

Fish response to hypoxia stress: growth, physiological, and immunological biomarkers

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Abstract Water quality encompasses the water physical, biological, and chemical parameters. It generally affects the fish growth and welfare. Thus, the success of a commercial aquaculture project depends on supplying the optimum water quality for prompt fish growth at the minimum cost of resources. Although the aquaculture environment is a complicated system, depending on various water quality variables, only less of them have a critical role. One of these vital parameters is dissolved oxygen (DO) level, which requires continuous oversight in aquaculture systems. In addition, the processes of natural stream refinement require suitable DO levels in order to extend for aerobic life forms. The depletion of DO concentration (called hypoxia) in pond water causes great stress on fish where DO levels that remain below

1–2 mg/L for a few hours can adversely affect fish growth resulting in fish death. Furthermore, hypoxia has substantial effects on fish physiological and immune responses, making them more susceptible to diseases. Therefore, to avoid disease outbreak in modern aquaculture production systems where fish are intensified and more crowded, increasing attention should be taken into account on DO levels.

Keywords Fish · Hypoxia · Performance · Innate immunity · Oxidative stress · Biomarkers

Introduction

The dissolved oxygen (DO) is a main limiting factor in fish farming because fish have aerobic metabolism requiring DO at efficient levels. The DO concentration in fish ponds depends generally on many factors including photosynthesis of phytoplankton, respiration of aquatic organisms, and/or the diffusion of atmospheric O₂. The solubility of O₂ in pond water relies on environmental conditions such as O₂ partial pressure, water temperature, and salinity (Campbell 1990; Withers 1992). Hypoxia is known as DO level low enough to negatively affect fish behavior, physiology, immunology, and growth (Jobling 1995; Lovell 1998; Mallya 2007; Pollock et al. 2007; Thorarensen et al. 2010; Burgos-Aceves et al. 2018). Most fishes have some capability to acclimate with fluctuations in DO levels, but if severe hypoxia persists, fishes will eventually die (Fitzgibbon et al. 2007; Cook and Herbert 2012). Hypoxia has been

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shown to induce primary, secondary, and tertiary stress responses in fish (Bernier and Craig 2005; Welker et al. 2007; Bernier et al. 2012; Segner et al. 2012). In captivity, fish always face repetitive and chronic stress situations (e.g., confinement, crowding, handling, variable water quality including hypoxia) from which they cannot escape. Hence, fish have to acclimate to any of these husbandry stressors. Therefore, the DO level should be maintained near the saturation level to enhance fish growth and feed intake, and increase the overall fish performance (Mallya 2007; Thorarensen et al. 2010). Brett (1979) suggested that a DO level of about 5 mg/L is critical for acceptable fish growth and feed utilization, and as DO level decreases, respiration and feeding activities also decrease. As a result of hypoxia, the physiological or metabolic activities of farmed fish would be negatively influenced (Wedemeyer 1996). Jobling (1995) suggested that the DO levels which limit the growth and feed intake in fish are usually 50–70% of air saturation. The prolonged exposure to hypoxia, regardless of the reason, causes low feed intake, growth retardation, and high tendency to disease (Kestemont and Baras 2001; Fitzgibbon et al. 2007; Portner 2010).

Effect of hypoxia on fish behavior

The swimming behavior of fish varied when held under hypoxic or normoxic conditions depending on fish species, rearing conditions, and the length and/or the level of DO and so on. Some fishes under hypoxic condition did not show any special activity to escape away from the hypoxic environment, and did not move upward to the water surface to resort to air breathing and remain static at the bottom of the tank to save their energy for facing the hypoxic condition (Israeli and Kimmel 1996; Wu 2002; Wu et al. 2007; Douxfils et al. 2012). This behavior may be due to frequent damage in gills of fish reared at hypoxic conditions (Araújo-Luna et al. 2018). In some other cases when the DO level was at its lowest, fish could be seen swimming rapidly in a circular motion with a wide mouth gape. This behavior attenuated to normal swimming behavior over time as DO returned to normoxic level (Bowyer et al. 2014). This behavior may be due to gills adaptation to hypoxia, including reduced gas diffusion distance (Saroglia et al. 2000) as well as increased total respiratory surface (Saroglia et al. 2002). In most of predatory fishes, the mouth

intermittently gaped and the operculum over the gills stayed incompletely open (Svobodova et al. 1993). On the other hand, Cook and Herbert (2012) reported that the swimming speed of yellowtail kingfish did not increase in response to inescapable hypoxia, but the fish show a rush and rest swimming behavior. This rush/rest behavioral response was related to anaerobic stress, obvious as increases in plasma lactate, glucose, and cortisol (Cook and Herbert 2012). The sensitiveness of fishes to hypoxia indicates that they may require a high DO concentration to ensure their survival. It has been perceived, in natural habitats, that hypoxia stress could have a strongly inverse impact on fish habitat quality and induced fish migrations (Breitburg 2002; Arend et al. 2011; Aboagye and Allen 2018).

Effect of hypoxia on gills performance

Fish gills are responsible for several physiological activities requiring behavioral, morphological, and physiological adaptations to hypoxic conditions (Randall and Daxboeck 1984; Olson 1991; Perry and McDonald 1993). However, fish gills are responsible for gas transfer, which is regulated by Fick's diffusion equation (Jobling 1994), their respiratory surface area (RSA) as well as their functional respiratory surface (FSA), and gas diffusion distance (GDD); all these factors are highly correlated with lifestyle and habitat of fishes (Perry and McDonald 1993). The GDD does not only vary considerably within fish species, but it also varies within the gill apparatus of the same fish species (Hughes and Morgan 1973). Prasad (1986) has shown a positive correlation between fish body mass and GDD in Indian flying barb (*Esomus danricus*). Environmental factors, such as water hardness (Greco et al. 1996) and water temperature and dissolved oxygen (Kisia and Hughes 1992; Randall and Daxboeck 1984; Saroglia et al. 2000), could also affect GDD. Similarly, the surface area of the lamella and the number of lamellae vary not only from fish species to another, but also within the same species, according to their size (Hughes 1984). Changes in the anatomy of the gill respiratory surface area during body development appears to be related to routine metabolism in Nile tilapia, *Oreochromis niloticus* (Kisia and Hughes 1993). Only a part of the total respiratory surface is perfused by blood during "quiet" ventilation (Booth 1978), compared with "strong" ventilation (Nilsson 1986), when the oxygen

uptake rate is increased. Duthie and Hughes (1987) observed that in rainbow trout, *O. mykiss*, whose functional gill area had been reduced by cauterization, when forced to swim showed a significant proportional reduction in maximum oxygen consumption. Oxygen consumption at rest and at subcritical swimming speeds was not affected. The authors concluded that the total gill area is utilized for oxygen uptake only under conditions of maximum aerobic demand and a direct limit is imposed on oxygen uptake at the gills independent of environmental oxygen partial pressure (PO_2), when elevated above normoxia.

