Asian Fisheries Science **19**(2006):411-422
ISSN: 0116-6514
https://doi.org/10.33997/j.afs.2006.19.4.008

Asian Fisheries Society, Manila, Philippines

Artificial Induction of Ovulation in Pond-raised Mahseer, *Tor khudree* Using Carp Pituitary and Ovaprim

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Abstract

A total of 102 induced breeding trials were carried out at Harangi, Karnataka between 1993 and 1997, with a view to standardize hatchery technology for pond-raised mahseer, *Tor khudree*. The initial seeds for raising the broodstock were brought from Lonavla, Maharashtra in 1990 and reared in mud-bottomed masonry ponds. Male and female brood fish were maintained separately and fed on improved diets. Female brood were injected with HCG at 5 mg•kg^{-1} once a month from December till the onset of monsoon in June, to improve fecundity. In all the trials, observations on water temperature, response of females to inducing agent, weight of females that responded, fecundity, time required for ovulation and fertilization rate were recorded. The following are the findings from the series of breeding trials conducted with different objectives. Between the two inducing agents evaluated, Ovaprim performed better in terms of response of female (40%), than pituitary extract (29.63%). Feeding broodstock with a diet having 24.5% crude protein resulted in higher ovulation response, shorter time for ovulation and higher fertilization rate of eggs, compared to 31.5% crude protein diet. Plankton weight recorded in ponds fed on low protein was less than in ponds receiving high protein diet, indicating better utilization of natural food in the former. Cryopreserved spermatozoa performed comparably ($P>0.05$) with that of normal spermatozoa in terms of fertilization rate and quality of hatchlings. The duration of egg hatching and yolk absorption were found to be temperature dependent. Fertilized eggs incubated in Mangalore at 27-28^{o}C took 60 hours for hatching and 95 hours for yolk sac absorption, compared to 120 hours

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taken for hatching and 238 hours for yolk absorption by those maintained at 20-24°C in Harangi.

Introduction

The population of the famous Indian sport and food fish, *Tor khudree* (Deccan mahseer) has dwindled over the years due to anthropogenic activities and it is now classified as an endangered species (Ogale 1994). Kulkarni (1971) pioneered artificial breeding of *Tor khudree*, making use of fully matured brood fish collected from Walwhan lake in Lonavla, Maharashtra. Through this technique, Kulkarni and Ogale (1978) have been successful in routinely producing mahseer fingerlings that have been used for the purpose of ranching natural habitats, to revive the stock. Raising captive broodstock of *Tor khudree* at the Harangi fish farm in Kodagu, a hill district of Karnataka and its induced breeding was attempted as part of a project initiated by the Government of Karnataka for the artificial propagation of mahseer. Even though carps mature in captivity, they do not breed, unless artificially induced. Intensive studies have been carried out on induced breeding of Indian and exotic major carps with success, but such studies are lacking in the case of Deccan mahseer. The present study was carried out with the following objectives.

- To compare the effect of carp pituitary with that of the synthetic ovulation inducer Ovaprim
- To evaluate diets with 31.5% and 24.5% crude protein vis-à-vis ovulatory response of broodstock
- To assess the performance of cryopreserved spermatozoa with the normal ones and
- To determine the impact of two temperature regimes (20-24°C and 27-28°C) on egg incubation.

Materials and Methods

Fingerlings of *Tor khudree* weighing 2-3 g transported from Tata Electric Company, Lonavla to Harangi in 1990 were used for raising the broodstock. They were reared for two years in masonry ponds of 400 m^2

area, having a soil bed of about 20 cm, providing the traditional feed mixture of rice bran and groundnut oil cake (1:1) at 3% body weight per day. The pond was fertilized every month using cattle dung at a rate of 2,000 kg ha^{-1}. Fish were segregated by sex in the third year. The females were injected with HCG at 5 mg•kg^{-1} at monthly intervals from December till the onset of monsoon in June every year, starting in 1992. Brood fish were fed improved diets (Tables 1 and 2) comprising of locally available ingredients. Feeding was done at 2% of body weight daily. Fresh feed was prepared every day using finely ground (less than 400 µm size) feed ingredients, mixing them with water and cooking in a pressure cooker for about 20 minutes. The vitamin and mineral sources were mixed thoroughly to the cooked and cooled dough. Proximate composition of the feed mixture was determined using AOAC (1975) methods.

