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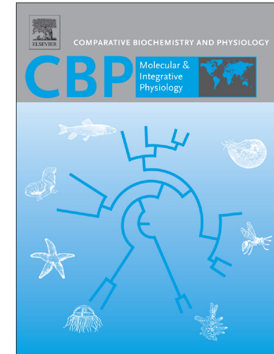

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Changes of oxidative status in yellowfin seabream larvae (*Acanthopagrus latus*) during development

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Running title: Ontogeny of oxidative enzymes in yellowfin seabream larvae

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Abstract

Dynamic changes of tissues, organs and growth that occur in fish larvae during the transition to the juvenile stage are accompanied by differences in metabolic, locomotor and feeding activities that can reflect on fish's oxidative status. In this study, we examine how body growth, antioxidant system (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and total antioxidant capacity (TAC) and oxidative damage (malondialdehyde-MDA) parameters change in larvae of yellowfin seabream larvae (*Acanthopagrus latus*) during early development (0, 7, 15, 22 and 30 day after hatching-DAH). Body growth (length and weight) starts to intensify from 15 DAH. We observed general increase in the antioxidant system (AOS) with the age of larvae from newly hatched and 7 DAH up to 15 and 22 DAH individuals. 15 and 22 DAH larvae had the greatest levels of TAC, SOD and GR activity, while 30 DAH larvae had higher CAT activity from 0, 7 and 15 DAH and MDA concentration in comparison to 15 DAH individuals. Several developmental events can be linked with observed results: lower AOS in 0 and 7 DAH individuals with low locomotor activity, growth, endo-exogenous feeding phase and cell differentiation; 15 and 22 DAH larvae are under pressure of fast growth, enhanced swimming and foraging capacity; while higher MDA production in 30 DAH larvae can be a result of shifts in muscle metabolism, changes in both quality and quantity of food and a significant increase in weight. The present study provides insight into the changes in redox status during the ontogeny of *A. latus*, fish species about which physiology is still little known but with a potential for use in marine culture. Ability to lower oxidative stress during critical developmental periods can enhance that potential.

Keywords: antioxidant system; fish larvae; ontogeny; oxidative stress; yellowfin seabream

1. Introduction

Fish through ontogeny face various physiological, biochemical and morphological requirements. In the early developmental stages, they are challenged with both internal and external stressors that can reflect on their well-being (Infante and Cahu, 2001). Larvae undergo changes that involve cell differentiation during the formation of tissues and organs, proliferation, and apoptosis during the replacement of larvae with adult-type tissue. They also experience intense somatic growth (Melendez and Mueller, 2021). Even though development is a continuous and dynamic process, there are several critical periods (Martínez et al., 2021). In fish larviculture, transition from endogenous to exogenous feeding at early larval development, as well as weaning from live food to compound feeds are considered two of the main critical periods (Hamre et al., 2013; Martínez et al., 2021). Modification of digestive mechanism and transition to novel food sources together with intense body growth and development are processes responsible for changes in metabolic activity and consequently reactive oxygen species (ROS) production (Ma et al., 2014; Pimentel et al., 2015). ROS, such as $O_2^{\cdot-}$ and H_2O_2, at lower concentrations are shown to act as intracellular signaling molecules regulating circulation, energy metabolism, and remodelling of cells, tissues and organs (Allen and Tresini, 2000). The redox balance during larval development is spatially and temporally dynamic to allow these processes to occur. However, higher concentrations of ROS can damage biomolecules (by oxidation of DNA, lipids and proteins) and cellular structures, contributing to cell senescence, tissue and organ dysfunction and organ loss, and thereby affecting overall organism performance (Alonso-Alvarez et al., 2007; Costantini, 2019). The occurrence of oxidative stress in fish species is often related to different pathological damages such as liver damage, the reduced bone cortex and bone strength,

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anemia and muscular dystrophy followed by poor survival and reduced growth, all factors are important in aquaculture (Ritchie and Friesen, 2022; Tsneg et al., 2023).

The antioxidant system (AOS) has a key role in physiological mechanisms that maintain redox homeostasis (Halliwell and Gutteridge, 2015), and the knowledge about its response at crucial phases of fish ontogeny would allow us to identify and quantify underlying costs of free radical production (Prokić et al., 2018). AOS includes different pathways that can act through both enzymatic (e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione S-transferase (GST)) and non-enzymatic components (total glutathione (GSH), vitamins E, C, thiol groups and others) (Costantini, 2014).