The DO demand and PO_2 fluctuation in water could be compensated by adapting the RSA, as well as FSA, and GDD in fish gills. In this regard, a positive correlation was found in European sea bass, *Dicentrarchus labrax*, between environmental DO and both GDD and RSA (Saroglia et al. 2000, 2002, 2007, 2010). However, sea bass reared under hyperoxic condition represented significantly higher GDD than fish reared under normal oxygen concentrations. This also occurred when an improvement in O_2 solubility was due to a decrease in seasonal water temperature. The increase in FSA causes a rise in water loss and ionic gain through the gills in fish in a hypertonic environment such as water salinity (Evans 1993). Fish respond to this by increasing their water intake, which requires an increase in energy in order to excrete the excess ions through the gills. In contrast, a higher ion loss occurs when fish are acclimated to a hypotonic environment such as freshwater and respond with adequate mechanisms consisting in an enlarged water uptake and elimination of excess water and ions via the kidney.

Hypoxia may lead to asphyxiation and fish mortality, relying on the DO requirements for each fish species and to a lesser extent on their rate of adaptation. The main pathological-anatomic alterations of fish gills involve a very pale skin color, congestion of the cyanotic blood in the gills, adherence of the gill lamellae, and small hemorrhages at the anterior of the ocular cavity and in the skin of the gill covers (Mallya 2007). In this regard, Rinaldi et al. (2005) reared sea bass, *D. labrax*, at mild hypoxia, normoxia, and mild hyperoxia conditions (respectively, 60–70%, 90–100%, and 120–130% of the saturation value), and modifications to PO_2 levels were studied with morphological and immune-histochemical techniques. In normoxia and mild hyperoxia conditions, the gills had the typical structure with rows of parallel filaments; nevertheless, hyperoxia condition altered the

pillar cell structure, leading to an enlargement of the vascular lumen. In hypoxia condition, gills lost all their regular structure and were disorganized, where the vascular lumen was reduced and the lamellae were disorganized, were twisted, and showed frequent apical bleb. Araújo-Luna et al. (2018) found that the most frequent damage in gills registered for primary parameters was hyperplasia and cellular anomalies (degeneration and necrosis), whereas for the secondary parameter was congestion in the secondary lamellae.

Effect of hypoxia on osmo-respiratory compromise in fish

The osmo-respiratory compromise in fish is the balance between “need of oxygen” and “need of osmotic regulation” (Nilsson 1986). There are two major scenarios in which the osmo-respiratory compromise may interfere with osmotic homeostasis: (1) when oxygen demand is high, and gill perfusion must be increased to favor gas exchange at the expense of ion regulation; and (2) when the epithelium must be thickened to defend ion balance at the expense of gas exchange. The osmoregulatory compromise within fish species was generally affected by changes in environmental conditions, such water temperature, salinity, and exercise (Sardella and Brauner 2007). In general, elevated metabolism associated with high temperature results in a partial loss of osmoregulatory control, and in other cases, exposure to condition requiring an upregulation of osmoregulatory characteristic of the gills (e.g., exposure to dilute water) could result in morphological changes in the gill that impair gas exchange (Henriksson et al. 2008). Randall et al. (1972) indicated that a large, permeable gill membrane is required for efficient gas transfer but that a small, impermeable epithelium is needed to minimize diffusive ion losses. They also demonstrated that rainbow trout accelerate Na^+ loss with an increase in oxygen consumption during exercise, which they attributed mainly to an increased FSA of the gills during activity.

It can be also argued that the different O_2 concentrations may cause an acid-base disturbance, affecting blood pH and gill morphology. In this regard, Cecchini and Caputo (2003) published a study on the acid-base balance in sea bass in relation to water oxygen concentration. Their study was carried out for 5 weeks under controlled conditions, the PO_2 conditions being assayed at 64, 97, 150, and 250% of the saturation values and at

a salinity and temperature. They found no significant differences in Na^+ concentrations and blood PO_2 existed, whereas the blood pH was only significantly reduced by the hypoxia condition and not in the range from “normoxia” to the highest hyperoxia experienced. The study of Saroglia et al. (2010) shows an important relationship between PO_2 and the efflux of Na^+ and Cl^- in fishes exposed to a hypo-osmotic challenge together with manipulation stress; the Na^+ and Cl^- efflux was elevated 1.9- and 2.5-fold, respectively, in fishes previously acclimatized at the low PO_2 conditions. This can be explained by the studies of Saroglia et al. (2000, 2002), who reported that hyperoxia is associated with discrete changes in morphology of the gill, including reduced RSA and increased GDD in the gills of sea bass; the reduced attitude to exchange gases with water (O_2 and CO_2) also caused a reduction in the ion efflux rate. Brauner (1999) and Brauner et al. (2000) found a dramatic change in acid-base equivalent exchanges across the gills during hyperoxia exposure in freshwater fishes and also found that hyperoxia impaired hypo-osmoregulation following seawater transfer of Atlantic salmon smolts.

The overall picture of osmotic regulation for both freshwater and marine fishes is reasonably clear since the osmoregulatory organs and tissues have been identified as digestive tract, gill, kidney, urinary bladder, and liver for ureotelic regulators. Both passive and active flux of solutes, mainly NaCl plus urea, in ureotelic tissues have been assessed in many species. The chloride cells, or mitochondria-rich cells, in the teleost gills have been shown to play an important role in ionic homeostasis by compensating passive epithelial ion flux through appropriate active ion uptake and excretion (Evans 1993). Interactive effects of salinity on physiology and behavior also have to be taken into account including, among others, tissue permeability to water and ions, gill ventilation, perfusion, and functional surface area (Swanson 1998).

