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Effect of Different Dietary Protein Levels on Egg Development and its Response to Inducing Agents during Induced Spawning of *Channa marulius*

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Authors' Contributions
MHR designed the experiment and performed experimental work. FA managed the brooders. GA formulated the feed. MA performed chemical analysis. KJI and SA analyzed the data. MHR wrote the article. NTN helped in preparation of manuscript.

Key words
Dietary protein, Egg development, Inducing agents, Spawning, *Channa marulius*.

ABSTRACT

Brood stock of *Channa marulius* with an average weight of 948.02±5.72g was randomly stocked in duplicate earthen ponds (90×70×4ft) and fed on 40%, 35% and 30% protein diet @5% of their live body weight. In 40% protein diet treatment, male fish was injected 1st dose with ovaprim + HCG (0.3+0.3ml) and female fish was given 2nd dose after 24 hrs of intervals with ovaprim (0.2ml), while 1st dose ovaprim (0.7ml)+HCG (1.0ml) and 2nd dose ovaprim (0.7ml) was given to the females. The treatment which was given 35% protein diet received 1st HCG+HMG (0.3+0.3ml) and ovaprim (0.2ml) to males and ovaprim (0.5ml)+fresh PG (1.0 ml), 2nd dose ovaprim (0.7ml) and 30% protein containing feed treatment 1st dose with ovaprim+HMG (0.3+0.3ml) and 2ndovaprim (0.2ml) to males and ovaprim (0.3ml) + HMG (1.0ml) with 2nd dose ovaprim (0.7ml kg^{-1}) to females were injected. The highest average fecundity with latency period of 47.70±0.54 h was observed in treatment 3 (40% CP) while treatment 2 (35% CP), in combination of ovaprim + fresh PG showed very short latency period when compared to the ovaprim+HCG injected fish. Treatment 1 and control failed to spawn. Fish fed on 30% protein diet (treatment 1) and the control had the lowest fecundity showing insufficiency of protein required for proper development of the ovary. These studies revealed that 40% protein diet not only improved fish growth and health but also enhanced egg fecundity, gonadal development and spawning.

INTRODUCTION

Channa marulius is an important member of snake headed fishes and is well liked in Pakistan due to high growth potential, peculiar taste and recuperating qualities for sick and feeble both physically and mentally. Though, this fish is carnivorous in nature but comfortably can consume and digest diets composed from different plant by-products with high protein contents. It can be successfully reared in mono as well as in polyculture set up with locally culturable commercial fish varieties.

Nutrition plays a significant role in the spawning performance of fish (Yaakub and Ali, 1992; Faturoti, 2000; Manissery *et al*., 2001; Muchlisin *et al*., 2006; Fasakin, 2007; Hafeez-ur-Rehman *et al*., 2016). Proper feeding of brood stock before the commencement of spawning can guarantee healthy brood stock and proper gonad development which results successful spawning. Several investigations have reported lot of differences in nutritional requirements of brood stock (NRC, 1983; Izquierdo *et al*., 2001). Best combination of both live and supplementary feed also influences the gonadal development and fecundity in brood fish. In addition, energy contents (Smith *et al*., 1979; Takeuchi *et al*., 1981), proper balance of essential fatty acids, amino acids (Santiago *et al*., 1983; Watanable *et al*., 1984a, b; Shim *et al*., 1989; Santiago and Reyes, 1993) and protein to carbohydrate ratio (Cerda *et al*., 1994) have pivotal role in the cascade of this process.

