# EMBRYONIC DEVELOPMENTAL STAGES OF *NOTOPTERUS NOTOPTERUS* IN INDUCED BREEDING

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## Abstract

The present study was conducted to investigate the embryonic developmental stages of *Notopterus notopterus* in the induced breeding. The breeders of *Notopterus notopterus* were collected from the Hlawgar fish farm, Yangon Region in September 2020. The injected hormone was buserelin acetate (Suprefact) 10µg per 1kg for the first injection and the second injection was 30 µg per 1kg. The hormone was introduced two times of injection to the female but it was introduced single dose to the male. After 52 hours of injection, female laid eggs on the surfaces of the bricks. The present work showed that the fertilized eggs had yellowish color. The eggs were enveloped with many ridges around the micropyle. The cleavage of fertilized eggs was at 1 hour after laying of the eggs. The early morula (1cell, 2 cells, and 4 cells) stage lasted 2:54 hours and morula (16 cells and 32 cells) stage was found in 4:50 hours. Blastula stages of the dome formation, late blastula and multicellular layer occurred at 6:54, 8:10, and 10:01 hours respectively. The latest cell division of embryonic stage was found at 40:25 hours. The embryonic phase was completed at 50:21 hours. The results of the present work indicated the different phases of embryonic stages of *Notopterus notopterus* and the time periods of cell division of the eggs.

**Keyword** Induced breeding, cleavage, fertilized eggs, embryonic developmental stages

## Introduction

In recent years, aquaculture had been recognized as an important strategy to meet the growing demands of fish protein all over the world. Artificial propagation of fish is the most promising and reliable way of ensuring availability of good quality fish seed all year round and sustainability of the aquaculture industry. It involved the use of natural (hypophysation) or synthetic hormones to induce ovulation and spawning in farmed fishes (Salami, *et. al*., 2003).

Aquaculture activities are suitable solutions to reduce fish exploitation from nature for human consumption. The oviparous bronze featherback (knife-fish), *Notopterus notopterus*, is a popular food fish with ornamental value, thrives well in freshwater rivers, ponds and lakes. Bronze featherback (*Notopterus notopterus*, Pallas 1769) is one of the native species which had high economic value.

Bronze feather knife fish have high economic value and favored by society to be consumed and made ornamental fish. It had a very hardy fish and can be reared in aquarium, stagnant water and aquaculture system on a variety of feeds. The fish *N. notopterus* in the wild had been categorically kept in the list of the threatened species.

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High-fat content makes bronze featherback had a delicious and distinctive taste, in addition this fish also had high protein and vitamin A content. In Myanmar, people consumed as traditional food by cooking, frying and making fish ball of bronze featherback knife fish.

Induced breeding is a producing of fish larvae through artificial stimulation using synthetic hormone. The artificial fecundation and ranching are the envisaged strategies for conservation and rehabilitation of endangered species. The stimulation promotes timely release of sperms and eggs.

In Myanmar, artificial spawning has been performed in fishes by giving hormone stimulation to the parent with buserelin acetate (suprefact). The use of buserelin acetate had been successfully performed on many fish species especially *Labeo rohita*, *Cyprinus mrigala*, *Cyprinus carpio, Clarias. spp* and *Anabas testudineus.* The use of buserelin acetate was also performed successfully in induced breeding of *Prochilodus lineatus* and *Osteobrama belangeri* recently (Kalayar Win Maung, *et. al.*, 2019, Than Than Myint, *et. al.*, 2019).

The aim of the present study was using synthetic hormone to perform induced breeding of *Notopterus notopterus* including embryonic developmental stages of *Notopterus notopterus*. The understanding on hormone stimulation on breeders of *Notopterus notopterus* is valuable for the commercially mass production of important fish species.

## Materials and Methods

### Study site

Breeders were collected from Hlawgar fish farm located at 16˚ 58' 15.58" N and 96˚ 06' 36.40" E. It is also situated in Minglagdon Township, Yangon Region (Fig. 1).