Plankton samples were collected at fortnightly intervals for biomass estimation, from January till the onset of monsoon in June every year, through a net made of no. 30 bolting silk cloth (mesh size 60 µm), by filtering 100 liters of water from different locations of each pond. The plankton was made free of excess moisture using blotting paper and transferred to a previously weighed aluminium foil and then weighed. For determination of dry weight, samples were taken in foils of known weight and dried in a hot-air oven at 60°C for 24 hours. After drying, the foils were cooled in a desiccator and weighed in an electronic balance.

The selection of brood fish was based on external characters. Males oozing milt on slight pressure on the abdomen and females with soft, swollen abdomen and reddish vent were selected for induced breeding. Carp pituitary and Ovaprim were used as inducing agents. After hormone administration, the fish were examined periodically at intervals of 3 hours. If the eggs were not freely oozing even after 24 hours of hormone treatment, the fish were classified under the 'no response' category. Whenever the eggs were freely oozing, the fish were stripped and the eggs were collected in enamel trays and mixed immediately with fresh milt obtained through stripping of males, following the dry method of fertilization.

After fertilization, the eggs were given a bath in malachite green solution (25 ppm) before keeping them for hatching. The eggs were kept for hatching in wooden trays with nylon net bottom, providing continuous flow of water. The hatchlings after yolk absorption were fed with egg white for a few days and then transferred to manure nursery ponds.

Comparison of effectiveness of carp pituitary and ovaprim

Starting from June 1992, brood fish were maintained on a 31.5% crude protein diet (Table 1). In the 1993 breeding season, 27 females were induced with pituitary in 2 split doses (6 and 12 mg•kg^{-1} body weight, respectively) with a gap of 6 hours, following the traditional hypophysation method adopted for Indian major carps (Chaudhuri and Alikunhi 1957). Males were injected with 2-3 mg pituitary kg^{-1} at the time of 2nd injection to the females. Ovaprim (Syndel Laboratories, Canada) containing 20 µg salmon gonadotropin releasing hormone analogue and 10 mg ml^{-1} domperidone, both dissolved in propylene glycol was injected to 10 females in a single dose at 0.8 ml•kg^{-1} to female and 0.2 ml•kg^{-1} to male simultaneously.

Table 1. Percentage composition (by weight) of ingredients and proximate composition of the feed mixture used for feeding *Tor khudree* brood (1992-1996 trials).

Ingredient	%	Proximate parameter	%
Rice bran	15	Moisture	8.60
Groundnut cake	15	Crude protein	31.49
Germinated horse gram	25	Crude fat	4.63
Fish meal	10	Crude fibre	9.82
Broken rice	9	Ash	13.26
Wheat flour	10	Nitrogen-free extract	32.20
Maize	10	Gross energy (kJ•g^{-1})	13.72
Black gram	5		
Vitamin and mineral mixture *	1		
Vimeral**	0.07		

* Supplevite M, Sarabhai Chemicals, Baroda, India

** Vimeral is a product of Glaxo India Ltd. Each ml contains 48-mg vitamin E, 12,000 IU vitamin A, 6000 IU vitamin D3 and 20 µg of vitamin B12.

Table 2. Percentage composition (by weight) of ingredients and proximate composition of the feed mixture used for feeding of *Tor khudree* brood (1997 trials).

Ingredient	%	Proximate parameter	%
Rice bran	30	Moisture	6.40
Groundnut cake	25	Crude protein	24.40
Horse gram	10	Crude fat	5.80
Fish meal	10	Crude fibre	12.41
Ragi flour	24.5	Ash	11.81
E Care Se*	0.5	Nitrogen-free extract	39.18
		Gross energy (kJ•g^{-1})	13.84

*E Care Se containing vitamin E and Selenium is a product of Vetcare, Bangalore, India.