Yellowfin seabream (*Acanthopagrus latus*) is an important carnivorous sparid species from the coasts of Japan, southern Korea, Taiwan, China, northern Vietnam and the Indo-West Pacific Ocean like the Persian Gulf. This species has the potential for use in marine culture, because of good performances such as spawning in captivity, tolerance to environmental factors, especially temperature, moderate growth rate and good feed conversion ratio (Zakeri et al., 2009). Development of *A. latus* larvae consists of anatomical changes with major differentiation in the digestive system associated with the transition to a new diet, but also with morphological changes and pigment production and reorganization (Morshedi et al., 2021). The larval period is also characterized by high growth rates between 16 and 30 days after hatchings. The diet of *A. latus* larvae is mainly based on live prey, such as rotifers and *Artemia* nauplii and metanauplii (Sangari et al., 2021). The nutritional quality of such foods may not contain all essential nutrients for the larvae, which can affect the oxidative status of individuals (Eryalçın et al., 2020; Hasanpour et al., 2021). Previous studies have suggested that fish during early life stages may display species-specific, larvae-age and diet-dependent

changes in levels of oxidative stress (Kalaimani et al., 2007; Liravi, et al., 2014; Pérez et al., 2020).

Oxidative stress is considered both a cost and a constraint of growth, that can induce trade-offs in various fitness traits (Smith et al., 2016). Therefore, the present research was conducted to provide a better understanding of the changes in the antioxidant system and level of oxidative damage during ontogeny of *A. latus* larvae from hatching up to 30 days after hatch (DAH). Results from this study can be used to improve the oxidative status and rearing condition of this fish species, to minimize oxidative stress through manipulation of feeding strategies during critical developmental periods.

2. Material and Methods

2.1. Fish husbandry

Fertilized eggs of *A. latus* were obtained from the laboratory of Aquatic Research, Persian Gulf University, Bushehr, Iran. The eggs were from wild-caught broodstocks that were stocked in a 4000-l circular tank connected to a recirculation system with constant conditions: water temperature 20°C, with 12L: 12D photoperiod. The weight range of *A. latus* broodstock between males and females varied from 250 to 450 g respectively, individuals were fed with squid and fresh fish every day. The broodstock process was simulated through continuous raising and lowering of water levels in the rearing tank.

Floating fertilized eggs were collected by a 120 µm diameter mesh net, calculated the number and incubated in 300-l cylindroconical fiberglass tanks. During incubation, water exchange was not carried out, while the air diffuser provided gentle aeration. On the second day of incubation, newly hatched larvae were randomly distributed into cylindrical polyethylene

tanks (initial density of 50 larvae/l), filled with 300 l of sand-filtered and UV-treated seawater.

The larvae feeding protocol was as followed: Microalgae, *Nannochloropsis* sp. (500000 cell/ ml), were added into the water of the larval tanks throughout the experiment (from day 1 up to 30 DAH); from day 2 to 20 DAH they were fed with rotifers with a density of 5–10 individuals/ml *Brachionus rotundiformis*, S-type and 10–16 individuals/ml *Brachionus plicatilis*, L-type, and from 18 to 30 DAH they were fed with 0.5 - 3 individuals/ml *Artemia sp*. nauplii and with microdiet (Skretting® GemmaMicro®; Netherlands, 200–300 µm: protein 55, lipid 15, ash 13.5 and fiber 5) from 25-30 DAH. The feeding frequency was twice per day (09:00 and 16:00) in order to maintain a constant density of live prey in the rearing tanks. During the night, the larvae were not fed; water change began at 7 DAH and was gradually increased from 20-100 % as larval growth increased. Moreover, the dead larvae and wastes were collected and siphoned out from the bottom of the tank daily from 7 DAH. The average water physicochemical parameters during the experimental period such as temperature, dissolved oxygen, pH and salinity were 23 ± 1 ºC, 6.8± 0.6 mg/L, 7.9 ± 0.2 and 40.0 ± 1.0 ppt, respectively.

2.2 Sampling

To assess the oxidative stress parameters in the early stages, yellowfin seabream samples were randomly collected in triplicate from the rearing tank at 0 (hatching), 7, 15, 22 and 30 DAH. A various number of larvae (20 to 1000, due to the DAH) were collected with a 100 µm mesh net, then anesthetized with 2-phenoxyethanol, and washed with distilled water to removed salt and anesthetic solution from the larval body, after that the larvae were instantly placed in liquid nitrogen and sotred at −80 °C for next analysis.

