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Production of Genetically Female Common Carp, *Cyprinus carpio*, through Sex Reversal and Progeny Testing

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Abstract

The common carp, *Cyprinus carpio* L., an introduced species to India, is an important species for aquaculture and enhanced fisheries in Karnataka state. Common carp has a number of advantages over the other carp species. However, one of the major disadvantages is that the present stock exhibits early sexual maturation and unwanted reproduction during grow-out, resulting in suppressed growth and small size at harvest. Considering the importance of the species, there is a need to find suitable solution(s) to this problem. The production of sterile or monosex populations are among the options. Fry of different age and size groups were treated with two androgens, namely 17α-methyl testosterone (MT) and 17α-methyl-di-hydroxy-testosterone (MDHT), in a series of trials. MT treatments at 100 $mg{\cdot}kg^{-1}$ yielded only partial sex reversal (77.14% male), while MDHT treatments at 50 and 100 $mg{\cdot}kg^{-1}$ resulted in complete masculinization (100% male) in "small" size 50 day old common carp. The hormone treated fish were crossed with normal females to identify neo-males, which produced all or predominantly female progeny. The results indicate the potential of MDHT for hormonal masculinization and the possibility of producing all female common carps through this approach.

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Introduction

Common carp is one of the most widely cultured carp species. It has been transplanted into many countries outside its natural range, so much so that it is now one of the most widely distributed freshwater fish species (Jhingran & Pullin 1988; FAO 2005). Its hardiness, fast growth, easy propagation, omnivorous feeding habit, ability to readily accept supplementary feed, resistance to disease and tolerance to a wide range of climatic conditions have made common carp a popular species in many countries. It was introduced in India in 1937 (Prussian strain) and in 1957 (Bangkok strain) (Jhingran 1991) to utilize the pond bottom food resources in composite fish culture and has now become an integral part of the Indian freshwater aquaculture. In the state of Karnataka located in South Central India, it represents 43% of the total fish seed stocked in different water bodies in the state and its contribution to inland fish production is around 35%. Unlike the Indian major carp species, it can be spawned throughout the year under controlled conditions, however, early maturation can be a major disadvantage since common carp can spawn in culture systems well before harvest, resulting in a large number of small sized fish, which can reduce the yield of marketable fish. Besides upsetting the stocking density, a considerable amount of energy is used for gonadal development and maturation. Early maturation in common carp appears to be common in tropical and subtropical environments, unlike in temperate climates where the species commonly matures at two years old or later. Even if fish do not spawn, gonadosomatic index (GSI) can exceed 20% of the harvested weight of an individual fish (Jhingran 1991; Basavaraju et al. 2000).

Considering the potential of this species, it is essential to find solutions to this problem of unwanted reproduction in production ponds. One potential method to overcome this problem is to eliminate one of the sexes or to produce sterile individuals. A technique to induce sterility in common carp through feeding fry with high doses of 17α-methyltestosterone (MT) has been developed (Rao & Rao 1983; Rao et al. 1984). In the present study, the approach of producing all female populations through sex reversal and progeny testing was investigated. Although the male and female genotypes are established at fertilization, phenotypic sex differentiation occurs later in development. There is a window of opportunity, which varies between fish species, during which phenotypic sex can be altered by administration of estrogens or androgens to produce all female or all male populations, respectively. This can result in fish with the sex opposite to that which their genotype would normally have determined.

Hormonal sex reversal can be used directly (where hormone treated fish are grown for consumption) or indirectly (where the sex determination system is manipulated to produce broodstock fish that will give rise to monosex offspring) to create monosex populations for aquaculture. This has been demonstrated to prevent spawning in pond culture in tilapia, where phenotypically monosex males can be produced via androgen treatment (Shelton et al. 1981; Rothbard et al. 1983) or genetically male tilapia can be produced by manipulation of the sex determination system (Mair et al. 1997). In other species "indirect" monosex female culture has been used where females grow faster or show some other advantages in aquaculture, e.g. in salmonids (Johnstone et al. 1978; Donaldson & Hunter 1982) and in the silver barb (Pongthana et al. 1999). Used in combination with gynogenesis or androgenesis, this technique can be used to establish inbred lines with fish of either sex (Streisinger et al. 1981; Nagy & Csanyi 1984; Sarder et al. 1999).