Evaluation of 31.5% and 24.5% protein diets for broodstock

During 1994, 1995 and 1996, a total of 51 females maintained on 31.5% crude protein diet (Table 1) were induced for ovulation using Ovaprim at the dose mentioned above. In 1997, 14 female broodstock maintained on a 24.5% crude protein diet during 1996-97 rest season were induced and the spawning response was compared with the data obtained during earlier years. A muscle relaxant 'Epidosine' was also injected to female fish at 0.2 ml•kg^{-1} following Ovaprim administration in the trials carried out in 1997.

Evaluation of the performance of cryopreserved and normal spermatozoa

In order to study the effectiveness of cryopreserved milt (spermatozoa), fertilization tests were carried out in the 1997 breeding season. Fresh milt was collected from healthy males of *Tor khudree* at Harangi and transported to Mangalore in liquid nitrogen (−196^{o}C). Modified fish Ringer's solution and dimethyl sulfoxide (DMSO) were used as extender and cryoprotectant, respectively (Basavaraja and Hegde 2003). Milt stored in liquid nitrogen was transported back to Harangi after 70 days and revived before conducting fertility studies. About 6,000 eggs obtained by inducing mahseer with Ovaprim administration were fertilized with cryopreserved spermatozoa. The percentage of fertilization obtained with cryopreserved spermatozoa and normal spermatozoa in the same year was compared.

Determination of the effect of egg incubation at 20-24^{o}C versus 27-28^{o}C

On three occasions, fertilized eggs were transported from Harangi in oxygenated plastic bags to the College of Fisheries, Mangalore, where the temperature ranged from 27 to 28^{o}C. The eggs were kept in aerated fiberglass tubs (125 L) for hatching and the bad eggs were removed constantly. After yolk absorption, the hatchlings were fed with plankton and 'Hitachi' aquarium fish feed for 5 days and then transferred to fertilized, butoxed (0.1ml m^{-2} for killing insects) cement tanks with soil base. Parallel groups were also maintained at Harangi (temperature 20-24 ^{o}C) for comparison of time required for hatching and yolk absorption.

One-way ANOVA technique was applied to data on plankton and time taken for ovulation. Fish body weight, fecundity and fertilization data

were subjected to log, square root and arc sine transformations respectively, followed by ANOVA. Least significant difference based on 't' test was carried out wherever applicable.

Results

Plankton biomass was high ($P<0.05$) in ponds receiving high protein diet (Table 3). Among the 102 females induced, only 35 spawned. No difference ($P>0.05$) in terms of fecundity, time for ovulation and fertilization percentage was recorded when pituitary and Ovaprim were used as inducing agents in 1993 (Table 5). However, the response of females (%) to the inducer was 40 with Ovaprim as compared to 29.63 obtained with pituitary. In both the cases, spawning occurred between 24 and 25.5 hours following administration (Table 4).

Table 3. Range of plankton biomass in experimental tanks.

Treatment	Wet weight (mg/L)	Dry weight (mg/L)
Low protein diet pond	30.07-53.54[a]	4.31-6.82[a]
High protein diet pond	62.09-71.83[b]	5.87-7.36[b]

Different superscripts for values in the same column indicate significant ($P<0.05$) difference.

When low protein (24.5%) broodstock diet was used, 57.14% females responded compared to 30.52% females (average) fed high protein (31.5%) broodstock diet (Table 4). Further, feeding low protein diet resulted in lesser ($P<0.05$) ovulation time (23 hours) and higher fertilization (97.38%) compared to feeding the high protein diet (Table 5).

The rate of fertilization recorded varied between 86.0% and 97.38% from 1993 to 1997, it being significantly higher in 1997 compared to that of earlier years. The fertilization rate with cryopreserved spermatozoa was comparable ($P>0.05$) with that obtained using normal spermatozoa 96.89% vs. 97.38%. Further, the fry obtained using cryopreserved spermatozoa were as normal as those produced from fresh sperms.

