

Alterations in antioxidant defence in the early life stages of silver carp,  
*Hypophthalmichthys molitrix*

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## ABSTRACT

Fish larvae experience major cellular and biochemical changes during their early life stages. The aim of the present study was to evaluate alterations in the antioxidant status and values of lipid peroxidation and vitamin C content during the different life developmental stages of *Hypophthalmichthys molitrix*. Eggs and larvae were sampled at fertilization, organogenesis, eyed egg, hatch, active feeding, and 14 and 21 days after active feeding. An age dependent significant variation in SOD activity was seen during the period of study as the highest activity recorded at the eyed egg stage ( $P < 0.05$ ). Meanwhile, the activity of GPX and CAT did not show any significant changes in this study ( $P > 0.05$ ). The overall trend of MDA concentration showed significant increase from fertilization toward 21 days after fertilization ( $P < 0.05$ ). Vitamin C content showed an opposite pattern and decreased during the period of study ( $P < 0.05$ ). It can be concluded that vitamin C plays a crucial role in the antioxidant defence system during the early life stages of *H. molitrix* as could prevent from increase of MDA content till active feeding.

**Keywords:** antioxidant enzymes, egg, *Hypophthalmichthys molitrix*, larvae, lipid peroxidation

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## Introduction

Fish, like other aerobic organisms, need oxygen to survive, but the use of O<sub>2</sub> results in the formation of reactive oxygen species (ROS) that could damage molecules. This situation, needing oxygen to live and at the same time undergoing the danger of oxidation, is known as the oxygen paradox (1). Production of ROS induces oxidative stress and can induce damage to cell membranes, inactivation of enzymes, damages to genetic material and other vital cell components (2). There must be effective antioxidant systems to maintain health in fish. Biological effects of highly reactive oxygen species are controlled by a wide spectrum of antioxidant mechanisms, consisting of antioxidant compounds such as NADH, NADPH, glutathione (GSH); dietary micronutrients such as vitamins E and C or carotenoids and antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) (2).

Relation between the antioxidant defence and detoxification system and age is widely studied in mammals, especially in humans, while in the case of fish, our knowledge is limited and controversial (2, 3, 4, 5). It has been shown that in the early life phases of some fish species, environmental stress such as increases in pollutants could develop a detoxifying system by activation of cytochrome P-450 and enzymes such GPX and glutathione transferase (GST) (6, 7).

The study of these protective enzymes during early life in fish can be vital to determine the origin of the formation of these protective mechanisms against ROS, as, during development, fish embryos and larvae are sensitive to the environment surrounding them like the content of oxygen (8). Increased uptake of exogenous oxygen may have the

potential to affect pro-oxidant processes in the early life stages of fish (7).

Carp species such as silver carp, widely cultivated warm water fish in Iran, have high economical importance and are widely sold and used in their fresh form (9). The aim of this work was to examine the antioxidant defence, main antioxidant enzyme activities (SOD, CAT and GPX), vitamin C and lipid peroxidation during early development of *H. molitrix* to understand protective mechanisms that prevent pathologic conditions in this species.

## Material and Methods

### Sampling

Eggs and larvae were obtained by induced spawning from brood stock at the facilities of Azadshahr, Golestan, Iran. Samples were collected from the fertilization, organogenesis, eyed egg, hatched, active feeding, and finally 14 and 21 days after active feeding stages. Times of sampling were detected by histological methods. Samples were captured with a net, which was washed in distilled water before capture. Larvae were transferred to the rearing tanks after hatch and were fed with soybean and egg yolk leachate twice a day. All samples were kept at -80 °C while waiting for analysis.

### Analysis

Samples (1 g×3 replicates) were homogenized in ice-cold buffer (100mM Tris-HCl, 0.1 mM EDTA and 0.1% triton X-100 (v/v), pH 7.8) at a ratio of 1:4 (w/v). Homogenates were centrifuged at 30,000×g for 30min in a Centrikon H-401 centrifuge. After centrifugation, the supernatant was collected and frozen at -80 °C until analysis (5).

The activity of SOD was evaluated with SOD detection kit (Cusabio, China) according

to the manufacturer's instructions. The role of SOD is to accelerate the dismutation of the toxic superoxide ( $O_2^-$ ) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. SOD levels were recorded at 505 nm and through a standard curve and expressed as unit per milligram of protein ( $U\ mg^{-1}\ protein$ ).

