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Status of Whole Body Cortisol and Total Protein Content in Eggs, Embryos and Larvae of Silver Carp (*Hypophthalmichthys molitrix*)

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Abstract

The status of whole body cortisol and total protein content in eggs, embryos and larvae of silver carp (*Hypophthalmichthys molitrix*) was investigated. After induced spawning, unfertilized and fertilized eggs were sampled and sampling was continued at an interval of two h up to 48 h of post fertilization. Unfertilized and fertilized eggs contained high level of whole body cortisol that decreased sharply and reached to significantly lower value after 2 h of post fertilization (hpf) and remained almost constant up to 8 -12 hpf. Later, it increased gradually and reached to peak value at 48 hpf. The total whole body protein content showed fluctuation during the development, higher content in fertilized and unfertilized eggs, decreased significantly after 2 hpf and remained low up to 6 hpf. At 8 hpf, the total protein content increased significantly and showed increasing trend up to 18 hpf, then sharply declined up to 48 hpf. The presence of cortisol and protein content in fertilized and unfertilized eggs confirm the view that they are maternal in origin, while higher concentration of cortisol in eggs may be due to inadequate handling of broodfish.

Keywords: Cortisol; Protein content; Eggs; Embryos; Larvae; *Hypophthalmichthys molitrix*

Introduction

Like many organisms, fish larvae development is influenced by the maternal hormones (Tagawa *et al.,* 2000; Özen and Balcı, 2011). Many investigators reported that maternal hormones related with development (e.g., T3 and T4), reproduction (like testosterone and estradiol) and metabolism (e.g., cortisol), are accumulated in eggs during oogenesis (Hwang *et al*., 1992; Tagawa *et al.,* 2000; Feist and Schreck, 2002; Simontacchi *et al.,* 2009; Li *et al.,* 2010; Ünver and Saraydın, 2012) and after fertilization, affects the success of the eggs in the production of viable offspring. From an ecological perspective two steroid hormones, cortisol and corticosteroid are of particular interest and used as an endocrinological indicator of stress (Pavlidis *et al.,* 2011). Cortisol is readily accumulated in the oocytes of cultured fish and significant amount is present in fertilized eggs, embryos and larvae (de Jesus *et al*., 1991; Hwang *et al.,* 1992; Auperin and Geslin, 2008; Simontacchi *et al.,* 2009; Pavlidis *et al.,* 2011). Fertilized and unfertilized eggs contained high content of cortisol (Stouthart *et al.,* 1998; Simontacchi *et al.,* 2009) that decreased rapidly between fertilization and hatching (Simontacchi *et al.,* 2009). Many scientists observed the affects of cortisol on hatching and different phases of growth of embryos (Sampath-Kumar *et al.,* 1995; Eriksen *et al.,* 2006, 2007; Leatherland *et al.,* 2010) and reported that cortisol promoted the survival of larvae in Asian sea bass, *Lates calcarifer* (Sampath-Kumar *et al.,* 1995), stimulated the growth of larvae in tilapia, *Tilapia mossambica* (Mathiyalagan, 1996) and stimulated the action of thyroid hormones in Japanese flounder, *Paralichthys olivaceus* (de Jesus *et al*., 1991).

It is well documented that if the cortisol concentration in the plasma of broodfish before ovulation increases then oocytes have higher content of cortisol (Leatherland *et al.,* 2010). Therefore, it might be possible that cortisol level that arises in broodfish fish due to husbandry and induced breeding practices effect the various aspects of fertilization, hatching and early embryogenesis (Auperin and Geslin, 2008; Mileva *et al.,* 2011). In humans and rodents evidences are available which confirm the negative effect of prenatal stress on the physiological and morphological aspects of offspring (Malaspina *et al.,* 2008; McGowan *et al.,* 2009). Increased fetus mortality, low birth weight, reduced immunity, decreased adrenal mass, reduced gonads, malfunctioning of nervous and neuroendocrine function in human are related to maternal stress (Rondó *et al*., 2003), while in Salmonids, the high concentration of cortisol in

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oocyte before fertilization adversely affect the early embryonic development and growth of larvae (Eriksen *et al.*, 2006, 2007; Auperin and Geslin, 2008). In rainbow trout, *Oncorhynchus mykiss* maternal stress resulted in smaller sized eggs and young (Contreras-Sánchez *et al*., 1998).