2.3 Survival and growth performance

Total length (measured to the nearest 0.1 mm), wet weight (measured to the nearest 0.1 mg) and survival percentage were calculated. Twenty larvae samples were caught on the same sampling days, and anesthetized with 2-phenoxyethanol. Total length was measured using a dissecting microscope and an image analyzer (Analysis, SIS Gmbh, Germany). The all-surviving larvae were collected by draining the tank at the end of the experiment (30 DAH), fixed in 4% buffer formalin solution and counted thereafter. The survival rate was calculated by the following formula: Survival (%) = [(number of larvae at 30 DAH + number of sampled larvae) / initial number of larvae)] × 100.

2.4 Antioxidant enzyme analyses

For Superoxide dismutase (SOD) activity was estimated by the method of Kono (1978). The principal of this method is based on the inhibitory effect of superoxide dismutase enzyme on reduction of nitroblue tetrazolium (NBT) by superoxide radicals which is generated by oxidation of hydroxylamine hydrochloride. It competes with enzyme SOD for superoxide anions. In presence of SOD in reaction mixture, NBT will produce less amount of coloured complex than control. The assay is carried out in 24 well plate. To each well of plate, 1ml of 50 mM sodium carbonate (pH 10), 0.5 ml of 96 µM NBT and 0.1ml of 6% Triton-X-100 was added and incubated at 37°C for 10 minutes. Next, 10 µl of enzyme extract and 0.1 ml of 20 mM hydroxylamine hydrochloride (pH 6) were added and immediately the difference in absorbance was recorded kinetically every minute for 10 minutes at 560 nm using a microplate reader. The SOD specific activity was expressed as inhibition unit per mg protein per minute. One unit is the amount of SOD that inhibits the rate of formazan formation by 50 percent. The catalase activity was recorded by measuring the reduction in absorbance for 3 minutes at 240 nm in 1.5 ml of reaction mixture consisting of 13.2 mM H_2O_2 prepared in 50mM phosphate buffer (pH 7.0) and 0.1 ml of homogenate. The absorbance was read kinetically every 20 second and the mean change in absorbance per minute was calculated.

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The control solution contains 50mM phosphate buffer (pH 7.0) and 0.1 ml of homogenate. The catalase activity was reported in micromoles of H_2O_2 dissociated during one minute per mg protein and the extinction coefficient used at 240 nm was 43.6 $M^{-1}cm^{-1}$ (Aebi, 1984). The glutathione reductase (GR) activity was measured using NagluRTM glutathione reductase assay kit (Navand Lab kit company, Iran). The kit enables the spectrophotometric measurement of glutathione reductase activity by the increase in absorbance caused by the reduction of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] at 412 nm to generate yellow colored nitro benzoic acid (TNB2-). Glutathione Reductase was measured using GSH calibration curve and the extinction coefficient of GSH at 412 nm was 0.017 $mM^{-1}cm^{-1}$. The total antioxidant capacity (TAC) was measured using NaxiferTM total antioxidant capacity assay Kit (Navand Lab kit company, Iran). The kit measures the antioxidant capacity in different samples, based on divalent iron reduction ability (FRAP) and with a single electron transfer mechanism. The color change resulting from the reaction was read at the wavelength of 593 nm.

The method used for the determination of lipid peroxidation was based on the reactivity of an end product of lipid peroxidation, malondialdehyde (MDA) with thiobarbituric acid TBA in the presence of trichloroacetic acid (TCA) to produce a red adduct. (Heathand Packer, 1968). In brief 100 mg larvae were homogenized in 1 ml of ice-cold 1% TCA and centrifuged for 10 minutes (15000 g, 4̊ C). One ml TBA solution was mixed with 0.5 ml of supernatant and heated in 95̊ C water bath for 30 minutes. The reaction mixture was cooled and transferred to a flat bottom 48 well plate. The OD of the supernatant was read at 532 and 600 nm using a microplate reader. The OD600 nm is subtracted from OD532 nm and the MDA concentration was calculated. The extinction coefficient of this MDA-TBA abducts at 532 nm is 155 $mM^{-1}cm^{-1}$. The result is expressed as µmole MDA g^{-1} fresh tissue.

2.5 Statistical analyses

Data were analyzed using SPSS ver.22.0 statistical software. All the data (total length, weight, %survival, oxidative stress parameters) are presented as means ± SE determined from three replicates. Arcsine transformations were conducted on all data expressed as percentages to get homogeneity of variance before statistical analysis. One-way ANOVA was performed with a significance level of 0.05 following confirmation of normality and homogeneity of variance. Tukey's procedure was used for multiple comparisons.

