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Embryonic development and growth performances of an endangered fish species *Nandus nandus*: effects of dietary polyunsaturated fatty acids supplementation

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Abstract

The present study explored the embryonic and larval development of an endangered fish species *Nandus nandus* and resolved larval growth performances with the dietary supplementation of different types of lipids. Fertilized eggs were collected from fiber glass tanks immediately after spontaneous spawning of *N. nandus*, which were fed with a 1% phospholipid (squid meal) supplemented diet for 3 months. Fertilized eggs were transparent, spherical, yellowish and sticky in nature. The first cleavages of eggs were observed 0.3±0.01 h post fertilization at 26°C water temperature. Hatching started around 18 h post - fertilization and newly hatched larvae were found to be 1.2±0.1 mm in length. First feeding started 64.0±0.30 h post hatching. After rearing for 10 days, they were divided into 4 groups and separately fed with only dry tubificid worms, 1% docosahexaenoic (DHA) supplemented with dry tubificid worm, 1% phospholipid supplemented with dry tubificid worm and live tubificid worms as treatment I, treatment II, treatment III and treatment IV, respectively. After 50 days of the trial, larvae of treatment II showed significantly ($p<0.01$) higher growth performances in length: 3.18±0.13 cm, weight: 339.8 ± 36.94 mg and survival rate: 78±2 % which were comparable to that of treatment IV, which showed the highest ($p<0.01$) length of 3.4±0.1 cm, weight of 406.6±27.99 mg and survival rate of 97±1 %. Larvae in treatment I showed the lowest growth performances in length: 2.73 ± 0.16 cm, weight: 259.8±29.97 mg and survival rate of 63±3 %. As this is the first record for the determination of embryonic and larval development of *N. nandus* with different lipid supplemented diets, it might be possible to save this endangered fish species by adopting this technology at field level.

Keywords: Docosahexaenoic acid, Embryonic development, Endangered fish, Larval growth, *Nandus nandus*

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Introduction

Nandus nandus (Nandidae) is one of the native fish species commonly known as Gangetic leaffish is declared as an endangered fish species (Ng, 2010). They are often found in ditches, undated paddy fields, muddy streams, rivers, pools and marshes (Bhuiyan, 1964), and marked as small indigenous fish species (SIS) (Ali, 1997). SIS contributes a lot to the vitamin A, calcium and iron intake in Bangladeshi people (Roos *et al*., 2003). Being an SIS, *N. nandus* also contains low fat and good protein percentages. The crude protein, fat, carbohydrate and mineral content estimated in *N. nandus* was found to be 52.5%, 2%, 0.208% and 5.7%, respectively (Ray and Dhar, 2012). In Bangladesh this is a high prized fish and sold at US $ 2.50-4.50/kg. Due to the severe reduction in its natural feeding and breeding grounds as a consequence of climate change, and human intervention since the last few decades, and because of its very sensitive feeding behavior, this species is faced with the threat of extinction. To date it has not been possible to collect the seeds of such an endangered fish species from the wild for further improvement of the stock. Although *N. nandus* has a very tasty, nutritious and enticing appearance, and despite the threat to its extinction, no reports are readily available on the developmental biology and larvae rearing technique of this species. Although, a few attempts have been taken previously to spawn *N. nandus* artificially, the survival rate of larvae was found to be very low (Pal *et al*., 2003). In order to save endangered fish species it is necessary that to produce the maximum number of quality fingerlings from the available broodstock. Therefore, it is imperative to develop techniques to get robust and good quality seeds so that the fingerlings can survive in the nature. To figure out the environmental predilection and dietary requirements, comprehending the embryonic and larval progress and organogeny is critically important (Koumoundouros *et al*., 2001; Borçato *et al*., 2004). Though "live feed" serve as "living capsules" of nutrition (Tiwari, 1986), due to climate change and environment pollution, assurance of regular availability of live feed is difficult. Hence, nutritional status can further be enhanced by using the technique known as "bioenrichment" so that nutritional status of the fishes, prawns and shrimps feeding on them can be increased. During organogenesis, dietary essential polyunsaturated essential fatty acids (PUFAs) play an important role in developing cell membranes, and may modulate processes involved with membrane transportation, receptor functioning, enzymatic activities and serve as precursors for highly active molecules such as eicosanoids (Navarro and Sargent, 1992; Tocher, 2003; Ganga *et al*., 2006). From previous experiments it was also found that developing fish require higher levels of n-3 HUFA than adult fish, and the efficacy of dietary docosehexaenoic acid (DHA) is superior in increasing the

growth, survival rate and tolerance of different fish species like pikeperch larvae (*Sander lucioperca*) and white fish fry (*Rutilus frisii kutum*) to various stress responses (Takeuchi, 1997; Gholami, 2010; Lund and Steenfeldt, 2011). Though it is demonstrated that fresh water fish species have bioconversion capacity of essential fatty acids (Henderson, 1996; Buzzi *et al*., 1997), it is assumed that fish larvae may have limited capacity in lipid biosynthesis due to the insufficient development of higher cells during larval growth (Fontagné *et al*., 1998; Sargent *et al*., 2002). So, it is important to provide fish larvae with dietary PUFAs for their optimum growth and proper functioning. In previous experiments for the growth of juvenile sea bass (*Dicentrarchus labrax*), at least 0.7 % n-3 PUFAs of the dry diet (Skalli and Robin, 2004) and for optimum growth and efficient feed utilization of juvenile starry flounder (*Platichthys stellatus*) 0.9 % of n-3 PUFAs in the diet (Lee *et al*., 2003) were recommended. In the present study, an approach is taken for the improvement of rearing techniques to get better quality fry and fingerlings of the endangered fish species *N. nandus* by revealing their embryonic and larval development, and rearing them with 1 % DHA and 1 % phospholipid (squid meal) supplemented with dry tubificid worms.

Materials and methods

Collection of broodfish and eggs

Broodfish (*N. nandus)*, caught by lift net and dip net from Old Brahmaputra River, Eastern side of Bangladesh Agricultural University, Mymensingh, Bangladesh during the winter season when the water level decreases, were collected from the local fisherman. Upon arrival at the faculty, fishes were sexed and maintained in the cisterns ($1.23\times2.44\times0.46$ m^3) adjacent to the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh. They were fed with 1 % squid extracted phospholipid supplemented diet and cultured for 3 months until they were ready to spawn (Reza *et al*., 2013). Groundwater was constantly circulated in a closed system and maintained at 18 cm at a flow rate of 0.25±0.05 L/s. During the study, temperature, pH and dissolved oxygen (DO) of groundwater recorded were 25.5±1.5°C, 7.3±0.1 and 6.5±0.5 ppm, respectively. The whole experiment was done under the natural photoperiod regime. From the ovulated *N. nandus*, four pairs of ready to spawn females and four pairs of male were selected at different time intervals (April 21, 24, 25 and 30, 2011), and were transferred in the ratio of 1:1 (female:male) to a fiberglass tank ($0.8 \times 0.5 \times 0.41$ m^3), where some water hyacinth were also placed as stimulous for spawning, and kept under regular surveillance. Total body length and weight of selected females recorded were 11.32±0.48 cm and 25.03±2.23 g. Total body length and weight of selected males recorded were 8.05±0.34 cm and 7.15±0.59 g. The water of fibreglass tanks was

maintained at 32 cm with a 0.106±0.01 L/s flow rate. The fish spawned spontaneously after midnight in the water hyacinth (Fig. 1) of the fibre glass tank and the eggs were seperated by washing the water hyacinth several times with slow water flow, collected with a fine mesh net, and placed in a plastic 500 ml beaker partially filled with water from the cistern (Reza *et al*., 2013).

Figure 1: Spontaneous spawning of *Nandus nandus* in water hyacinth in captive condition.

Observation of embryonic and larval development

For further observation of embryonic and larval development, the eggs of *N. nandus* were transferred to 5 incubation trays (1.016×0.406×0.127 m^3), each tray containing 4,000±50 eggs. The eggs were sticky in nature, which was facilitated with the continuous flow of water from the porous PVC pipes as inlet along with outlet facilities. During the study water was maintained at 7cm at 0.072±0.03 L/s flow rate to ensure proper oxygen circulation and good water quality. Photomicrographs of the embryonic and larval development were done by using a photomicroscope (OPTIKA B-350, Italy).

