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Sambid Swain
[1]ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India
[2]Centurion University of Technology and Management, Odisha, India

Paromita Banerjee Sawant
ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India

Narinder Kumar Chadha
ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India

JK Sundaray
ICAR-Central Institute of Freshwater Aquaculture, Odisha, India

Chandra Prakash
ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India

Corresponding Author:
Sambid Swain
[1]ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India
[2]Centurion University of Technology and Management, Odisha, India

Effect of water pH on the embryonic development of Discus, *Symphysodon aequifasciatus*, Pellegrin, 1904

Sambid Swain, Paromita Banerjee Sawant, Narinder Kumar Chadha, JK Sundaray and Chandra Prakash

Abstract
The study was conducted to determine the influence of varying incubating water pH of 5.26 ± 0.14, 6.60 ± 0.12, 7.36 ± 0.08, 8.60 ± 0.15 on hatching percentage, incubation period, survival and initial egg development of discus, *Symphysodon aequifasciatus*. The study illustrated that discus eggs required a water pH between5.26 to 8.60 for hatching. The egg incubated at acidic pH had smaller eggs diameter compared to alkaline pH treatments. Highest embryonic survival was recorded at pH 6.60. It can be noted that there is a profound influence of incubation water pH on the embryonic stage as it influences from cleavage stage till eclosion, the eggs incubated in acidic pH developed faster than those incubated in alkaline pH from cleavage stage till successful hatching. It can be concluded that pH affects the initial eggs development and pH 6.60 can be considered best for discus egg development with minimum incubation duration and highest survival along with high hatching percentage, which can be helpful to promote successful hatchery operation.

Keywords: Discus, pH, embryology, Ornamental Fish

Introduction
The global ornamental fish industry is a fast growing industry with international export touching around 347.5 million US dollar and the wholesale value is estimated about 1 billion in 2014 (Dey, 2016) [1]. Discus, is considered as a popular freshwater ornamental fish fetching high price and has a considerable market demand for its attractive and colorful appearances (Lim and Wong, 1997 [2]; Chong *et al*., 2002) [3], is one of the member of Cichlids family. It is native to the Amazonian basin, where it is mainly found in unpopulated small streams and creeks (Giovanetti, 1991 [4]; Farias and Hrbek, 2009) [5]. Discus possess an unique and complex reproductive behaviour which involves establishment of breeding territories by male, selection of mate for pair formation, selection of suitable site or substrate for egg laying and its cleaning and subsequent spawning (Chellappa *et al*., 2005 [6]; Chong *et al*., 2005 [7]. The embryonic development of fishes from fertilization till hatching follows the same chronological pattern (Falk Peteren, 2005 [9]; Anjos and Anjos, 2006) [5]. Ecolusion and embryonic developmental stages depends on the species and the prevailing environmental condition in which the eggs are incubated. The abiotic factors such as temperature, pH, water flow, water hardness and photoperiod plays an important factor crucial for growth and reproduction in aquatic organisms (Menni *et al*., 1996 [10]; Mazerolle, 2005 [11]; Lacoul & Freedman, 2006) [12]. The pH of water not only influence the early life stages, mortality and disease resistance but also the growth and reproduction in fishes. Extreme water pH may causes mass mortality in cultured fishes (Doudoroff 1956[13]; Kwain 1975[14]; Jezierska and Witeska 1995 [15]; Zweig *et al*. 1999 [16]; Zaniboni-Filho *et al*. 2002 [17]; Scott *et al*. 2005 [18]; Zaniboni-Filho *et al*. 2009) [19]. Sensitivity to extreme values of pH condition varies accordingly to fish species and age, most of the fishes are highly sensitive to extreme pH condition during its early embryonic and larval stages (Lloyd and Jordan, 1964 [20]; Laurence and Howell, 1981) [21]. Earlier studies reported that a decreased pH can abruptly increase the metal toxicity by reducing the availability of essential substances (Leduc *et al*., 2013) [22]. Despite being a popular ornamental fish which is widely distributed around the world, there are very scanty records about its early development. Given its high market value and popularity, studies on this species have become increasingly relevant particularly those related

to embryonic and larval development of the species, which will improve larval rearing and also serve as a basis for conservation work in the species. Investigation of the effect of pH the embryology of the fish is scare. In the view of this, the present study was undertaken to determine the effect of varying pH on the various morpho-physiological events in embryonic development of discus.