The activity of GPX was evaluated with a GPX detection kit (Cusabio, China) according to the manufacturer's instructions. In the presence of glutathione reductase (GR) and NADPH, the oxidized glutathione (GSSG) is immediately converted into the reduced form with a concomitant oxidation of NADPH to  $NADP^+$ . The decrease in absorbance was measured spectrophotometrically against blank at 340 nm. One unit of GPX was defined as 1 mol of oxidized NADPH per min per milligram of tissue protein. The GPX activity was expressed as  $U\ mg^{-1}\ protein$ .

Catalase activity was measured according to the method of Aebi (1984). Briefly, tissue sections were homogenized in triton X-100 1% (Merck, Darmstadt, Germany) and the homogenates were diluted with phosphate buffer (pH 7.0). The reaction was initiated by the addition of hydrogen peroxide to the reaction mixture and the level of enzyme activity was quantitated according to the ability of the tissue catalase to decompose hydrogen peroxide, by monitoring the decrease in absorbance at 240 nm against a blank containing phosphate buffer instead of substrate. The value of  $\log A1/A2$  for a measured interval was used for unit definition owing to the first-order reaction of enzyme. One unit of CAT is the amount of enzyme that decomposes 1.0 mM of hydrogen peroxide per minute at pH 7.0 and 25° C. MDA was measured as described by Buege and Aust (1978). Briefly, one volume of plasma was mixed with two volumes of a

stock solution of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 mol) hydrochloric acid thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the precipitate is removed by centrifugation at 1000 g for 10 min. The absorbance of the clear supernatant determined at 535 nm. Protein content of supernatants for enzyme analysis was determined using a colorimetric method of Lowry with bovine serum albumin as standard (12). Ascorbic acid was estimated by the method of Omaye *et al.* (1979). In brief, 1 ml of 10% liver homogenate and 0.6 ml of blood sample and 0.4 ml 10% TCA were mixed. It was centrifuged at 3500 g for 10 min and 1 ml of the supernatant was mixed with 0.5ml of 2,4-dinitrophenylhydrazine reagent and incubated at 37°C for 3 h. The tubes were removed and 2.5 ml of ice-cold 85%  $H_2SO_4$  was added. It was mixed well and kept at room temperature for 30 min and the absorbance was measured at 520 nm. Ascorbic acid values were expressed as  $\mu g$  per mg of protein.

### Statistical analysis

Statistical analysis was performed using SPSS software (version 16.0, Chicago, IL) at the significant level of 95%. The results were presented as means  $\pm$  standard error. Data were tested for normality using the Shapiro-Wilk test, and one-way ANOVA was employed to reveal significant differences in measured variables among experimental groups in each sampling time. When a difference was detected ( $P < 0.05$ ), Duncan multiple comparison test was used to discriminate differences between the treatments.

## Results

The activities of various enzymes of the antioxidant system showed different patterns during the period of study. Activity of SOD during the study period is shown in Figure 1. Activity of SOD was unchanged during organogenesis but increased in eyed egg in

comparison to fertilization. Its value decreased during hatch but increased after the start of active feeding. SOD activity was decreased substantially again, 14 and 21 days after active feeding. Some insignificant variations in the activity of CAT were recorded during the period of study (Figure 2).