Analysis of feed composition

Composition of protein, lipid, ash and nitrogen-free extract of live and dry tubificid worms were analysed following the method of Association of Official Analytical Chemists, (AOAC) (1980). DHA and squid meal phospholipids were generous gifts from Nippon Chemical and Feed Co. Ltd., (Hakodate, Hokkaido, Japan). The experiment of fatty acid analysis was performed in the laboratory of Biomolecular Chemistry, Faculty of Fisheries, University of Hokkaido,

Hakodate, Japan. For the analysis of fatty acid composition, the samples were converted to methyl ester derivatives individually following the method of Prevot and Mordret (1976). The samples were dissolved in 1ml n-hexane, and 0.2 ml of methanolic 2 M NaOH solution was added. The mixture was shaken, kept at 50 ^{o}C for 20 s, and then 0.2 ml of methanolic 2 M HCl solution was added. The n-hexane layer was collected, concentrated, and subjected to gas chromatographic analysis using a 0.5 µm PEG-20 M liquid phase-coated 40m×1.2mm diameter G-300 column (Chemicals Evaluation and Research Institute, Saitama, Japan) and flame ionization detection. The instrument was a Hitachi 163 gas chromatograph (Hitachi Co. Ltd., Ibaraki, Japan). Flow rate of helium used as carrier gas was 10 mL/min. The temperatures of the column, detector and injector were 170, 250 and 240°C, respectively. The identification of fatty acids was established by comparing the peak retention times with authentic standards (St. Louis, MO, USA) and following the method of Takahashi *et al*. (1988).

Preparation of larval diet

For larvae rearing, dry tubificid blocks (Tai Grim Aquarium Co. Ltd., Taiwan) were soaked in the lipid solution to absorb the lipids. Four types of feeds were prepared. Dry tubificid worms with no fatty acid supplementation, 1% DHA supplemented with dry tubificid worms, 1% phospholipid (squid meal) supplemented with dry tubificid worms, and chopped live tubificid worms that were considered as treatment I, II, III and IV, respectively.

Determination of larval growth

Upon absorbance of 75 % yolk sac of the larvae, they were offered chopped live tubificid worms, and reared for 10 days upto transition from sac-larvae to fry. Thirty, 10-day old fry were weighed and stocked into each of the 4 trays (1.016×0.406×0.127 m^3) with 3 replications. In the trays shelters were made with broken earthen pots locally known as "chara" as the larvae had a tendency to cluster under the shelter. The water in trays was also facilitated with continuous water flow of 0.072±0.03 L/s from the porous PVC pipes as inlet along with outlet facilities, and maintained at 7cm in a circulatory system. All the fry were almost of same size at initial stocking. At the primary stocking of treatment I, the fry stocked were 1.19±0.074 cm in length and 39.4±10.33 mg in weight; in treatment II they were 1.21±0.080 cm in length and 37.3±6.79 mg in weight; in treatment III they were 1.22±0.077 cm in length and 41.1±8.90 mg in weight; and in treatment IV were 1.25±0.083 cm of length and 38.8±7.96 mg of weight. The fry were fed to satiation under natural photoperiod twice daily (09:00 and 19:00 h) according to the feeding trials of previous experiments (Ribeiro *et al*., 2002; Mollah *et al*., 2009; Olurin and Oluwo, 2010; Olurin *et al*., 2012). To assess the growth performances of fry at every ten day interval from initial

stocking, the mean length and weight were measured from random samples of ten fry after collection from each tray. Weight (mg) was taken by digital electric balance and the length (cm) by placing the fry on a petridis placed on a graph paper (Mollah *et al*., 2009; Hossen *et al*., 2014). Specific growth rate (SGR) of fry was recorded at the time of harvesting. Sampling was done in the morning (08:00 h) before feeding. Changes in length, weight, SGR and survival rate were determined by using the following formulas:

Length gain (cm)=Average final length - Average initial length

Weight gain (mg)=Average final weight - Average initial weight (Mollah *et al*., 2009; Hossen *et al*., 2014)

Specific growth rate (mg/day) = {ln (final wt (mg) – ln (initial wt (mg)/time (days)} × 100 (Hossen *et al*., 2014)

Specific growth rate (cm/day) = {ln (final wt (cm) – ln (initial wt (cm)/time (days)} × 100 (Musa *et al*., 2012)

% of survival = (No. of fry alived / Total no. of fry stocked) × 100 (Oyero *et al*., 2009).

Statistical analysis

Statistical analyses of larval growth were performed using statistical software package MSTAT-C (Michigan State University, USA) by Randomized Complete Block design 1 Factor (RCBD 1 Factor). Differences were considered significant at an alpha of 0.01 with Duncan's multiple-range test ($p<0.01$). Statistical analysis of survival rate of larvae was performed using SPSS (SPSS, Chicago, IL, USA). The comparisons of the treatments were made by one-way analysis of variance (ANOVA), and Tukey's post hoc test was conducted to determine specific differences in treatment means.

Results

Embryonic development

Unfertilized eggs: Unfertilized eggs measured were 0.7±0.05 mm in diameter (Fig. 2a). The eggs were opaque, spherical and whitish in color.

Fertilized eggs: The fertilized eggs were sticky in nature. They were transparent, spherical and brownish yellow in color. Immediately after fertilization the diameter of the eggs was found to be 1.0±0.05 mm (Fig. 2b).

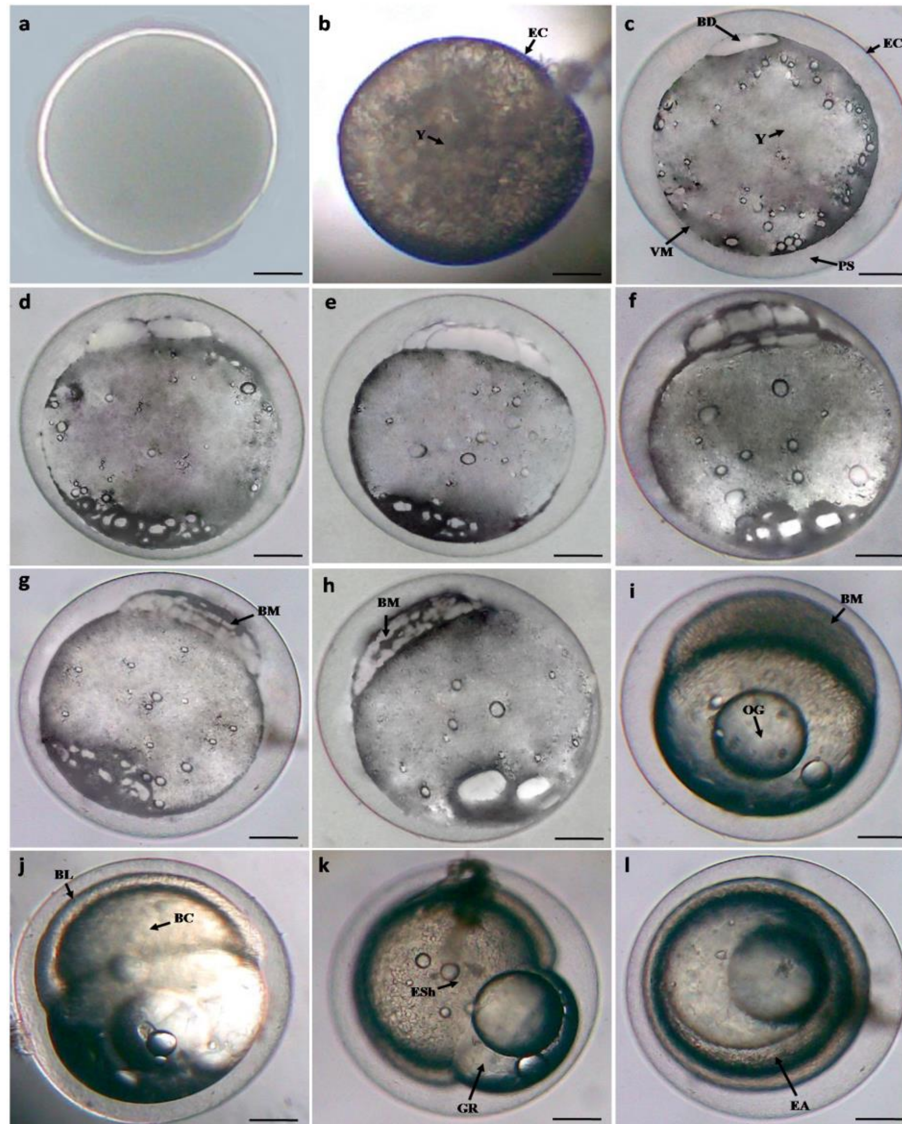

Figure 2: Embryonic developmental stages of *Nandus nandus*. (a) unfertilized egg, (b) fertilized egg, (c) blastodisc, (d) first cleavage, (e) second cleavage, (f) third cleavage, (g) fourth cleavage (h) fifth cleavage (i) morula stage and (j) blastula stage, (k) early gastrula stage, (l) late gastrula stage (EC- Egg capsule; Y-Yolk; BD-Blastodisk ; VM-Vitelline membrane; PS - Perivitelline space; BM-Blastomeres; OG-Oil Globule; Bd-Blastoderm; BC-Blastocoel; VH-Vegetal hemisphere; ESh-Embryonic shield; GR-Germ ring; EA-Embryonic axis). Bar=0.5 mm.