Material and Method

Broodstock Maintenance

The brooders of *Symphysodon aequifasciatus* were maintained in a 1000 L capacity glass aquarium tank which was equipped with power filter and heater for maintaining a conducive environment for breeding. Temperature was maintained at 28.5^{0}C and photoperiod was set at 13h light; 11hr dark. Fishes were allowed for pair and after pairing the pair was shifted to individual aquaria of 1000 L capacity. The brooders (103.4± 9.32g) were fed with commercial feed (Tetrabits) and moist feed (Minced Chicken, Beef Liver) @ 6% and 8% body weight respectively every alternate day.

Assessment of Water quality parameters

Physico-chemical parameters *viz*. temperature, pH, dissolved oxygen, Free carbon dioxide, Total hardness, ammonia (NH_3-N), nitrite (NO_2-N) and nitrate (NO_3-N) were measured daily as per the standard protocols.

Spawning, Egg collection and the Experimental design

The paired fishes were kept in spawning tanks of 1000 L capacity which was fitted with thermostat, heater, sponge filter and a PVC pipe of 30 cm length as substrate. After spawning the substrate along with the eggs were transferred to four incubators of 5 litre capacity having water of varying pH levels, 5.26 ± 0.14, 6.60 ± 0.12, 7.36 ± 0.08, 8.60 ± 0.15 for evaluation of embryonic development. These incubators were fitted with automatic thermostats to maintain a stable temperature along with aeration facilities. For lowering the water pH, concentrated sulphuric acid was added to the bore water having 7.8 pH and for alkaline pH treatments sodium hydroxide was added till the desired pH was obtained.

Observing the developmental stages

For observation of different larval stages the eggs at different duration were collected from the substrate using scalpel and were counted. The developmental stages of eggs were analysed for single spawning and pH trails were done for three consecutive spawning, from incubation period till eclosion. For investigating the different stages of embryonic development following procedure were followed: (i) Until the end of gastrula period observation were done every 30 minutes (ii) From the start of organogenesis till the emergence of heart every 60 minutes the observation were made (iii) Every 90 minutes emergence of the heart until the time of hatching under olympus SZX16 strereozoom microscope (15X magnification). During the observation the eggs were also inspected for their quality, those unfertilized and have fungal infections were discarded. Based on the classification of embryonic stage proposed by Mattos *et al*., 2014 [23], Radael *et al*. (2013) [24]; Fujimoto *et al*., 2004 [25]; Humphrey *et al*., 2003 [26]; Fujimoto *et al*. (2006) [27]; Reid & Holdway, 1995 [28] the embryonic development stages were identified and characterized at the time when >50% of the eggs observed reached a certain stage of embryonic development. For measuring the diameter of hydrated egg, perivitelline space and size of yolk sac the recommendation of Ahlstrom *et al*., 1976 [29] was followed and for fertilization rate and hatching time Godinho., 2007 [30] recommendation were followed.

Statistical Analysis

The effect of water pH on the egg size and perinuclolar space, incubation period, hatching success, mortality and degree of abnormalities were analyzed by one way analysis of variance (ANOVA), which was carried out by SPSS 16. Difference between the treatment means for various parameters were tested using Duncan multiple range test and were considered significant if p-value is <0.05.

Results

Water Quality

The experiment was carried out in closed environmental conditions in order to restrict abrupt changes in temperature variation and other water quality parameters. All the physiochemical parameter of water were monitored regularly. Oxygen levels were always higher than 7.8 mg l^{-1}, total ammonia and nitrite levels were never greater than 0.3 mg $l^{-1}$and 0.01 mg l^{-1}, respectively over the entire course of the experiment. The alkalinity and hardness averaged at 30 mg $CaCO_3$ l^{-1} and 50 mg $CaCO_3$ l^{-1} respectively. The water pH values remained as per the experimental design. (Table 1)

Table 1: The range of the water quality parameters observed during experimental period.

