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Stages of Embryonic Development of the Zebrafish *Danio rerio* (Hamilton)

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Abstract
We describe a series of stages for development of the embryo of the zebrafish, *Danio rerio***.** We define seven broad periods of embryogenesis-the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. These divisions highlight the changing spectrum of major developmental processes that occur during the first 3 days after fertilization, and we review some of what is known about morphogenesis and other significant events that occur during each of the periods. Stages subdivide the periods. Stages are named, not numbered as in most other series, providing for flexibility and continued evolution of the staging series as we learn more about development in this species. The stages, and their names, are based on morphological features, generally readily identified by examination of the live embryo with the dissecting stereomicroscope. The descriptions also fully utilize the optical transparancy of the live embryo, which provides for visibility of even very deep structures when the embryo is examined with the photomicrography.

Keywords: *Danio rerio*, Morphogenesis, Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, Hatching.

Introduction
The zebrafish *Danio rerio* is one of the most important vertebrate model organism in developmental biology (Grunwald and Eisen, 2002). Zebrafish eggs are large relative to other fish (0.7mm in diameter at fertilization) and optically transparent the yolk being sequestered in to a separate cell. Furthermore, fertilization is external so live embryos are accessible to manipulation and can be monitored through all developmental stages under a dissecting microscope (Kimmel *et al* 1995). Development is rapid, with precursors to all majororgans developing within 3 hr and larvae displaying food seeking and active avoidance behaviours within five days post fertilization, 2-3 days after hatching (Kimmel *et al* 1995).
A popular aquarium species, the zebrafish has been used in developmental biology for many years (Cg, Creaser, 1934). A staging series is a tool that provides accuracy in developmental studies. Still knowledge of early ontogeny is of critical importance in understanding the biology of a species and the functional trends and environmental preferences of the different developmental stages (Koumoundouros *et al* 2001; Borcato *et al* 2004). A detailed understanding of the ontogeny is therefore essential to identify species- specific adaptations and their ecological consequences (Verreth *et al* 1992). This part of the embryo study provides a detailed illustration of the normal development of zebrafish D. *rerio* and an integral part of the guideline for the embryo-toxicity tests. Knowledge of normal early developmental stages of zebrafish as a important guideline for the ecotoxicological test.

2. Materials and methods
2.1 Zebrafish maintenance for breeding
The adult wild type zebrafish (*Danio rerio*) is easily obtainable, readily maintainable and under appropriate conditions yields a larger number of non-adherent, fully transparent eggs (Laale, 1977). Data given in Table 1 are adopted as typically relevant characters for the maintenance of breeder fish in the present study. Care was taken to select a wild type zebrafish strain with continuously high egg production. Adult wild type zebrafish for breeding stock were purchased from Annai aquarium Azhagiamandapam. Approximately 100 fish were divided evenly in five 50 L glass aquaria and they were maintained in typically relevant dechlorinated tap water. Aeration and filtration were provided to the aquaria. Fish were fed with dry flake food or brine shrimp (Artemia) 3 times daily. The excess fecal were removed approximately one hour after feeding. Fish were acclimated to laboratory conditions for one month prior to breeding. Male fish can easily be distinguished from females by their slender body shape and an orange to reddish tint in the silvery bands along the body.

2.2 Parental breeding procedure

One month after acclimation to laboratory conditions, fish were grouped based on gender and kept in fully aerated 25 L glass tanks filled with tap water. The culture conditions are 25 $\pm 1^0$C at a 14 hr day/10 hr night. The fish spawning was conducted in spawning tray to obtain eggs for the exposure experiments. A larger spawning trays covered by meshed lid were placed at the bottom of the normal maintenance tank. The afternoon of the day before spawning, eight females and four males were placed in glass tank with spawning trays on the following day, at 1 hr after start of light cycle, spawning trays were removed from the tanks. Eggs were collected and placed into plastic petridishes containing sterile salt-based egg water (60 mg/l instant ocean salts) (Westerfield, 1995) and assessed for viability under a dissecting microscope. Fertilized eggs are translucent while non-fertilized ones appear opaque. Fertilized eggs were then divided into 5 ml sterile glass petridishes with approximately 50 eggs per dish and rinsed using egg water to remove any waste matter. 1hr PF viable eggs were selected and used for embryo toxicity test exposed in Alkylbenzene sulphonate.

Table 1: Maintenance, breeding and condition for embryo toxicity test of zebrafish *Danio rerio*

S.NO	Character	Description
1	Origin of species	India, Burma, Malakka, Sumatra
2	Sexual dimorphism	Females: Protruding belly, when carrying eggs Males: More slender, orange tint between blue longitudinal stripes.
3	Feeding	Dry flake food, Artemia twice daily
4	Approximate of adult fish	Female: 0.79 ± 0.18g Male: 0.68 ± 0.11g
5	Water criteria a. Water temperature Water quality	25.0 ± 1.0^0 C Hardness: 32- 218 (mg/l $CaCo_3$), residual chlorine <11µg/l, pH= 7.9 ± 0.3, particulate matter < 20 mg/l.