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In the maturation stage, some of the non-saturated fatty acids are present in the ovaries which increases the level of protein and dry matter which is evident of their integral role in reproductive processes (Lie *et al*., 1993). Feeding management of prepared feed like ration size demands independent and careful manipulation (Tyler and Dunn, 1976; Hislop *et al*., 1978; Moitra *et al*., 1979; Springate *et al*., 1985). Therefore, both quality and quantity of feed are equally important for vegetative and reproductive functions of fish life. For efficient induction of ovulation and spawning in murrels, ovaprim, ovatide, HCG and carp pituitary gland have been used as inducing agents (Thakur, 1976; Haniffa *et al*., 1996, 2000; Marimuthu and Haniffa, 2007). Although, hypophysation is difficult and complicated technique but sometimes gives good results in some of the fish species. One of the main disadvantage is its proper collection and determination of appropriate stage with maximum potency. Other synthetic hormones such as human chorionic gonadotropin (HCG) (Mollah and Tan, 1983; Zairin *et al*., 1992; Inyang and Hettiarachchi, 1994), luteinizing hormone (Billard *et al*., 1984; De Leeuw *et al*., 1985; Fermin, 1992) and Ovaprim (Alok *et al*., 1993; Francis, 1996; Haniffa *et al*., 1996; Hafeez-ur-Rehman *et al*., 2016) have been variably used with very little success.

The purpose of this study was, therefore, to investigate the role of varying protein levels in artificial feed on growth and reproductive performance of *Channa marulius*, in terms of fish response to various inducing agents (fresh PG, HCG, ovaprim, HMG) when administered independently and/or in various preconceived or on site manipulated ratios.

MATERIALS AND METHODS

Maintenance of brood stock

Thirty brood fishes of *C. marulius* having an average weight of 948.02±5.72g were randomly stocked in earthen ponds (90×70×4ft). Three different diets (Table I) were prepared independently containing protein levels of 40%, 35% and 30%, hereafter named as treatment 3, 2 and 1, respectively. Six ponds were randomly allotted to each dietary treatment (2 ponds per treatment). Feed ingredients were analyzed for crude protein, crude fiber, crude lipid, nitrogen free extract, ash and moisture contents (AOAC, 2003). Fish were fed @5% of their live body weight twice a day at 08:00 A.M. and 04:00 P.M for 365 days. Water was regularly monitored for accidental changes in pH and dissolved oxygen.

Selection of brood stock

Sexually mature brooders were selected from each pond on the basis of external maturity signs (Haniffa *et al*., 1996). Mature males had the soft pectoral fins, the lower jaw of the mouth was slightly hard like sand paper and anal genital papilla was round in shape. Unlike carps *marulius* females had hard pectoral fins, lower jaw was soft, abdomen was swollen, and genital papilla was swollen and slightly oval in shape. Like carps slight pressure the female belly did not ooze out eggs. Maturity stage in both sexes was, however, further confirmed by opening up their belly and taking out gonads and observing them under microscope. Peripheral dislocation of nucleus verified maturity of eggs and readiness of female for hormonal administration. Confirmation of maturation was followed by collection of desired brood stock and their further preparation for hormonal injections.

Table I.- Formulation of experimental diets containing different protein concentration*.

Ingredients	T1 30% CP		T2 35% CP		T3 40% CP	
	%	C.P	%	C.P	%	C.P
Fish meal	15	7.5	15	7.5	25	12.5
Soybean meal	20	8.4	20	8.4	20	8.4
Maize gluten	20	12	25	15	30	18
Rice polish	40	4.8	35	4.4	20	2.4
Molasses	4	-	4	-	4	-
Vitamins	1	-	1	-	1	-
Total	100	32.7	100	35.3	100	41.3

*Control has received the natural food with 30% CP as reference diet that are widely used for major carps.

Artificial propagation

Two years old, *C. marulius* were randomly divided into three treatments: T1 (30% CP), T2 (35% CP), T3 (40% CP) and control in duplicates. Each treatment had four females and two males (Jhingran and Pullin, 1985). Four females of 1615±86.98g each were stocked in control circular tanks, while females weighing 1750±68.49g, 1817.5±218.84g and 1702.5±68.49g were stocked in T1, T2 and T3 tanks, respectively. In the same tanks two males with an average weight of 1500±35.35g, 1750±49.49g, 1800±113.13g, and 1800±282.84g were paired with females in control, T1, T2 and T3 tanks, respectively. Fishes were anaesthetized and then weighed for dose calculation. Both male and female fishes simultaneously were administered with various hormonal preparations (Table II). The various hormones injected were; carp fresh pituitary (cPG) of *Cyprinus carpio*, human chorionic gonadotropin (LG Laboratories-HCG-5000-PK-0506) (5000 IU kg), human menopausal gonadotropin (HMG Massone, FSH-75IU, LH 75 IU) and

ovaprim (Syndel Laboratories, Vancouver, BC, Canada). All the fish were administered second dose of ovaprim after 24 h (Table II) and released into their respective concrete circular spawning tanks (2m diameter and 2000 L water holding capacity) provided with continuous fresh aerated well water. Breeding behavior of fishes was observed every 6 h in the first half and then every one h or less. Continuously running water maintained dissolved oxygen and temperature at 5-6mg/l and 28-30°C, respectively.