Water quality parameters	Range
Temperature(^{0}C)	28-31.5
pH	As per Experimental Design
Dissolved oxygen (mgl^{-1})	5.2-6.8
Free carbon dioxide (mgl^{-1})	0-4
Total hardness (mgl^{-1})	48-62
Ammonia-nitrogen (NH_3-N)	0.01-0.04
Nitrite-nitrogen (NO_2-N)	0-0.02
Nitrate-nitrogen (NO_3-N)	0.48-2.13

Newly released egg

Newly released yellow coloured eggs were oval shaped with an average diameter of 1.37 ± 0.14 mm were found attached to the substrate by cementing substances. The discus eggs are considered to be polilecitos as they have large amount of yolk, which is mostly concentrated at the vegetal pole and all the organelles are concentrated at the animal pole. The unfertilized eggs are off white in colour and its chorion is very touch sensitive compared to fertilized eggs (Mattos *et al*. 2014) [22]. The embryonic stages of discus has been given in plate 1

Effect of pH on egg diameter and perivitelline space

The eggs incubated at acidic pH of 5.26 and 6.60 had an average diameter of 1.29 ± 0.17 mm and 1.34 ± 0.23mm respectively which was significantly smaller than the eggs incubated in alkaline pH,7.36 (1.37 ± 0.15 mm) and 8.60 (1.44 ± 0.20 mm). Apart from egg diameter the perivitelline spaces was also significantly smaller in acidic pH 5.26 (0.22 ± 0.020 mm) and 6.60 (0.29 ± 0.017 mm) compared to alkaline Ph7.36 (0.32 ± 0.029 mm) and 8.60 (0.35 ± 0.014 mm). (Fig 1)