Such studies support the hypothesis that the energy cost for osmoregulation is lower in an isosmotic medium, where the gradients between blood and water are minimal, and that these energy savings are substantial enough to increase growth. For instance, it was estimated that osmoregulation might consume as much as 54–68% of the non-swimming metabolic output in two species of tunas (Bushnell and Brill 1992). Even in species with lower metabolic rates, osmoregulation appears to use a high proportion of

the available energy (Nilsson 2007), while Boeuf and Payan (2001) reported that it ranges from 20 to 50% of the total energy expenditure, roughly 100–150 mL/h/kg O_2 depending on the environmental salinity. Theoretically, a gill ideally designed to absorb oxygen for energy requirements should be highly permeable, with quite a large surface area, leading to an ascending metabolic cost of osmoregulation (Grau et al. 1994). These conflicting demands may represent the major constraint in the overall metabolic capacity of all fishes, which appear to be limited by the need for an “osmo-respiratory compromise” (Nilsson 1986).

A central process in osmotic regulation is active Na^+ transport; a great deal of energy is consumed in moving ions through a Na^+ and K^+ pump on the basolateral side of the transporting epithelia. The ratio of Na^+ transported to ATP consumed in this process is generally known for most transport epithelia (Boeuf and Payan 2001). It would also be possible to take into account the cost of Cl^- extrusion through the gill, involving complex mechanisms (Marshall et al. 1999; Chen et al. 2001). Morgan and Iwama (1999) specified that the energy required by freshwater and saltwater gills only represents a relatively small (4%) portion of the fish's total energy budget. However, it is important to consider potential problems posed by only using Na^+ flux data to calculate associated ATP usage. For instance, it can be questioned whether Na^+ transport is 100% efficient. NaCl transport by mitochondria-rich chloride cells in the gills has not yet been fully elucidated. Na^+ is not thought to be directly and exclusively excreted by Na^+ and K^+ -ATPase, but rather via the basolateral tubular network, through either diffusion or an active bulk flow mechanism; thus, Boeuf and Payan (2001) argued that the energy required could be underestimated. Many of the ions transported by ATPases may simply diffuse back out of the basolateral network into body fluids or, if the bulk flow hypothesis is correct, the energy needed to drive it would also have to be estimated. Another element reported by the latter authors is that accepting a low cost for osmoregulation mechanisms would contradict the demonstrated effect of water salinity, especially in the case of saltwater, on the growth of teleosts (Imsland et al. 2001).

Under severe hypoxia, fish reduce locomotion or feed intake to conserve energy, which may not be sufficient. This can result in the slowing down of ATP production through aerobic respiration, forcing the

animal to utilize anaerobic pathways to contribute to its energy requirements (Pichavant et al. 2002). But anaerobic metabolism is not as efficient as aerobic respiration and a significant raise in the glycolytic flux is needed to avoid a detrimental fall in cellular ATP (Routley et al. 2002; Chabot and Claireaux 2008). To that, blood glucose level rises for anaerobic fuel supply in many teleosts subjected to environmental and functional hypoxia and anaerobic metabolism further results in lactate accumulation in muscle tissue (Pichavant et al. 2002; Routley et al. 2002; Lushchak et al. 2005). When juvenile sea bass subjected to chronic hypoxia, glycogen content and lactate concentration in the liver decreased, and the expression of phosphoenolpyruvate carboxy kinase raised, indicating a stimulation of anaerobic glycolytic pathways (Cadiz et al. 2017).

Effect of hypoxia on fish performance and production

Great concern has been paid to DO levels in ponds' water, as low ambient DO levels are known to affect growth, feed consumption, and physiological status of fish (Jobling 1994). In coho salmon, *Oncorhynchus kisutch*, and sockeye salmon, *O. nerka*, largemouth bass, *Micropterus salmoides*, and common carp, *Cyprinus carpio*, growth is affected when DO concentrations were < 4–5 mg/L (Brett 1979; Brett and Blackburn 1981). In terms of managing fish ponds, it is always suitable to increase the aeration when fish start to swim near the surface, opening their mouth. The aeration of fish ponds will rise the DO level to be satisfactory and of course make the environmental conditions more comfy for fish. According to Mallya (2007), the minimum DO requirements are as follows: cold water fish, 6 mg/L; tropical freshwater fish, 5 mg/L; and tropical marine fish, 5 mg/L. It is worthy to mention that these values are minimum requirements for healthy growth, tissue repair, and reproduction (Svobodova et al. 1993). Lovell (1998) reported that DO requirements per unit of fish weight significantly decline with increasing the individual weight and this reduction for common carp, *C. carpio*, may be represented by the subsequent ratios: yearling = 1, two-year-old carp = 0.5–0.7, marketable carp = 0.3–0.4 mg DO/g. Significant differences in DO requirement were also found for different fish species. Using a coefficient of 1.0 to represent the oxygen demand of common carp, relative values for few species else are

as next: trout 2.83, peled 2.20, pike perch 1.76, roach 1.51, sturgeon 1.50, perch 1.46, bream 1.41, pike 1.10, eel 0.83, and tench 0.83 mg DO/g (Lovell 1998).

Growth is commonly related to the quantity of feed intake in fish, which is reflected by inhibition of weight gain due to low feed intake caused by hypoxia in post-smolt Atlantic salmon (Remen et al. 2012); big sea bass, *Micropterus salmoides*; common carp, *C. carpio*; turbot, *Scophthalmus maximus*; silver salmon, *O. kisutch* (Brett and Blackburn 1981; Pichavant et al. 2001; Ruyet et al. 2003); and Nile tilapia, *Oreochromis niloticus* (Tran-Duy et al. 2008; Abdel-Tawwab et al. 2014, 2015; Li et al. 2018). Studies examining the impact of hypoxic conditions on fish production have shown that even partial periods of hypoxia could lead to a decline in fish growth (Pichavant et al. 2000, 2001; Foss et al. 2002). Fish under hypoxic conditions tend to decrease their feed intake although some studies have reported that reduced DO levels did not affect feed conversion (Caldwell and Hinshaw 1994; Thetmeyer et al. 1999). Other studies have reported variable feed conversion efficiencies (Pichavant et al. 2000, 2001; Foss et al. 2002) suggesting that the fish capability to utilize feeds under hypoxic conditions may be compromised. However, some fish species are much more resistant for low DO than others, causing in differential survival (Poon et al. 2007).