At Harangi, where the temperature was 20-24^{o}C, the eggs took 119.69 $\pm$ 4.73 hours for hatching and yolk absorption took 238 $\pm$ 7.21 hours, while in Mangalore at the temperature range of 27 to 28^{o}C, they started hatching at 60 hours and the process of yolk absorption was complete in 95.67 $\pm$ 3.05 hours. Fungal infection of developing eggs (despite

Table 4. Spawning response of Tor khudree during 1993-1997 trials

Year	No. of females induced	No. responded	Response (%)	Wt. of females responded (g)	Pituitary ($mg \cdot kg^{-1}$) Dose I	Pituitary ($mg \cdot kg^{-1}$) Dose II	Ovaprim ($ml \cdot kg^{-1}$)	Time for ovulation (hr)	Number of eggs per 100 g female	Fertilization (%)
1993	27	8	29.63	600	6	12		24.0	58	75
				450	6	12		24.0	168	85
				500	6	12		24.5	109	91
				400	6	12		25.5	9	84
				500	6	12		24.0	193	96
				400	6	12		24.0	226	94
				420	6	12		24.5	123	78
				470	6	12		25.5	159	85
1993	10	4	40.00	600			0.8	25.0	145	96
.				550			0.8	24.0	63	75
				400			0.8	25.5	154	80
				450			0.8	25.0	102	96
1994	20	5	25.00	450			0.8	25.0	42	60
				450			0.8	24.0	37	68
				600			0.8	24.5	128	88
				500			0.8	24.0	228	94
				500			0.8	24.0	17	74

Year	No. of females induced	No. responded	Response (%)	Wt. of females responded (g)	Pituitary ($mg \cdot kg^{-1}$) Dose I Dose II	Ovaprim ($ml \cdot kg^{-1}$)	Time for ovulation (hr)	Number of eggs per 100 g female	Fertilization (%)
1995	19	5	26.32	520		0.8	25.5	168	90
				470		0.8	25.0	96	60
				500		0.8	24.5	76	75
				550		0.8	24.0	159	90
				450		0.8	24.0	203	94
1996	12	5	41.67	380		0.8	24.0	161	93
				310		0.8	24.5	260	95
				420		0.8	24.0	48	84
				500		0.8	25.0	190	90
				550		0.8	24.0	196	85
1997	14	8	57.14	2500		0.8	22.0	40	96
				1500		0.8	22.0	60	97
				1500		0.8	22.0	73	96
				1250		0.8	22.0	80	98
				2000		0.8	25.5	90	99
				2250		0.8	23.5	160	99
				1500		0.8	24.0	113	97
				2500		0.8	23.0	200	97

treatment of eggs with malachite green solution) due to poor water quality, especially turbidity, was frequently encountered at Harangi, resulting in mortality of eggs.

Table 5. Average values and F-ratios for the different parameters

Year	Body weight (kg)	Fecundity (no per 100 g female)	Time for ovulation (hr)	Fertilization rate (%)
1993a	$467.5^{a}\pm23.66$	$130.63^{a}\pm25.28$	$24.50^{b}\pm0.23$	$86.00^{a}\pm2.60$
1993b	$500.0^{a}\pm45.64$	$116.00^{a}\pm20.99$	$24.88^{b}\pm0.31$	$86.75^{a}+5.44$
1994	$500.0^{a}\pm27.39$	$90.40^{a}\pm39.33$	$24.30^{b}\pm0.20$	$76.80^{a}+6.28$
1995	$498.0^{a}\pm17.72$	$140.4^{a}\pm23.61$	$24.60^{b}\pm0.29$	$81.80^{a}+6.34$
1996	$432.0^{a}\pm42.59$	$171.0^{a}\pm34.74$	$24.30^{b}\pm0.20$	$89.40^{a}+2.16$
1997	$1875.0^{b}+176.78$	$102.0^{a}\pm18.98$	$23.00^{a}\pm0.45$	$97.38^{b}+0.42$
F-ratio	65.581*	0.915	4.551*	5.019*

1993a - Pituitary, 1993b - Ovaprim

*P<.0.05.

Different superscripts for values in the same column indicate significant ($P\leq0.05$) difference.