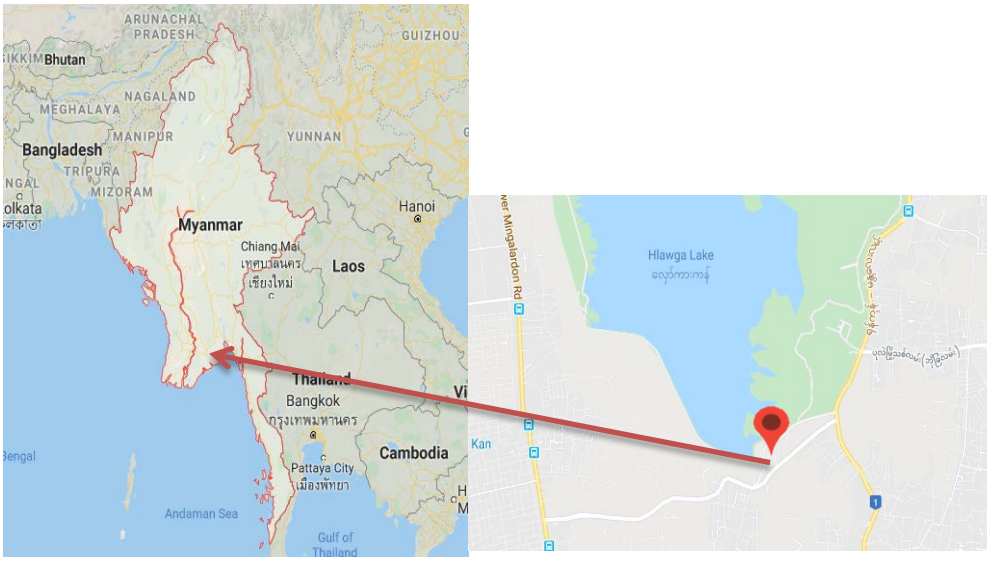



**Figure 1** Map showing Hlwagar Fishery Station. (Source: Google)

### Study period

The study period was from 2019 May to 2020 January.

### Collection of Breeders

*Notopterus notopterus* was collected from the earthen ponds of Hlawga fishery station. The collected breeders included 23 females and 45 males. They were then transferred to concrete tank before preparation for the induced breeding.

**Maintenance of Breeders**

The breeders were maintained in the concrete tank for 14 days before induced breeding. The measurement of concrete tanks was (240 x 240 x 90) $cm^3$. The water level was maintained at 45 cm with running system. All the breeders were fed with formulated feed produced by Green Feed Co. Ltd with 10% of body weight. Nutrient contents in Green Feed were crude protein 40%, fiber 11 %, calcium 2.5% phosphate 1.5% and lysine 2.0%. The ingredients contained soybean meal, fish meal, squid meal, wheat, broken rice, amino acid, minerals and vitamins. They were fed twice a day, 9 am in the morning and 7 pm at night.

**Preparation of Spawning Sites**

A tank (240 x240 x 90) $cm^3$ was prepared for spawning after injection. Before using the tank, the concrete tank was cleaned with (30%) of salt water for disinfection. In the tank, four different spawning sites were created at four corners for the nesting and hiding places of fish. Bricks, stone and wooden blocks were used for hiding.

**Selection of Breeders**

Among the 68 individuals of collected breeders, four healthy males and four females were selected for induced breeding. The sexes of *Notopterus notopterus* were differentiated by the shape of the genital papillae (Weitkamp, 2005). The male had narrow genital papilla of reddish color and longer than the rudimentary pelvic fin. Female had broader and whitish color papilla and shorter than its pelvic fin (Plate 1).

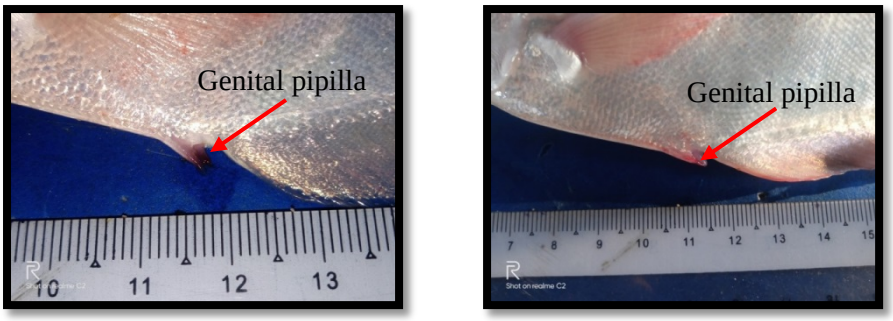
**Hormone preparation and Injection of *Notopterus notopterus***