Blastodiscs: Fertilized eggs had a spot (blastodisc) on one pole 0.1 - 0.2 h after fertilization and were readily recognizable with naked eyes within 20 min after fertilization (Fig. 2c). Cleavage period: The first cleavages of eggs were partial or meroblastic, forming a transitory blastula stage. The blastodisc divided into two distinct cells by vertical cleavage within 0.3 ± 0.01 h post fertilization (Fig. 2d). The second cleavage was observed to form four cells within 0.5±0.01h post fertilization, which was at right angles to the first

(Fig. 2e). Consecutively, the third cleavage forming eight cells (Fig. 2f); the fourth cleavage forming sixteen cells (Fig. 2g) and the fifth cleavage, forming thirty two cells (Fig. 2h) was recorded within 0.8±0.01h, 1.05±0.05h and 1.30±0.05h post fertilization, respectively. All along the cleavage periods the eggs were measured 1.0±0.05mm in diameter.

Morula: The blastomeres, after repeated cleavage, resulted in 64 celled early morula stage within 2.0±0.15h after fertilization. A cap like structure was seen over the animal pole. Eggs were measured to be 1.0±0.05mm in diameter (Fig. 2i).

Blastula: The cap like structure over the animal pole gradually increased in size as the development progressed. Blastula stage was observed 3.30±0.15h post fertilization. At this time eggs were found to be 1.0±0.05mm in diameter. Following the morula stage the blastoderm started invading the yolk by spreading over the yolk in the form of a thin layer (Fig. 2j).

Gastrula: Early gastrula stage appeared at 6.30±0.15h after fertilization. The formation of germinal ring around the yolk was visible and that about half of yolk was occupied by blastoderm. The mean diameters of the eggs were 1.1±0.01mm (Fig. 2k). 10±0.15h after fertilization, the late gastrula stage was observed when blastoderm covered 3/4 of the yolk. During this stage the embryonic axis started to form, and the optic rudiment appeared (Fig. 2l). The diameters of the eggs were found to be 1.1±0.01mm all through the stage.

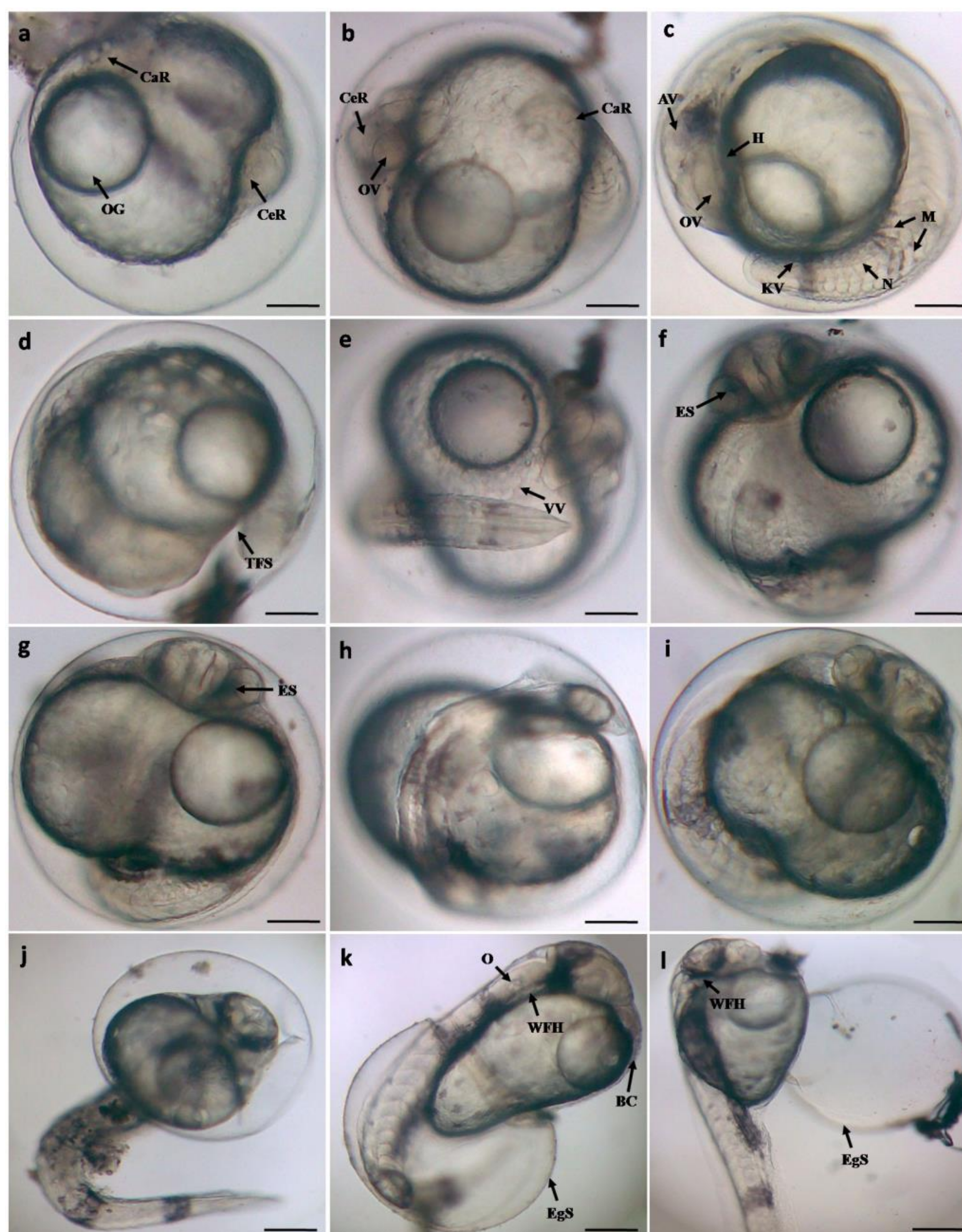

Figure 3: Embryonic developmental stages of *Nandus nandus*. (a) yolk plug stage, (b-h) organogenesis, (i) before hatching, and (j-l) Hatching. (CeR-Cephalic Region; CaR-Caudal Region; OG-Oil Globule; OV- Optic vesicle; AV-Auditory vesicle; M-Myomeres; N-Notochord; H-Heart; KV-Kupffer's vesicle; VV- Vitelline vessels; TFS-Tail Free Stage; Es-Eye spot; O-Otoliths; WFH-Well Functional heart; Bc-Blood circulation; EgS-Egg Shell). Bar = 0.5 mm.

Yolk plug stage: In this stage, the yolk invasion was completed by gradual spreading over the germ layer. The cephalic region and caudal region are differentiated which was observed 11.0±0.15 h post fertilization. The eggs measured 1.1±0.01mm in diameter (Fig. 3a).

Organogenesis: The head and tail end of the embryo were differentiated and the optic vesicle was observed at 12.30±0.15h post fertilization (Fig. 3b).

