

Influence of 17-alpha methyl testosterone on the production parameters of common carp (*Cyprinus carpio* L.) fry

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SUMMARY

During our research we aimed at finding an answer as to what extent the different concentrations of 17-alpha methyl testosterone incorporated in the diet of common carp fries can influence the production parameters of the species, as well as how efficient their sex-reversal can be with the use of this method. To this end, an aquarium experiment was conducted in the course of which four different hormone treatments were set and monitored. The fish feed was enriched with 17-alpha methyl testosterone in 50 ppm, 75 ppm, 100 ppm, 500 ppm dosages.

The obtained figures revealed that the hormone treatments had no influence on the production parameters and conservation of the common carp fries. Further on, our team is to determine the sex of the fish through the examination of gonads during autopsy when they reach the 500 g average weight.

Furthermore, a male specific test method which was supposed to be of great help in our attempt to select the sex-reversed specimens in the subsequent processes was also put to the trial. During the experiment the DNA-isolation of different sample types (muscle tissue, fin, mucus) of common carp with identified sex was successfully carried out. The extracted PCR product was examined with agarose gel. Our results indicated that the ccmf2 marker was applicable, however, the obtained figures were not reliable.

Keywords: common carp, sex-reversal, methyl testosterone, performance

INTRODUCTION

Common carp (*Cyprinus carpio* L.) is a native fish species in Europe and Asia. The carp is a type of freshwater fish, which -due to the natural geographical features of Hungary- is bred in some of the most significant ponds of the country (Molnár 2009). The ever-intensifying fish breeding in pond farms calls for a better understanding and application of production increasing methods. One of the possible solutions to this matter may be the exploitation of better somatic growth abilities in mono-sex seed carp stocks as opposed to those of the mixed-sex populations (Cherfas et al. 1996, Kocour et al. 2003, 2005). Establishing mono-sex seed carp stocks occurs on the basis of common protocols, which also applies to mother fish breeding. The production of motherfish stocks is accomplished by artificially induced gynogenesis and by the technique of sex-reversal through hormone treatment (Komen et al. 1988, 1991, 1992, 1993; Cherfas et al. 1993, Horváth and Orbán 1995, Ben Dom et al. 2001, Gomelsky 2003, Komen and Thorgaard 2007).

In the course of sex-reversal the aim is to convert the female fish specimens into phenotype males. This type of sex-reversal can be effectuated by steroid-based hormones (Shelton et al. 1995, Colborn et al. 1993, Damstra et al. 2002) The steroids are ingested into the body generally by oral administration, i.e. supplemented in the fish food or by dissolving in water (Piferrer 2001, Devlin and Nagahama 2002, Mubarik et al. 2011). The most frequently used androgen hormone is the 17-alpha methyl testosterone. It is a synthetic hormone which has fast absorbing

qualities, does not accumulate in the body and quickly eliminates from the organism (Sumpter 2005).

The hormone treatment must be applied in the spawning phase, which is a sensitive period in the sexual development of the fish. During the treatment of gynogenic female common carp. Researchers found that along with a 20% mortality rate, a 80% sex-reversal efficiency rate can be achieved if the fish are fed on ad-libitum of fish food supplemented with 100 ppm dosage of 17-alpha methyl testosterone for 44.–80. days after hatching (Nagy et al. 1981, Hulak et al. 2007, Singh 2013, Jensi 2016). Other scientists, however, carried out treatments on mixed-sex stocks in a similar way. By applying different hormone concentrations, they obtained a 100% male rate together with a 100% stock conservation rate, on the basis of which the efficiency of the hormone treatment could be assessed (Mubarik et al. 2011).

Investigating the above problem, in our current research we seek to get an answer to the question as to what extent the different concentrations of 17-alpha methyl testosterone incorporated in the fish food can influence the production parameters of common carp fries as well as what effect this method has on their conservation. In the future we target at determining the success of the hormone treatment on the basis of female and male proportions. Furthermore, the usability of sample types (muscle tissue, fin, mucus) from different common carp specimens with identified sex is to be revealed by testing PCR markers (ccmf1, ccm2, ccm3) described by Chen et al. (2009, 2010).