All the breeders were injected with Buserelin acetate (suprefact). The sex ratio of breeders was (1:1). Two doses were prepared for female while one dosage was prepared for male. Females were injected 10µl/kg in first injection. In second injection, dose of 10 µl/kg was injected for male while 30µl/kg for female. Weight of breeders and concentration of hormone were described in Table 1.

**Hormone injection of *Notopterus notoprerus***

Hormone injection of *Notopterus notopterus* was carried out in 17 July, 2019. The hormone stimulant was injected at the base of pectoral fin. First injection was carried out in the evening (6 pm) at water temperature of 27˚C. The second injection was conducted in 12 am at the water temperature of 29˚C. Courtship behavior was observed after the injection.

A. Breeder of *Notopterus notopterus*




B. Male C. Female

**Plate 1** Breeder *Notopterus notopterus* and genital Papilla of Male and Female

**Table 1 Preparation of hormone dosage for female and male of *Notopterus notopterus***

| No. | Sex (M=Male F=Female) (g) | Body weight (g) | 1st doses (Suprefact) | | Interval (hr) | 2nd doses (Suprefact) | |
|---|---|---|---|---|---|---|---|
| | | | (Suprefact) µg/kg | DOM (mg/kg) | | (Suprefact) µg/kg | DOM (mg/kg) |
| 1. | F | 250 | 2.5 | 1.25 | 6 | 7.5 | 1.25 |
| 2. | F | 323 | 3.2 | 1.61 | 6 | 9.7 | 1.61 |
| 3. | F | 198 | 1.9 | 1.05 | 6 | 5.9 | 1.05 |
| 4. | F | 230 | 2.3 | 1.15 | 6 | 6.9 | 1.15 |
| 5. | F | 130 | 1.3 | 0.65 | 6 | 3.9 | 0.65 |
| 6. | M | 280 | - | - | - | 2.8 | 1.4 |
| 7. | M | 220 | - | - | - | 2.2 | 1.1 |
| 8. | M | 314 | - | - | - | 3.14 | 1.57 |
| 9. | M | 270 | - | - | - | 2.7 | 1.35 |
| 10. | M | 302 | - | - | - | 3.02 | 1.51 |

## Results

### Mating Behavior of *Notopterus notopterus*

Among the five pairs of fish, only one pair showed mating. The female displayed a swollen belly, indicating female genital papilla was ready to mate, while the male appeared visibly drawn to the female. Around fourteen hours after the second injection, the male approached the female, swimming alongside. Male and a female stayed in a corner of the tank where bricks were set up. The successful spawning event of the breeding was observed 52 hours after hormone injection and fish spawning was found only in bricks set. The female laid eggs on the surface of brick (Plate 2). The female deposited approximately 355 eggs. The eggs laid by female were attached to the surface and bottom side of the bricks .The diameter of fertilized egg had 4 mm.

**Plate 2** Eggs attached to the surface of brick

**Embryonic Developmental Stages of Eggs**

The fertilized eggs displayed a spherical shape, measuring 4 mm in diameter, and were characterized by a yellowish coloration. The egg envelope presented multiple external ridges, predominantly clustered around the micropyle (Plate 3-a, b).

**The embryonic period**

**Zygote-one cell** - After fertilization, cell division in the zygote began at 1:00 hour, resulting in the emergence of a distinct brownish pattern on the top of the eggs (Plate 3-c). The germinal disc exhibited characteristic bipolar differentiation, with the cytoplasm tightly connected to the yolk through the formation of the prospective internal yolk layer.

**Two-blastomere -** The germinal disc underwent its initial cleavage, was found at 2:00 hours, resulting in the division of the germinal disc into two identical blastomeres, marking the formation of the two-cell stage (Plate 3-d).