At 13±0.15h post fertilization, the embryo was elongated and encircled the yolk materials and both the tail and head ends were clearly differentiated. During this stage the Kupffer's Vesicle, myomeres, notochord and beating heart were clearly visible. (Fig. 3c). The tail started to separate from yolk materials after 14±0.15h of fertilization (Fig. 3d). Complete separation of tail from yolk materials and its to and fro movement started after 15±0.15h of fertilization. During this period the embryo occupied 80 % of the egg peripheral space (Fig. 3e). With the continuous development, at 15.30±0.15h after fertilization the embryo occupied 85% of the egg peripheral space, the eye spot was formed and the movement of the tail increased (Fig. 3f). At 16±0.15h post fertilization, and with continuous development, the embryo occupied 90% of the egg periphery space; blood circulation was visible during this period (Fig. 3g). Continuous wriggling movement and black spots were observed all over the body at 16.30±0.15h post fertilization. After 17.0±0.15h of fertilization the embryo occupied almost all the egg peripheral space and tried to rupture the egg shell (Fig. 3h).

Just before hatching: following development, the embryo was ready to hatch at 17.30±0.15h post fertilization (Fig. 3i). The embryo occupied almost all the egg periphery space and continued its endeavor to rupture the egg shell. Embryonic development stages of *N. nandus* are summarized in Table 1.

Table 1: Summary of the embryonic development stages of *Nandus nandus*.

Phase	Diameter (mm)	Time after fertilization (h)	Developmental landmarks
Unfertilized egg	0.7±0.05	0	Opaque, spherical and whitish in color
Fertilized egg	1.0±0.05	0	Sticky, transparent, spherical and brownish yellow in color
Blastodiscs	1.0±0.05	0.1-0.2	Recognizable spot at one pole
First cleavages	1.0±0.05	0.3±0.01	Partial or meroblastic 2 celled stage, formation of transitory blastula stage, division of blastodisc into 2 distinct cells by vertical cleavage
Second cleavage	1.0±0.05	0.5±0.01	4 cell stage which was at right angle to the first
Third cleavage	1.0±0.05	0.8±0.01	8 cell stage
Fourth cleavage	1.0±0.05	1.05±0.05	16 cell stage
Fifth cleavage	1.0±0.05	1.30±0.05	32 cell stage
Morula stage	1.0±0.05	2.0±0.15	Cap like structure over animal pole, blastomeres cleaved into 64 celled 3 - 4 layers
Blastula stage	1.0±0.05	3.30±0.15	Blastoderm spread over the yolk in the form of a thin layer
Early gastrula stage	1.1±0.01	6.30±0.15	Germinal ring formed around yolk and about half of yolk was occupied by blastoderm

Table 1 continued:

Late gastrula stage	1.1±0.01	10.0±0.15	Blastoderm covers 3/4 of the yolk, clearly visible embryonic shield and germinal ring. Embryonic axis started to form. Optic rudiment appeared.
Yolk plug stage	1.1±0.01	11.0±0.15	Complete yolk invasion by gradual spreading over the germ layer. Different cephalic and caudal region
Organogenesis	1.1±0.01	12.30±0.15	Optic vesicle, distinguished head and tail end.
	1.1±0.01	13.0±0.15	Elongated embryo encircled the yolk materials. Clearly visible Kupffer’s Vesicle, myomeres, beating heart and notochord
	1.1±0.01	14.0±0.15	Tail started to separate from yolk materials
	1.1±0.01	15.0±0.15	Tail completely separated from yolk materials and started to swing. Embryo occupied 80% of egg periphery space
	1.1±0.01	15.30±0.15	Formation of eye spots, increased movement of tail.
	1.1±0.01	16.0±0.15	Embryo occupied 90% of egg periphery space, visible blood circulation.
	1.1±0.01	16.30±0.15	Continuous wriggling movement, black spots all over the body
Just before hatching	1.1±0.01	17.30±0.15	Embryo occupied almost all the egg peripheral space and continued its endeavor to rupture the egg shell
Hatching	1.2±0.1	18.0-20.0	Most of the larvae emerged with tail and some emerged with head region. Well functional heart.

Hatching: embryo’s vigorous wriggling movement inside the egg and continuous beating on the egg shell by the caudal region made the egg shell fragile. Hatching started 18 h after fertilization (Fig. 3j, k, l). As the entire embryo did not hatch out at one time, it took around 1-2 h for the completion of the total hatching procedure of embryos. Most of the larvae emerged with their tail portion (Fig. 3j) and some of them emerged with their head region (Fig. 3k). Well functional heart was visible after hatchlings came out of the egg shell. Newly hatched larvae were 1.2±0.1 mm in length.

Larval development

Larvae were 1.3 - 1.4 mm in length, and were brownish in color at 1.3 - 2 h post hatch. Yolk sac was oval in shape and the anal region was clearly distinguished. During this time the digestive tract was observed, which appeared as a tube like structure from the posterior dorsal side of yolk sac. The lens was clearly visible while eyes were unpigmented. Caudal, dorsal and ventral fin folds were visible (Fig. 4a). After 8±0.30h of hatching, the dorsal and ventral fins started to develop. Larvae were still 1.4-1.5mm in length (Fig. 4b). Pigmented eyes appeared after 16±0.30h of hatching, when the average length of the larvae was 1.5-1.6mm. At this time a prominent

depression was clearly visible in place of mouth and dark spots were observed at the anterior part of the body. Yolk sac was partially reduced and became dark brownish in color due to pigmentation (Fig. 4c). After 24±0.30h of hatching, when the larvae reached 1.7-1.8mm of length, the upper and lower jaw could be clearly distinguished, reduction of yolk sac continued, and the pectoral fin started to develop and appeared as a protuberance. Development of other fin folds proceeded at this time (Fig. 4d). With the continuous development, mouth was clearly distinguished and pigmentation spread throughout the body after 36±0.30h of hatching. Absorption of 45-50% yolk sac was observed and larvae were 1.9-2.0mm in length at that time (Fig. 4e). When larvae had reached a length of 2.2-2.3mm (48±0.30h post hatching), and 70-75% yolk sac was absorbed, movement of pectoral fin and dark brown spots all over the body were observed (Fig. 4f).