3. Results

3.1 Larval growth and survival

The biometric measurements of *A. latus* larvae displayed an exponential increase of wet weight (WW) and a linear increase of length during larval stages (Fig. 1). The mean larval wet weight (mg) ranged from 0.17 ± 0.02 mg (Newly hatched larvae (day 0)) to 5.92 ± 0.3 mg (30 DAH) and the mean larval length (mm) from 2.6 ± 0.09 mm (Newly hatched larvae; day 0) to 7.46 ± 0.6 mm (30 DAH). As shown in Fig. 1, there are two phases of growth during yellowfin seabream larvae development. In the first phase, a slow growth was detected from 0 (0.17 ± 0.00 mg WW) until 15 DAH (0.77 ± 0.21 mg WW). The second phase is identified coinciding with the onset of *Artemia* sp. nauplii in the feeding protocol from 18 DAH with a marked increase in WW to the end of the experiment. The survival rate was estimated to 49.9 ± 0.4 %.

3.2 Oxidative stress

The SOD showed the highest activity in 15 DAH larvae, followed by 7 DAH and 22 DAH larvae, the lowest activities were observed in newly hatched larvae (0 DAH) and 30 DAH larvae. Catalase values were significantly higher in 30 DAH larvae in comparison to other groups, with the only exception of the 22 DAH larvae. 15 DAH and 22 DAH larvae showed significantly higher activity than earlier developmental days (0 DAH and 7 DAH). The

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results of GR indicated higher activity in 15 DAH and 22 DAH larvae than in 30 DAH larvae, while 0 DAH and 7 DAH larvae exhibited significantly lower activity as compared to other larvae. The MDA results showed a significant difference between 30 DAH and 15 DAH. The MDA concentration of 30 DAH larvae was higher than the 15 DAH larvae. Total antioxidant capacity was highest in 15 and 22 DAH larvae, followed by TAC values in 7 DAH and 30 DAH larvae, the lowest was observed in 0 DAH larvae.

4. Discussion

The transitional period from larvae to juveniles in fish is followed by substantial developmental changes and intense growth (Smith et al., 2016; Prokić et al., 2019). Different pressures during ontogeny reflect on biological processes (metabolic activity, oxygen consumption, cell proliferation and apoptosis) that can result in different levels of oxidative damage and antioxidative responses (Metcalfe and Alonso-Alvarez, 2010; Prokić et al., 2021). Studies on fish larvae redox status during development often shows that dynamic of oxidative events can vary among species (Kalaimani et al., 2007; Hamre et al., 2014; Liravi et al., 2014; Sole et al., 2004). Differences in oxidative stress can be related with various factors (habitats, life histories, development and diet) that larvae experience in this sensitive life stage (Perez et al., 2020). Adding more study species and systems to research will improve our understanding of the mechanisms behind this process. Our results suggest that the oxidative status of yellowfin seabream larvae varies during development, from a low antioxidant response and oxidative damage during early development (0-7 DAH), high AOS response from 15-22 DAH to greater oxidative damage of lipids seen in individuals at 30 DAH.

The lower AOS response in newly and 7 days after hatched yellowfin seabream larvae can be related to low locomotor activity, growth and endo-exogenous feeding phase. Even though

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ROS are observed in various marine fish larvae just after hatching, their concentrations tend to increase as larvae develop. It has been suggested that ROS in newly hatched individuals can act as an intrinsic defense system that protects larvae from pathogens present in the new aquatic environment (Covarrubias et al., 2008; Mugoni et al., 2014). In 7 DAH larvae, AOS can have a role in maintaining ROS levels necessary for the activation of the apoptosis/proliferation-related pathways and the modulation of biological systems, avoiding any oxidative damage during this sensitive phase (Hamre et al., 2014). Most of the larval activities are dedicated to physiological development including the splach-nocranium (Sarvi et al., 2019) and the gastrointestinal system (Pittman et al., 2013), rather than to somatic growth (Nazemroaya et al., 2015). A study on larvae of Atlantic cod suggested that a well-balanced cellular redox environment is important for the regulation of gene expression included in cell differentiation (proliferation and apoptosis) during early development (Hamre et al., 2014).