**Four-blastomere and Eight-blastomere** At 2:32 hours, the subsequent cleavage furrow formed at a right angle to the first cleavage furrow, resulting in the emergence of four equivalent blastomeres. The third cleavage took place at 2:54 hours, giving rise to eight blastomeres arranged in two parallel rows of four cells. (Plate 3-e, f)

**Early morula and Late morula** - The fourth cleavage displayed an orientation that was approximately horizontal to oblique. By the 4-hour, the germ comprised 16 blastomeres. The fifth cleavage occurred at 4:58hr. Subsequent cleavages involved the nonsynchronous development of furrows, resulting in the production of blastomeres of unequal sizes. At this stage, the germ consisted of approximately 32 blastomeres (Plate 3-g, h).

**Blastula** - At 6:54 hours, the germinal disc exhibited a distinct pebbled appearance, with the upper exterior side forming a dome shape. The yolk cell displayed a ring-like projection, indicating the formation of the internal yolk layer (Plate 3-i).

**Flat blastula and Late blastula** - The germinal division underwent into two distinct types of cells, at 8:10 hours. These cells primarily originated from the superficial cells of the blastoderm, forming an epithelial sheet consisting of a single layer. The deep layer of cells resided beneath the internal surface of the enveloping layer. The margin of the blastoderm expanded outward. The deep cells had undergone multiplication, resulting in the formation of a multicellular layer at 10:01 hours. As cleavage progressed, surface of the yolk cell underlying the blastoderm became flattened, and most of the yolk vacuoles were located internally (Plate 3-j, k).

**The embryonic phase**

**50% Epiboly and 75% epiboly notochord-formation** - The blastoderm expanded and enveloped approximately 50% of the yolk surface. As the blastoderm elongated vegetally, the nuclei of the external yolk syncytial layer migrated beneath the blastoderm, dispersing throughout the yolk mass. The boundary between the blastoderm and the germ ring became distinguishable at 26:14 hours. At 28:08 hours, the blastoderm had covered approximately 75% of the yolk surface, marking the completion of this stage. The neural ridges in the prospective head region rose above the epidermal yolk sac covering. The notochord was already visible along the midline of the neural plate, establishing the morphological definition of the future embryo's rostral-caudal axis. The initial three somites appeared (Plate 3-l, m).

**Embryonic shield** – At 29:15 hours, the periphery of the blastoderm continued to expand across the yolk boundary, leading to the emergence of a noticeable embryonic shield as a slender protrusion. The longitudinal orientation of the embryonic shield was subsequently identified as the potential embryo (Plate 3-n).

**Wedge-shaped neural plate -** The blastoderm underwent further expansion, spreading and encompassing nearly 90% of the yolk surface, while the yolk plug noticeably diminished in size compared to the previous stage. The neural plate continued its lateral extension and transformed into a wedge shape at 40:25 hours (Plate 3-o).

**Latest epiboly -** The blastoderm enveloped nearly the entirety of the yolk surface, leaving a small exposed yolk plug. The embryonic ring was enlarged all around and the main yolk sac cavity was elongated anteriorly and posteriorly beneath the complete neural plate to form the segamentation cavity at 42:35 hours (Plate 3-p).

**Spoon-shaped embryo** - Epiboly was accomplished, and the yolk mass was completely enveloped by both the blastoderm and the yolk syncytial layer. Progression of a neural groove became apparent along the central axis of the plate, forming a concave shape at the front end. Shortly thereafter, the neural folds had converged at the midline, resulting in the neural plate acquiring a shape resembling a dumbbell (Plate 3-q).

**Early trunk-tailed bud** - The lateral boundaries of the neural plate were conspicuous and bent inward towards the central line of the developing organism during a specific time period. Most of the posterior segment of the neural precursor and the underlying mesoderm extended from the surface covering of the skin-like yolk sac, giving rise to an early stage of the trunk-tail region. At this stage, the embryo possessed approximately 10-13 somites. The initial muscular movements were observed in the posterior part of the trunk-tail area at 64:05 (Plate 4-a).

**Tail bud-bent** - During this stage, the distinguishing feature was the independent trunk-tail formation. The posterior tail section of the developing organism detached itself from the yolk sac and arched downward, following the contour of the egg membrane, at the time of 90:00 hours (Plate 4-b).