In majority of teleost during ontogeny, content of whole body cortisol decreases before the development and activation of the HPI and then increases gradually [*Paralichthys olivaceus*: de Jesus *et al.* (1991); *O. keta*: de Jesus and Hirano (1992); *Cyprinus carpio*: Stouthart *et al.* (1998); *Lates calcarifer*: Sampath- Kumar *et al.* (1995); *P. dentatus*: Veillette *et al.* (2007); *O. mykiss*: Auperin and Geslin (2008); *Danio rerio*: Alsop and Vijayan (2008)]. According to many reports de novo synthesis of cortisol usually starts soon after hatching (Hwang *et al.*, 1992; Sampath-Kumar *et al.*, 1995; Szisch *et al.*, 2005) whilst, many investigators including Veillette *et al*. (2007) reported that HPI axis is not efficient before the absorption of yolk sac in many fish species. Therefore, depending upon species, cortisol level increased in response to stressor within a day or weeks, during ontogeny of teleost (Applebaum *et al.*, 2010; Pavlidis *et al.*, 2011).

In teleost, beside hormonal status, nutrition status of broodfish also influences the survival and growth of embryo (Heras *et al.*, 2000; Powell *et al.*, 2002). In most of the vertebrate including fish, nutrition status of female determine the biochemical composition of eggs that effect the embryonic development and survival of larvae (Ronnestad *et al.*, 1998; Garcia-Guerrero *et al.*, 2003). During embryonic development lipids particularly triglycerides are the main source of energy (Heras *et al.*, 2000; Roustaian and Kamarudin, 2001) and high level reserves in eggs enhances the survival of larvae (Powell *et al*., 2002). The protein contents in general are used as structural components of embryonic tissue and in some conditions for the source of energy (Lemos and Rodriguez, 1997). The continuous depletion of lipid and significant variation in the content of protein during embryonic development was observed in of *C. quadricarinatus* (Garcia-Guerrero *et al*., 2003). In crayfish, *C. quadricarinatus*, during first 30 days after fertilization protein was used but at lower rate than lipid but after that protein consumption increased, while lipid used at the same rate (Garcia-Guerrero *et al.*, 2003). The increase in consumption and decrease in protein content during hatching is related to increase demand of energy for differentiation and growth process. The same trend was also reported by many researchers in various crustaceans and fish species (Biesiot and Perry, 1995; Roustaian and Kamarudin, 2001), while approaches for energy use are varied and depend on extrinsic and intrinsic factors. According to Pandian (1970) loss of chorion after hatching also contributes to the decrease in total protein content.

Throughout the world induced spawning program at hatcheries is playing a significant role in enhancing the fish production by providing fish seed and fry for culturing and restocking purpose. In induced breeding practice broodfish endure capture, handling, crowding, confinement, transport and hand stripping stresses and the level of maternal stress determine the quantity and quality of fish seed. Considering the management practices at hatcheries, the present study is designed to report the status of whole body cortisol and protein contents in eggs (unfertilized and fertilized), embryos and larvae of silver carp *Hypophthalmichthys molitrix*, artificially bred at local fish hatchery. Silver carp is one of the most popular indigenous culturable species that is actively induced bred almost all fish hatcheries in Pakistan.

Materials and Methods

Experimental Fish

The experiment was performed at Fish Seed Hatchery, Rawal Town, Islamabad, Pakistan during May, 2011. A total of six females and eight males broodfish of silver carp. *H. molitrix*, mean body weight 2.2±0.5 and 2.5±0.3 kg, respectively were harvested from the earthen ponds and transferred to hatchery building and placed in concrete holding tanks with well aerated water. Ripeness of broodfish was accessed by several indicators like in female, the abdomen was round, soft and genital opening was swollen, protruding and pinkish red, while in male, secondary sexual characteristics was obvious.

Induced Spawning

After 2-3 h of acclimatization period, to induce ovulation and spermiation, female and male broodfish were weighed and injected with Ovaprim-C intramuscularly at a dose of 0.6 and 0.2 mL kg^{-1} body weight respectively. The injected males and females were shifted to a circular tank having slow moving water. The temperature of water was 24-26ºC, pH ranged from 7.5 to 8.11, oxygen concentration was ~6-6.8 mg L^{-1} and ammonia was less than 0.25 ppm.