Randolph and Clemens (1976) discussed that the feeding style of channel catfish, *Ictalurus punctatus*, differs with DO availability and found that when the DO content dropped below 59% saturation, fish started to lose their appetite. The rainbow trout, *O. mykiss*, appetite reduced when oxygen saturation dropped below 60% saturation (Jobling 1995). Similar results have been observed in the case of European sea bass, *D. labrax* (Thetmeyer et al. 1999); blue tilapia, *O. aureus* (Papoutsoglou and Tziha 1996); channel catfish, *I. punctatus* (Buentello et al. 2000); juvenile turbot, *S. maximus* (Pichavant et al. 2001); and Nile tilapia, *O. niloticus* (Tsadik and Kutty 1987; Tran-Duy et al. 2008; Abdel-Tawwab et al. 2014, 2015; Li et al. 2018). All those fishes showed reduced growth when exposed to low DO levels. Likewise, at different temperatures, the DO levels changed over increasing the water temperature. Bowyer et al. (2014) investigated the effects of water temperature (21, 24, or 27 °C) and DO regime (normoxic vs. hypoxic) on growth, feed intake, and digestive enzyme activity of the yellowtail kingfish,

Seriola lalandi, for 5 weeks. They notified that specific growth rate (SGR) of fish exposed to hypoxia at 21, 24, and 27 °C were 13, 20, and 17% lower, respectively, than SGR recorded for the fish reared under normoxic conditions. This study also clearly demonstrated that growth rate and feed intake of yellowtail kingfish were negatively affected by hypoxic conditions. Hansen et al. (2015) compared the impact of decreasing DO levels from 100 to 70% of air saturation (hypoxia condition) on parameters of production performance (feed intake, growth, feed conversion ratio, mortality) in triploid versus diploid Atlantic salmon kept at high seawater temperature (19 °C) for 51 days. They found that triploidy fish showed low feed consumption and growth more than diploid fish by reducing DO from 100 to 70%. Araújo-Luna et al. (2018) reared gilthead seabream, *Sparus auratus* (316.3 ± 1.73 g), at DO levels of 44.3, 65.0, and 84.1% for 6 weeks. They found that fish growth was significantly lower in low DO level than other DO levels; meanwhile, a negative linear relationship was observed between FCR and increasing DO levels indicating to better food conversion at higher DO levels. Also, under hypoxia conditions, fish reproduction is restrained, and both fertilization success and larval survival are compromised. Fish, under this situation, reduce their activity, feeding reproduction, and so on. Bera et al. (2017) exposed goldfish, *C. auratus* (25.0 ± 0.5 g), to four cyclic hypoxia (H) and normoxia (N) treatments of 6 h H/8 h N, 9 h H/15 h N, 12 h H/12 h N, and 24 h H. The control groups (C) were maintained at 24-h N condition (6.4 ± 0.2 mg O₂/L). They found that cyclic hypoxia (0.8 ± 0.2 mg/L DO) for 9 h or more per day suppressed ovarian growth and viable spermatozoa production. They also found that plasma steroid concentrations particularly of 17 α -hydroxyprogesterone (17-HP), estradiol (E2), and testosterone (T) in female fish, and T and 11-ketotestosterone (11-KT) in male fish, were attenuated under diel hypoxic conditions. The reduced gonadosomatic index, decreased number of tertiary oocytes, and motile spermatozoa in hypoxic fish clearly indicate suppression of gametogenesis. Thereby, prolonged diel cyclic hypoxia may affect valuable fishery resources and fish population structure by impairing reproductive performances and inducing estrogenic effects in fish males.

All these responses are aimed to preserve cellular oxygen homeostasis and reduce energy expenditure, thereby augmenting fish survival during hypoxia. To

keep away from this, aquaculture systems must be supplied with sufficient DO saturation via artificial aeration or oxygenation. Besides aeration or oxygenation to overcome hypoxia effects on fish farming, diets with high carbohydrate and fat contents should be given to fish; that may help in reducing negative effects of critical hypoxia stress. Fish could use carbohydrate and lipids as fundamental energy sources during acute hypoxia stress, and metabolize more lipid during long-term hypoxia stress (Li et al. 2018).

Effect of hypoxia on fish biochemistry and physiology

The biochemical and physiological status of cultured fish is an essential part of evaluating their health status where their changes might be an evidence for unsuitable environmental conditions or the existence of stressing factors such as feed restriction, crowding, toxic chemicals, excess organic compounds, and even usual procedures in aquaculture (Barcellos et al. 2004; Cnaani et al. 2004; Bartoskova et al. 2013; Abdel-Tawwab 2016; Abdel-Tawwab and Wafeek 2017; Aliko et al. 2018; Faggio et al. 2018; Gobi et al. 2018; Schonova et al. 2018). In many fish species, sub-lethal hypoxic level leads to a complicated sequence of neural, behavioral, and physiological modifications through the primary and secondary stress responses in an attempt to re-establish homeostasis (Caldwell and Hinshaw 1994; Delaney and Klesius 2004; Welker et al. 2007; Terova et al. 2008), for example, common carp, *C. carpio*, rised breathing frequency (Glass et al. 1990) and rainbow trout, *O. mykiss*, rised hemoglobin concentration levels (Soivio et al. 1980).

Douxflis et al. (2012) investigated the physiological responses of two generations (F1 and F5) of Eurasian perch, *Perca fluviatilis* (68 ± 28 g), exposed to 4-h hypoxia or normoxia conditions as follows: F1 + normoxia, F1 + hypoxia, F5 + normoxia, and F5 + hypoxia. For each of these experimental conditions, DO concentration in hypoxia was stabilized to 2.0 ± 0.5 mg/L and in normoxia was stabilized to 7.44 ± 0.37 mg/L during the trial period. They found that in both generations, single and repeated hypoxia resulted in glucose elevation and reduction of spleen somatic index. On the other hand, Muusze et al. (1998) studied effects of progressive and stepwise hypoxia in Amazon fish (*Astronotus ocellatus*) and detected no differences in cortisolemia, while