MATERIALS AND METHODS

The preparation of 17-alpha methyl testosterone solution

The 17-alpha methyl testosterone (69240-5G, Sigma-Aldrich) was placed into 50 ml Falcon test tubes (525-0159, VWR) and 30 ml 70% pharmacy alcohol was added to it. During the preparation of the solutions, XB 120A Precisa analytical balance scales were used. The dissolution of the hormones was accelerated with the help of a vortex test tube shaker (Velp Scientifica, zx³) used for 1 minute at maximum speed. The solutions were stored in the tubes covered with aluminum foil.

The application of 17-alpha methyl testosterone to the feed

The previously prepared 30 ml solutions with different concentrations (50 ppm, 75 ppm, 100 ppm, 500 ppm) were applied separately to 0.5 mm and 1.1 mm fish feed (Mubarik et al. 2011). The application method involved spreading the feed evenly in the extraction machine (Biobase) and continually sprinkling it with a sprayer. It took approximately 20 minutes for the alcoholic spray to dry. Then the evenly sprayed dry feed was thoroughly turned over by hand.

Parameters of treatments

The experiment was carried out in the laboratory of fish biology at the University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management. The fish fries under examination were from the local population of Szeged landrace common carp and they were provided by NAIK-HAKI from the artificial reproduction of 16 May 2017. The fish hatched on 19 May 2017. The experiment took place from the 45 to 80 days after hatching, which is in accordance with what is described in the literature (Nagy et al. 1981). Each treatment was repeated three times. The experiment was conducted in 15 pieces of

180 l capacity aquariums, all equipped with a unique biological filter and an air diffuser. 20 pieces of 45-day-old mixed-sex common carp fries were placed in each of the aquariums. The aquariums also went through a daily 20% water change and substrate cleaning. Water temperatures and dissolved oxygen levels were also inspected on a daily basis (Hach HQ 30d) in three randomly selected aquariums. The average water temperature was 24.42±1.5 °C and the average dissolved oxygen level was 7.96±0.6 mg per l during the experiment. These figures are considered optimal for common carp fry rearing. In the first 24 days the fish were given feed of 0.5 mm particle size Biomar Inico Plus (58% crude protein, 15% crude fat), and for an additional 11 days on feed of 1.1 mm particle size (58% crude protein, 18 crude fat) (*Table 1*). In the research we applied ad-libitum demand-feeding three times a day (Csorvási 2015).

Table 1

Feed composition		
Granule size	0.5 mm	1.1 mm
Crude protein	58%	58%
Crude fat	15%	18%
Ash	11.20%	10.80%
Crude fibre	0.40%	0.40%
Phosphorus	1.70%	1.71%
Calcium	2.38%	2.31%
Sodium	1.04%	0.94%

The examined four groups were placed in block layout so that the effects of external environmental factors (sunlight, air temperature) could be reduced (*Table 2*). The individual body weight of the fish was measured (with VWR SE 422 – 0.01 g precision) both at the beginning and at the end of the experiment during all the treatments and repetitions.

Table 2

Aquariums arrangement

Control	50 ppm	75 ppm	100 ppm	500 ppm	Control	50 ppm	75 ppm
100 ppm	500 ppm	Control	50 ppm	75 ppm	100 ppm	500 ppm	

Post-rearing

The experimented groups of the separate treatments were relocated into 5 pieces of 350 litre recirculation aquaculture system (RAS). Depending on the survival rate we created 53-60 pieces per 350 l stock density groups. The post-rearing of the animals took place at this location up to their age of 5 months. During the post-rearing process, we measured the average weight by taking 20 sample pieces per treatment which also allowed to draw conclusions on the biomass. At the beginning of the research ad-libitum demand-feeding was performed with 1.1mm, later on 3 mm and 4.5 mm feed particle sizes Biomar Efico Alpha in accordance with the growth of the fish (*Table 3*). Post-rearing is continued until the groups

reach the 500 g average weight. For specimens of this size sex differentiation can be detected with great certainty (Nagy et al. 1981, Mubarik et al. 2011).