Even though MT, the most commonly used synthetic hormone in masculinization of fish has been used extensively in direct sex ratio manipulation (mostly in tilapia), and is known to be eliminated rapidly from the body of treated fish (Johnstone et al. 1983; Rao et al. 1990), active residues and metabolites may persist for some time (Gomelsky et al. 1994) and consumers may be apprehensive about consuming hormone treated fish. The combination of neo-male (genetically female but functionally male) production through sex reversal and the use of these neo-males to produce all female populations appears to be a safe and practical approach for the common carp (Gomelsky 2003). Earlier studies in this species did however indicate effects of size and age of fry at the start of treatment on the efficiency of androgen sex reversal (reviewed by Gomelsky 2003). The present study investigated this approach, using MT and 17α-methyldihydroxy testosterone (MDHT) for sex reversal with treatments of fry of different ages and sizes. Unlike MT, MDHT is not susceptible to aromatisation, which can lead to paradoxical feminization (Devlin & Nagahama 2002), and may therefore prove to be more efficient for hormonal masculinization in some cases.

Materials and Methods

A group of male and female common carps (10 breeding pairs) was used for producing the fry for experimental purposes with *Hydrilla* plants as the spawning substrate for egg deposition. The fry were reared in $4m^2$ concrete nursery ponds with red soil base until the start of the experiment. The 17α-methyltestosterone, MT ($C_{20}H_{30}O_2$) and 17α-methyldihydroxy-testosterone, MDHT ($C_{20}H_{32}O_2$) (both from Sigma, U.S.A) were the two androgens used in the present study.

Hormone incorporated feed was prepared at the beginning of the trial using the alcohol evaporation method (Guerrero & Shelton 1974). The ingredients, groundnut oil cake and rice bran in powder form, were mixed in a ratio of 1:1 w/w. The required amount of hormone(per kg of feed) was dissolved in 500 ml of 96% ethanol and sprayed over the powdered feed, kept in a tray and mixed thoroughly, to ensure uniform distribution of the hormone. The hormone-incorporated diet was then air dried at room temperature. The control diet was prepared in the same manner using only the solvent. Diets containing different levels of the hormone were prepared separately, sealed in plastic bags and stored at room temperature. This feed was used throughout the experimental period.

Hormone treatment of fish was carried out in 100 litre fiberglass tanks. The tanks were kept inside the field laboratory to avoid algal growth in water due to direct sunlight. The tanks were disinfected with potassium permanganate, flushed with water, dried and later filled with fresh water prior to the start of the experiment. In the first experiment using MT, two size and two age groups of common carp were stocked at 100 per tank (i.e. one $fish \cdot l^{-1}$) for hormone treatment (Table 1). The fish were fed *ad libitum* four times a day and the experiment was conducted in triplicate. Excess feed was removed and 75% of the water was exchanged every day. The fish were sampled and weighed every week to assess the growth of the fry and to record mortality. Based on earlier reports, 100 $mg \cdot kg^{-1}$ MT was used for a duration of 40 days (Nagy et al. 1981; Gomelsky et al. 1994). For comparison, ungraded controls were maintained and fed on hormone free diet.

In the second experiment, using MDHT, two different concentrations (50 and 100 mg $hormone \cdot kg^{-1}$ feed) of hormone were assessed (Table 2). Only one age group (50 day old) with two size groups (small and large) of common carp fry, derived from the same spawning set, were used.

Table 1. Hormone concentration (17α - methyltestosterone), treatment period, treatment group and mean stocking weight of common carp, *Cyprinus carpio*

Treatments	Hormone concentration. ($mg \cdot kg^{-1}$ feed)	Period of treatment / duration (days)	Group	Nos. stocked	Mean weight (g) of replicates
Control	Nil	45-100 (40)	Random	100 100 100	0.21[b] ±0.001
MT-1	100	45-84 (40)	Small	100 100 100	0.10[c] ±0.003
MT-2	100	45-84 (40)	Large	100 100 100	0.32[a] ± 0.006
MT-3	100	61-100 (40)	Small	100 100 100	0.10[c] ±0.002
MT-4	100	61-100 (40)	Large	100 100 100	0.32[a] ±0.007

Values within a column with different letter notations are significantly different ($P < 0.05$).