**Optic placode and heart-beat -** The auditory placode formed its initial appearance. Heart contraction was observed. The straightening of the developing organism and the existence of blood cells facilitated the circulation of blood. The blood cells circulated through a series of structures in a specific arrangement: a heart with two chambers, a dorsal aorta extending from the heart to the rear boundary of the yolk at 94:20 hours (Plate 4-c).

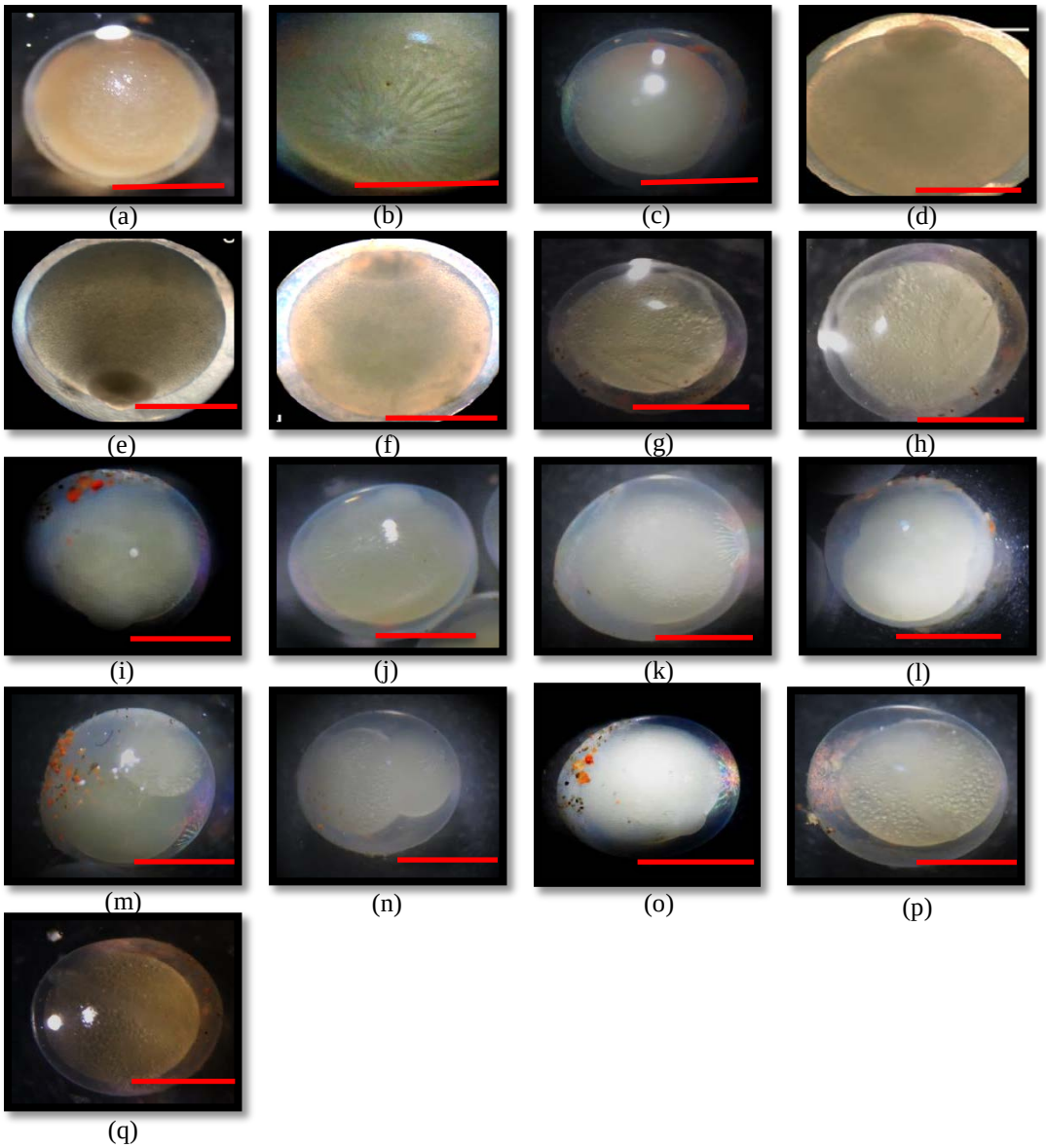



**Plate 3** The cleavage phase and embryonic phases of *Notopterus notopterus* (a. fertilized egg, b. micropylar ridges, c. one blastomere, d. two two blastomere, e. four two blastomeres, f. eight blastomeres, g. early morula, h. late morula, i. blastula, j. flat blastula, k. late blastula, l. 50% epiboly, m. 75% epiboly, n. embroyoic shield, o. Wedge-shaped neural plate, p. latest epiboly and q. spoon-shaped embryo.) Scales bar= 2mm

**Eye pigment** - The eye exhibited widespread pigmentation. Numerous tiny unbranched melanophores, which harbored dispersed melanin pigment, were evenly dispersed across the entirety of the head. The muscular contractions of the developing organism transformed into vigorous motions encompassing the entire body. The vascular fin fold network expanded throughout the entire fin fold area at 122:05 hours (Plate 4-d)

**Pre-hatching** - The yolk cavity had expansion, while the entire front portion of the developing organism remained attached to the yolk sac covering. Under these circumstances, the tip of the curved trunk-tail area extended as far as the uppermost part of the head. During movements, it was possible for this tip to come into contact with or even surpass the head. The embryo executed robust and energetic rotations and movements. These actions proved

adequate to alter the positioning of the embryo within the egg envelop. The embryo assumed a twisted posture at 146:50 hours (Plate 4-e).

**Hatchling** – Hatching occurred approximately at 192 hours after spawning. Despite a consistent temperature, the hatching process exhibited variability across different reproductive occurrences, and even within the same group of eggs, spanning a duration of several minutes to half an hour. Typically, the tail emerged initially, and following a partial breakage of the egg membrane, the unhindered embryos, often with the head portion still enclosed, remained within the egg envelope (Plate 4-f).