Table 3

Feed composition at post-rearing		
Granule size	3 mm	4.5 mm
Crude protein	40%	43%
Crude fat	23%	14%
Ash	3.4%	5.7%
Crude fibre	6.2%	4.5%
Phosphorus	0.85%	0.86%
Calcium	0.96%	0.84%
Sodium	0.29%	0.15%

DNA isolation and polymerase chain reaction (PCR)

The sex identification of common carp specimens was carried out by DNA isolation. E.Z.N.A tissue kit (D3396-01) was used for the protease digestion of 30mg muscle tissue [h], fin [ú], mucus [n] in water for 90 minutes. The samples were stirred up with the aid of a vortex test tube shaker (Velp Scientifica, zx³) at the beginning of the isolation and shaken every 30

minutes for 10 seconds continuously. In the procedure RNase A digestion was used. A one-time centrifugation was conducted at 13000 g (Eppendorf 22 °C) with 50 µl 70 °C Elution Buffer. The DNA concentration of the samples was defined by a NanoDrop 1000 (Thermo Fisher Scientific) spectrophotometer. The resulting genomic DNA samples were stored at -20 °C until further use.

Table 4

Photometry results

Pattern identification	Concentration (ng per ul)	A260*	A280**	260/280***	260/230****
h_ú1	964.29	19.286	10.191	1.89	2.36
h_ú2	1633.28	32.666	17.304	1.89	2.40
n_ú1	1374.27	27.485	14.397	1.91	2.42
n_ú2	1047.64	20.953	10.887	1.92	2.40
h_h1	803.37	16.067	8.365	1.92	2.38
h_h2	133.70	2.674	1.448	1.85	2.18
n_h1	259.93	5.199	2.830	1.84	2.29
n_h2	287.23	5.745	3.146	1.83	2.23
h_n1	480.32	9.606	5.208	1.84	2.29
h_n2	542.80	10.856	5.995	1.81	2.27
n_n1	269.52	5.390	2.851	1.89	2.59
n_n2	118.70	2.374	1.272	1.87	2.09

Note: * 260 nm absorbance (nucleic acids), ** 280 nm absorbance (proteins), *** with this ratio the purity of the isolated DNA solution can be measured for proteins (above 1.8 above pure), **** 260/230 (organic solvents, these can inhibit the PCR reaction) above 2.0 "clean" the isolate.

The three pairs of primers for sex-specific markers (Ccmf1, Ccmf2 and Ccmf3) were synthesized on the basis of sequences by Chen et al. (2009, 2010) described in their publication (Integrated DNA Technologies). The composition of the PCR mix relative to 10 µl reaction medium: 1× GoTaq G2 buffer (Promega), 150 µm dNTP mix (Fermentas), 2 mm MgCl₂ (Promega) 0.2 µm forward primer, 0.2 µm reverse primer, 0.05 U/µl GoTaq G2 polymerase, distilled water.

The parameters of the applied PCR program: (PTC-200 PCR instrument, Bio-Rad): initial denaturation 94 °C, 2 minutes; denaturation 94 °C, 0.5 minute; primer adhesion 60 °C, 0.5 minute; synthesis 72 °C, 1 minute; final synthesis 72 °C, 5 minutes. The number of cycles was 30. The resulting PCR product was verified by means of agarose gel electrophoresis. The agarose gel used in the procedure was 1.5 m/v% in 1× TAE buffer. The operation was run at 4V/cm voltage), which was stained with ethidium bromide (Biotium) DNA dye. The stained PCR products were made visible by UV light.