significant impacts on lactate concentration and metabolic rate were observed. Contrariwise, other studies on Siberian sturgeon (*Acipenser baeri*), spotted wolffish (*Anarhichas minor*), and crucian carp (*Carassius carassius*) reported rapid and significant cortisol rise following an acute hypoxic disturbance (Maxime et al. 1995; Lays et al. 2009; Sula and Aliko 2017). In Eurasian perch, it has been found that cortisol peaked earlier than 1.0 h following an intense stressor and quickly returned to pre-stress level within 1–6 h (Acerete et al. 2004; Milla et al. 2010; Douxfils et al. 2012). In turbot, Pichavant et al. (2002) did not find any cortisol raise during a 6-h period of slight or moderate hypoxia but an increase of cortisolemia was seen under severe hypoxia throughout the 6-h period and up to 6 h after healing in normoxic water. Thus, the severity of hypoxic stress may be an important contributing factor of the cortisol release. Many studies demonstrated rapid increases in red blood cells (RBCs), hemoglobin (Hb), and/or hematocrit (Ht) following hypoxia in fish (Affonso et al. 2002; Wells and Baldwin 2006; Abdel-Tawwab et al. 2014, 2015). Araújo-Luna et al. (2018) found that gilthead seabream reared at normoxic condition (85.4% DO) presented significantly lower levels of Ht when compared to fish at low DO level, meanwhile no significant differences in Hb, glucose, and cortisol among DO levels. The increase in RBCs count, under hypoxic condition, may be because fish spleen contracted releasing a considerable amount of RBCs into the circulating blood system to enhance its oxygen carrying capacity (Douxfils et al. 2012). Interestingly, it appeared that RBC counts have high metabolic maintenance costs that are fueled by both glucose and lactate (Wells and Baldwin 2006). Use of these molecules by metabolically active erythrocytes is expected to contribute to the maintenance of an efficient Hb-oxygen transport system (Wells and Baldwin 2006). Consequently, the raised glucose detected in fish under hypoxia stress might also be explained by the release of spleen erythrocytes requiring glucose substrate for optimal oxygen transport activity.

Myoglobin (Mb) is an oxygen-binding hemoprotein that was once thought to be exclusively expressed in oxidative myocytes of skeletal and cardiac muscle where it serves in oxygen storage and facilitates intracellular oxygen diffusion. Qi et al. (2018) reported that severe hypoxia induced significant expression of Mb at the mRNA and protein levels in the heart of schizothoracine fish species, *Schizopygopsis pylzovi*, which suggests Mb had

a major role in the supply of oxygen to the heart of Tibetan Plateau fish.

Acute hypoxia was recognized to elevate the levels of catecholamines, activating glycogenolysis and gluconeogenesis with a net result of increasing plasma glucose levels (Wright et al. 1989). Similar results were found on gilt-head seabream, *Sparus aurata* (Henrique et al. 1998); Nile tilapia, *O. niloticus* (Delaney and Klesius 2004; Abdel-Tawwab et al. 2014, 2015); channel catfish, *I. punctatus* (Thomas et al. 2007); and grass carp, *Ctenopharyngodon idella* (Gan et al. 2013), stressed by hypoxic condition. The rise in glucose concentrations was due to the mobilization of energy storage under fatigued conditions of low DO, as a source of fuel for anaerobic metabolism (Faggio et al. 2016; Prokic et al. 2018). On the other hand, through fish exposure to acute hypoxia, cardiac ATP concentration of tilapia (*Oreochromis hybrid sp.*) was unchanged compared with normoxia and anaerobic glycolysis contributed to ATP supply as evidenced by considerable accumulation of lactate in the heart and plasma (Speers-Roesch et al. 2010). Gracey et al. (2001) observed that when longjaw mudsucker, *Gillichthys mirabilis*, was exposed to short hypoxia, genes involved in the glycolytic metabolic pathway, muscle contraction, and locomotion were all downregulated in the muscle cells.

On the other hand, Bowyer et al. (2014) noticed that the digestive enzyme activities (i.e., trypsin, lipase, and amylase) were not affected by DO concentrations. Hansen et al. (2015) compared the impact of lowering DO from 100 to 70% of air saturation (hypoxia) on parameters of physiological status (plasma K^+ , Cl^- , Na^+ , osmolality, glucose, creatinine (Cr), bilirubin, triacylglycerol (TAG), and alkaline phosphatase (ALP) levels in triploid versus diploid Atlantic salmon kept at high seawater temperature (19 °C)). They reached that analysis from blood samples pulled drawn on day 51 shows that plasma levels of Cl^- , TAG, ALP, and bilirubin were lowered in triploids in general, and that plasma Cr levels trebled and plasma K^+ levels declined in triploids exposed to 70% DO for 29 days.

The hypoxia stress could disorder the physiological homeostasis where a complex process of physiological and biochemical changes is involved in fish to cope with hypoxia stress (Welker et al. 2007; Terova et al. 2008), including declined metabolic rate, high ventilation and anaerobic respiration, and high Hb- O_2 affinity (Rahman and Thomas 2007). Hypoxia also affects nutrient metabolism (Mahfouz et al. 2015; Polymeropoulos et al.

2017). When DO in water is insufficient to the oxygen demands for aerobic glycolysis, the normal physiological function and metabolic rate cannot be maintained (Richards 2011). Under hypoxia stress, fish usually decline oxygen consumption by slowing down movement and improving oxygen-carrying capacity through the increase of RBCs and Hb values (Cossins and Crawford 2005; Roesner et al. 2006; Xia et al. 2016). Moreover, hypoxia is correlated with the activation of anaerobic metabolism, and anaerobic glycolysis would meet the high energy requirement of fish during hypoxia stress (Muusze et al. 1998; Bartrons and Caro 2007; Speers-Roesch et al. 2010). Because of the low ATP yield of anaerobic glycolysis, the substrates such as glycogen and glucose will be substantially consumed, leading to accumulation of lactate (Richards 2011; Genz et al. 2013). In this regard, Li et al. (2018) exposed Nile tilapia, *O. niloticus* (6.3 ± 1.2 g), to acute hypoxia stress with DO of 0.7 ± 0.1 mg/L for 6 h and chronic hypoxia stress with DO of 1.1 ± 0.1 mg/L for 4 weeks to evaluate its reaction towards nutritional metabolic pathways. They noticed that fish in the acute and chronic trials had various adaptive mechanisms. At stress of acute hypoxia, glycogen contents either in liver or in muscle tissues decreased significantly; however, there was no significant difference in triglycerides (TG). The lactate dehydrogenase (LDH) activity was exceeded subsequently with acute hypoxic stress. Nevertheless, the response of fish to long-term hypoxia stress was different from acute hypoxia. On the other hand, the crude fat in fish decreased in the hypoxia fish group; furthermore, TG either in the liver or in the muscle tissues were significantly lower. Beta oxidation of the liver tissues was elevated under the hypoxic conditions, whereas the hepatic glycogen content increased in the hypoxia-treated fish. Transcriptomic test displayed that the gene expression regarding the synthesis of carbohydrate and lipolysis increased in the hypoxia group, while genes responsible for either carbohydrate catabolism or fat synthesis exhibited the reverse action. The mRNA expression of genes involved in glycolysis and glycogenolysis was significantly upregulated by acute hypoxia stress. Under hypoxic or anoxic conditions, the metabolic inhibition stratify well to fish, where aerobic metabolism of fish declines and the fish depend upon anaerobic glycolysis for energy production (Virani and Rees 2000). In this concern, Mahfouz et al. (2015) studied the effect of short term for 1.0 day (trial 1) and long term for 1-month hypoxia (trial 2) on some glycolytic enzyme