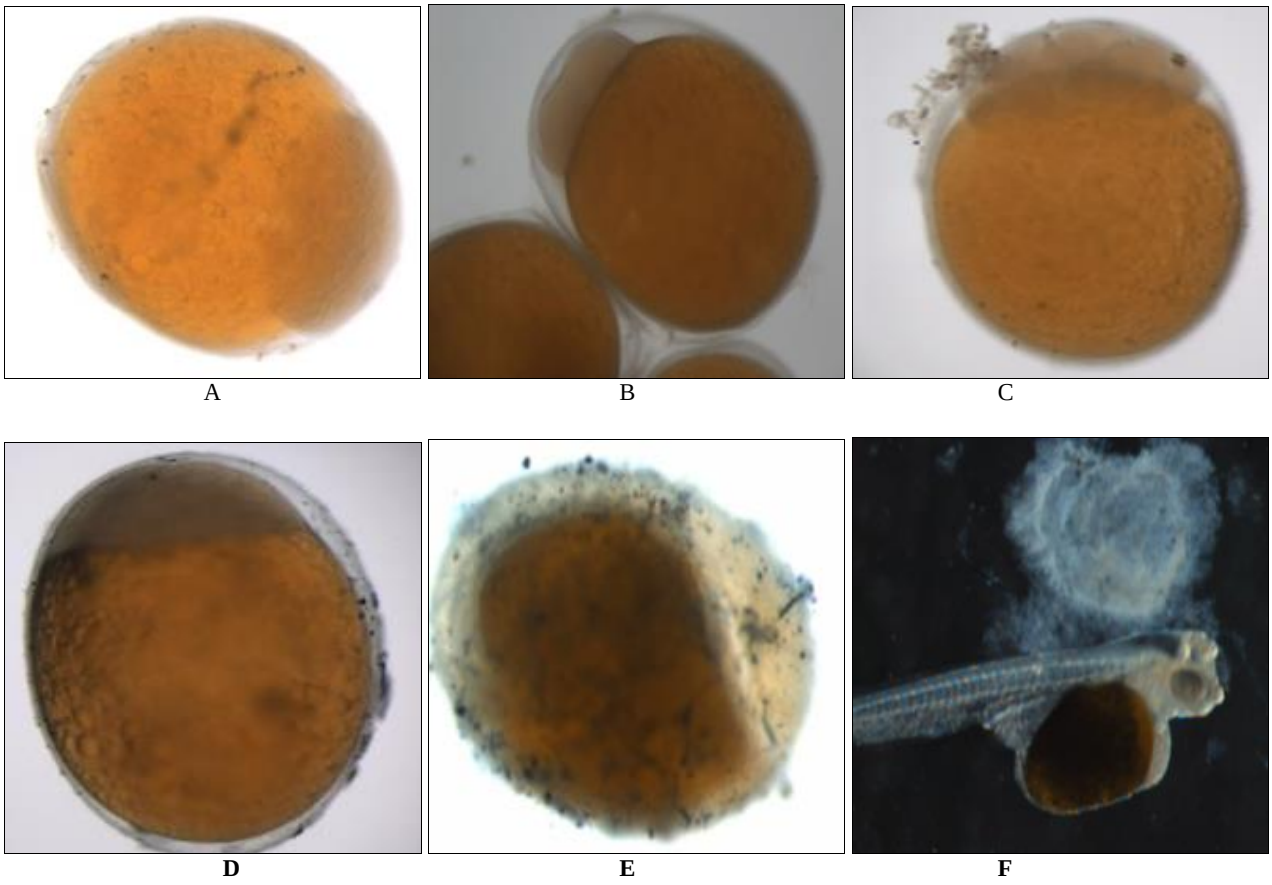

Fig 1: A Recently fertilized egg with only one blastomere (B) 4 cell embryonic stage (C) Blastula Stage (D) Gastrula (E) Organogenesis (F) Hatchlings.

Effect of pH on egg diameter and perivitelline space

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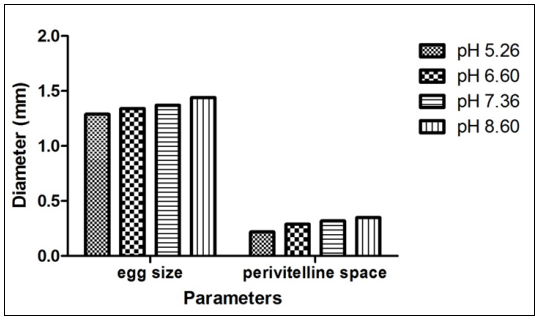

Fig 2: Egg ddiameter and perivitelline space diameter of eggs in relation to varying pH levels

Hatching percentage

There was a marked relationship between the hatching percentage and the pH of the incubating media as significant difference among the treatments were observed ($p < 0.05$). Highest hatching rate was observed at pH 6.60 (82.33 ± 1.45%) followed by pH 7.36 (79.12 ± 1.52%) and pH 8.60 (52.33 ± 2.18%) the lowest being pH 5.26 (20.00 ± 1.86%). The hatching percentage with respect to varying pH is graphically represented in fig 2.

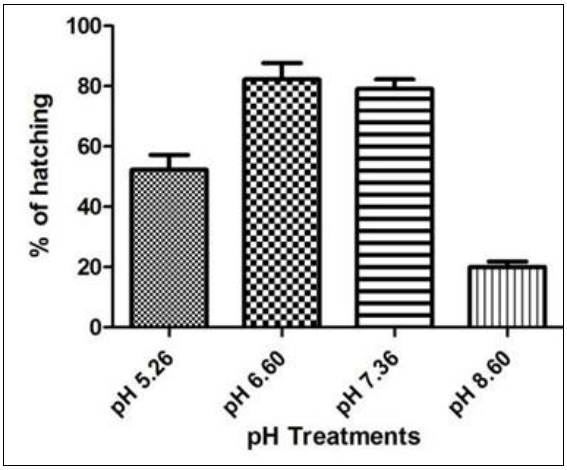

Fig 2: Hatching percentage of eggs in relation to varying pH levels

Mortality and Percentage abnormalities of early larval stage:

The cumulative mortality varied broadly based on the pH of the incubating media. Among the treatment group higher mortality was observed in alkaline pH than in acidic pH. For acidic treatment highest mortality was observed in pH 5.26 (65.00 ±1.15) followed by 6.60 (17.66 ±1.45) and for alkaline media highest being pH 8.60 (47.66 ±2.18) followed by 7.36 (35.00 ± 1.52).At pH 5.26 about 8% of eggs were observed with incomplete formation of perivitelline space although for all other treatment there was complete formation of perivitelline space. Mortality of the eggs in relation to varying pH levels is represented in fig 3.