Hand Stripping

Spawning started 10 h after ovaprim treatment. The female that released some eggs from the urinogenital pore with slight pressure, was removed from the circular tank with the help of dip net and dried with towel and weighed. From hand stripping technique, eggs were collected in a clean and dry plastic bowl and fertilized without delay with milt obtained by hand stripping method from the ovaprim treated males. To ensure fertilization mixture of sperm and eggs was gently mixed with hen feathers. While stirring well water was added for hardening of eggs. After 3-5 min., fertilized eggs were washed carefully with well water. Repeated the washing of eggs several time. When fertilized eggs swell and started to float then transferred in concrete circular tank.

Egg Incubation

In circular tank, fertilized eggs were incubated at ambient temperature for hatching and canvas screen were used to cover the circular tanks. The radius and depth of circular tank was 32 inch and 26 inch respectively and the rate of flow of water was 23-25 L min^{-1}, the temperature during incubation period was 22.5°-23.5°C. Hatching started after 20 h of post fertilization and completed in 7.0 h. Newly hatched yolk sac larvae were kept in the incubation tank with continuous flow of water for about two days or till the yolk of the yolk sac larvae was absorbed.

Sampling

Sampling procedure was carried out during the course of the experiment with care and caution. 1 g sample of unfertilized eggs in triplicate was taken after hand stripping of silver carp *H. molitrix* broodfish and immediately frozen in liquid nitrogen. Two-three sub samples were also taken, weighed and number of eggs were counted and then calculated the number of eggs in 1 g sample. Fertilized eggs were also sampled and frozen in liquid nitrogen. After the incubation of eggs in circular tanks for hatching, sampling procedure was continued at an interval of 2 h. The sampling was terminated at 48 h of post fertilization. All samples were collected in triplicate and immediately frozen in liquid nitrogen and then stored at -20ºC.

Cortisol Extraction

Whole-body cortisol extraction method (Ramsay *et al.*, 2006), with some modification was adopted. Samples were thawed and homogenized with 1 mL of phosphate buffered saline(PBS) by using glass/glass hand homogenizer and then disrupted by ultrasonication for 2 min. Probe of sonicator was rinsed with an additional 500 µL PBS into the sample tube. The homogenized contents were transferred to a separating funnel and sample tube was rinsed twice with 2-3 mL of diethyl ether, which was also transferred to the separating funnel. Hormone was extracted from each sample thrice with 8 volume of diethyl ether. For extraction of hormone, separating funnel was strongly shaken for 30 sec and then placed on stand. After 2-3 min the aqueous and ether layers were separated. The bottom aqueous layer was removed and top ether layer containing hormone was decanted in to the test tube. The ether was evaporated and sample was dried at 45°C under a gentle stream of nitrogen gas. The extract was stored at –20°C. Analysis of free cortisol was performed by using commercially available Immulite cortisol Simens Kit and all the samples were run in duplicate.

The efficiency of extraction (% recovery) from whole body sample was assessed by adding redioinert cortisol in charcoal stripped whole body homogenate at predicted concentrations of 5, 10 and 20 ng g^{-1}. These samples were then extracted with diethyl ether and quantified with Immulite cortisol Simens Kit. The extraction efficiency was found greater than 95%. All values of cortisol from the whole body of fish were corrected accordingly.

Protein Estimation

Each sample of 90 mg weight was homogenized in 3ml Phosphate buffer saline (pH 7.4) and centrifuged at 13000 g for 15 min at 4ºC. The supernatant was collected and stored at -20ºC for protein analysis. For the estimation of total protein content in the eggs and larvae of Silver carp, *H. molitrix*, Lowry method (Lowry *et al.*, 1951), was adopted and crystalline bovine serum albumin (BSA) was used as a standard.

Statistical Analysis

Data obtained from the experiment were expressed as mean ± SE. The results were analyzed by one-way analysis of variance followed by Tukey test using SPSS version 16.0.Values $P<0.05$ were considered statistically significant.