After the hormone treatment period, the fish were reared in cement cisterns, following standard rearing protocols. When the fish attained sexable age (2.5-3 months post hatching) and size (5-10g), twenty-five fish from the control and hormone treated groups were randomly selected and sacrificed for sex assessment. The sex was assessed by squashing the gonadal tissue using the acetocarmine squash technique (Guerrero & Shelton 1974). The data on survival and sex ratio was subjected to t-test after arcsine transformation to determine differences within the treatment (between replicates) and subsequently one way ANOVA using SPSS statistical package (ver. 7.5).

Progeny testing

Males from selected sex reversal treatments (groups with >95% of males in sampled fish) and control groups were maintained as future breeders in earthen ponds after tagging using PIT tags. As and when these males matured and started oozing milt, they were crossed with normal control females in single pair crosses for progeny testing. Control (normal males crossed with normal female) progeny were also produced for comparison. Progeny from 27 MDHT treated males in two batches of 12 in the first batch (five fish from 50 mg and seven from 100 mg) and 15 (nine fish from 50 mg and six from 100 mg) in the second batch and four control males were produced. The progeny were reared in cement cisterns following standard rearing protocols and later sexed after attaining sexable age and size to identify the neo-males giving all female progeny.

Results

Sex reversal experiments using MT

In the experiment using 17α-Methyl Testosterone (MT), there were no significant differences in weight at stocking between the "small" fish at 45 days and 61 days or between the "large" fish at these two ages, while there were significant differences between the different size groups at both ages ($P<0.05$) (Table 1). The survival of these common carp fry assessed at the same time varied during the hormone treatment period (Tables 2 and 3). Compared to the control (85.0%), the survival was low in small size groups (58.3% in MT1 and 76.3% in MT3), while it was high in large size groups (95.6% in MT2 and 98.6% in MT4). This indicates that survival was directly proportional to the size and age of the fish at stocking (Table 2).

Table 2. Summary of common carp survival during hormone treatment and post hormone treatment period and sex ratios using 17α-methyltestosterone (MT)

Treatment	Period of treatment (days)	Nos. stocked per replicate	Survival during treatment		Survival during post hormone treatment period (90-days)		Sex ratio (male:female+ intersex)
			Nos. survived	% Mean	Nos. survived	% Mean	
Control	45-100	3 (300)	255	85.00^{ab} (SD 5.19)	188	62.66^{ab} (SD 8.38)	$1.22:1^{b}$
MT-1	45-84	3 (300)	175	58.33^{c} (SD 15.30)	107	35.66^{c} (SD 14.04)	$2.93:1^{ab}$
MT-2	45-84	3 (300)	287	95.66^{a} (SD 2.51)	228	76.00^{a} (SD 2.65)	$1.40:1^{b}$
MT-3	61-100	3 (300)	229	76.33^{b} (SD 4.5)	134	44.66^{b} (SD 5.86)	$5.09:1^{a}$
MT-4	61-100	3 (300)	229	76.33^{b} (SD 1.15)	134	44.66^{b} (SD 11.59)	$5.09:1^{a}$

Values within a column with different superscripts are significantly different ($P< 0.05$).

Table 3. Details of common carp survival during treatment and post treatment-rearing period in the experiment using 17α-methyldihydroxytestosterone (MDHT)

Treatment	Period of treatment (days)	Replicates/ nos.	Survival during post treatment		Survival during post rearing period (90-days)		Sex ratio (Male:Female + Intersex)
			Nos. survived	% Mean	Nos. survived	% Mean	
Control	45-100	3 (300)	258	86.00 [a] (SD 4.36)	185	61.67 [b] (SD 4.62)	2.16:1 [b]
MDHT-1	45-84	3 (300)	127	42.33 [c] (SD 3.22)	85	28.33 [d] (SD 6.66)	1.00:0 [c]
MDHT-2	45-84	3 (300)	284	94.66 [a] (SD 4.04)	237	79.00 [a] (SD 3.00)	5.25:1 [a]
MDHT-3	61-100	3 (300)	169	56.33 [b] (SD 4.93)	134	44.67 [c] (SD 1.15)	1.00:0 [c]
MDHT-4	61-100	3 (300)	282	94.00 [a] (SD 3.00)	226	75.33 [a] (SD 8.14)	2.84:1 [b]

Values within a column with different superscripts are significantly different ($P< 0.05$).