For older larvae (15- 30 DAH) we noticed a greater increase in body length and especially in body mass for individuals at 22 and 30 DAH. Larvae at this age are faced with pressure to obtain sufficient energy necessary for fast growth that will increase their survival chances during later life stages (Mogensen and Post 2012). Larvae enhance swimming and foraging capacity that together with a developed digestive system and feeding on live prey and microdiet allows a gain in body size (rotifers up to 20 DAH, *Artemia* nauplii from 18-30 DAH and microdiet from 25-30 DAH) (Pérez et al., 2020; Morshedi et al., 2021). However, greater activities and energy demands are also associated with the increase of metabolic activity, ATP amounts, oxygen uptake, and consequently higher unavoidable production of reactive oxygen species (ROS) (Selman et al., 2012; Smith et al., 2016). Our results revealed that intense metabolic processes and somatic growth coincide with larvae AOS response.

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Total antioxidant capacity, coupled with GR and CAT activities increase in 15 and 22 days old larvae. Individuals 15 DAH also experienced high activity of SOD. High total antioxidant capacity can act as a protective mechanism against ROS generation. Activities of SOD and CAT, enzymes of the first line of defense, point to increased mitochondrial activity due to greater overall physical activity, while the GR points to the importance of maintaining the glutathione redox system in larvae 15 and 22 old (Halliwell and Gutteridge, 2015). Providing adequate nutrition is also necessary for maintaining antioxidant system as some antioxidants (such as ascorbate, tocopherol, ubiquinones, and β-carotenes) and micronutrient (selenium, cysteine) are obtained directly from food (Vo et al., 2022). Adding new food (composition of protein, lipids, as well as micronutrients) can positively contribute to AOS of 15 and 22 DAH larvae, for example the taurine from Artemia. It was suggested that taurine can enhanced growth and glutathione system, lowering MDA concretions in Asian seabass *(L. calcarifer*) (Morshedi et al., 2022).

From 30 DAH the morphological transition from the larval to the juvenile stage in *A. latus* begins (Leu and Chou, 1996). In those individuals, we reported the greatest MDA concentration and CAT activity. Changes in oxidative status (higher H_2O_2 concentration) and oxidative damage in these larvae can be related to several events: shifts in muscle metabolism, changes in both quality and quantity of food and a significant increase in weight (Wieser, 1995; Killen et al., 2010; Edworthy et al., 2018). According to Edworthy et al. (2018), both standard and active metabolic rates rise significantly after notochord flexion, a change that appears after 22 DAH in *A. latus*. During metamorphosis, an increase in LPO was also seen in Asian seabass (*Lates calcarifer*) as a result of altered metabolic rate and oxidation of PUFAs (Kalaimani et al., 2007). *Artemia* nauplii also contain higher lipid levels compared to rotifers (Evjemo and Olsen, 1997), which may contribute to greater production

of lipid peroxides. A significant increase in CAT activity was not enough to counteract ROS formation and prevent higher LPO production in *S. senegalensis* larvae at 28 DAH (Sole et al., 2004) that it seems the CAT activity in the present research also has the same conditions.

Overall, this study presents physiological changes that occur during the development of *A. latus* species with the potential to be used in aquaculture. The characterization of the oxidative status in *A. latus* during early development can be implemented to improve the growth and life quality of fish larvae by lowering oxidative stress and associated pathologies and disease.

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Ethical approval

This study was carried out in accordance with the principle of the Basel Declaration and recommendations of Iranian Fisheries Science Research Institute and the Faculty of Veterinary Medicine at University of Tabriz, the FVM.REC.1396.939.

Deceleration of competing interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1. Larval growth of *A. latus* in total length (mm) and wet body weight (mg) from 0 to 30 days after hatching (DAH). Results are presented as mean ± standard error of the mean (N=6).

Fig. 2. Specific activities (U mg^{-1} protein) of superoxide dismutase (SOD) during the larval development of *A. latus*. Results are expressed as mean ± standard error of the mean (N=6). Different letters indicate significant differences among different larval ages (ANOVA, $P < 0.05$).

Fig. 3. Specific activities (μmole mg^{-1} protein) of catalase (CAT) during the larval development of *A. latus*. Results are expressed as mean ± standard error of the mean (N=6). Different letters indicate significant differences among different larval ages (ANOVA, $P < 0.05$).

Fig. 4. Glutathione reductase activities (nmole min^{-1} ml^{-1}) during the larval development of *A. latus*. Results are expressed as mean ± standard error of the mean (N=6).

Different letters indicate significant differences among different larval ages (ANOVA, $P < 0.05$).

Fig. 5. Lipid peroxidation of tissue (nmole g^{-1} tissue). The nmole malondialdehyde (MDA) produced per gram of tissue during the larval development of *A. latus*. Results are expressed as mean ± standard error of the mean (N=6).