Results

During the embryonic development, the whole body cortisol content of silver carp *H. molitrix*, showed great fluctuation. Unfertilized eggs contained high level of cortisol, 155.72±0.47 ng g^{-1} that decreased to some extent 150.87±0.63 ng g^{-1} in fertilized egg (Fig. 1). The cortisol level decreased sharply and reached to significantly lower value 32.52±0.58 ng g^{-1} at 2 hpf and remained almost constant up to 8 -10 hpf. After this it gradually increased and reached to 18.53±0.80 ng g^{-1} at hatching (20 hpf) and 38.59±0.86 ng g^{-1} at larval stage (48 hpf).

The total protein content in fertilized and unfertilized eggs was 26.83±1.03 mg g^{-1} and 26.81±1.40 mg g^{-1} respectively, considerably comparable (Fig. 2). After fertilization there was significant decreased in protein content, 15.46±1.77 mg g^{-1} eggs at 2 hpf and remained low up to 6 hpf. After this, there was an increasing trend and it reached to 26.66±0.55 mg g^{-1} at 18 hpf. The significant decrease in total protein 10.59±0.43 mg g^{-1} was observed at hatching. The decreasing trend continued up to 48 hpf and the total protein content reached to 4.93±0.93 mg g^{-1} larvae.

Discussion

It is well documented that corticosteroids play distinctive functions throughout the early life of vertebrates including fish (Tagawa *et al.*, 2000; Feist and Schreck, 2002; Eriksen *et al.*, 2006). Cortisol that transferred from broodfish to eggs promote the survival and growth of larvae (Eriksen *et al.*, 2006, 2007; Leatherland *et al.*, 2010), while higher concentration in eggs due to maternal stress showed deleterious effect on early embryogenesis and development (Auperin and Geslin, 2008; Mileva *et al.*, 2011). Many researchers reported the existence of cortisol hormone

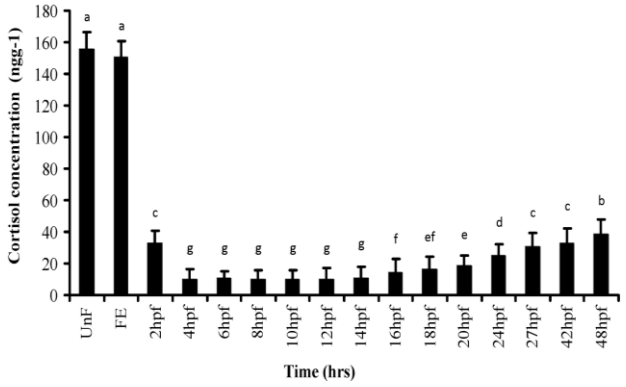

Fig. 1: Status of cortisol (ng g^{-1}) in eggs and during early development of silver carp *Hypophthalmichthys molitrix.* Mean ± SE followed by the same letters are not significantly different (P< 0.05) when compared with fertilized eggs (n= 3). Mean ± SE followed by the same subscript are not significantly different (P<0.05)

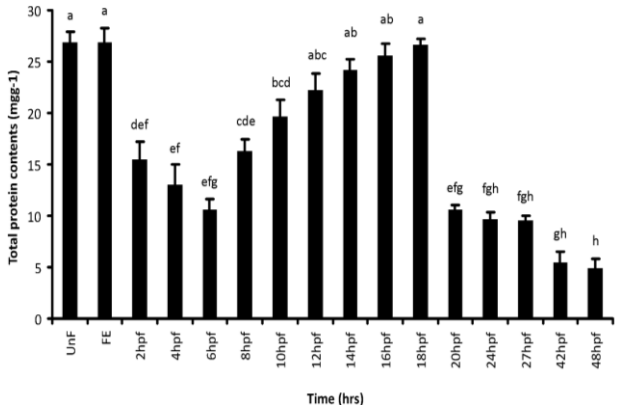

Fig. 2: Total protein content (mg g^{-1}) in eggs and during early development of silver carp *Hypophthalmichthys molitrix.* Mean ± SE followed by the same letters are not significantly different (P< 0.05) when compared with fertilized eggs (n=3). Mean ± SE followed by the same subscript are not significantly different (P<0.05)

in unfertilized and fertilized eggs of number of fish species (de Jesus and Hirano, 1992; Hwang *et al.,* 1992; Sampath-Kumar *et al*., 1995; Pavlidis *et al*., 2011; Zuberi *et al*., 2002) and confirm the view that cortisol in eggs is of maternal in origin that is actively involved in embryogenesis (Tagawa *et al.,* 2000).