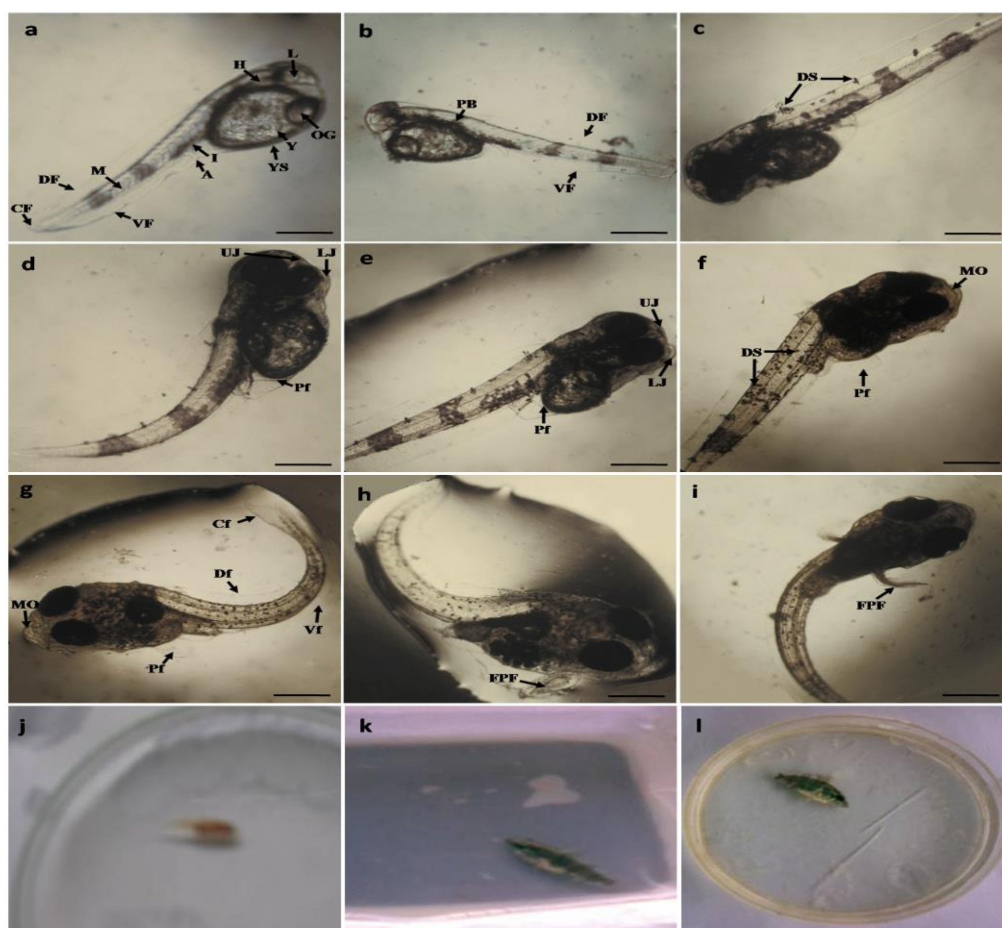

Figure 4: Larval developmental stages of *Nandus nandus*. (a-f) larval stages, (g-i) post larval stages, (j) fry and (k and l) fingerling. (L-Lens; H-Heart; OG-Oil globule; Y-Yolk; YS-Yolk sac; A-Anus; I-Intestine; VF- Ventral finfold; M-Myomeres; DF-Dorsal finfold; CF-Caudal finfold; PB-Pectoral bud; DS-Dark Spot; LJ-Lower jaw; UJ-Upper jaw; Pf-Pectoral fin; MO-Mouth opening; Vf-Ventral fin; Df-Dorsal fin; Cf-Caudal fin; FPF-functional pectoral fin). (a-i) Bar = 0.5 mm.

Post larval stage: after 64±0.30h of hatching a functional dorsal, caudal and ventral fins were observed. The yolk sac was almost absorbed. Melanophores were more concentrated at the head region than on the body. Functional mouth and pouch like stomach were observed. Larvae required supplementation of diet for further development. Larvae were 2.4-2.5mm in length at this time (Fig. 4g). Anus became fully functional and larvae started to swim freely at 72±0.30h post hatch when they were found to be 2.6-2.7 mm in length (Fig. 4h). Larvae were able to swim against the slow water current when they were capable of moving the caudal and pectoral fins vigorously, and had free moving eyeballs after 96±0.30h of hatching when they were 3.0-3.1mm in length (Fig. 4i).

Fry: ten days after hatching larvae were found to be 1.1±0.1cm in length. Pigmentation appeared all over the body. During this stage, organogenesis was completed and except for their color patterns the juveniles were morphologically similar to the adults (Fig. 4j). After 35-40 days of hatching the color pattern also resembled the parents (Fig. 4k, l). Larval development stages of *N. nandus* are summarized in Table 2.

Table 2: Summary of the larval development stages of *Nandus nandus*.

Stages	Time after Hatching	Length (mm)	Developmental landmarks
Larval stage	1.3-0.20 h	1.3-1.4	Brownish color, oval yolk sac and clearly distinguished anal region. Tube like digestive tract appeared from the posterior dorsal side of yolk sac. Lens was clearly visible and eyes were found unpigmented. Caudal, dorsal and ventral fin folds were visible.
	8±0.30 h	1.4-1.5	Dorsal and ventral fin started to develop.
	16±0.30 h	1.5-1.6	Pigmented eye and prominent depression in place of mouth were clearly visible. Dark spots were observed at the anterior part of the body. Due to the pigmentation partially reduced yolk sac became dark brownish in color.
	24±0.30 h	1.7-1.8	Clearly distinguished upper and lower jaw and continuation of the yolk sac reduction. Pectoral fin appeared as a protuberance. Development of other fin folds proceeded.
	36±0.30 h	1.9-2.0	Pigmentation spread throughout the body. Mouth was clearly distinguished. 45-50% yolk sac was absorbed.
	48±0.30 h	2.2-2.3	Dark brown spots were observed all over the body. Larvae started to move pectoral fin. 70-75% yolk sac was absorbed.
Post larval stage	64±0.30 h	2.4-2.5	Functional dorsal, caudal fin and ventral fins were observed. Yolk sac was almost absorbed. Melanophores were more concentrated at the head region than on the body. Functional mouth and pouch like stomach were observed. Larvae required supplementation of diet for further development.
	72±0.30 h	2.6-2.7	Anus became fully functional. Larvae started to swim freely.
	96±0.30 h	3.0- 3.1	Free movement of the eyeballs and vigorous movement of the caudal and pectoral fins were observed. Larvae were able to swim against slow water current.
Fry	10 days	1.1±0.01	Pigmentation appeared all over the body, complete organogenesis, incomplete body color patterns.

Proximate composition of live and dry tubificid worm

Among the live and dry tubificid worms, dry tubificid worms contained higher amounts of protein (64.38 %) as compared with the protein content (61.82 %) of live tubificid worms. Lipid content of live tubificid worms was 17.24% which was higher than the lipid content (8.12%) of dry tubificid worms. Protein, lipid, ash and nitrogen-free extract contents of live and dry tubificid worm are shown in Table 3.

Table 3: Composition of live and dry tubificid worms (n=3)[a].

Composition (%)	Live tubificid worm	Dry tubificid worm
Protein	61.82	64.38
Lipid	17.24	8.12
Ash	9.47	12.06
Nitrogen-Free Extract	6.36	9.14

[a]Results are mean percent content of protein, lipid, ash and nitrogen-free extract

Fatty acid composition of squid meal phospholipids

In the analysis, among the fatty acids, the highest amount found was DHA (22:6n-3) at 39.7%. The second highest among the PUFAs was eicosapentaenoic acid (EPA) (22:5n-3) at 11 %. Among the saturated fats palmitic acid (16:0) was the highest at 32.6 %. The total fatty acid composition is shown in Table 4.

Table 4: Fatty acid composition of squid meal phospholipids.

Type	Isomer	Systematic name of fatty acids	Common name of fatty acids	%
Saturated Fats	16:0	Hexadecanoic acid	Palmitic acid	32.6
	18:0	Octadecanoic acid	Stearic acid	1.5
Monounsaturated fats	18:1	Octadecenoic acid	Oleic acid	2.5
Polyunsaturated fats	18:2 (n-6)	Octadecadienoic acid	Linoleic acid	1.0
	20:2 (n-6)	Eicosadinoic acid		0.5
	20:4 (n-6)	Eicosatetraenoic acid	Arachidonic acid	1.0
	22:5 (n-3)	Eicosapentaenoic acid (EPA)		11
	22:6 (n-3)	Docosahexaenoic acid (DHA)		39.7

Determination of larval growth performance

PUFAs had significant positive effects on the growth performances of larvae and fry. Best growth performances (3.4±0.1cm in length and 406.6±27.99mg in weight) were found in larvae fed live feed of (p<0.01). Larvae fed 1 % DHA supplemented with dry tubificid worms also showed better growth performances attaining a length of 3.18±0.13cm and weight of 339.8±36.94mg, which was comparable to those live feed. Then sequentially

better growth performance in length (2.91±0.15cm) and weight (306.67±42.22mg) was found in case larvae fed phospholipids supplemented with dry tubificid worms. The larvae in the control group fed only on dry tubificid worms with no supplementation of fatty acids showed significantly lowest growth performances in length (2.73±0.16cm) and weight (259.8±29.97mg) (p<0.01). The effects of PUFAs supplemented diets on larval length and weight are shown in Fig. 5 and Fig. 6, respectively.

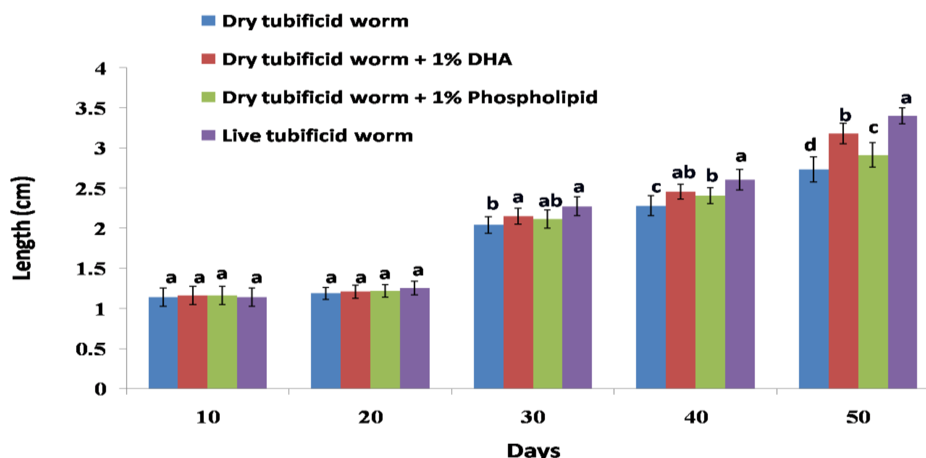

Figure 5: Length (cm) measurement of *Nandus nandus* fry fed only dry tubificid worms, 1% DHA enriched with dry tubifcid worms, 1% phospholipid (squid meal) enriched with dry tubifcid worms, and live tubificid worms for 50 days. Data were represented as means±S.D. Different letters denote significant differences (*p*<0.01) between groups, analyzed by RCBD 1 Factor with Duncan's multiple-range test.