3. Result

The zebrafish egg is telolecithal, cleavage is meroblastic and discoidal, the observed selected major stages of zebrafish development details are given in Table 2. Entire embryogenesis was described in *D.rerio* (from fertilization to the end of hatched out yolk-sac larvae) with focused on the organogenesis. The cleavage period (45 min to 1.30 hrs), the blastula (4 hr), gastrulation (10 hr), segmentation (10.30 to 20 hr), pharyngula period (24 to 36 hr) and hatching (48 to 96hr) were observed Table 1 and Fig 1.

3.1 Cleavage phase

First cleavage occurred at the animal pole (discoidal cleavage) at 45 min post fertilization (PF), forming two equal sized blastomeres. The 4 and 8 cell blastomeres stage appeared 1hr and 1.25 hr PF, respectively. The 16 cell blastomeres stage was observed at 1.5 hr PF followed by the blastula stage at 2- 4 hr PF.

3.2 Blastula

At 3.45 hr PF, flattening of the cellular materials occurred, leading the formation of the blastula
(Fig 1.A and 1.B).

3.3 Gastrula

Cells of the disc spread over the yolk mass towards the vegetal pole (4hr PF) replacing the blastoderm margin and initiating gastrulation. At 5.15 hr PFR epiboly covered nearly half of the yolk (50% epiboly stage). 10 hr PF the process of epiboly was completed and the embryonic shield was formed (Fig 1.C and 1.D).

3.4 Segmentation

The process of segmentation started at 10. 5 hr PF formed with first somite furrow. At 12 hr PF somites are developed, mesodermal component of the early trunk was formed and tail was segmented. At 20 hr PF, the tail well extended (Fig 1.E).

3.5 Hatching

The hatching process started at 48 hr PF and end at 96 hr PF. The embryo showed twisting movement inside the eggs few hours before hatching. 48 hr after fertilization certain egg membranes were ruptured with caudal region of the embryo and the tail emerged out followed by the rest of the body (Fig 1.F & 1.G).

3.6 Newly hatched embryo

The body of the newly hatched embryo remained in a curved position for few hours after hatching, with the head bend down over the yolk. The newly post hatched (PH) embryo was transparent, light yellowish colour. Mouth and anus were not opened and the eyes were still translucent. A thin membranous fin fold surrounded the caudal region (Fig 1.H).

3.7 24 hr old post hatched larva

The size of the yolk sac was reduced and the membranous fin fold expanded. The tail curved and barbells appeared. The alimentary canal could be seen as a straight tube emerging from post dorsal part of the yolk sac.

3.8 48 hr old post hatched larva

The membranous fin fold surrounded the entire area from behind the head region. Optic and auditory vesicle could be distinguished. The mouth and jaws began to differentiate and the barbells become elongated.

3.9 72 hr old post hatched larva

The yolk sac was reduced. The barbells became larger around the well developed mouth. The eyes further differentiated (Fig 1.I).

3.10 120 hr old post hatched larva

The yolk sac were gradually replaced by the developing alimentary canal until the yolk sac was completely reserved (Fig 1.J).

Table 2: Stages of embryonic development of the control zebrafish *Danio rerio*

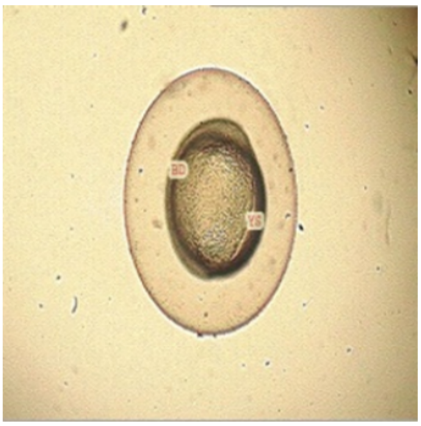
Time (h. min)	Stage	Characterization
	Fertilization	Zygote
	Zygote period	Cytoplasm accumulates at the animal pole, one cell stage.
	Cleavage period	Discoidal partial cleavage division.