Table II.- Proximate composition of experimental diets.

Ingredients (%)	Con-trol	T1 30% CP	T2 35% CP	T3 40% CP
Crude protein	30.8	30.5	35.21	40.34
Crude fibers	3.15	3.20	7.35	3.89
Crude lipid	3.55	3.75	4.02	5.52
Dry matter	85.10	86.30	89.55	92.07
Moisture	14.40	14.10	13.70	13.20
Ash	17.05	18.02	16.90	14.08
Nitrogen free extract (NFE)	42.35	45.30	36.52	26.24
Gross energy (MJg^{-1})	14.40	15.20	17.97	19.33

Spawning, fecundity and egg fertilization rate

Numbers of eggs were counted gravimetrically (Haniffa and Sridhar, 2002). One gram of egg sample was randomly withdrawn from the bulk and weighed. Sampling and subsequent weighing was repeated thrice. All the weighed egg samples were counted manually and then averaged.

$$\text{Fecundity} = \frac{\text{Total no. of eggs}}{\text{Total weight of fish (g)}}$$

Fertilization of eggs was very much obvious soon after sprinkling but was confirmed after 6-8 h. Egg development was very slow, however it was easily differentiable from unfertilized eggs. Percent fertilization was calculated following (Muir and Robert, 1985):

$$\text{Fertilization rate (\%)} = \frac{\text{Fertilized eggs}}{\text{Total number of eggs}} \times 100$$

Egg development

During egg development, samples were randomly collected at regular interval of 4 h. The collected eggs were fixed in 4% formalin and examined under a binocular microscope (Nikon Eclipse E400).

Statistical analysis

The data obtained from the induced spawning of *C. marulius* with administration of different hormone sources was subjected to analyses of variance (ANOVA) to determine if significant difference ($P<0.05$) occurred among the treatments. The differences among treatment means were differentiated by applying Duncan's Multiple Range Test ($P\leq0.05$) using SAS-9.2 statistical package.

RESULTS

Crude protein contents of all the diets of this experiment were within the range of 30.5% and 40.34% while crude fibers ranged between 3.15% and 3.89%. Gross energy was highest in the T3 (19.33 MJg^{-1}) and least in control as reference diet (14.40 MJg^{-1}). Table II shows the proximate composition of the experimental diets.

The brooders of *C. marulius* showed the aggressive behavior 23 h after 2nd dose of hormones. During courtship, the male bent its body close to the female and ejected milt in close proximity to the release of eggs by females with concomitant initiation of fertilization. There was complete harmony and synchronization in breeding activities of both sexes. Water temperature, dissolved oxygen, pH were invariably maintained at 28-30°C, 5.5-6mg/l and 6.7-7.6, respectively with continuous supply of fresh aerated water. Treatment 2 with inclusion of fresh PG though comparatively with low protein diet spawned successfully.

The highest ($P<0.05$) average fecundity (1427.50±94.67) with latency period of 47.70±0.54 was observed in treatment 3 (40% CP) while treatment 2 (35% C.P) had comparatively low fecundity (1277.50±124.18) with latency period of 49.25±0.12 (Table IV). *Channa marulius* did not spawn in Treatment 1 and control tank. Fishes in these treatments were dissected, ovaries were removed and eggs were counted after mulching the ovaries and releasing the eggs free. Fecundity was 1051.88±95.76 and 965.00±136.06 in treatment 1 and control, respectively. Though, Treatment 2 (ovaprim in combination with fresh pituitary hormone) showed long latency period but fertilization (%) (49.12±7.23) was low, while Treatment 3 (ovaprim + HCG) showed very short latency period when compared to the pituitary-injected fish (Table IV). Breeding performance of fish was closely observed during whole 48 h of spawning process. Fertilized eggs were yellow in color, spherical, non-adhesive and translucent. Blastodisc divided after 35-60 min into two blastomeres. Segmentation was meroblastic. Second cleavage proceeded after 1-2 h, and then multicellular blastodic was formed.