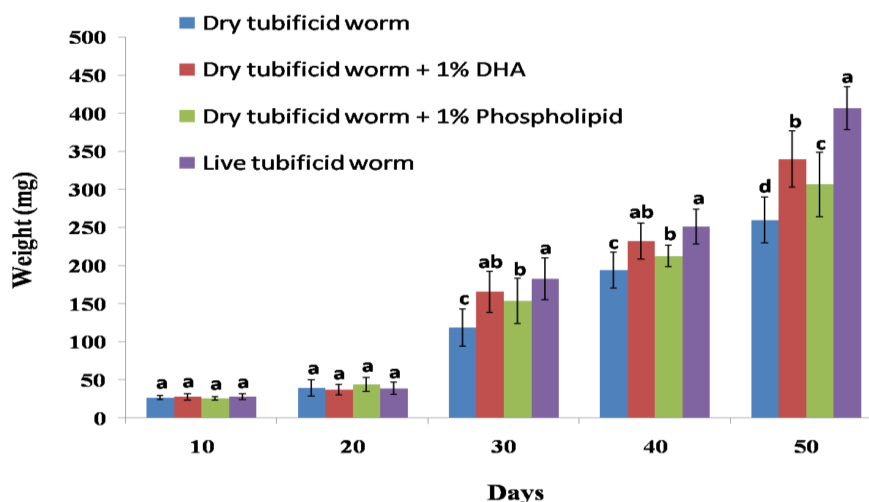

Figure 6: Weight (mg) measurement of *Nandus nandus* fry fed only dry tubificid worms, 1% DHA enriched with dry tubifcid worms, 1% phospholipid (squid meal) enriched with dry tubifcid worms, and live tubificid worms for 50 days. Data were represented as means±S.D. Different letters denote significant differences (*p*<0.01) between groups, analyzed by RCBD 1 Factor with Duncan's multiple-range test.

SGR also showed similar results as those found for length and weight gain (Table 5). Here larvae fed with live tubificid worms showed the best results

followed by the larvae fed with 1% DHA enriched with dry tubifcid worms, 1% phospholipid enriched with dry tubifcid worms and dry tubificid worms.

Table 5: Mean specific growth rates (SGR) of *Nandus nandus* fry fed only dry tubificid worms, 1% DHA enriched with dry tubifcid worms, 1% phospholipid (squid meal) enriched with dry tubifcid worms, and live tubificid worms for 50 days.

Parameter	Treatment			
	Dry tubificid worms	**1% DHA enriched with dry tubifcid worms**	**1% phospholipid (squid meal) enriched with dry tubifcid worms**	**live tubificid worms**
SGR (mg/day)	5.472±0.183[d]	6.366±0.105[b]	6.04±0.119[c]	6.728±0.042[a]
SGR (cm/day)	2.032±0.147[d]	2.524±0.088[b]	2.294±0.075[c]	2.748±0.067[a]

Different letters in a row indicated statistically significant differences (p<0.01) between groups, analyzed by RCBD 1 Factor with Duncan's multiple-range test.

Determination of survival rate in N. nandus larvae

PUFAs also had significant positive effects on the survival rates in larvae of *N. nandus*. DHA supplemented with dry tubificid worms fed larvae showed a significant survival rate of 78±2 % (p<0.01), which was comparable to 97±1% in larvae fed with live tubificid worms. Squid meal phospholipid supplemented with dry tubificid worms also showed better survival rate of 73±3% (p<0.01) while the larvae fed dry tubificid worms with no fatty acid supplementation showed the lowest survival rate of 63±3%. Survival rate of larvae of *N. nandus* fed with different PUFAs containing feeds is shown in Fig. 7.

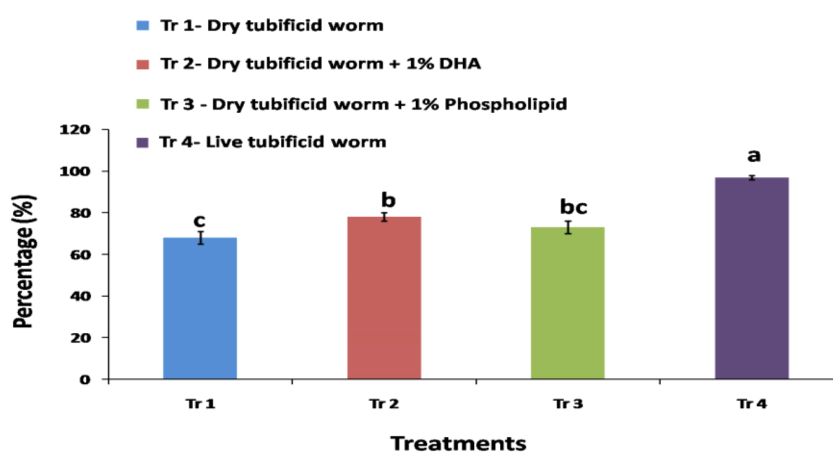

Figure 7: Measurement of survival rate of *N. nandus* fry fed only dry tubificid worms, 1% DHA enriched with dry tubifcid worms, 1% phospholipid (squid meal) enriched with dry tubifcid worms, and live tubificid worms for 50 days. Data were represented as means±S.D. Different letters denote significant differences (p<0.01) between groups, analyzed by one-way ANOVA with Tukey's post hoc test.

Discussion

The embryonic stages of *N. nandus* transpired inside the chorion and ended with hatching. The egg membrane was fully detached from the egg and had a small perivitelline space, which was filled with fluids that may protect the eggs and the embryo from any external damages. These features were supported by the early developmental stages of other fish species like *Anabas testudineus, Pangasius sutchi, Labeo bata* etc. (Islam, 2005; Miah *et al*., 2009; Zalina *et al*., 2012).

In the present study, *N. nandus* showed a discoidal meroblastic cleavage pattern where timing of the cell cycle during the first cleavage events was found highly synchronous, with each cycle having approximately the same length of 20-30 min, which was comparable to the previous early cleavage pattern of *Labeo bata* (Miah *et al*., 2009). The diameter of the fertilized eggs of *N. nandus* was 1±0.05mm. In case of *Channa punctatus* fertilized eggs ranged between 0.9 and 1.1 mm in diameter, and timing of cell cycle during first cleavage was around 15-25min (Haniffa *et al*., 2003).

In this experiment, the invasion of *N. nandus* yolk was completed 11±0.15h after fertilization, whereas in a previous experiment yolk invasion of the *A. testudineus* was found to be completed 10 h after spawning (Munshi and Hughes, 1991). Kupffer's vesicle of *N. nandus* was clearly observed around 13±0.15h after fertilization, which has been compared to that of *Danio rerio*, where Kupffer's vesicle appeared 10.33h-11.66h after fertilization during 1 - 4 somite stages (Thisse *et al*., 2001).

In this study, hatching of *N. nandus* started 18h after fertilization, which was supported by the result of previous experiments where *N. nandus* hatched 18-20 h after fertilization (Pal *et al*., 2003). In other studies, *L. bata, C. punctatus* and *Heteropneustes fossilis* hatched 18.0-20.0 h, 24 h and 23-24 h after fertilization, respectively (Haniffa *et al*., 2003; Miah *et al*., 2009; Puvaneswari *et al*., 2009). In the case of *Mastacembelus pancalus*, larvae emerged with its tail portion first in 34.0 to 35.0 h after fertilization (Rahman *et al*., 2009), which supported the early hatching behavior of *N. nandus* embryos, that showed twisting movements inside the egg envelops and finally some of the embryos emerged with their head region and others with tail portion.