0.45 1 1.15 1.30		Two-cell stage; (median, vertical, division) Four cell stage; (Vertical division) 8 cell stage; (Vertical and parallel of the plane of the first division) 16-cell stage; (Vertical and parallel of the plane of the first division)
2 3 4	Blastula period	Start of blastula stage Late cleavage; (Blasto disc contains more number of blastomere) Flat interface between blastoderm and yolk.
5.15 8 10	Gastrula period	50% of the epibolic movements; blastoderm thins and blastoderm become curved. 75% of epibolic movement. Epibolic movement ends.
10.30-20	Segmentation period	Somites are developed undifferentiated mesodermal component of the early trunk, tail segmented. Tail well extended.
24 30 36	Pharyngula period	Spontaneous movements, tail is detached from the yolk, early pigmentation. Reduced spontaneous movement; retina pigmented, cellular degeneration of the tail end. Tail pigmentation; heart beating.
48-96	Hatching period	Heart-beat regular; yolk extension beginning to taper; dorsal and ventral pigmentation stripes meet at tail; foregut development.

4. Discussion

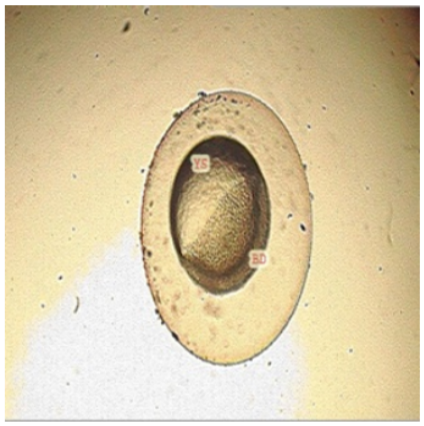
Studies on the normal embryonic development of *D. rerio* are important not only to increase the knowledge about the developmental process but also to understand the time specific developmental process in course of particular fish species. The zebrafish embryo has become major model in neuro biology and developmental biology (Westerfield, 2000; wixen, 2000). Previously development of zebrafish has been described in most detail (Roosen-Runge, 1938; Thomas and Waterman, 1978; Kimmel *et al* 1995). The variation in egg size and time of developmental stages in zebrafish was recorded (Thomas and Waterman, 1978; Kimmel *et al* 1995).

1. A. Blastula stage (3.45 hr)



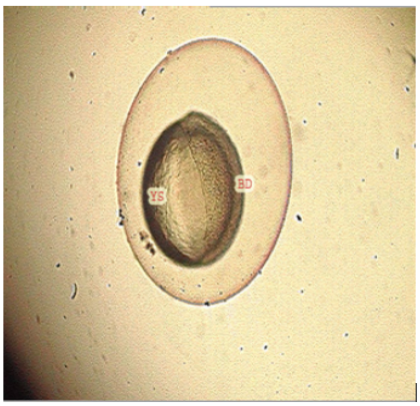
BD – Blasto Disc, YS – Yolk Sac

1. B. End of blastula stage (4 hr)



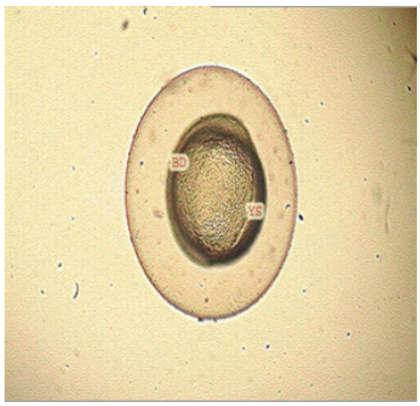
BD – Blasto Disc, YS – Yolk Sac

1. C. Gastrula stage 50 % Epibolic movement (5.30 hr) 1



BD – Blasto Disc, YS – Yolk Sac

1. D. Gastrula stage 75 % Epibolic movement (8 hr)

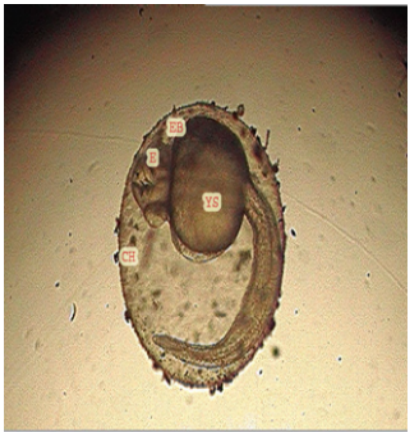


BD – Blasto Disc, YS – Yolk Sac

1. E. 18 hr old embryo stage

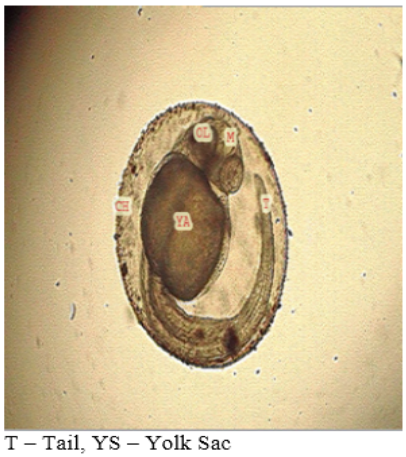
E – Eye, YS – Yolk Sac
SB – Segmented Body

