated XY males produced ratios slightly skewed to male. The total sex ratio from all 93 XY males was 52.4% male, highly significantly greater than the predicted 1:1 ratio ($\chi^2_{[1]} = 13.14$; $p < 0.001$).

Over all progeny tests, excluding those males from YY x YY crosses, fish designated as YY genotypes produced sex ratios ranging from 67.0 to 100% male with a mean of 95.63 ± 7.87 % male. It was noted that the 'YY' males from the YY x YY cross gave a higher mean sex ratio of 98.90 ± 3.1 % male. The 'YY' males and females used in these YY x YY crosses were selected based on the sex ratio of their initial progeny tests (all had produced 100% male progeny). These results therefore indicate a possible response to selection against the occurrence of small proportions of females.

Based on the hypothesis of a genetic basis for the occurrence of small proportions of females in the putative all-male progeny of 'YY' males, a number of males were selected for repeat progeny tests. Twenty-six males which had produced initial progeny test sex ratios of >95% male (mean = 99.67%) were selected and repeat mated one or more times (sex ratios were pooled for males repeat mated). The mean sex ratio of these repeat progeny tests was 99.66% male, higher than the 95.63 mean sex ratio for all 'YY' males, this difference being almost significant ($P = 0.0502$) as determined by a Mann-Whitney non-parametric test.

Table 1. Summary of progeny testing for potential ‘YY’ males (crossed to normal XX females) in progeny from XYΔ ♀ x XY ♂ and XYΔ ♀ x YY ♂ crosses.

Summary Parameter	XY x XY	XY x YY
No of families from which males were tested	7	3
Number of potential ‘YY’ males tested	54	158
Number of progeny sexed (mean per family)	3,753 (69.5)	10,842 (68.6)
Number of males classed as XY (%)	27 (50.0)	66 (41.8)
Number of males not classified (%)	6 (11.1)	6 (3.8)
Number of males classed as YY (%)	21 (38.9)	86 (54.4%)
Expected number of ‘YY’ males (%)	18 (33.3)	79 (50.0)
χ^2 for observed vs. expected number of ‘YY’ males	0.75 ns	1.24 ns
Range of sex ratios from XY males	37.2 - 70.0	34.2 - 70.0
Mean of sex ratios from XY males (\pm sd)	52.9 (\pm 8.1)	56.6 (\pm 7.9)
Heterogeneity Chi-squared for XY males (df)	36.33 (26)ns	90.89 (65)ns
Range of sex ratios from ‘YY’ males	67.0 - 100.0	71.7 - 100.0
Mean of sex ratios from ‘YY’ males (\pm sd)	95.31 (\pm 8.4)	96.4 (\pm 7.1)
Heterogeneity Chi-squared for ‘YY’ males (df)	50.21 (20)*	103.90 (78)ns
Total heterogeneity chi-squared for all males (df)	775.64 (53)***	2,168.58 (157)***

ns = not significant; * - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$

Table 2. Results from progeny testing of females resulting from feminization of progeny from XY x YY matings

Family number →	1	2	3	4	5	6	7	8	9	Total
Source cross ($\Delta \text{♀ XY} \times \text{♂ YY}$) ^a	1 x 1	1 x 4	2 x 2	2 x 3	3 x 3	4 x 2	5 x 3	5 x 5	6 x 6	6D ♀ x 6♂
Sex ratio following DES treatment	16:24	16:15	5:27	0:11	0:14	2:3	1:64	--	--	40:158
Male genotype used in progeny test	XY	XX	XX	XX	XX	XX	XX	XX	XX	--
Number of females tested	8	15	10	4	5	3	50	12	6	113
Number of progeny sexed	420	732	423	242	238	124	2,450	589	373	5,591
Number of females classed as XY	8	8	7	1	0	2	23	3	5	57
Number of females not classified	0	1	0	0	0	1	1	1	1	5
Number of females classified as YY	0	6	3	3	5	0	26	8	0	51
Expected proportion of YY $\Delta \text{♀}$ ^b	4	7.5	5	2	2.5	1.5	25	6	3	56.5
Exact probability for binomial test	0.008 ***	0.790 ^{ns}	0.344 ^{ns}	0.625 ^{ns}	0.063 ^{ns}	0.500 ^{ns}	0.775 ^{ns}	0.228 ^{ns}	0.062 ^{ns}	0.54
Range of sex ratios from XY $\Delta \text{♀}$ ♀ ^c	63.6-85.0	35.0-65.0	37.0-61.0	n\a	n\a	39.5-48.9	42.4-65.0	44.4-54.7	27.0-66.0	35.0-85.0
Mean sex ratio from XY $\Delta \text{♀}$ ♀	75.1	50.4	50.9	48.0	n\a	44.2	53.6	50.4	47.3	52.5
Range of sex ratios from YY $\Delta \text{♀}$ ♀ ^c	n\a	92.0-100.0	97.4-100.0	85.0-100.0	n\a	n\a	74.0-100.0	80.5-100.0	n\a	74.0-100.0
Mean sex ratio from YY $\Delta \text{♀}$ ♀	n\a	98.7	99.2	94.4	100.0	n\a	94.0	97.4	n\a	97.3