Different letters indicate significant differences among different larval ages (ANOVA, $P < 0.05$).

Fig. 6. Total antioxidant activities (nmole ml^{-1}) during the larval development of *A. latus*. Results are expressed as mean ± standard error of the mean (N=6).

Different letters indicate significant differences among different larval ages (ANOVA, $P < 0.05$).

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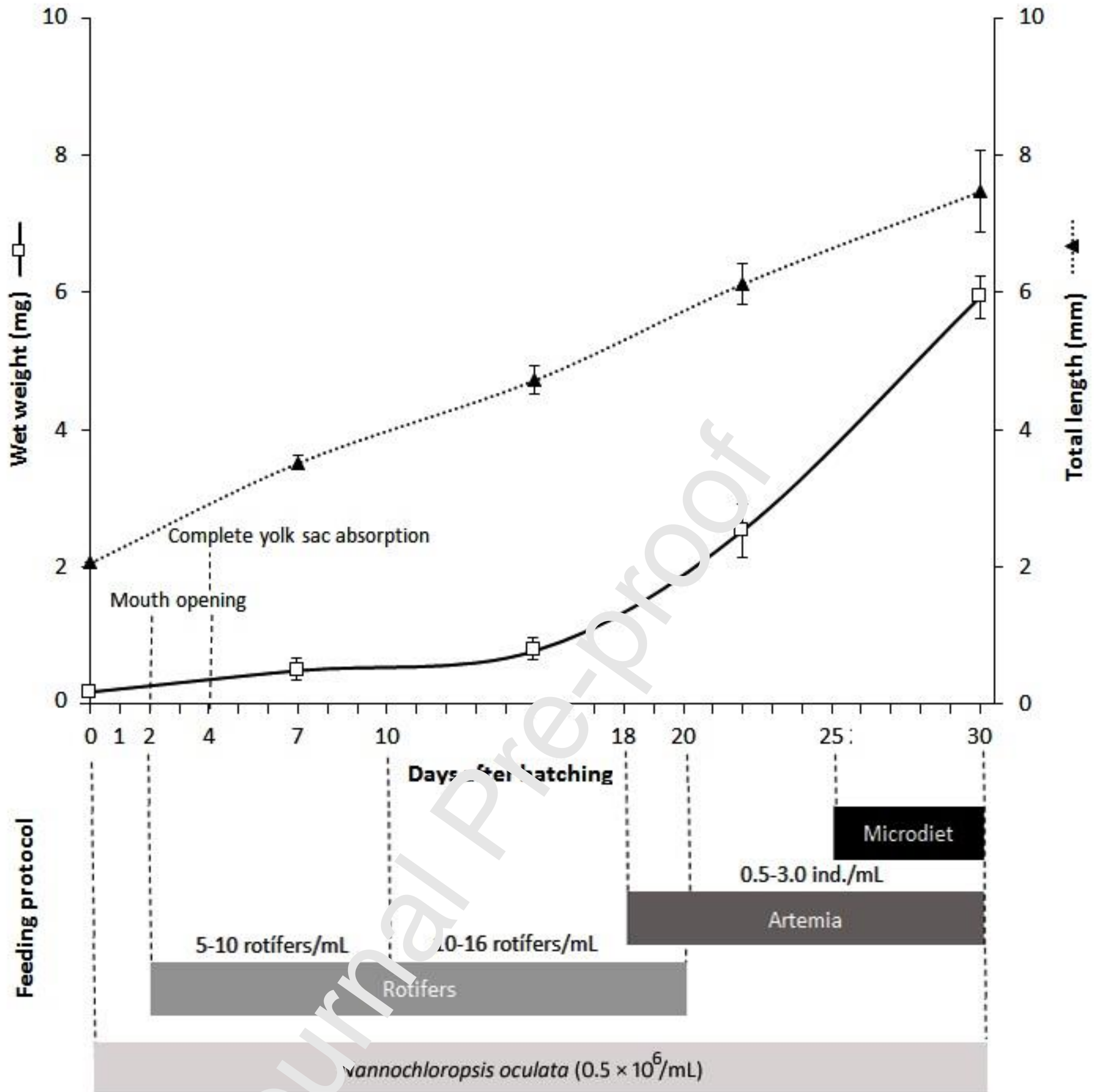