activity and mRNA expression in liver and white muscle of Nile tilapia (10 ± 1.2 g) that was subjected to 0.5, 1.0, or 2.0 mg DO/L for comparison with a normoxia group (8 mg DO/L). They concluded that, in fish liver, subsequent to short-term hypoxic exposure, the particular activities of phosphofructokinase (PFK) and pyruvate kinase (PK) decreased, while lactate dehydrogenase (LDH) activity was elevated at all DO concentrations compared to normoxia. This may be proportionate with a primary function of this organ in glucose export during hypoxia in order to supply fermentative fuel to other vital organs. It has been proposed that through short-term hypoxia, catecholamines regulate glucose availability in rainbow trout by inhibiting PK activity in the liver; that suggests an activation of gluconeogenesis and an inhibition of glycolysis (Wright et al. 1989). Furthermore, the raising in liver LDH activity is in accordance with Kraemer and Schulte (2004) who reported a significant increase in liver LDH activity of the teleost killifish after 48 h of hypoxic exposure. On the other hand, no changes in liver LDH activity of the common estuarine fish *Leiostomus xanthurus* were shown after exposure to different levels of hypoxia for 12 h (Cooper et al. 2002), and a slight decrease in LDH activity in Nile tilapia exposed for 10 h to severe hypoxic exposure (Ishibashi et al. 2002).

Previous studies reported that exposure to long-term hypoxia increases liver LDH activity of the killifish through the first 28 days of exposure up to here to hypoxia (Greaney et al. 1980), PFK, PK, and LDH activities of tench (*Tinca tinca*) throughout the first 42 days of exposure to hypoxia (Johnston and Bernard 1982) and gulf killifish after 4 weeks from exposure to hypoxic stress (Martinez et al. 2006). Even though PK and PFK enzymes are specific to catabolic reactions (glycolysis) under hypoxia, LDH enzyme is shared between catabolic and anabolic reactions (glycolysis and gluconeogenesis) and rising increased activities of all these enzymes imply a futile cycle whose net result is ATP turnover (Martinez et al. 2006). The increased activity of the examined enzymes in the white muscles following long-term hypoxia is in accordance with Johnston and Bernard (1982) and Martínez et al. (2011) who concluded that activity of PFK in skeletal muscle of tench, subjected to hypoxic conditions for 6 weeks and African fish *Barbus neumayeri* exposed to hypoxia for a month, was higher than that of normoxic fish. This reflected an adaptation response for enhanced anaerobic glycolysis during exposure to

hypoxic water. In contrast, some previous studies reported that exposure to long-term hypoxia either decreases PFK, PK, and LDH activities in skeletal muscle of gulf killifish after a month from exposure to hypoxia (Martinez et al. 2006) or have no change on LDH activity of killifish during hypoxic for 28 days (Greaney et al. 1980). Bera et al. (2017) found that cyclic hypoxia (0.8 ± 0.2 mg/L DO) for 9 h or more per day caused such alterations in plasma lipid and sex steroid profiles in hypoxed fish, which in turn directly or indirectly suppressed ovarian growth and viable spermatozoa production. They also found that hypoxia decreased significantly total cholesterol and high-density lipoprotein, while it elevated significantly triglycerides in both fish sexes. Plasma steroid concentrations particularly of 17α -hydroxyprogesterone (17-HP), estradiol (E2), and testosterone (T) in female fish, and T and 11-ketotestosterone (11-KT) in male fish, were attenuated under diel hypoxic conditions. Intriguingly, both diel and continuous hypoxia elevated plasma E2 and vitellogenin levels in males.

Effect of hypoxia on fish immunity and bacterial infection

The DO level in pond water is especially important because it is closely related to disease outbreaks (Null et al. 2017; Domenici et al. 2017; Gallage et al. 2016, 2017). As a result of hypoxic condition, fish growth and production will be reduced and the possibility of a disease outbreak increases (Lovell 1998; Shoemaker et al. 2000). Adverse water quality in terms of anthropogenic activity or adverse environmental conditions including hypoxia may prejudice the immune system, leading to lowered resistance to pathogen infections (Di Marco et al. 2008). Hypoxia has shown to modify the innate and adaptive immune responses in fish (Boleza et al. 2001; Cecchini and Saroglia 2002; Ortuno et al. 2002; Cuesta et al. 2003; Kvamme et al. 2013; Abdel-Tawwab et al. 2014, 2015). For example, previous reports demonstrated high mortality as a result of streptococcal infection in tilapia *Oreochromis* sp. subjected to hypoxic conditions (Bunch and Bejerano 1997; Evans et al. 2003). Fukuda et al. (1997) and Evans et al. (2003) demonstrated that long periods of low DO in water up to 1.0 mg DO/L increased a stress response in yellowtail jacks, *Seriola lalandi*, and Nile tilapia leading to weakening immune response and reduced their resistance

versus pathogenic bacteria *Enterococcus seriolicida* and *Streptococcus agalactiae*, respectively. Cecchini and Saroglia (2002) confirmed that antibody responses against human γ -globulin in hypoxic European sea bass, *D. labrax*, were weaker than those in hyperoxic ones. Air exposure-induced hypoxia lowered the respiratory burst in gilthead seabream, *S. auratus* (Ortuno et al. 2002), and also retarded head kidney natural cytotoxic cell activity in gilthead seabream (Cuesta et al. 2003). Reduction in lysozyme activity and abundance of C3 components in the serum of hypoxia-stressed fish may have resulted from the energetic demand associated with long-term stress responses. Admittedly, if a portion of the fish's energy budget is required to cope with stressors, then less energy will be available for other biological functions, including immunity (Douxflis et al. 2012; Segner et al. 2012). It now remains to be determined whether these immune changes would have harmful effects on the fish overall resistance to disease through achievement of bacterial challenge tests. Furthermore, since the effects of stressors might be tissue-specific (Milla et al. 2010), not only serum immune actors but defense mechanisms in inner organs (e.g., head kidney, spleen, thymus) and on the primary barrier (i.e., gut, gills, mucus, and skin) should also be assessed to go through the immunological results of hypoxia. Even if harmful immune modifications occur at the circulatory level, this may not necessarily result in an increased susceptibility to disease depending on the integrity and immune capabilities in these abovementioned tissues. In the study of Ni et al. (2014), fish subjected to hypoxia stress (6 h after hypoxia stress) showed a significant rise in the levels of the serum total protein. It is speculated that fish may increase specific proteins such as lysozyme or complement to enhance the immunity level to cope with stress (Abdel-Tawwab et al. 2014, 2015).