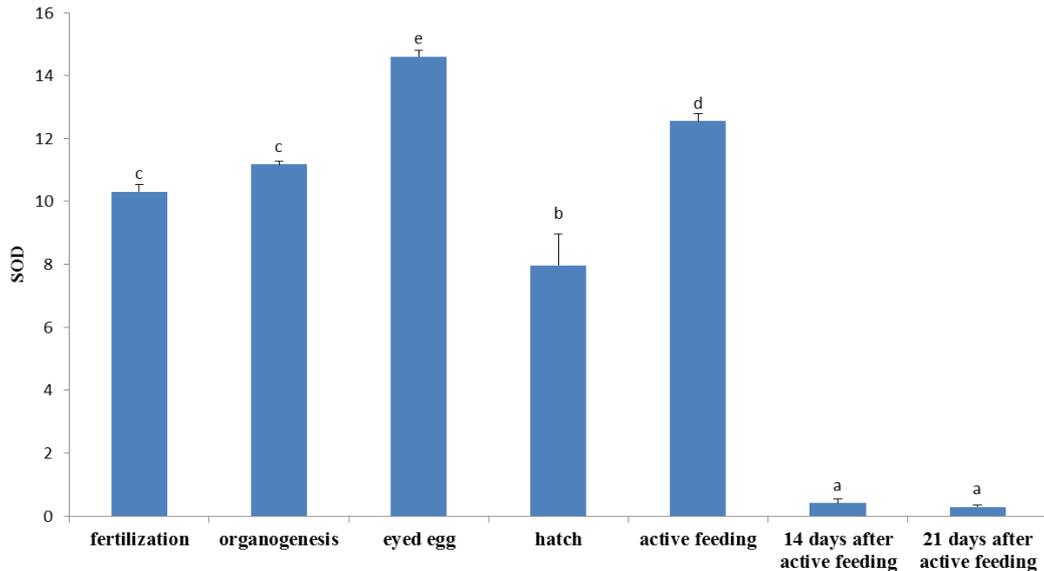


Figure 1. Activity of SOD (units/mg protein) in different sampling times (fertilization, organogenesis, eyed egg, hatch, active feeding, 14 days after active feeding and 21 days after active feeding) in *Hypophthalmichthys molitrix*. Different letters show significant difference ( $P < 0.05$ ).

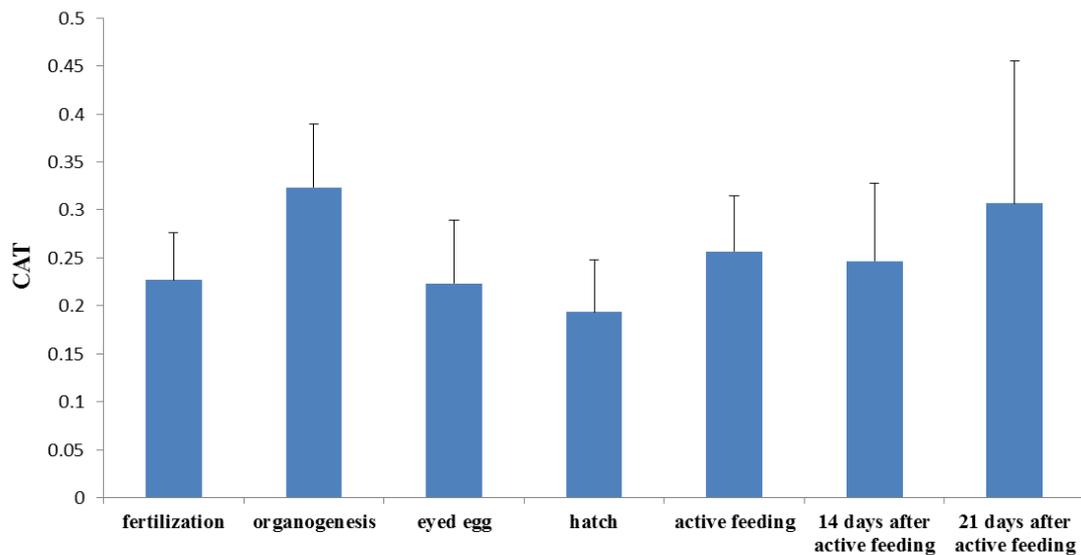


Figure 2. Activity of CAT (units/mg protein) in different sampling times (fertilization, organogenesis, eyed egg, hatch, active feeding, 14 days after active feeding and 21 days after active feeding) in *Hypophthalmichthys molitrix*

Like CAT, no significant age-related differences in activity of GPX were recorded during the early life stages of *H. molitrix* (Figure 3). The MDA content of egg and larvae of *H. molitrix* remained constant until active feeding period, and then increased sharply, approximately 15-fold, 14 and 21 days after active feeding, as the highest value

( $7.18 \pm 0.19$  nmol/mg protein) was recorded 21 days after active feeding. Vitamin C content of egg and larvae decreased during this study as the highest value ( $8.78 \pm 0.23$  mg/dl) was measured at fertilization and the lowest ( $0.34 \pm 0.09$  mg/dl) was seen 21 days after active feeding (Figure 4).