Figure 1

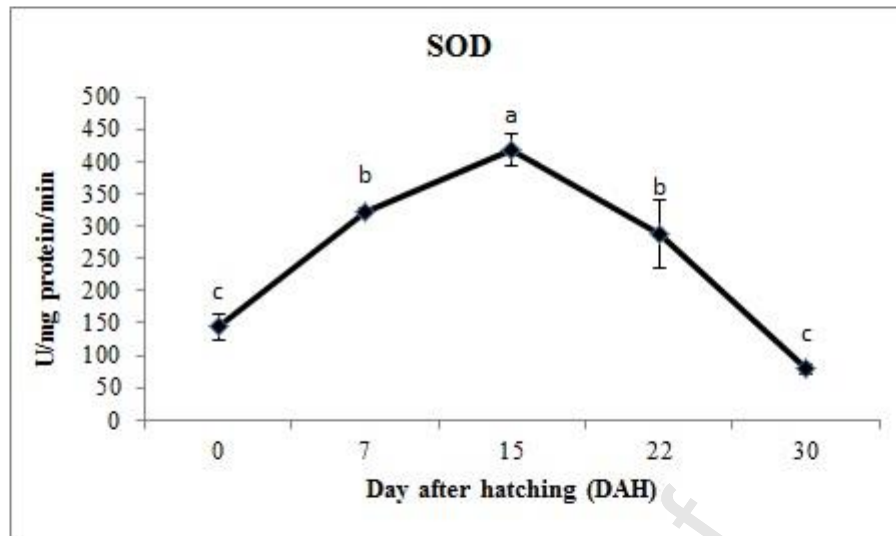

Figure 2

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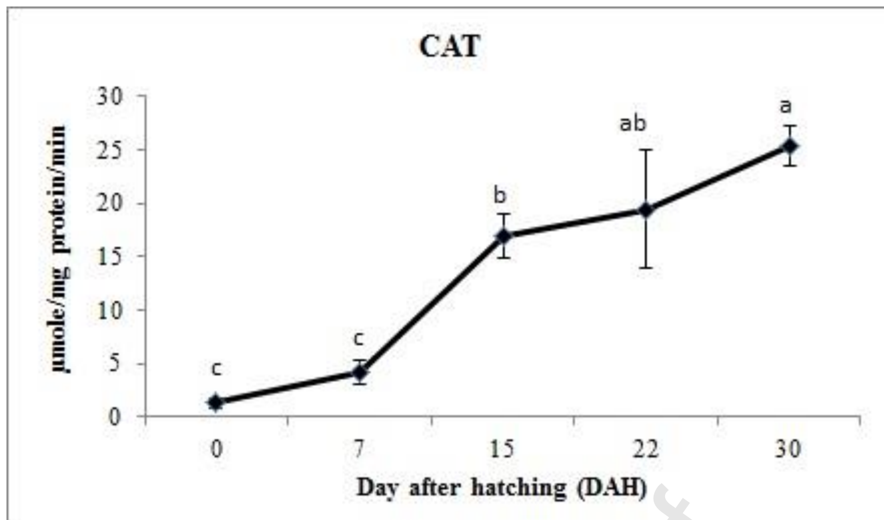

Figure 3

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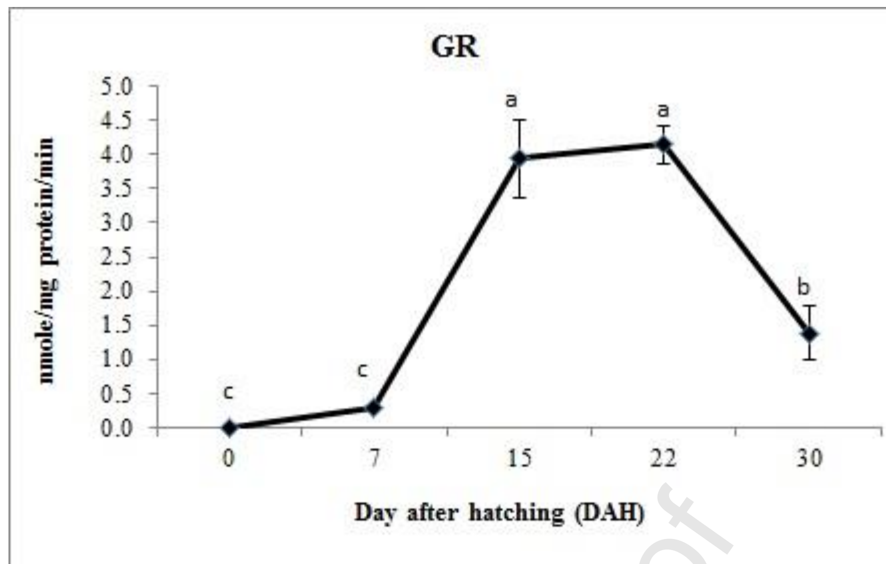