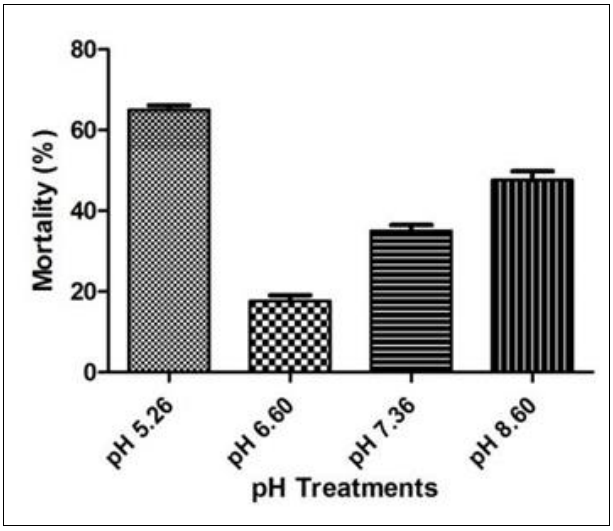

Fig 3: Mortality of the eggs in relation to varying pH levels.

Effect of pH on developmental rate

No significant difference in the timing of the developmental stage was observed till the early gastrula phase after which alkaline pH, 7.36 and 8.60 showed a significantly delayed developmental rate in developing embryo compared to other treatment. Similar tread in the timing of developmental stage for eggs from fertilization till the heart beat stage for eggs incubated at pH 7.36 and 8.60, after which the rate of development was faster for pH 7.36 till hatching. Significant changes in the timing of developmental stages was recorded for all the treatments which negatively correlated for alkaline treatments (Table 2).

Table 2: Embryonic developmental stages in relation to varying pH levels

Stage of Development	Characters	pH 5.26 (hpf)	pH 6.60 (hpf)	pH 7.36 (hpf)	pH 8.60 (hpf)
Zygote	Recently fertilized	0.0	0.0	0.0	0.0
Clevage	2 cell	1.0	1.0	1.0	1.0
	8 cell	5.30	3.0	3.0	3.5
	32 cell	10.30	5.0	7.0	8.0
	64 cell	18.40	9.5	14.5	16.0
Blastula	128-1028 cell	26.0	18.5	20.5	22.0
Gastrula	10%	31.30	22.0	24.0	27.5
	50%	34.50	28.0	31.0	32.5
	90%	40.30	32.5	37.5	38.5
Organogenesis	Appearance of head and tail	46.00	34.5	40.0	42.0
	Optical primodium	54.30	39.5	46.5	48.0
	Heartbeat	62.30	45.5	55.5	58.5
	Muscle contraction	67.0	53.0	60.5	64.5
Eclosion	Hatching	76.05	61.10	69.06	74.00

Period of discus eggs

The pH of the incubating media has an considerable effect on the incubation period of the eggs. Hatching occurred at 76:05, 61: 10, 69:06hr and 74:00 (h:min) hours post fertilization (hpf) for eggs incubated at pH 5.26, 6.60, 7.36 and 8.60 respectively (Fig 4).