**Jaws and branchial arches -** The concentrated clusters of mesenchyme forming the jaws and branchial arches were seen in the region between the head and yolk mass. Upon stimulation, hatched embryos performed swift and conspicuous motions, spinning in circles with the yolk sac movement. The newly hatched embryos were deliberated directional movement within the first 10 days (Plate 4-g).

**The Protopterygiolarval phase**

**Mouth opening** - The head process had already experienced a substantial elevation. Numerous structures are now more distinctly delineated. The dorsal and caudal fins emerged concurrently as more concentrated masses of mesenchyme within the fin fold. Melanophores dispersed considerably, not only on the cranial section but also made their initial appearance on the trunk and tail region. The cranial region was elevated and experienced additional growth and alignment. The mandible had moved forward in a straightened manner. The mouth aperture exhibited “&” configuration, with evident demarcation between the upper and lower jaws in day 10 (Plate 4-h).

**Progressive median fin-fold regression and formation of distinct fins** - The dorsal and caudal fins were extended along the embryonic fin fold. The continuous decline of the dorsal median fin fold assisted in defining the form of the body, including the dorsal, anal, and caudal fins. The embryos progressively gained mobility and appeared to be drawn towards clusters of nourishing organisms. The embryo attained the ability to completely elevate its head and actively initiate the opening or closing of its oral cavity. The yolk-sac's capability had markedly reduced in days 12 (Plate 4-i).

**Late embryo** - The height of the dorsal embryonic fin fold was a substantial reduction. The pectoral fins were generated with a proximal bud, and a fan was sustained by the segmented lepidotrichial rays. They were operational and employed, as in numerous other teleostean larvae, for directional impulsion. At this phase, the initial movement of active respiration of the gill cover occurred in day 20 (Plate 4-j).

**The larval period**

**Exogenous feeding** – In the initial stage of the larval stage, external exogenous feeding took place simultaneously with internal nutrient utilization. The residual yolk sac was still evident but nearly absorbed. The dorsal fin had undergone growth, displaying formed fin-rays. The definitive form of the tail fin was achieved through the prominent narrowing and regression of the remaining dorsal fin fold at the tail's end. The pectoral fins were adequately developed and exhibited high mobility by day 25 (Plate 4-k).