Statistical analysis

Statistical studies were performed using IBM SPSS Statistics 22. Within the groups, the homogeneity test was performed with a Levene test (P <0.05) (Fehér et al. 2014). The effects of treatments

and repetitions were determined by single-factor analysis of variance (ANOVA) for the initial and final body weight of the carp fries. Significant differences between the body weights of the stocks were determined by LSD (Nieoczym and Kloskowski 2014) and Tukey-test (P <0.05) (Csengeri et al. 2011).

The method used in the experiment to examine the survival rate of the fish was adopted by OMMI (OMMI 2001). For the calculation of feed conversation the most frequently used indicator was FCR (Rad et al. 2003, Przybyl and Mazurkiewicz 2004, Nasir 2016). In the formula $FCR (g/g) = F / (W_f - W_i) (g/g)$, F refers to the amount of dry feed consumed during the experiment (g), W_f refers to the final body weight (g), and W_i stands for the initial body weight (g). To examine the growth rate of the fish, the SGR indicator was used (Busacker et al. 1990, Hopkins 1992, Lee et al. 1999). In the formula $SGR (%) = (\ln W_f - \ln W_i) / t \times 100$, W_f refers to the final body weight (g), W_i stands for the initial body weight (g), and t indicates the number of days.

RESULTS

The parameters we measured for water quality did not indicate any harmful values for the fish. The overall results of the groups are shown in Table 6.

Table 6

Results (mean + standard deviation) (P <0.05)

	T _{Control}	T ₅₀	T ₇₅	T ₁₀₀	T ₅₀₀
Initial average weight (g)	0.16 ±0.06 ^a	0.16±0.04 ^a	0.17±0.08 ^a	0.17±0.13 ^a	0.16±0.11 ^a
Final average weight (g)	4.59±1.81 ^a	4.70±1.70 ^a	4.30±1.67 ^a	4.63±2.56 ^a	4.24±2.61 ^a
Survival %	100±0	100±0	95±8.66	100±0	96.67±5.77
FCR	0.71	0.75	0.82	0.73	0.85
SGR %	9.52±0.14	9.45±0.22	9.03±0.20	9.35±0.17	9.18±0.48

The average weight per treatments is illustrated in *Figure 1*. The survival rate does not correlate with the average weight in our case, which is due to low population density. The highest survival rates were obtained in treatments T_{Control}, T₅₀, T₁₀₀ with a 100% ratio. The mortality rate was not significant in any of the treatments. The lowest survival rate was measured in treatment T₇₅ with a 95% ratio. The survival rates are illustrated in *Figure 2*.

Figure 1: Initial and final average weights

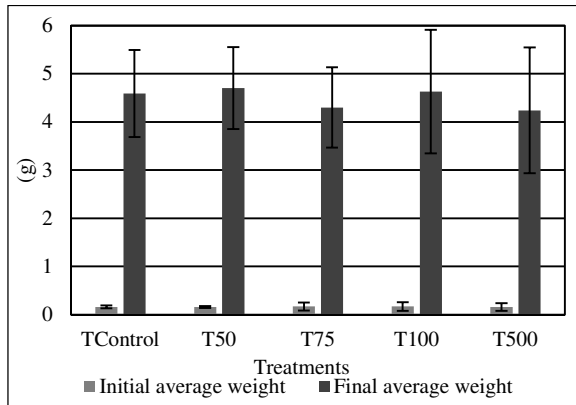
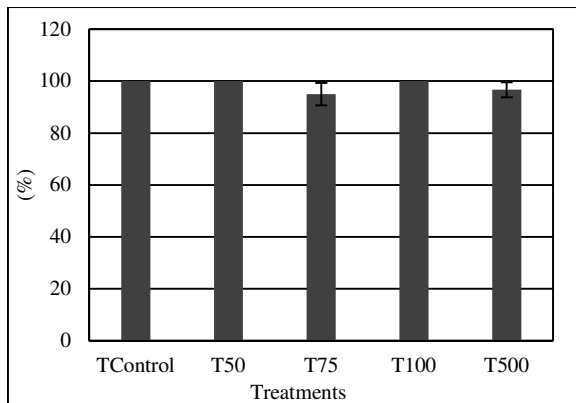
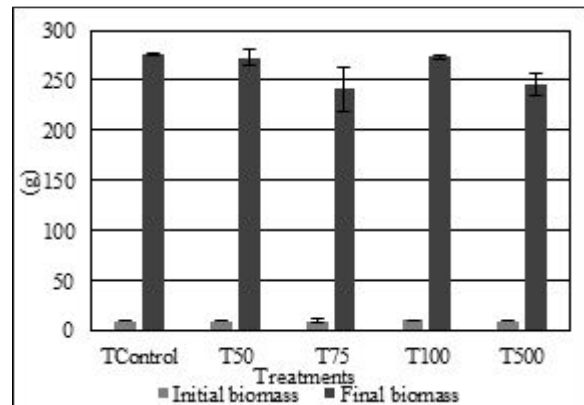