Previous studies have reported that the innate immunity and specific antibody titer decreased as DO level decreased when fish were exposed to pathogenic bacteria *Edwardsiella ictaluri*, *Aeromonas hydrophila*, or *S. agalactiae* (Welker et al. 2007; Abdel-Tawwab et al. 2014, 2015; Gallage et al. 2016, 2017). The immune parameters and defensive competence vary greatly between fish species, even between closely related species (Schroder et al. 1998). Abdel-Tawwab et al. (2014, 2015) studied the effect of different DO levels on innate immunity of Nile tilapia, which were reared at 0.1–1.5 (low), 3.0–3.5 (medium), and 6.5–7.0 mg/L (high) for

12 weeks and further injected by pathogenic bacteria (*A. hydrophila*). They found that nitroblue tetrazolium (NBT) values and lysozyme activity as well as fish resistance to *A. hydrophila* infection decreased as DO level decreased.

Wang et al. (2018) investigated the effect of intermittent hypoxia under different temperatures on the immunomodulation in vaccinated Nile tilapia. Fish (20.0 ± 3.0 g) were acclimatized to intermittent hypoxic (4.0 ± 1.0 mg DO/L) or normoxic (8.0 ± 0.5 mg DO/L) conditions at 30 ± 0.5 or 35 ± 0.5 °C. Interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), and gamma interferon (IFN- γ) mRNA expressions in spleen and head kidney were significantly lower in vaccinated hypoxic fish compared to those in the vaccinated normoxic fish. The activities of superoxide dismutase, catalase, and glutathione peroxidase were significantly lower, while malondialdehyde levels were significantly higher in vaccinated hypoxic fish. Additionally, the phagocytic activity of peripheral blood leucocytes (PBLs) and intracellular reactive oxygen species (ROS) production in head kidney cells declined significantly, while nitric oxide levels in tissues cells increased significantly under hypoxic at either 30 or 35 °C conditions. Taken together, intermittent hypoxia at 30 °C and 35 °C could suppress immunomodulation in vaccinated Nile tilapia resulting in higher cumulative mortality due to *S. agalactiae* infection. The results of Wang et al. (2018) suggest fish were not getting the expected level of protection from vaccination when vaccinated fish kept at intermittent hypoxic under different temperature conditions.

Effect of hypoxia on DNA and genes expression

In hypoxed fish, a complex set of biochemical and physiological alterations is employed to cope with hypoxia stress (Nikinmaa 2002). Many of these adjustments depend on a large extent on changes in the expression of genes that encode diverse groups of physiologically relevant proteins. Gracey et al. (2001) recently identified alterations in the expression of over 120 genes in hypoxic longjaw mudsucker, *Gillichthys mirabilis*. Genes that are induced by hypoxia appear to share a common mode of transcriptional regulation. This induction depends upon activation of a transcription factor, the hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer composed of α and β subunits. HIF-1 β is

generally found to be constitutively expressed in the nucleus and to be insensitive to changes in O₂ availability, whereas stabilization of HIF-1 α and its nuclear accumulation are acutely regulated by hypoxia (Uchida et al. 2004). Since the initial characterization of HIF-1 α in humans, several additional cDNAs have been isolated in different vertebrates, whereas orthologues from fish have only been identified in a few species. On the other hand, although recent technological developments have made it possible to measure patterns of gene expression, only few published reports are available on tissue expression patterns of HIF-1 α in fish exposed to hypoxia. Terova et al. (2009) utilized the real-time PCR technology to monitor dynamic changes in levels of HIF-1 α transcripts, in response to acute and chronic hypoxic stress. They found that the number of HIF-1 α mRNA copies increased significantly in response to both acute (1.9 mg/L, DO for 4 h) and chronic (4.3 mg/L, DO for 15 days) hypoxia in European sea bass, whereas it remained unchanged in fish exposed to hyperoxic (DO 13.5 ± 1.2 mg/L) conditions.

The capability of a fish to tolerate hypoxic conditions is a complex trait, governed by multiple genes (Terova et al. 2008; Majmundar et al. 2010; Jha et al. 2015). Therefore, to understand the regulation of gene expression, collections of differentially expressed cDNA under stressed conditions would be a useful genomic resource (Rodrigues et al. 2012). This acclimatization process, during physiological and metabolic changes in organisms prompted by hypoxic stress, is mediated by hypoxia-inducible factors (HIFs) (Majmundar et al. 2010). These factors bind to the regulatory regions of the hypoxia-inducible genes, which results in HIF-regulated gene activation (Ortiz-Barahona et al. 2010). The two alpha subunits, i.e., HIF1-alpha and HIF2-alpha, have unique and complementary roles in adaptive response to tissue hypoxia (Mohindra et al. 2013, 2016; Tripathi et al. 2013) and provide a distinctive mechanism for hypoxia tolerance in Atlantic croaker (Rahman and Thomas 2007) and grass carp (Law et al. 2006). Poon et al. (2007) evaluated the response of the intact liver of common carp, *C. carpio*, to hypoxia, for 42 days at 0.5 mg DO/L, and realized an extensive DNA damage in liver cells, especially during the first week of exposure. The authors attributed their results to cellular mechanisms that seemed to be directed towards preventing apoptosis in the face of DNA damage and promoting DNA repair. Al-Salahy (2006) found that hypoxia significantly increased DNA fragmentation in

the liver but not in the white muscles of African catfish, *Clarias gariepinus*, exposed to hypoxic conditions (from 5.1 to 0.6 mg DO/L). Mahfouz et al. (2015) also confirmed an increase in DNA damage that was directly proportional to increasing hypoxic concentrations in a concentration-dependent manner. The hypoxia impact was more noticeable in short-term exposure than in long-term. They also explained that the liver was more vulnerable to hypoxic effect than the muscle where the latter appears to be relatively hypoxia-tolerant compared to the liver as a guide to the reduction in the percentage of DNA tail and tail moment. They suggested that Nile tilapia cope better with long-term hypoxic conditions, possibly as an adaptive response.