**Eye differentiation** - The larvae exhibited high mobility and actively sought out nourishment. The pigmented outer layer of the eye had been formed, appearing as deep-black, with visible ocular motion as the larvae swim in close proximity to the seabed. On day 25, a vivid yellow-orange cluster occupied the digestive tract (Plate 4-l).

**Anal and caudal lobe formation** - The dorsal fin was detached from the fin fold near the tail and the elevated count of bony rays became evident in the fold of the fin on the posterior side, merging with the caudal fin. The concentration of melanins had significantly increased around the entire body, particularly beneath the pectoral fins and the region above the digestive tract in day 40 (Plate 4-m).

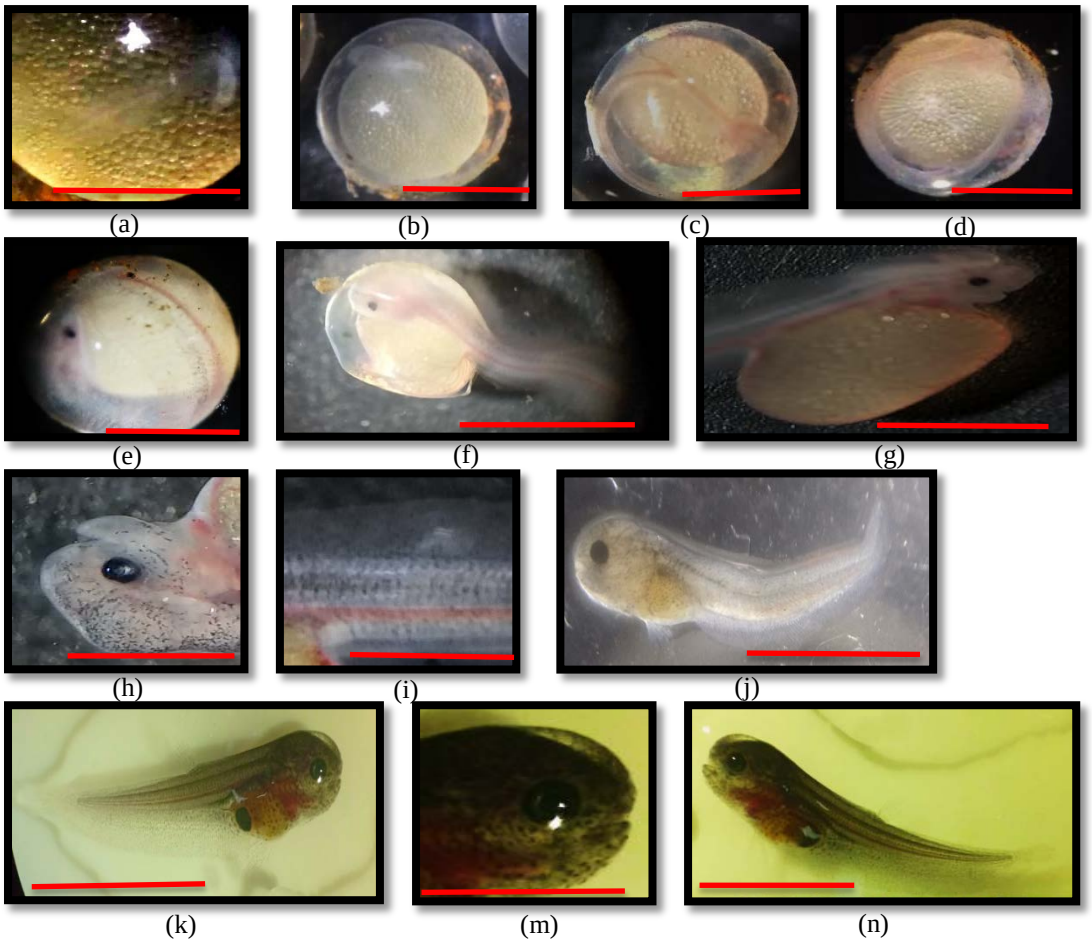



**Plate 4** Embryonic phases, protopterygiolarval phase, and larval period of *Notopterus notopterus* (a. early trunk bud, b. tail-bud bent, c. otic placode, d. eye pigmentation, e. prehatchling, f. hatchling g. branchial arches formation, h. mount opening, i. median fin-fold regression and formation, j. late embryo, m. eye differentiation, n. fin lobe formation.) Scale bar = 2mm

## Discussion

In the present study, Induced breeding performed in one native indigenous fish species *Notopterus notopterus.* Female fish laid eggs after 52 hours of the second injection.