Figure 2: Survival %



In *Figure 3* we indicated the amount of the original biomass at the start of the research by showing the total weight of the common carp per treatment. At the beginning of the treatment we tried to create homogeneous stocks by sorting out male and female specimens, it can be clearly seen in *Table 1* and *Figure 1*. The individual body weights of the assorted groups were tested by the IBM SPSS Statistics 22 program which proved that the groups were homogenous. The results of growth efficiency after the applied hormone treatments compared to those of the control group and to each other had no significant differences.

Figure 3: Biomass changes under 35 days



By examining the treatments separately, it becomes obvious that the fish were growing nearly at the same rate in each group, which is supported by the values of the specific growth rate (SGR). By examining the treatments collectively, there is no significant difference in the FCR values. *Table 7* reveals the amount of feed consumed by the groups.

The values for specific growth rate (SGR) and feed conversion ratio (FCR) are shown in *Table 8*.

Table 7

Granule size	Consumed feed (g)				
	T _{Control}	T ₅₀	T ₇₅	T ₁₀₀	T ₅₀₀
0.5 mm	93.3	94.21	89.43	95.28	96.34
1.1 mm	95.5	102.00	99.00	97.00	105.00
Total	188.8	196.21	188.43	192.28	201.34

Table 8

Feed Conversation Ratio and Special Growth Rate values

	SGR (%)	FCR (g/g)
T _{Control}	9.52	0.71
T ₅₀	9.55	0.75
T ₇₅	9.17	0.82
T ₁₀₀	9.35	0.73
T ₅₀₀	9.28	0.85

Table 9 demonstrates that in the post-rearing phrase we experienced continuous biomass growth as well as low mortality rate with each group compared to the initial values of the experiment.