a- Number represent different individual males and females

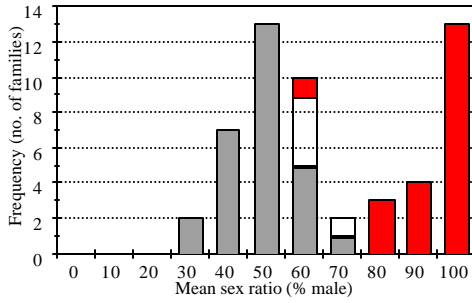
b - Expected proportion based on hypothesis of 1:1 ratio of XY to YY genotypes, with equal feminization rates.

c - Sex ratios given as percentage males

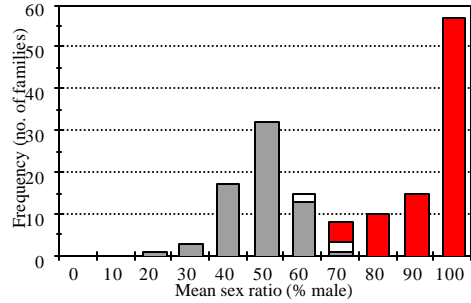
Table 3. Summary of progeny testing for putative ‘YY’ males (crossed to normal XX ♀ ♀) and ‘YY’ females (crossed to sex reversed XXΔ ♂ ♂) in progeny from YYΔ ♀ x XY ♂.

Summary Parameter	‘YY’ males	‘YY’ females
No of families from which males were tested	4	3
Number of putative YY genotypes tested	61	12
Number of progeny sexed (mean per family)	3,723 (61.0)	652 (54.3)
Number of males classed as YY (%)	61 (100.0)	12 (100.0)
Expected number of ‘YY’ males (%)	61 (100.0)	12 (100.0)
χ^2 value of observed vs. expected number of ‘YY’ males	0.0	0.0
Range of sex ratios from ‘YY’ males	79.5 - 100.0	100.0
Mean of sex ratios from ‘YY’ males (\pm sd)	98.9 (\pm 3.1)	100 (\pm 0)

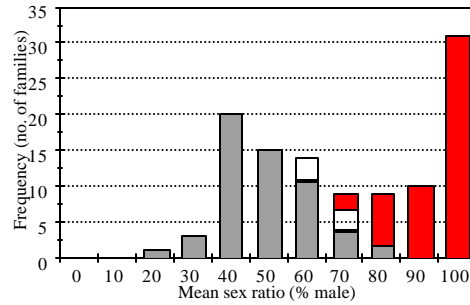
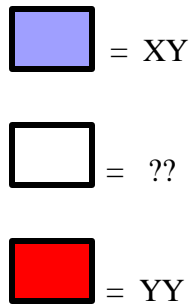
Figure 2. Histograms showing frequency distributions for family sex ratio in the progeny testing for ‘YY’ males from $XYD \text{♀} \times XY \text{♂}$, $XYD \text{♀} \times YY \text{♂}$, and $YYD \text{♀} \times YY \text{♂}$ crosses and for ‘YY’ females from $XYD \text{♀} \times YY \text{♂}$ and $YYD \text{♀} \times YY \text{♂}$ crosses (10% class intervals were used except that the rightmost column represents only 100% males).