The eggs of *Notopterus Notopterus* possessed an adhesive characteristic, and the present study confirmed the species as a button spawner, producing relatively sizable eggs. The eggs were partially transparent in nature, encased within a yellowish yolk mass. It was documented that *Chitala chitala* exhibited an adhesive egg envelope (Axelrod and Burgess, 1981).

The egg of *Notopterus notopterus* was found with external ridges which were centered round the single micropyle located at the animal pole. Based on the four classifications of micropyle of *N*otopterus *notopterus*, it exhibited a spiral arrangement of raised lines that partially

terminated in the micropylar regoin (Kunz, 2004). The configuration grooves on the surface of the species followed a radial pattern, extending from the animal pole to the vegetal pole. The eggs of the Loricariidae family's *Sturisoma aureum* and the cyprinid *Barbus conchonius* also possessed a micropyle with furrows and ridges directed towards the micropylar canal (Amanze and Iyengar 1990).

Spawning in *N. notopterus* occurred mostly at night during study. Friese (1980) also reported that spawning in *Notopterus notopterus* tended to occur in the early morning. Similarly, Pinxteren (1974) reported the spawning of *Chitala* and *Notopterus* species occurring mainly at night. The number of the eggs laid by the female *Notopterus notopterus* was 355 eggs.

Southwell and Prashad (1919) reported that the higher number of eggs laid by *Chitala chitala* was 300–500 eggs. In the present study, it was found that *Notopterus notopterus* possessed the longer body than the other fish species. The fertilized eggs of *Notopterus notopterus* hatched after 192 hrs (8 days) at 23˚C. Yanwirsal, *et. al.,* (2017) had shown that *N. notopterus* hatched at 168 hrs (7 days) after spawning at temperature of 27 °C. Srivastava, *et. al*., (2012) reported that the hatching period of *Notopterus notopterus* was 5–6 days after spawning.

According to present results, eye differentiation in *Notopterus notopterus* occurred within five day. Eye pigmentation of *Notopterus notopterus* lasted five or six days. Diedhiou *et. al*., (2007b) showed that eye pigmentation of *Notopterus notopterus* was found on the fourth day after hatching.

The melanophore or pigment melanin was diffused on the integument of *Notopterus notopterus* when the embryo was in 5 days old. In fishes of the *Paramormyrops magnostipes*-complex, black melanophores developed one day after hatching (Nguyen, 2011). Similarly, Yanwirsal *et al*. (2017) had shown the onset of melanophore development in the integument of *N. notopterus*. It became apparent on the frontal region before hatching, which occurred at five to six days.

Induced breeding of *Notopterus notopterus* was conducted using busereline acetate (suprefact). Yulindra *et. al*., (2017) examined the effect of different dosage of ovaprim in induced breeding of knife fish (*Notopterus notopterus*) and they reported that ovaprim 1.5 ml/kg is the best dosage for *N. notopterus*. According to the present study, busereline acetate (suprefact) can be used for induced breeding of *Notopterus notopterus.*

## Conclusion

Induced breeding of *Notopterus notopterus* was succeeded using Buscerelin acetate (suprefact). This experiment was conducted to study on embryonic and larval development of Bronze featherback knife fishes by artificial propagation. The embryonic developmental stages from the morula to the hatchling lasted (146-192) hours which was longer than approximately 120 hours of carp species.

## Acknowledgements

We would like to express the profound gratitude to Dr. Aye Mi San, Professor and Head (Retired), Department of Zoology, University of Yangon for her encouragement. Our thanks go to Daw Thin Thin Oo, Deputy Director, U Ye´Htun Naing, Assistant Director and all staff of Hlawgar Fishery Station for their help and permission to conduct the experiment in Hlawga Fishery Station.

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