Discussion

Initial attempts made at the College of Fisheries, Mangalore in the 80's to bring pond-raised mahseer to maturity using traditional feed containing a mixture of rice bran and groundnut cake yielded only partial success. Even though a large number of feeding trials have been carried out at the College, employing mahseer fingerlings (Shyama and Keshavanath 1991; Keshavanath 2000), studies on broodstock diets have not been conducted. The quality and quantity of dietary protein plays an important role in the maturity of *Tor khudree*. In this investigation, two improved diets with different protein levels were tested for inducing maturity. The two diets had a large number of plant based ingredients, fish meal forming the sole animal protein source. Both the test diets were effective in inducing maturity in mahseer. Somashekarappa et al. (1990) reported advancement of maturity in the Indian major carp *Catla catla* through the provision of an improved diet consisting of different locally available ingredients. Sreenivasamurthy and Keshavanath (1986) found the protein requirement of *Tor khudree* fingerlings to be 40% as against 30% required by the Indian major carps (Renukaradhya and Varghese 1986). Results of this study indicate that mahseer brood can be successfully produced even on a 24.5%

protein diet. Nonetheless, the contribution of protein by natural food should not be overlooked as the broodstock was maintained in manured ponds. The wet and dry weight of plankton recorded in the low protein fed broodstock ponds was significantly ($P<0.05$) less compared to the plankton biomass in ponds receiving high protein diet, indicating maximum utilization of natural food in the former. The fat level in the test diets was kept low (4.63 and 5.80%) to avoid accumulation of fat in the fish, which is known to affect spawning. Vimeral/E Care Se was added to the diet to supply vitamin E, which plays a vital role in the maturation of gonads as well as the quality and quantity of eggs in fishes (Watanabe 1985; Gupta et al. 1987). In addition to the provision of improved diet, female fish were also injected with HCG at monthly intervals from December to June as it is reported to improve maturity in carps (Somashekarappa et al. 1990). Incidentally, the brood fish fed on 24.5% protein diet during the rest season of 1996-97 and induced bred in 1977 were much larger in size (av. wt. 1875 $\pm$ 176.78 g) than those fed on 31.5% protein diet and used for breeding in the earlier years (Table 4).

Kulkarni and Ogale (1986) reported successful spawning of *Tor khudree* through injection of carp pituitary at 18 mg•kg^{-1} female. Our results tally with this observation. According to them, *Tor khudree* can be bred when the female is about 900 g. In the trials conducted during 1993-1996, female fish used were 600 g or less in weight. The breeding success achieved with these fish indicates that even smaller sized brooders can be bred. The results of the 1993 breeding trials show that the spawning response of mahseer was better to Ovaprim as compared to pituitary. Further, use of Ovaprim eliminates the necessity of handling the brood fish twice. Pandey et al. (1998) succeeded in induced spawning of *Tor putitora* employing Ovaprim and obtained a fertilization rate of 70-80%. A minimum of 22 hours was required for spawning after hormone administration as seen from the results of the 1997 trials. Time taken for ovulation in these trials was less than those of earlier years. This could be attributed to the larger size of the fish used and the muscle relaxant Epidosin. The rate of egg fertilization was also higher in the 1997 trials (Table 5). The fecundity of *Tor khudree* recorded in our trials was low as compared to that reported by Desai (1970). Kulkarni (2000) observed that in pond-raised mahseer the fecundity is generally low. The prolonged hatching time and yolk absorption at Harangi can be related to the prevailing low temperature. Species of *Tor* are reported to hatch within 60-80 hours at a temperature of 24-28^{o}C (Kulkarni and Ogale 1978). Poor water quality, especially turbidity was responsible for lower survival of the developing eggs and spawn at

Harangi, suggesting the need for clean, aerated and warm water in hatching and larval rearing.

Cryopreserved spermatozoa have been successfully employed in induced breeding of a few fish species (Gupta and Rath 1993). Ponnaiah et al. (1999 a,b) have successfully cryopreserved spermatozoa of *Tor khudree* and *Tor putitora* respectively. The 70-day cryopreserved spermatozoa yielded 96.89% fertilization which is comparable to that obtained with fresh milt (97.38%). This implies that even in the absence of male brood, induced breeding of *Tor khudree* can be taken up with cryopreserved milt.

The successful induction of mahseer to maturity in confinement and its induced breeding with either pituitary or Ovaprim and the use of cryopreserved milt for fertilization would go a long way in the artificial propagation of mahseer.

Acknowledgements

We thank the Director of Fisheries, Government of Karnataka for extending the facilities at the Harangi Fish Farm. We also thank the unknown referees whose comments helped in improving the quality of this paper.

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