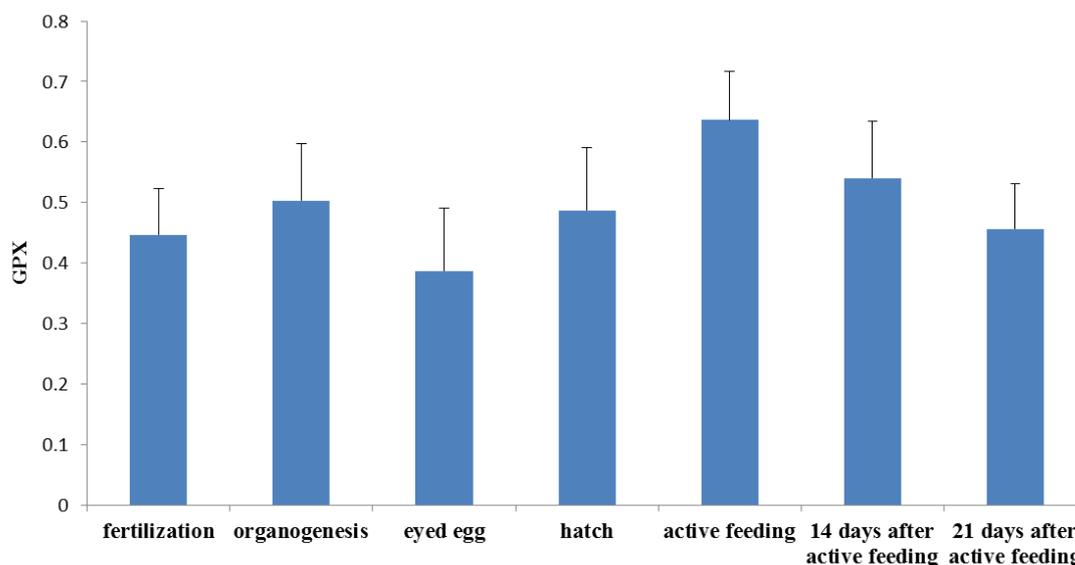


Figure 3. Activity of CAT (units/mg protein) in different sampling times (fertilization, organogenesis, eyed egg, hatch, active feeding, 14 days after active feeding and 21 days after active feeding) in *Hypophthalmichthys molitrix*

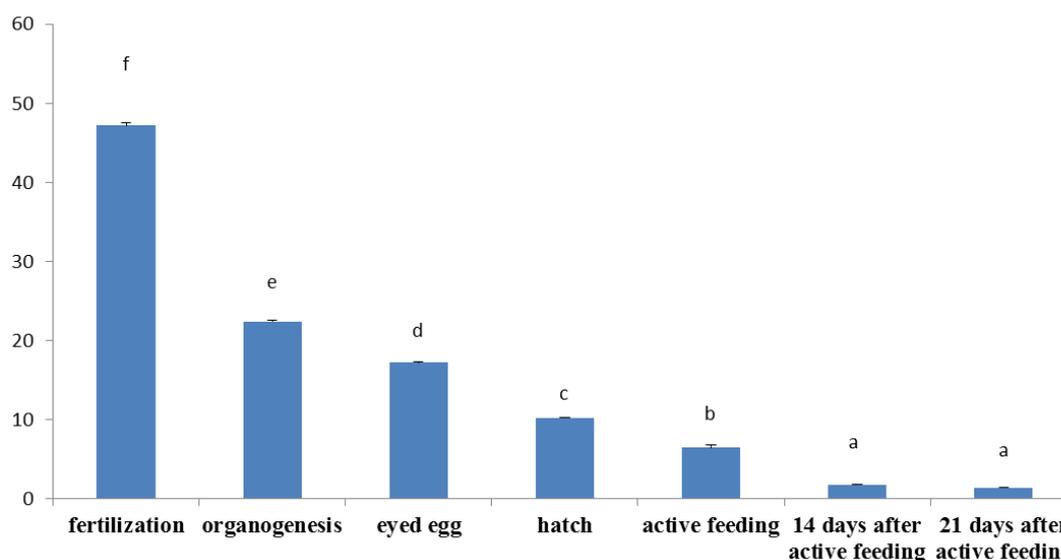


Figure 4. Vit. C concentration (mg/dl) in different sampling times (fertilization, organogenesis, eyed egg, hatch, active feeding, 14 days after active feeding and 21 days after active feeding) in *Hypophthalmichthys molitrix*. Different letters show significant difference ( $P < 0.05$ ).

## Discussion

Characterizing antioxidant defence and oxidative status could describe mechanisms included in the development and survival of fish and their progeny in farms. Few works have evaluated antioxidant defence development in the early life stages of fish.

This study showed that measurable amounts of antioxidant system enzymes were present in *H. molitrix* eggs prior to hatching. Activity of SOD, CAT and GPX was recorded in eggs and, in combination with vitamin C, it could be concluded that the *H. molitrix* is well protected against peroxidation from early stages of life. Protection against oxidative stress from fertilization had been reported previously in eggs of *Dentex dentex* (2), *Lates calcarifer* (4) and *Acipenser naccarii* (5).

In our study, differences in the activity of antioxidant enzymes were only significant for SOD, and GPX and CAT both remained unchanged during ontogenesis. In most of the species studied, significant changes in

antioxidant enzyme activity were observed when exogenous feeding started (2, 7, 14), but in this study only SOD showed a significant increase in the start of exogenous feeding that decreased in the next samplings. Diaz *et al.* (2010) reported that activity of SOD, CAT and GPX increased after exogenous feeding in *Acipenser naccarii*. These changes may be diet-dependent as shown in *Solea senegalensis* previously (15). Sharifi *et al.* (2014) also showed that in *O. mykiss*, activity of SOD, CAT and GPX increased significantly until exogenous feeding. This may be caused by different patterns of expression of these enzymes in different species.