1. F. 40 hr old embryo stage



E – Eye, YS – Yolk Sac
EB – Ear Bud, CH – Chorion

1. G. 48 hr old embryo stage



T – Tail, YS – Yolk Sac
OL – Optical Lens, CH – Chorion
M – Melanophore

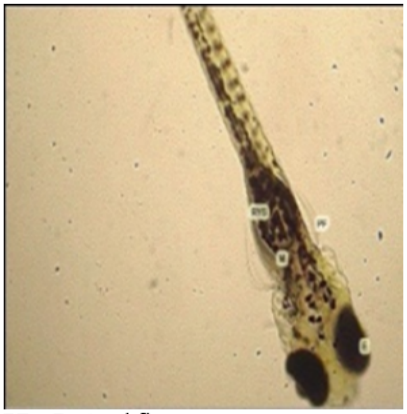
1. H. 62 hr old newly hatched embryo

M – Melanophore
G – Gut, A – Anus
P - Pericard

1. I. 72 hr old post hatched embryo

G - Gut
RYS – Reabsorbed yolksac
M – Melanophore
E – Eye, A – Anus

1. J. 120 hr old post hatched embryo



PF – Pectoral fin
RYS – Reabsorbed yolksac
M – Melanophore
E - Eye

Fig 1: Normal embryonic development of Zebrafish *D. rerio*

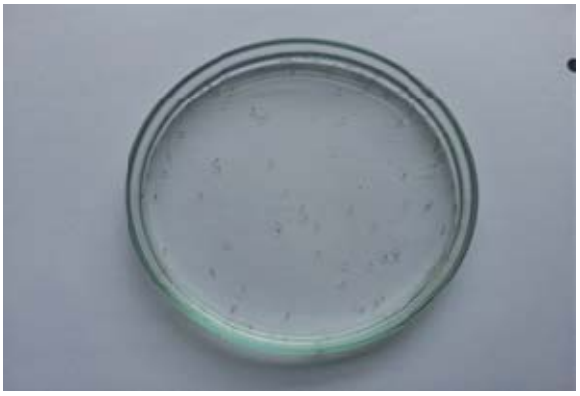


Fig 2: Zebrafish *D. rerio* newly hatched young ones

For the first time some of the cells now become completely cleaved from the others. These "complete" cells are the four most central blastomeres, the quartet that is entirely surrounded by other cells in Figure B. Their complete cleavage occurs near the end of the 16- cell stage because of the way the cleavage furrows undercut the blastodisc from the center, going outward toward the blastodisc margin. Indeed, the undercutting furrows still do not reach the margin, and the 12 cells surrounding these four central ones, the so-called marginal blastomeres, remain connected to the yolk cell by cytoplasmic bridges (Kimmel and Law, 1985a). From this stage onward until the midblastula period the cleavages completely partition most or all of the nonmarginal blastomeres, but still incompletely partition the marginal ones.

Epiboly, beginning in the late blastula (Solnica- Krezel and Driever, 1994), is the thinning and spreading of both the YSL and the blastodisc over the yolk cell, as you might model by pulling a knitted ski cap over your head. Eventually, at the end of the gastrula period, the yolk cell becomes engulfed completely. This is accomplished by the streaming outward, toward the surface, of the deepest blastomeres. As they move, they mix fairly indiscriminately among more superficial cells along their way (Wilson *et a1*1993). Active cell repacking by these so-called radial intercalations (Keller, 1980) may be a part of the driving force of early epiboly. The intercalations do not drive deep cells into the EVL, which remains a compartmentalized monolayer (Kimmel *et al* 1990b). Additionally, deep blastomeres at the margin mix together to a considerably lesser extent than do the central ones (Helde *et al* 1994).

Presuming that events specifying mesoderm begin to occur before epiboly, as they seem to do in *Xenopus*, then lack of mixing in the marginal deep cell population during epiboly could be important to maintain regions with different cellular "specifications" (Kimmel *et al* 1991) or identities.

Epiboly appears to depend on functional microtubules (Strahle and Jesuthasan, 1993) and might be under control of early-acting zygotic genes (Kane, 1991). Axial mesoderm appears to have the potential to induce a second embryonic axis, as revealed in transplantation experiments involving cells of the embryonic shield of the early gastrula (Ho, 1992b).

The earliest cells to elongate into muscle fibers appear to derive from a part of the medial somitic epithelium, the "adaxial" region (Thisse *et al* 1993) adjacent to the developing notochord, and in the middle, dorsoventrally, of each somite. The precocious subset of adaxial cells may include a special cell class, the muscle pioneers (Felsenfeld *et al* 1991), recognizable by enhanced expression of specific markers (Hatta *et al* 1991a).

5. Conclusion

Therefore, the developmental biology of control zebrafish was undertaken as a part in present work of toxicological testing on zebrafish embryo.

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