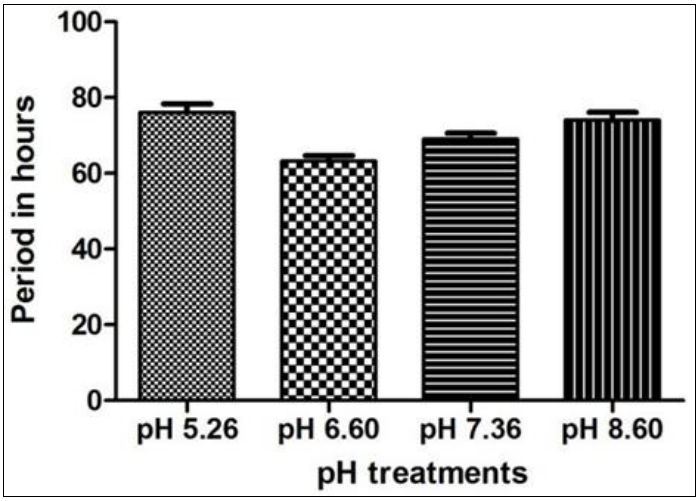

Fig 4: Incubation period (hrf) in relation to varying pH levels

Discussion

Most of the physiochemical parameters of the incubating medium were within favourable ranges for fish culture throughout the experimental period (Witeck *et al.,* 2011 [31]; Oba *et al*., 2009) [32]. Earlier studies showed that water pH has an influence on the egg quality and the early developmental stages (Lopes *et al*., 2001 [33]; Townsend & Baldisserotto, 2001 [34]; Baumgartner *et al*., 2008) [35]. Most of the gametes and early embryos of fishes are sensitive to changes in water pH; the degree which may vary depending on the type of species and its stages (Depeche and Billard, 1994 [36]; Woolsey and Ingermann, 2004) [37]. Normally the early life stages of fishes are more sensitive than juveniles and adult (Depeche and Billard, 1994 [36]; Zaniboni-Filho *et al*., 2002) [17]. The pH of the incubating media significantly interfere with the exocytose of the cortical alveoli and also affects the osmotic capacity of the perivetelline fluids which resulted in the alteration of the size and shape of the eggs (Depeche and Billard, 1994) [36]. In this study highest egg diameter with larger perivetelline space were found in eggs in alkaline incubating media compared to acidic treatment which may be due to changes in the egg hydration mechanism, which corroborates with the work in *Leporinus obtusidens* (Mário Augusto gosmann and Alex Pires de Oliveira Nuñer, 2015) [38] *Prochilodu slineatus* (Sanches EA *et al.,* 2015) [39]; *Microhyla ornate* (Padhye AD and Ghate HV., 1988) [40]; New Jersey frogs (Gosner and black, 1957) [41], *Ambystoma* salamanders (Pough and Wilson 1977) [42], Ambibians (Freda and Dunson, 1985) [43].

The present study shows that at slightly acidic water pH may yield higher hatching percentage which is concurrent with the studies of Martti Rask (1984) [44], Edward and Gjedrem (1979) [45] and Runn *et al*.,(1977) [46]. Studies has also shown that in extreme waters pH of 4.5 and 9.5 trout eggs rarely hatches (Depêche billard, 1994 [36]; Ferreira, *et al*., 2001) [47]. Further the delayed in hatching and decrease in hatching percentage in very low acidic water can be attributed to the inactivation of enzyme chorianase, which prolongs the duration of hatching at pH lower than 6.5 (Reynalte-Tataje *et al*., 2015) [48]. The current study depicts that hatching time in alkaline and slightly acidic incubating media were faster compared to very low acidic pH of 5.5 as there may be physical alterations of the chorin i.e. the reduction of chorin permeability. Similar result was also recorded for Zebra fish (Zahangir *et al*., 2015) [49] *Salmo truttafario* (Brown and Lynam, 1981) [50], brook trout *Salvelinus fontinalis* (Swarts *et al*., 1978) [51] and *P*. *promelas* (Mount, 1973) [52] contradictions to the present study was reported by Daye and Garside (1979) [53] and Menendez (1976) [54] who stated that there is no influence of pH on incubation period in atlantic salmon and *S*. *fontinalis* respectively. Even rapid development of *S*. *fontinalis* at pH levels lower than pH 5.0 was reported by Trojnar (1977) [55]. Under optimal pH condition the perivetelline fluid of egg allows the passage of Many authors have attributed that anoxia is a major cause of low survival at low pH less than 6.5 which may be due to loss of sodium that disturbs homeostatic condition in fish (Robinson *et al*., 1976) [56] or any change in the internal condition of the egg cell lead to post hatch deformities and mortalities (Melzner *et al*., 2009) [57]. Higher mortality rate along with embryonic deformities was observed for acidic treatment than that of alkaline treatments. Similar result of were also observed in Atlantic Salmon (Daye and Garside, 1979 [53]; Peterson *et al*.,1989) [58]. cations such as K+, Ca_2+,Mg_2+ and H+ but in adverse condition the passage is blocked and most detrimental effect is observed when the pH of the incubating media is very low lesser than 6.5 (Alderdice, 1988) [59]. Very low pH leads to decrease in ion exchange which ultimately decreases the accumulation of ion in egg as a result of which the overall development of the fertilized egg gets retarded. In the present study the pH of the media influenced the embryonic development from early cleavage period which preceded further till hatching. Similar observations are recorded *Rana pipiens* (Schlichter, 1981) [60], sea urchin (Todgham and Hofmann, 2009[61]; Donnell *et al*., 2010) [62].

Conclusion

From this experiment it can be noted that circumneutral pH is considered best not only for increased survival and hatching percentage but also improved development of embryo (within perivitelline space) was seen, because an increase in hydrogen ion concentration impair the formation and composition of the perivitelline space and chorion. The growth and development of embryo will be negatively affected by such changes. Taking these observations in to account it may be concluded that pH 6.60 is considered essential for successful hatchery phase of *Symphysodon aequifasciatus.*

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