The present experiment was induced spawning, hand stripping without giving anaesthesia to broodfish, and dry fertilization method was adapted to obtain the progeny. Hatching occurs at 20 hpf, which is in agreement with other reports on Indian and Chinese carps (Naeem *et al*., 2011).

In present study, unfertilized eggs of silver carp *H. molitrix* contained high level of cortisol, 155.72±0.47 ng g^{-1} (Fig. 1) that decreased to some extent 150.87±0.63 ng g^{-1} in fertilized egg. The cortisol level decreased sharply and reached to significantly lower value 32.52±0.58 ng g^{-1} after 2 hpf and remained almost constant up to 8-10 hpf. After this it gradually increased and reached to 24.89±0.57 ng g^{-1}at hatching and 38.26±0.86 ng g^{-1} larvae at 48 hpf. Present study showed that cortisol was present in all stages of development examined, while higher cortisol level was observed in the unfertilized and fertilized eggs of silver carp *H. molitrix* (Fig. 1), which indicated that cortisol present in the eggs was likely of maternal origin and our findings are in consistent with the work of several others investigators (Feist and Schreck, 2002; Li *et al*., 2010; Pavlidis *et al.,* 2011). Scanty of literature is available related to the concentration of whole body cortisol in unfertilized eggs, while several researchers measured the cortisol in fertilized eggs (Sampath-Kumar *et al.,* 1995; Szisch *et al*., 2005).

In present study, cortisol pattern during ontogeny of Silver carp *H. molitrix* was comparable to other studies but the concentration of cortisol at different development stages showed variation and higher amount of cortisol was observed in unfertilized and fertilized eggs as reported in other teleost. In rainbow trout whole body cortisol content in unfertilized and fertilized eggs were 3.0±0.4 and 3.2±0.3 ng g^{-1}, respectively (Li *et al.,* 2010), much less, as compare to observed in this study. Others investigators also reported the low or high levels of cortisol in fertilized eggs such as 2.5 ng g^{-1} in Japanese flounders (de Jesu *et al*., 1991), 1.65 ng g^{-1} in *Lates calcarifer*a (Sampath-Kumar *et al.,* 1995) and much higher levels in chum salmon, 20 ng g^{-1} wet weight (de Jesus and Hirano, 1992). The cortisols in eggs are seemingly originated from the maternal derived yolk, where they are accumulated during oogenesis (Tagawa *et al*., 2000). It seems that cortisol plays a major role in oocytes hydration during ovarian development and effect the hatching and different phases of the growth of embryos (Eriksen *et al.,* 2006, 2007; Leatherland *et al.,* 2010). Many investigators showed the rise in plasma cortisol during vitellogensis (Dawson and Grimm, 1980) that may rise further before ovulation in the mother, during oocyte development (Bry, 1989). It shows that elevation of plasma cortisol of broodfish before ovulation is related to the increased in concentration of cortisol in oocyte. In Coho salmon, the status of cortisol in ovarian fluid was paralleled to the cortisol concentration in the unfertilized eggs (Feist and Schreck, 2002). There are numerous factors including species variation, genetic makeup of broodfish, physical factors like temperature, handling of broodfish during induced spawning are related to maternal status of cortisol, which in turn related to status of cortisol in the eggs. The higher amount of cortisol in fertilized eggs in present study was might be due to our handling procedure of broodfish, or hand stripping without anaesthesia etc. In aquaculture industry, use of different types of anaesthesia is a common practice, for minimizing the handling stress, since stress reduced egg quality and spermatocrit and exert negative impact on fish production (Iwama *et al*., 1997). During induced spawning handling appears to further enhance the