The survival rate during the post hormone treatment rearing period (90 days) also indicated a similar trend to that seen during the hormone treatment period. The smaller fish showed low survival (mean survival 35.6% in MT1 and 44.6% in MT3) compared to large sized fish (76.0 % in MT2 and 64.3 % in MT4) and the control (62.6%). There was no significant difference in the survival rate (P>0.05) between the large size groups of different treatment ages (MT2 and MT4) at the end of this period, but there was a significant difference between the small size groups of different ages (MT1 and MT3) (P<0.05) (Table 2).

The control group showed >22 % more males so skewed to males, which is more towards males than the expected normal sex ratio but statistically does not differ from the normal sex ratio of 1:1. Fully mature gonads were observed in the control group, showing normal development. In this experiment the hormone treated groups contained male, female and intersex fishes (Fig. 1). A maximum of 77.1% males was obtained in MT3, the smaller, older group, followed by MT1 (68.5%) and MT4 (60.8%). There was no significant difference between the sex ratios of the control (55.0%) and MT2 (57.8%) or between the small size groups of different age (MT 1 and MT3). However, there was a significant difference between the large size groups of different ages (MT2 and MT4), which implies that both age and sex have effects on the sex ratio.

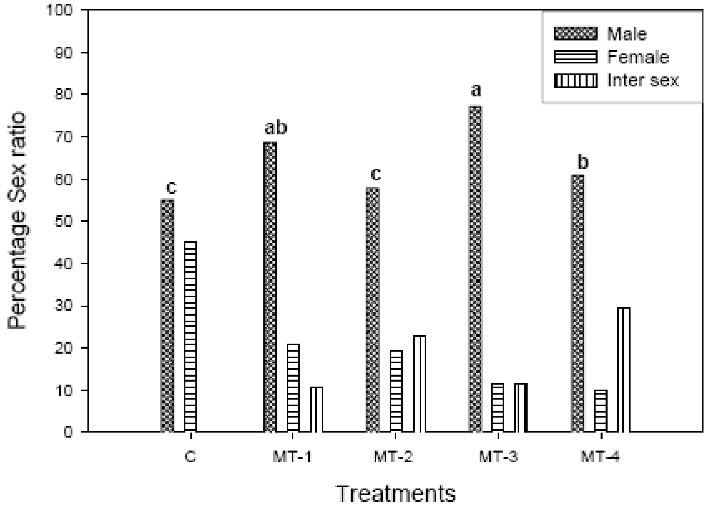

Figure 1. Sex ratios in control and hormone treated groups of common carp in the experiments using 17α-methyltestosterone at 100 mg•kg^{-1} feed

The percentage of intersexes was highest in MT4 (29.3%) followed by MT2 (22.8%), MT3 (11.4%) and MT1 (10.7%). However, there was no intersex fish in the control group. The presence of females and hermaphrodites in treated fish showed that the treatments did not totally reverse sex under the given experimental conditions.

Sex reversal experiments using MDHT

Since the experiment with MT did not yield sex ratios close to 100% male in the treated groups, another experiment was carried out using MDHT. The details of weight and age at stocking are presented in table 4. Survival of the fish varied significantly between treatments. Survival (Table 3) was lower in the small sized treated groups (42.3% in MDHT1 and 56.3% in MDHT3) and significantly different ($P<0.05$) from the control groups (86.0%) during the hormone treatment period, while the survival was comparatively high in the large size groups (94.6% in MDHT2 and 94.0% in MDHT4), although not significantly different from the controls ($P>0.05$). The survival rate during the post treatment period also followed a similar trend to that seen during the hormone treatment period.