In the current study, 70-75% yolk sac was absorbed after 48±0.30h of hatching. Around 50-60h after hatching they were searching for food which was supported by a previous experiment where *N. nandus* larvae were searching for food after 56 h of hatching (Pal *et al*., 2003).

In the current study, during feeding trials with chopped live tubificid worms, chopped dried tubificid worms supplemented with 1 % DHA, chopped dried tubificid worms supplemented with 1 % phospholipid (squid meal) and only chopped dry tubificid worms, survival rates of larvae were 97±1%,

78±2%, 73±3% and 68±3%, respectively. In the previous experiment, only 16% of *N. nandus* larvae were found alive during feeding trial with hard boiled chicken egg yolk, pasted tubificid worms by screening, and small sized zooplankton captured with 200 µm mesh sized plankton net from the pond (Pal *et al*., 2003). As *N. nandus* larvae were observed to be very sensitive up to 10-15 days after hatching and they preferred to feed upon live feed, they may not consume hardboiled egg yolk at all. When tubificid worms are pasted by screening, a large portion of them is mixed with water as blood and the existing portion remain motionless. So, the pasted tubificid worms through screening may not be sufficient to fulfill the requirements of larvae as their first feeding diet. Survival rate was 16% when they were fed zooplankton only. In ponds, there are different types of zooplanktons of which some crustacean zooplankton having sharp organelles, that might be harmful during early stages of larval development as *N. nandus* larvae is very sensitive to surviving under stressful condition. Other than selective zooplankton (e.g. Artemia, Rotifer etc.), feeding trials of zooplankton might be more effective at 10-15 days after hatching. However, more experiment is recommended for zooplankton related feeding trials. In the present study, larvae were fed chopped tubificid worms for up to 10 days after starter feed. Then they were transferred for different feeding trials. Chopped dry tubificid worms look alive due to the circular water movement with motion and larvae also fed upon them.

In the current study, the protein concentration of dried tubificid worms was 64.38% which was higher than that of live tubificid worms (61.82%), but in case of live tubificid worms the highest growth in length (3.4±0.1cm), weight (406.6±27.99mg) as well as survival rate (97±1%) was recorded. SGR also showed a similar trend in growth with 6.728±0.042 mg/day in weight and 2.748±0.067 cm/day in length in live tubificid worms fed larvae, which was significantly higher than other treatments fed dry tubificid worms. This may be due to the larval preference for live tubificid worm than for dry tubificid worms. It was supported by other experiments where formulated feeds have been observed to be richer in protein than live feeds but survival rate was revealed higher in case of *L. rohita* larvae that prefer live feed to formulated feed (Bakhtiyar *et al*., 2011). Moreover, the minerals and micronutrients needed by fish in the early stages of life are not yet fully understood and hence cannot be incorporated in formulated feeds. On the other hand, the natural live food organisms supply all these micronutrients which otherwise are not known to us. Since live feed is rich in proteins, carbohydrates and fats along with various types of vitamins and minerals, live feed is always preferable (Singh *et al*., 1994). Larvae fed with 1% DHA supplemented with dry tubificid worms showed significant

higher growth performances of length (3.18±0.13cm) and weight (339.8±36.94mg) which was comparable to those fed live feed. In comparison to dry tubificid worms, phospholipid supplemented with dry tubificid worms also showed better growth performances of length (2.91±0.15 cm), and weight (306.67±42.22 mg). Trends in survival also reflected those seen in growth, with larval survival in the DHA (78±2%) and squid meal phospholipid (73±3%) treatment being the best which was comparable to the highest survival (97±1%) in the live feed treatment, while that in the control was the lowest (63±3%). In the previous experiment, Furuita *et al*. (1999) found that larvae receiving the high DHA diet demonstrated better 'viability' after exposure to stress tests (high salinity water: 65%). Other studies revealed that long-chain essential fatty acids have significant effects on growth and stress tolerance of pikeperch larvae (Lund and Steenfeldt, 2011). In the current study, higher survival rate of *N. nandu*s larvae, reared on DHA and phospholipid supplemented with dry tubificid worms was due to the increase of its tolerance against stress conditions. The result of the present study was also supported by a previous experiment, where yellowtail larvae (*Limanda ferruginea*) fed with high DHA diet were found significantly larger and had a higher survival rate (Copeman *et al*., 2002). Other studies have shown that high levels of DHA are present in the eyes and brains of Atlantic cod (*Gadus morhua*), European sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*) and herring (*Clupea harengus*) (Bell and Dick, 1991; Mourente *et al*., 1991; Bell *et al*., 1996). Diets deficient in DHA have been shown to change the fatty acid composition of neural tissue and decrease foraging efficiency at low light intensities in juvenile herring (Bell *et al*., 1995). *N. nandus* larvae are also visual predators, and it is therefore probable that enrichment with dietary DHA could directly affect their early foraging behavior and thus growth and survival.

Supplementation of diets with PUFAs can ensure the quality of broodstock of *N. nandus* that results in spontaneous spawning in captive conditions. If it is possible to make these valuable lipids available to the farmers, by adopting this technique, *N. nandus* would be considered as a valuable cultured species that must mitigate the threat of extinction. It is hoped that the culture of this high demand fish species will cut a good figure in total aquaculture production and be available to consumers. On the other hand, continuous supply of live feed is always difficult to rear any fish larvae in captivity and thus demands an alternative to live feed. PUFAs have many health beneficial effects to get better growth performances and survival rates in cultured fish species without any hazardous effects on fish health and consumers. The success of

this experiment may thus not only help in saving the fish from probable threat of extinction but will also find a way towards improved socio-economic conditions of the poor fish farmers of developing countries.

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References

Ali, M.Y., 1997. Small indigenous fish species culture in Bangladesh, Proceedings of National Workshop on Small Indigenous Fish culture in Bangladesh, IFADEP-SP 2, Dhaka, Bangladesh.

AOAC, 1980. Official methods of analysis. 13th ed., Association of official analytical chemists (ed. W. Hoewitz), Washington. D.C. 78P.

Bakhtiyar, Y., Langer, S., Karlopia, S.K. and Ahmed, I., 2011. Growth, survival and proximate body composition of *Labeo rohita* larvae fed artificial food and natural food organisms under laboratory condition. *International Journal of Fisheries and Aquaculture*, 3(**6**), 114-117.

Bell, M. V. and Dick, J. R., 1991. Molecular species composition of the major diacylglycerophospholipids from muscle, liver, retina, and brain of cod (*Gadus morhua*). *Lipids*, 26(**8**), 565-573.

Bell, M.V., Batty, R.S., Dick, J.R., Fretwell, K., Navarro, J.C. and Sargent J.R., 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids*, 30(**5**), 443-449.

Bell, M.V., McEvoy, L.A. and Navarro, J.C., 1996. Deficit of didocosahexaenoyl phospholipid in the eyes of larval sea bass fed an essential fatty acid deficient diet. *Journal of Fish Biology*, 49(**5**), 941-952.

Bhuiyan, A.L., 1964. Fishes of dacca. Asiatic Society of Pakistan. 148P.

Borçato, F.L., Bazzoli, N. and Sato, Y., 2004. Embriogenesis and larval ontogeny of the pirugordura, *Leporinus piau* (Fowler) (Pisces, Anostomidae) after induced spawning. *Revista Brasileira de Zoologia*, 21(**1**), 117-122.

Buzzi, M., Henderson, R.J. and Sargent, J.R., 1997. The biosynthesis of docosahexaenoic acid (22:6n-3) from linolenic acid in primary hepatocytes isolated

from wild northern pike. *Journal of Fish Biology*, 51(**6**), 1197-1208.

Copeman, L.A., Parrish, C.C., Brown, J.A. and Harel, M., 2002. Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. *Aquaculture,* 210(**1-4**), 285-304.

Fontagné, S., Geurden, I., Escaffre, A.M. and Bergot, P., 1998. Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio* L.) larvae. *Aquaculture*, 161(**1-4**), 213-223.

Furuita, H., Konishi, K. and Takeuchi, T., 1999. Effect of different levels of eicosapentaenoic acid and docosahexaenoic acid in *Artemia nauplii* on growth, survival and salinity tolerance of larvae of the Japanese flounder, *Paralichthys olivaceus*. *Aquaculture*, 170(**1**), 59-69.

Ganga, R., Tort, L., Acerete, L., Montero, D. and Izquierdo, M.S., 2006. Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*. *Journal of Endocrinology*, 190(**1**), 39-45.