stress and greatly increased the cortisol levels among numerous fish species (Mousavi and Yousefian, 2012). Our findings of sharp declined of cortisol after fertilization at blastula and gradual increased around the time of hatching has also been documented in the Japanese flounder (de Jesus *et al*.,1991), milkfish *Chanos chanos* (Hwang *et al*., 1992), *Lates calcarifer* (Sampath-Kumar *et al.,* 1995), gilthead sea bream *S. aurata* (Szisch *et al*., 2005), summer flounder (Veillette *et al.,* 2007), zebrafish *Danio rerio* (Alsop and Vijayan, 2008), red drum *Sciaenop ocellatus* (Applebaum *et al.,* 2010) rainbow trout (Li *et al.,* 2010) and European sea bass *Dicentrarhus labrax* (Pavlidis *et al.,* 2011). In *Danio rerio*, there was a balanced increased in the amount of mRNA encoding for melanocortin 2 receptors and StAR protein, 11β-hydroxylase from fertilization to hatching (Alsop and Vijayan, 2008). These are the indicators of the development of several components of the HPI axis. Whilst, ample evidences in fisheries indicated that in several fish species HPI axis is not efficient before absorption of yolk sac (Veillette *et al.,* 2007), a crucial phase of embryogenesis, where embryo is vulnerable to stressors. Conversely, development of inter-renal function varies among fish species (Hwang *et al.,* 1992). De novo cortisol synthesis begin as early as one week after fertilization in rainbow trout *O. mykiss* but as late as two week after hatch in Japanese flounders *P. olivaseus* (de Jesus *et al.,* 1991) and in European sea bass *Dicentrarhus labrax* it occurs near the transition period when dependence on endogenous feeding lessen and exogenous feeding start (Pavlidis *et al.,* 2011).

It is well documented that in human and rodent the prenatal stress adversely influenced the physiological and morphological aspects of progeny and altered its phenotypic traits (Malaspina *et al.,* 2008; McGowan *et al.,* 2009). In human, enhanced mortality of fetus, decreased in birth weight, altered immune responses, reduction in adrenal and gonad mass, altered nervous and neuroendocrine systems are linked to prenatal maternal stress, while in salmonid species, higher concentration of cortisol in plasma of broodfish before fertilization adversely affect embryogenesis and growth (Eriksen *et al.,* 2006, 2007; Auperin and Geslin, 2008).

It is also reported that the success of embryonic development and viable fry production from the teleost egg depends on the biochemical composition of the egg, the time to fertilization after ovulation and the availability of the balanced micro diet (Kjorsvik, 1994; Ronnestad *et al*., 1998), whereas nutrient composition of egg is species-specific and the exact order of consumption varies both qualitatively and quantitatively (Finn *et al.,* 1995). In many teleosts, vitellogenesis is the main event liable for the massive growth of oocytes. During vitellogensesis most nutritive products are taken up and stored for future use by the developing embryo. In salmonid fish, for example, vitellogenesis may account for over 90% of the final volume of an oocyte (Tyler, 1991). In most fish studied, the `building blocks' for the later embryo, includes amino acids, lipid and calcium, that are derived from the plasma during vitellogenesis. Most of them are originating from the uptake of a large complex molecule called vitellogenin (VTG) (Tyler, 1991). According to Ng and Idler (1983) VTG is synthesized by the liver in response to circulating estradiol-17ß from the ovary and receptor-mediated endocytosis is involved in the uptake of VTG by the oocyte (Tyler and Lancaster, 1993).

In the present study, the total protein content in fertilized and unfertilized eggs of silver carp *H. molitrix* were 26.83±1.03 and 26.81±1.40 mg g^{-1} (Fig. 2) respectively, considerably comparable. After fertilization there was significant decreased in protein content, and it remained low after 6 hpf. After this there was an increasing trend and it reached to 26.66±0.55 mg g^{-1} at 18 hpf. The significant decreased in total protein was also observed at hatching. The decreased in protein after fertilization may be related to catabolism of amino acid for satisfying the energy demand (Finn *et al.,* 1995; Sivaloganathan *et al.,* 1998). Although, according to Heras *et al.* (2000) and Roustaian and Kamarudin (2001) lipid particularly triglycerides are the main energy supplier during early embryogenesis but protein at lower rate is also used as a fuel, while after hatching protein demand increased due to differentiation and growth (Garcia-Guerrero *et al.,* 2003). The similar decreased in protein content during embryonic development of marine fishes was also observed by many investigators (Ronnestad and Fyhn, 1993; Ronnestad *et al.,* 1994; Finn *et al.,* 1995) and suggested that during embryogenesis free amino acid act as an important substrate for protein synthesis and energy metabolism.

In conclusion, presence of cortisol and protein content in fertilized and unfertilized eggs confirm that they are maternal in origin, while higher concentration of cortisol in eggs may be due to inadequate handling of broodfish. It seems that there is a need of improvement in management practices at local hatchery for minimizing the detrimental effect of maternal stress on survival and growth of fish larvae.

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