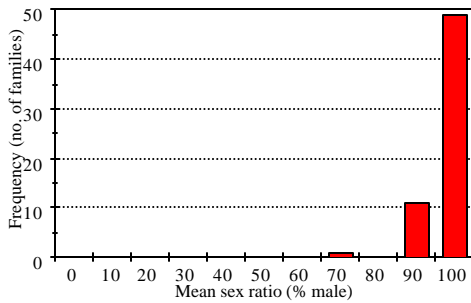
a) Males from XY x XY crosses



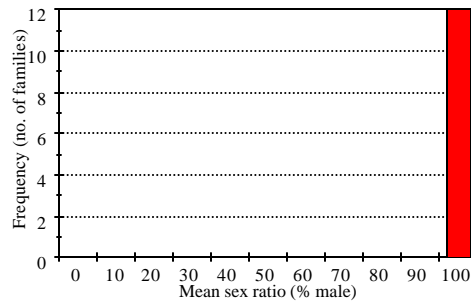
b) Males from XY x YY crosses



c) Females from XY x YY crosses



d) Males from YY x YY crosses



e) Females from YY x YY crosses

Discussion

Sex determination in *O. niloticus*

Our results provide unequivocal evidence for an underlying basic monofactorial mechanism of sex determination in this strain of *O. niloticus* and also for the viability of novel 'YY' males. The results from the progeny testing for 'YY' males from XY x XY crosses, in which 'YY' males were present in expected proportions, indicates that the 'YY' males have viability and fertility equivalent to normal males. A similar pattern was observed with the progeny testing of males from the XY x YY crosses, with the number of 'YY' males not differing significantly from that expected based on a theory of segregation of a single male determining factor (gene or supergene).

The distinct bimodal sex ratio distribution of the families from these two sets of progeny tests, and the relatively low proportion of unassigned genotypes, supports the validity of the arbitrary criterion used in the designation of genotype. This is further supported by the highly significant heterogeneity of the sex ratios of all families, considered together, compared to the relative homogeneity of sex ratios from the groups of males designated separately as XY and YY genotypes. Progeny sex ratios of males designated as normal XY males varied from 34.2 to 70.0% but with a mean close to 50% male, with a slight but significant overall excess of males. These sex ratios, thus, appear to be normally distributed about the expected 1:1 ratio of males to females. This distribution is similar to, but slightly more heterogeneous than that observed for the same Egyptian strain of *O. niloticus* by Mair et al. (1991a) and slightly more homogenous than those observed by Shelton et al. (1983) in an Ivory Coast strain of *O. niloticus*. Lester et al. (1989) observed considerably more heterogeneity in the sex ratios of families collected from a mix of strains, some of which were known to be introgressed with *O. mossambicus* (Macaranas, et al. 1986). The authors interpreted these high levels of heterogeneity for sex ratio as evidence for polyfactorial sex determination in this species and it is possible that some strains of "*O. niloticus*" may have such polyfactorial mechanisms, especially when introgressed with other species.

The sex ratios produced by the males that were designated as YY genotypes were not quite in accordance with those predicted by the hypothesis of simple monofactorial sex determination, many of them being somewhat lower than the expected 100% male. This observation is in accordance with the results of Mair et al. (1991a) who observed a single female in the progeny of one of four 'YY' males progeny tested. Scott et al. (1989), on the other hand observed no females, in the sexing of 285 progeny of a single YY male crossed to ten separate females. Similarly, Varadaraj and Pandian (1989) observed no females among the progeny of eight 'YY' females in *O. mossambicus*. It is probable that the greater sample sizes and the larger number of fish tested are responsible for the more numerous observations of females in progeny of 'YY' males in this study. The overall sex ratio from 'YY' males was greater than 95 % male with the majority of ratios being 100%. There was no apparent trend in the occurrence of these aberrant sex ratios that would indicate the segregation of a single

autosomal sex modifying locus as postulated for *O. niloticus* by Hussain et al. (1994) and demonstrated for *O. aureus* by Mair et al. (1991b).

The increase in the overall proportions of males in the progeny testing of YY genotypes derived from crosses of selected (on the basis of the 100% male sex ratios in initial progeny tests) 'YY' males indicates some form of response to this selection and thus a genetic basis to the occurrence of these aberrant females. Similarly the higher than average proportions of males in repeat matings of "selected" (i.e. produced >96% male sex ratios in initial progeny tests) 'YY' males provides further support for this hypothesis of a genetic basis. It cannot be discounted however, that the mean sex ratios of all fish designated as YY was lower than that from the repeated matings of 'selected' 'YY' males due to the erroneous inclusion of one or more sex ratios from XY males, incorrectly designated as YY.