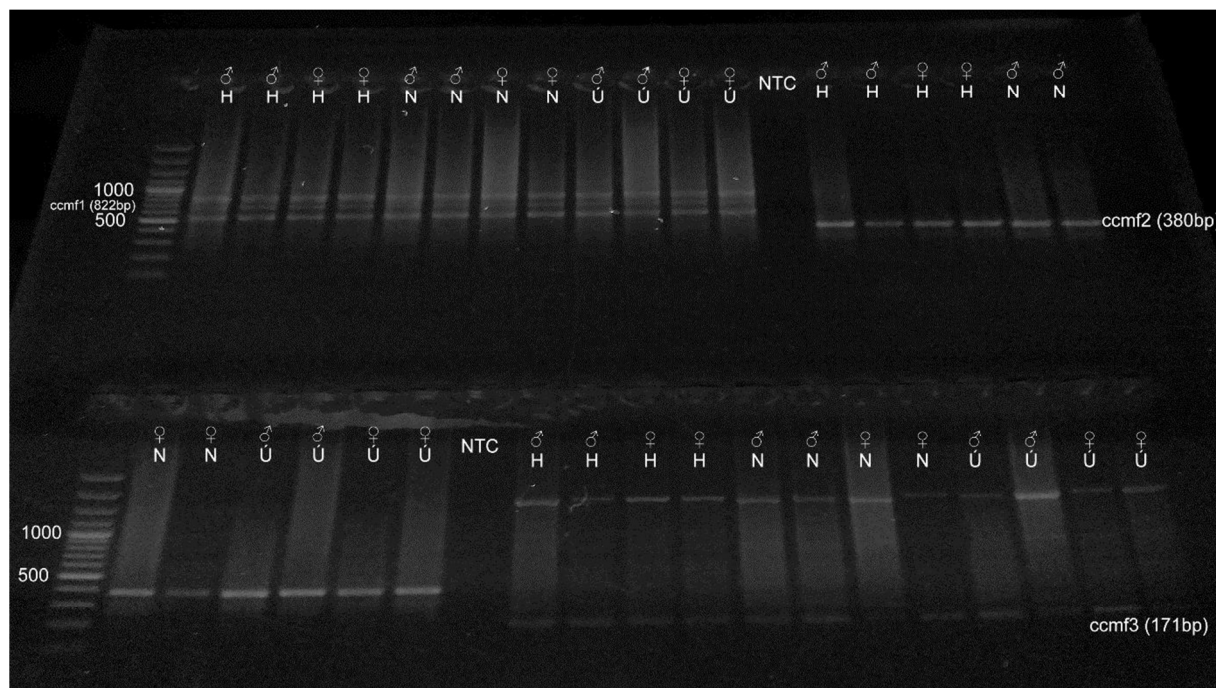
As part of the supplementary work sex-specific identification was tested by PCR as described by a Chen et al. (2009, 2010), however, the use of this method proved unreliable. DNA isolation from all the three sample types (muscle tissue, fin and mucus) was successful. The ccmf1 and ccmf3 markers did not function based on the described method because not only the PCR product size was visible on agarose gel (Table 4). For the ccmf2 marker, a single product was obtained, which in turn was not sex-specific. The ccmf2 marker was repeated, but the male marker continued to appear on the phenotype, even on female subjects.

Table 9

Biomass (kg) and survival % changes at post-breeding

Date	T _{Control}	T ₅₀	T ₇₅	T ₁₀₀	T ₅₀₀	Total
06 September	1.68	1.67	1.85	2.02	1.62	8.84
20 September	3.21	3.07	3.07	3.38	3.09	15.81
04 October	4.59	4.23	3.51	3.52	3.95	19.80
18 October	5.91	4.79	5.31	5.75	5.44	27.21
Survival %	96.6	96.6	80.0	95.0	91.6	91.96

Figure 4: Male-specific identification



Explanation: In the picture the examination of the three sample types, muscle tissue [h], fin [ú], mucus [n] can be seen together with the ccmf1, ccmf2 and ccmf3 markers. Ccmf2 is usable, however, there is no visible difference between the male [♂] and female [♀] samples. NTC (No Template Control), Generuler 50 bp DNA marker was used (Thermo Fisher Scientific).

CONCLUSIONS

The results of the experiment showed that statistically significant differences in body weight gain were not detectable. The uniformity of homogeneous stocks at the start of the experiment remained unchanged by the end of the research too. For each group, 95% survival rate or even higher was observed at the end of the hormone-treated period. On the basis of the biomass harvested at the end of the experiment,

the FCR and SGR figures show that the hormone treatment did not affect the production parameters of the fish at this stage of fry rearing. Even the post-rearing stage is characterized by low mortality rate and continuous weight gain, which means that common carp can be introduced to our recirculation aquaculture system.

The aim of the experiment is to find out how successful hormonal sex reversal can be with the method we applied. The sex of the fish can be identified by autopsy and by microscopic gonad test after the groups reach the 500 g average weight. On the basis of the resulting sex ratios, we can make conclusion about the efficiency of sex-reversal.

During the PCR experiment, it was found that with the tested *ccmf2* male-specific marker, male specimens could not be identified with reliable results. The failure of the test is due to the fact that a

successful experiment conducted by the authors can only be used for a local variant of common carp, for the Yellow River population. The sex-specific identification of the common carp with other PCR markers and other non-invasive sampling technology requires further research in the future.

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