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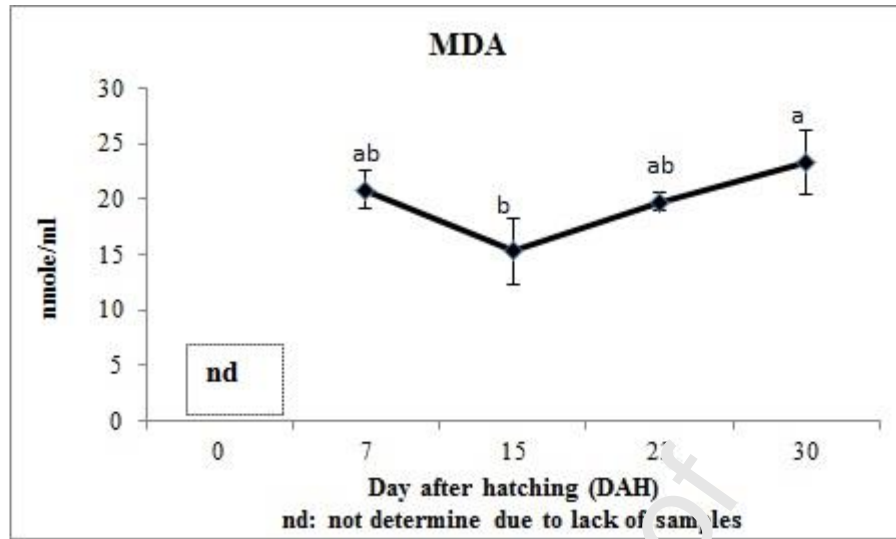

Figure 5

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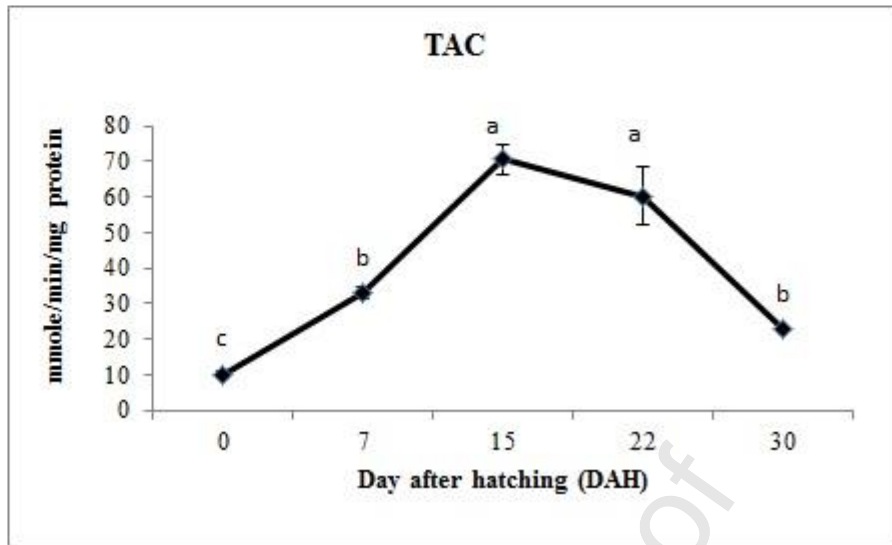

Figure 6

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☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

All persons listed as authors have read, contributed to preparing the manuscript as given below: Vahid Morshedi1*, Farzaneh Noori2, Marko D. Prokić3, Tamara G. Petrović3, Fateme Jafari2, Seyed Hossein Moradian 4 Vahid Morshedi and Seyed Hossein Moradian carried out the experimental design, fish maintenance and sample collection Vahid Morshedi carried out Conceptualization; Data curation; Funding acquisition; Investigation; Project

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Highlights

- ▸ Oxidative status in yellowfin seabream larvae during critical developmental periods was studied.
- ▸ Intense metabolic processes and somatic growth in yellowfin seabream larvae coincide with larvae the antioxidant system (AOS) response.
- ▸ The oxidative status of yellowfin seabream larvae varies during development, low AOS response from 0-7 DAH, high AOS response from 15-22 DAH to greater oxidative damage of lipids at 30 DAH.

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