In a recent study Capili (1995) demonstrated that these "rare" females, arising in progenies of 'YY' males, conform to the expected XY genotype in progeny tests with known genotypes, in the same strain. In a separate study Capili (1995) also noted the existence of significant paternal and maternal effects on the occurrence of rare females in the progeny of 'YY' males. It seems likely that a series of autosomal sex modifying genes, acting as a threshold trait, may be responsible for the occurrence of these rare females, and that these autosomal genes could be selected against to further increase the proportion of males in the progeny of 'YY' males. It is also likely, however, that there is a temperature effect on sex differentiation. This has been demonstrated in the occurrence of males in the putative all female progeny of sex reversed $XX\Delta\sigma\sigma$ (Baroiller et al. 1995 and J. S. Abucay FAC, CLSU, unpublished data) and may also play a role in the occurrence of these rare females. The seasonally higher temperatures encountered in the Philippines, which are more extreme than those in the natural range of this species, may also be a factor in the slight excesses of males in the progeny of normal XY males observed in this and other studies.

Potential for application in aquaculture

The successful feminization of the YY genotype is the first to be reported in tilapia, and is a vital step in the development of the YY technology on a large scale, as it makes possible the production of 'YY' males without the need for time consuming progeny testing. In their work on *O. mossambicus* in the 1970s, Yang et al. (1980) produced up to 600 'YY' males in XY x YY matings, although it is not clear whether these were identified in progeny testing. There are no reports of 'YY' males being used commercially for the production of all-male *O. mossambicus* and it seems likely that these 'YY' males were not produced on a commercial scale, possibly due to a failure to produce 'YY' females by feminization. We hypothesised that 'YY' males may be more resistant to feminization. This was investigated in a study by Abucay and Mair (in press), in which feminized females and non-feminized males from DES treated progeny of XY x YY crosses were progeny tested; evidence was presented for differential feminization of XY and YY genotypes in one of three families from which males were tested. Our data do not support the hypothesis of differential feminisation of XY and YY genotypes, although it is possible that this occurred in two of the nine families from which males were tested.

The successful mating of 'YY' males and 'YY' females (all the progeny of which were male) and the confirmation of the YY genotype of the males and DES treated females from these crosses, establishes the potential for development of this technology on a commercial scale. 'YY' males and females can now be produced in large numbers for use as broodstock to mass produce 'YY' males. In turn these can be crossed with females, which can also be mass produced from XXΔ♂ x XX♀ crosses, to generate commercially applicable numbers of Genetically Male Tilapia (GMT) for culture.

The progeny of the 'YY' males ("GMT" distinguishes them from sex reversed male tilapia), have been comprehensively evaluated in on-station and on-farm trials. Results from on-station trials indicate that GMT have very considerable benefits under culture, significantly increasing yields by up to 58%, compared to mixed sex tilapia of the same strain (Mair et al., 1995). Yields of GMT are also consistently greater than those for sex reversed male tilapia. In addition to the negligible recruitment in GMT populations, they have the further advantages of more uniform harvest size distribution, higher survival, and better food conversion ratios.

There are several important comparative advantages and disadvantages of the YY male technology as a means for production of monosex male tilapia, as compared to other commonly used alternatives such as manual sexing, hybridisation and sex reversal. The technique can be considered environmentally friendly as no hormones are applied to fish that are consumed and overall, hormone application to broodstock is very low. Species\strain purity is maintained and the fish produced for culture are normal genetic males. Although the development process is time consuming and labour intensive, once developed the production of monosex males can be maintained through occasional feminisation of YY genotypes. Provided that broodstock purity can be maintained, the technology can be applied (at the level of spawning 'YY' males with normal females) in existing hatchery systems without any special facilities or labour requirements. Discounting the initial development costs, additional costs for application of this technology at the hatchery level would be minimal, while the potential economic advantages to growers have been demonstrated to be very considerable.

Attempts are being made to extend this technology to the tilapia culture industry in the Philippines, as a unique example of application of genetic manipulation technology in developing country aquaculture.

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