Table III.- Hormone sources and their dosages in both male and female *Channa marulius.*

Treatment	Male body weight (g)	Female body weight (g)	Male *Channa marulius*				Female *Channa marulius*			
			Hormone dose (ml kg^{-1} BW) 1st dose		Hormone dose (ml kg^{-1} BW) 2nd dose		Hormone dose (ml. kg^{-1} BW) 1st dose		Hormone dose (ml. kg^{-1} BW) 2nd dose	
Control	1445.00± 42.03^{a}	1495.00± 156.02^{a}	-	-	-	-	-	-	-	-
Treatment - 1 (30% CP)	1587.50± 175.0^{a}	1632.50± 103.61^{a}	Ovaprim+HMG	0.3+0.3	Ovaprim	0.2	Ovaprim + HMG	0.3+1.0	Ovaprim	0.7
Treatment -2 (35% CP)	1702.50± 216.08^{a}	1613.75± 179.54^{a}	HCG+HMG	0.3+0.3	Ovaprim	0.2	Ovaprim+Fresh PG	0.5+1.0	Ovaprim	0.7
Treatment - 3 (40% CP)	1655.00± 154.16^{a}	1608.75± 282.15^{a}	Ovaprim+HCG	0.3+0.3	Ovaprim	0.2	Ovaprim + HCG	0.7+1.0	Ovaprim	0.7

Table IV.- Outcomes of induced spawning with administration of different hormone sources.

Treatments	Male body weight (g)	Female body weight (g)	Latency period(h)	Spawning success	Fertilization rate (%)	Incubation period (h)	Fecundity rate (natural)	Fecundity (after dissection)	Ova diameter (mm)
Control	1445.00± 42.03^{a}	1495.00± 156.02^{a}	Nil	Nil	Nil	Nil	Nil	965.00± 136.06^{b}	1.47±0.03^{d}
Treatment-1 (30% CP)	1587.50± 175.0^{a}	1632.50± 103.61^{a}	Nil	Nil	Nil	Nil	Nil	1051.88± 95.76^{a}	1.51±0.03^{c}
Treatment-2 (35% CP)	1702.50± 216.08^{a}	1613.75± 179.54^{a}	49.25± 0.12^{a}	Complete	49.12± 7.23^{b}	Nil	1277.50± 124.18^{b}	Nil	1.77±0.02^{b}
Treatment-3 (40% CP)	1655.00± 154.16^{a}	1608.75± 282.15^{a}	47.70± 0.54^{b}	Complete	65.25± 5.36^{a}	Nil	1427.50± 94.67^{a}	Nil	1.84±0.03^{a}

Data figures with different superscript letters are significantly different at P<0.05

DISCUSSION

In this study, crude protein contents of different diets used were 30.5%, 35 and 40.34%, crude fiber ranged from 3.15% to 3.89% gross energy was the highest in T3 (19.33 MJg^{-1}) and the lowest in control (14.40 MJg^{-1}). In the present study, Treatment 3 (40% CP and GE 19.33 MJg^{-1}) showed the maximum fecundity and egg fertilization which favorably corroborate with previous studies. These results are also quite in line with Sotolu (2010), Pathmasothy (1985) and Shim *et al*. (1989) who investigated that increased protein levels from 30% to 40% in the diets increased size and weight of fish, ovary size and hatchability in freshwater fish species. In addition, Cerda *et al*. (1994) and Muchlisin *et al*. (2006) reported that rise in dietary protein level in brood stock diets significantly improved weight gain, quantity and quality of eggs and larval viability. In our study, ovaprim+HMG treated females yielded low quality eggs and in treatment 1 and in control even failed to spawn and those spawned did not hatch. Eggs collection after fish dissection were of poor quality. This might be the result of protein malnutrition. Egg clumping and clustering inside the ovary was also common in the females of this treatment.