The sex ratios observed in the different treatment groups are given in figure 2. In this trial 100% males were recorded in the small size groups from both the concentrations (50 and 100 mg•kg^{-1}). In the larger size groups male, female and intersex fishes were recorded, and the percentage of males in both these groups and the controls were significantly lower than in groups MDHT-1 and MDHT-3. The percentage of intersex fish was much lower in this trial as compared to the previous experiment with MT. In the control group there were no inter sex fishes.

Table 4. Size, age of the common carp, hormone concentration and duration of the hormone treatment using 17α-methyldihydroxytestosterone

Treatment	Hormone concentration (mg•kg^{-1})	Period (days)	Group	Rep	No. stocked	Mean weight (g)	Mean weight (g) of replicates
Control	-Nil-	51-100 (50)	Random	1 2 3	100 100 100	0.147 0.149 0.150	0.148[b] ±0.001
MDHT1	50	51-100 (50)	Small	1 2 3	100 100 100	0.053 0.054 0.050	0.052[c] ±0.001
MDHT2	50	51-100 (50)	Large	1 2 3	100 100 100	0.244 0.243 0.235	0.24[a] ±0.003
MDHT3	100	51-100 (50)	Small	1 2 3	100 100 100	0.050 0.050 0.053	0.051[c] ±0.001
MDHT4	100	51-100 (50)	Large	1 2 3	100 100 100	0.242 0.241 0.240	0.241[a] ±0.006

Values within a column with different letter notations are significantly different (P< 0.05).
Rep = replicate

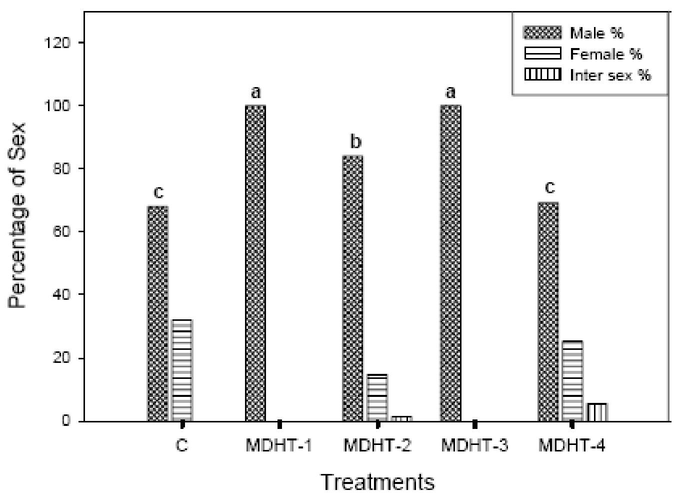

Figure 2. Sex ratios in control and hormone treated groups of common carp in the experiments using 17α-methyldihydroxytestosterone

Progeny testing

Males from selected sex reversed fish (100% male treated with MDHT) were crossed with normal females. Progeny from 27 treated males (in two batches of 12 and 15) and 4 control males were produced. Twelve males of MDHT batch-I that were progeny tested in individual pair crosses to control females did not give satisfactory results. Among the sexed progeny only one fish (100 mg MDHT) had a sex ratio significantly different from 1:1 ($p<0.05$). The first batch of 12 fish did not yield satisfactory results whereas in the second batch two of nine fish (50 mg MDHT) yielded 100% females, while two of six (100 mg MDHT) yielded greater than 85% females (Table 5).

Table 5. Summary of sexing from the progeny of MDHT treated fish

Treatment	Hormone treated males	Batch-1		Batch-2	
		No. females (%)	No. males (%)	No. females (%)	No. males (%)
MDHT-1 50 mg•kg^{-1}	1	11(44)	14(56)	12 (48)	13(52)
	2	14(56)	11(44)	25(100)	0(0)
	3	12(48)	13(52)	14(56)	11(44)
	4	14(56)	11(44)	15(60)	10(40)
	5	15(60)	10(40)	16(64)	9(36)
	6	-	-	25(100)	0(0)
	7	-	-	11(44)	14(56)
	8	-	-	20(80)	5(20)
	9	-	-	16(64)	9(36)
Control	1	-	-	12(48)	13(52)
MDHT-1 100mg•kg^{-1}	1	13(52)	12(48)	18(72)	7(28)
	2*	7(28)*	18(72)	15(60)	10(40)
	3	16(64)	9(36)	24(96)	1(4)
	4	11(44)	14(56)	20(80)	5(20)
	5	14(56)	11(44)	16(64)	9(36)
	6	9(36)	16(64)	22(88)	3(12)
	7	16(64)	9(36)	-	-
Control	1	10(40)	15(60)	9(36)	16(64)
Control	2	-	-	12(48)	13(52)