Gholami, M., 2010. Effects of n-3 HUFA enriched *Daphnia magna* on growth, survival, stress resistance and fatty acid composition of white fish fry (*Rutilus frisii kutum*). *Journal of Fisheries and Aquatic Science*, 5(**1**), 49-55.

Haniffa, M.A., Nagarajan, M., Marimuthu, K. and Raj, A.J.A., 2003. Embryonic and larval development of spotted murrel, *Channa punctatus* (Bloch). *Indian Journal of Fisheries*, 50(**3**), 355-362.

Henderson, R.J., 1996. Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. *Archives of Animal Nutrition*, 49(**1**), 5-22.

Hossen, M.S., Reza, A.H.M.M., Rakhi, S.F. and Hossain, Z., 2014. Performance of different dietary lipids on growth indices and survival of striped gourami, *Colisa fasciatus* (Perciformes: Osphronemidae) fry. *International Journal of Biochemistry and Biophysics*, 2(**1**), 1-7.

Islam, A., 2005. Embryonic and larval development of Thai Pangas (*Pangasius sutchi* Fowler, 1937). *Development, Growth and Differentiation*, 47(**1**), 1-6.

Koumoundouros, G., Divanach, P. and Kentouri, M., 2001. Osteological development of *Dentex dentex* (Osteichthyes: Sparidae): Dorsal, anal, paired fins and squamation. *Marine Biology*, 138(**2**), 399-406.

Lee, S.M., Lee, J.H. and Kima, K.D., 2003. Effect of dietary essential

fatty acids on growth, body composition and blood chemistry of juvenile starry flounder (*Platichthys stellatus*). *Aquaculture*, 225(**1-4**), 269-281.

Lund, I. and Steenfeldt, S.J., 2011. The effects of dietary long-chain essential fatty acids on growth and stress tolerance in pikeperch larvae (*Sander lucioperca* L.). *Aquacultue Nutrition*, 17(**2**), 191-199.

Miah, M.I., Harun, M.S., Rahman, M.M., Haque, M.R. and Hossain, M.A., 2009. Study on the embryonic and larval development of an endangered species of bata (*Labeo bata*). *International Journal of Sustainable Crop Production*, 4(**1**), 72-82.

Mollah, M.F.A., Roy, A. and Mamun, M.S.A., 2009. Growth performance of larvae produced from vitamin E treated female *Clarias batrachus* (Linnaeus). *Progressive Agriculture*, 20(**1-2**), 107-116.

Mourente, G., Tocher, D.R. and Sargent, J.R., 1991. Specific accumulation of docosahexaenoic acid (22:6n-3) in brain lipids during development of juvenile turbot *Scophthalmus maximus* L.. *Lipids*, 26(**11**), 871-877.

Munshi, J.S.D. and Hughes, G.M., 1991. Air breathing fishes of India. Oxford & IBH Publishing Co pvt. Ltd., New Delhi, pp. 181-208, 289-294.

Musa, S.M., Aura, C.M., Ngugi, C. C., and Kundu, R., 2012. The effect of three different feed types on growth performance and survival of African catfish fry (*Clarias gariepinus*) reared in a hatchery. *ISRN Zoology*, 2012, 6.

Navarro, J.C. and Sargent, J.R., 1992. Behavioural differences in starving herring *Clupea harengus* L. Larvae correlate with body levels of essential fatty acids. *Journal of Fish Biology*, 41(**3**), 509-513.

Ng, H.H., 2010. *Nandus nandus*. In: IUCN 2013. IUCN red list of threatened species. Version 2013.1. <www.iucnredlist.org>. Downloaded on 30 October 2013.

Olurin, K.B. and Oluwo, A.B., 2010. Growth and survival of African catfish (*Clarias gariepinus*) larvae fed decapsulated *Artemia*, live Daphnia, or commercial starter diet. *Israeli Journal of Aquaculture -BAMIGDEH*, 62(**1**), 50-55.

Olurin, K.B., Iwuchukwu, P.O. and Oladapo, O., 2012. Larval rearing of African catfish, *Clarias gariepinus* fed decapsulated *Artemia*, wild copepods or commercial starter diet. *African Journal of Food Science and Technology*, 3(**8**), 182-185.

Oyero, J.O., Awolu, T.E. and Sadiku, S.O.E., 2009. Comparative study of artemia and liqui-fry in the rearing of *Clarias gariepinus* fry. *Pakistan Journal of Nutrition*, 8(**2**), 176-180.

Pal, S., Rashid, H., Tarafder, M.A. K., Narejo, N.T. and Das, M., 2003. First record of artificial spawning of *Nandus nandus* (Hamilton) in Bangladesh using

carp pituitary gland: an endangered fish bred in captivity. *Pakistan Journal of Biological Sciences*, 6(**18**), 1621-1625.

Prevot, A.F. and Mordret, F.X., 1976. Utilisation des colonnes capillaries de verre pour l'analyse des corps gras par chromatographie en phase gazeuse. *Revue Française des Corps Gras*, 23(**7-8**), 409-423.

Puvaneswari, S., Marimuthu, K., Karuppasamy, R. and Haniffa, M.A., 2009. Early embryonic and larval development of Indian catfish, *Heteropneustes fossilis*. *EurAsian Journal of BioSciences*, 3(**12**), 84-96.

Rahman, M.M., Miah, M.I., Taher, M.A.H. and Hasan, M.M., 2009. Embryonic and larval development of guchibaim, *Mastacembelus pancalus* (Hamilton). *Journal of the Bangladesh Agricultural University*, 7(**1**), 193-204.

Ray, N. and Dhar, B., 2012. Study of bioenergetics, proximate composition and microbiological status of leaf fish *Nandus nandus* (Ham.1822). *Keanean Journal of Science*, 1, 76-82.

Reza, A.H.M.M., Rakhi, S.F., Hossen, M.S., Takahashi, K. and Hossain, Z., 2013. Enhancement of reproductive performances of *Gangetic leaffish*, *Nandus nandus* through up regulation of serum Ca^{2+} concentration, improved morphological alteration of liver and ovary with dietary polyunsaturated fatty acids. *Fish Physiology and Biochemistry*, 39(**4**), 779-791.

Ribeiro, L., Zambonino-Infante, J.L., Cahu, C. and Dinis, M.T., 2002. Digestive enzymes profile of *Solea senegalensis* post larvae fed *Artemia* and a compound diet. *Fish Physiology and Biochemistry*. 27 (**1-2**), 61-69.

Roos, N., Islam, M.M., and Thilsted, S.H., 2003. Small indigenous fish species in Bangladesh: contribution to vitamin A, calcium and iron intakes. *Journal of Nutrition*, 133(**11**), 4021S-4026S.

Sargent, J.R., Tocher, D.R. and Bell, J.G., 2002. The lipids. In: Halver J. E. and Hardy R. W. (Eds.), Fish nutrition 3rd ed., Academic Press, San Diego, pp. 181-257.

Singh, D.K., Kumar, A. and Reddy, A.K., 1994. Role of live feed in fish and prawn seed production and their culture. *Fishing Chimes*, July, pp. 13-16.

Skalli, A. and Robin, J.H., 2004. Requirement of n-3 long chain polyunsaturated fatty acids for European sea bass (*Dicentrarchus labrax*) juveniles: growth and fatty acid composition. *Aquaculture*, 240(**1-4**), 399-415.

Takahashi, K., Hirano, T. and Saito, M., 1988. Application of partition chromatographic theory for the analysis of marine triglyceride molecular species. *Nippon Suisan Gakkaishi*, 54(**3**), 523-528.

Takeuchi, T., 1997. Essential fatty acid requirements of aquatic animals

with emphasis on fish larvae and fingerlings. *Reviews in Fisheries Science*, 5(**1**), 1-25.

Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q. and Thisse, C., 2001. Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402). ZFIN Direct Data Submission http://zfin.org.

Tiwari, V.K., 1986. Live feed culture, Silver jubilee celebrations. Hi tech Aquaculture, open house, May,15P.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science*, 11(**2**), 107-184.

Zalina, I., Saad, C.R., Christianus, A. and Harmin, S.A., 2012. Induced breeding and embryonic development of climbing perch (*Anabas testudineus*, Bloch). *Journal of Fisheries and Aquatic Science*, 7(**5**), 291-306.