Fish fed on 30% protein containing diet (treatment 1) and the control had the lowest fecundity showing insufficiency of protein required for proper development of the ovary. Cerda *et al*. (1994) and Al Hafedh *et al*. (1999) reported similar findings stressing the importance of quality diet. Although, studies with tilapia and grass carp showed no relationship with dietary protein and egg size (Gunasekara *et al*.,1997; Khan *et al*., 2004), but the findings of Manissery *et al*. (2001) contradicted these studies and showed that dietary protein level may affect quality of common carp eggs.

Mean egg diameter of all fish which ovulated in the four experiments ranged from 1.47±0.03mm to 1.84±0.03 mm. Germinal vesicle broke down and an increase in the size of oocytes due to hydration indicated the changes in the nucleus and cytoplasm during final maturation (Goetz, 1983; Guraya, 1986). The lowest ova diameter (1.47±0.03 mm) was observed in the control tank. The highest ova diameter

(1.84±0.03) was observed in ovaprim+HCG injected *C.marulius* while those injected with ovaprim+fresh PG had comparatively smaller ova diameter (1.77±0.02 mm). The early action of steroidogenesis through hormonal influence might have resulted in increased ova diameter. Due to early steroidogenesis, the batch of oocytes might not have obtained sufficient yolk and hence resulted in reduced diameter of ova.

Latency period of brooders in treatment 3 (40% CP) was 47.70±0.54 h where ovaprim+HCG was injected while it was 49.25±0.12 h in treatment 2 (35% CP) which received ovaprim + fresh pituitary hormones. The egg fertilization rate was 65.25% and 49.125% in former and later treatment. Oladosu *et al*. (1993) artificially induced *H. bidorsalis* (mean weight 707.72±4.28.22g) for breeding with carp pituitary produced 33460±20.571 eggs. Contradictory to the previous studies showing low fertilized eggs similar in current studies might have been due to the weight differences of breeders. The latency period reported by many researchers are 23-24 h for *C. striata* (Haniffa *et al*. 2000), 6-25 h for *C. punctatus* (cf. Banerji, 1974), 22-25 h for *Heteropneustes fossilis,* (Kohli and Goswami, 1987), 14 h (Rao *et al*., 1989) and 16-20 h (Munshi and Hughes, 1991) for *Clarias gariepinus.* With regard to pituitary extract Parameswaran and Murugsesan (1976b) reported 28-100% fertilization in *C. striatus* and Kohli and Goswami (1987) observed 45% fertilization in *H. fossilis*. Fertilization was high in diet containing 40% CP and injected with ovaprim and HCG when compared with brooders fed diet containing 30% CP. After 59 h of release during hatching fungus severely attacked developing eggs and converted into clusters losing their integrity.

Egg development followed the same trend as has been reported by Marimuthu and Haniffa (2007) and Parameswaran and Murugsesan (1976b). In *H. fossilis*, the first cleavage was started in about 30 min after fertilization and 16 celled stages in about 70-80 min and morulla stage was observed in about 100 min (Thakur, 1976). In *C. punctate,* however, on the other hand fertilized eggs attained 16 cell stages after 45 min (Banerji, 1974). During the development of *C. marulius* eggs, blastodisc divided after 35-60 min into two blastomeres. Segmentation was meroblastic. Second cleavage preceded after 1-2 h, and then multicellular blastodic was formed. These findings are in agreement with the results of Haniffa *et al*. (2000) and Khan *et al*. (2004).

CONCLUSIONS

This study revealed that 40% CP containing diet not only improved health of brood stock but also enhanced female fecundity. This level of protein in diet improved growth, fish maturation, gonadal development and spawning of mature fish far better than 35% crude protein containing feed. So it can be concluded that 40% protein is required for proper breeding. Induced spawning on this fish species was a revolutionary success in the history of fisheries and aquaculture in Pakistan. Further studies are, however, required to investigate the possible factors which hampered egg development and invited pathogens which spoiled all the eggs before hatching.

Statement of conflict of interest

Authors have declared no conflict of interest.

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