Discussion

Among the various androgens used for the manipulation of sex in fish, MT has been the most common one found to be effective in producing male dominated populations by sex reversal (Devlin & Nagahama 2002). Basavaraja (1984) could not induce sex reversal in the local strain of common carp with MT, but obtained a male dominated population by the suppression of ovarian development. In the present investigation, administration of MT resulted in male dominated populations along with some intersex fish and females, which agrees with most of the earlier studies. However, treatment of “small” 50 day old fish with MDHT for 50 days yielded 100% male populations. The poor survival of the fry during the hormone treatment period in the small size fish group was probably due to frequent handling while cleaning left out feed and dependence of fish solely on artificial feed. The occurrence of ovo-testis (intersex) in some of the androgen-treated groups is an indication of incomplete sex reversal, as observed by Nagy et al. (1981) in MT treated gynogenetic females.

Previous histological studies have shown that the critical androgen-liable developmental stage in cyprinids (Gomelsky 1985) seems to occur after anatomical gonad differentiation but prior to cytological sex differentiation. For routine application of sex inversion, it is important to choose some practical indicators to determine the onset of this period. The data from the present investigation suggests that it is not only the age but also the weight of the fish that is equally important in determining the appropriate period of androgen treatment. This result is in agreement with the work of Gomelsky (1985) in common carp, using MT, although the fish used in the present experiments were smaller at the start of the hormone treatment. Mirza & Shelton (1988) have also reported the differential effects of this hormone in sex reversal of silver carp depending on the age/size of the seed.

Excessive androgen treatments have been reported to cause sterility or paradoxical feminization, the latter occurring in the case of aromatizable androgens such as MT but not in the case of non-aromatizable androgens such as MDHT (reviewed by Devlin & Nagahama 2002).

Based on the findings of the present study, it could be said that the oral administration of MDHT (50 $mg{\cdot}kg^{-1}$) to common carp fry (50 day old) is more potent than feeding with MT for complete sex reversal as both hormones were tested under almost identical conditions. The present study demonstrated that MDHT could completely sex reverse females to males in common carp at a dose of 50 $mg{\cdot}kg^{-1}$ fed to 50 day old small fry for 50 days. The labile period for sex reversal appears to be related to both size and age of the fish.

Studies on progeny testing for indirect production of all female common carp progenies by mating normal XX female with inverted XX neo-males were carried out by Nagy et al. (1981), Gomelsky (1985) and Cherfas et al. (1996) and also for selective breeding in association with induced gynogenesis (Cherfas & Tzoy 1984; Nagy & Csanyi 1984). The results from the progeny testing in the present study showed that some of the fish in the MDHT treatment groups were neo-males. In these preliminary findings the first batch of progeny testing did not yield satisfactory results hinting that the first batch of 12 fish tested (five fish from 50 mg MDHT and seven fish from 100 mg MDHT) may be genetically males. In the second batch of progeny testing, two of nine fish from 50 mg MDHT yielded 100% all female progeny and two of six fish from 100 mg MDHT yielded 90% female progeny. The results obtained in the present study indicate that 50 mg•kg^{-1} MDHT may be the most suitable option for complete sex reversals in common carp.

Conclusion

Treatment of common carp fry (50 day old) with MDHT (50 mg• kg^{-1}) produced some groups showing 100% male sex ratios. Progeny testing indicated that some of these fish were functional XX neo-males, the basis for a breeding programme to produce genetically female common carp. The production of mono-sex females from crosses between neo-males and ordinary females can be used to prevent reproduction during growout, and would not involve any hormone treatment of fish intended for consumption. It is likely that there would also be a small increase in production from a mono-sex female culture (Gomelsky 2003).

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