The highest value of vitamin C level was recorded at fertilization and decreased progressively until 21 days after fertilization. During early larval development, vitamin C level counterbalances the oxidative damage and lipid peroxidation. The decrease in vitamin C suggests a high rate of lipid peroxidation as seen in Figure 5 (MDA graph).

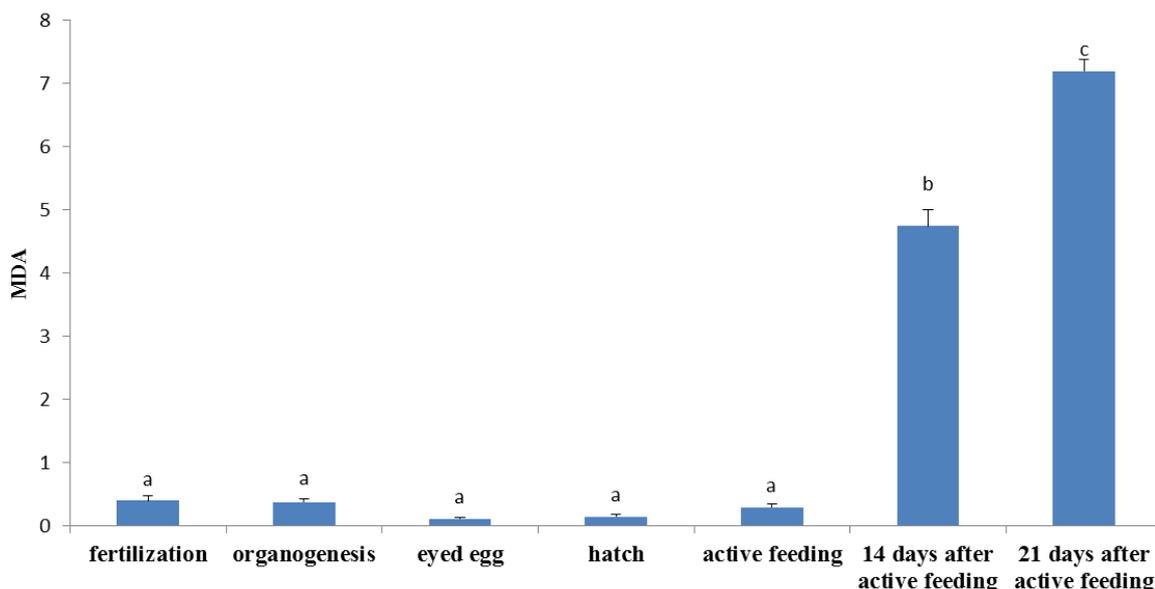


Figure 5. MDA concentration (nmol/mg protein) in different sampling times (fertilization, organogenesis, eyed egg, hatch, active feeding, 14 days after active feeding and 21 days after active feeding) in *Hypophthalmichthys molitrix*. Different letters show significant difference ( $P<0.05$ ).

In *Lates calcarifer* vitamin C was at its highest three days after hatch and decreased significantly until 30 days post-hatch (4). Vitamin E reacts with lipid peroxy and alkoxy radicals and forgives its labile hydrogen, generating vitamin E in its radical form. Vitamin C reduced back vitamin E radical to vitamin E (17). Thus, the gradual discharge of vitamin C level is related to a reduction of vitamin E, which confronts with lipid radicals. Vitamin E level is strictly correlated to metabolic acceleration or high rates of ROS generation, as demonstrated in animal systems (18, 19). Thus, an increase or decrease in vitamin C level has an inverse relation with vitamin E. In Black Sea animal embryogenesis, the activities of most of the antioxidant enzymes examined tend to increase, especially in eggs and hatching

larvae, while the contents of low molecular weight antioxidants decrease (14).

MDA did not change significantly from fertilization until exogenous feeding, but after that increased significantly. The increased value of MDA may be related to increased metabolic rate as reported previously (4). Mourente *et al.* (1999) found that in *D. dentex* the highest level of MDA was recorded at fertilization, and then it decreased until nine days after hatch. They concluded that high values of MDA were associated with the chorion and/or perivitelline fluid.

Vitamin C plays a key role in the antioxidant defence system during the early life stages of *H. molitrix* as it can prevent from increase of MDA content until active feeding.

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