The heme oxygenase (HO)-1 is a cytoprotective enzyme that can be involved in cytoprotection against hypoxia stress. In the study of Guan et al. (2017), they cloned duplicated HO-1a and HO-1b cDNAs in hypoxia-sensitive blunt snout bream (*Megalobrama amblycephala*). HO-1a and HO-1b encode peptides with 272 amino acids and 246 amino acids, respectively, and they share a low sequence identity of 55%. HO-1a and HO-1b mRNAs were maternally deposited in the zygote, and the mRNAs decreased to the lowest levels at 8 hpf. Both mRNAs were significantly expressed from 12 hpf and fluctuated but maintained a high level after 16 hpf. Using in situ hybridization, HO-1a and HO-1b mRNAs were ubiquitously expressed in embryos at 12 hpf. At 24 and 36 hpf, HO-1b transcripts were detected in the mid- and hind-brain, respectively, whereas HO-1a was mainly transcribed in the eyes and endoderm at 24 hpf and in the brain at 36 hpf. In adult fish, HO-1a was abundantly expressed in the heart, liver, gill, kidney, spleen, and brain, while HO-1b mRNA was detected mainly in the kidney. After exposure to hypoxic stress, both HO-1a and HO-1b mRNAs were upregulated significantly in the gill and liver but downregulated significantly in the brain. These findings suggest that duplicated HO-1 genes have evolved divergently and yet play overlapping biological roles in regulating the response to hypoxia in blunt snout bream, *M. amblycephala*. In addition, HO-1 genes can effectively mediate respiratory responses to hypoxia in goldfish (Tzaneva and Perry, 2014). Since teleosts experience an additional genome-wide duplication event (Taylor et al. 2003), it is possible that many teleost genomes have two HO genes.

Interaction between DO and husbandry conditions

The response of fish to hypoxic stress differed from one species to another depending on their response to the husbandry conditions. The hypoxic stress might disturb the balance and harmony between fish and the husbandry conditions, causing a stress response in fish (Barton and Iwama 1991; Barton 2002; Pollock et al. 2007; Douxfils et al. 2012; Fazio et al. 2012). Tran-Duy et al. (2008) studied the effect of different levels of DO and fish size on growth and feed utilization of Nile tilapia, *O. niloticus* (L.), which was exposed to two levels of DO either 3.0 or 5.6 mg/L and two fish sizes (21 and 147 g). The results of this study displayed that feed intake and fish growth at high DO level were significantly higher than those at low DO level. It is also proposed that the limitation of the gill surface area due to DO limitation caused an increase in gill respiratory surface with a reduction in gas diffusion distance (Saroglia et al. 2000, 2002, 2007, 2010); this results in lower feed intake and lower fish growth.

Duan et al. (2011) investigated effects of DO concentration and stocking density on growth, energy budget, and body composition of juvenile Japanese flounder, *Paralichthys olivaceus*. Fish (14 ± 2.1 g) were subjected to a normal and a high DO of 5.5 ± 0.5 and 14 ± 2 mg/L, as well as four stocking densities per each DO concentration (100, 200, 300, and 400 fish/m² for the normal DO and 200, 400, 600, and 800 fish/m² for the high DO). They found that feed utilization increased significantly with increasing DO concentration irrespective to the fish density. They also noted that the maximum fish weight was achieved under a high DO level. Bowyer et al. (2014) evaluated the interactive impacts of water temperature (21, 24, or 27 °C) and DO regime (normoxic vs. hypoxic) on growth, feed consumption, and digestive enzyme activity of yellowtail kingfish, *Seriola lalandi*, for 5 weeks. They stated that SGR of fish exposed to hypoxia at 21, 24, and 27 °C were 13, 20, and 17% lower, respectively, than SGR recorded for the fish reared under normoxic conditions. Abdel-Tawwab et al. (2014) examined the effect of different levels of DO and fish density on the performance of Nile tilapia, *O. niloticus* (L.). Fish were exposed to 0.8 ± 0.1 , 2.5 ± 0.3 , and 6.5 ± 0.5 mg/L and two stocking densities (15 and 30 fish per 100-L aquarium) for 12 weeks. They found that fish growth and feed consumption increased significantly with increasing DO levels irrespective to fish density. They also reported that glucose, activities

of AST and ALT, creatinine, and uric acid in fish plasma decreased significantly with increasing DO levels. Meantime, total protein and total lipid in plasma increased significantly by increasing DO. These results indicate that high DO could improve fish performance; however, the optimum level of DO is 6–6.5 mg/L diet.

Abdel-Tawwab et al. (2015) studied the impact of different levels of DO and fish size on growth, feed utilization, physiological alterations, and innate immunity of Nile tilapia, *O. niloticus* (L.), which was exposed to 0.1–1.5, 3.0 ± 3.5, and 6.5 ± 7.0 mg/L and two fish sizes (3.7 and 12.9 g) for 12 weeks. They found that final weight, weight gain, and feed intake increased significantly with increasing DO level irrespective to fish size. They also reported that glucose, activities of AST and ALT, creatinine, and uric acid in plasma declined significantly with raising DO concentration. Meanwhile, total protein and total lipid in fish plasma increased significantly by increasing DO level. The fish resistance to *A. hydrophila* infection and NBT values and lysozyme activity increased as DO level increase. These results displayed that high DO and low FS could improve fish performance; however, the optimum level of DO is 6–6.5 mg/L diet. Ni et al. (2014) examined the effects of stocking density and hypoxia on the immune and physiological responses of juvenile Amur sturgeon (*Acipenser schrenckii*). In this investigation, fish (42.0 ± 2.3 g) were cultured in nine square concrete ponds at three stocking densities (3.7, 6.9, and 9.0 kg/m³) for 50 days and DO was maintained at 7, 5, or 3 mg/L for 0, 0.5, 1.5, 3, and 6 h after hypoxia stress. The results pointed that the levels of cortisol, glucose, and hematological parameters elevated significantly after hypoxia stress. The count of RBCs in this study also elevated significantly after hypoxia stress. Rapid elevations in RBC count were also observed in hypoxia in juvenile Tambaqui (*Colossoma macropomum*; Affonso et al. 2002) and silver trevally (*Pseudocaranx dantex*; Wells and Baldwin 2006). The increase in RBC counts may raise blood oxygen capacity and improves oxygen delivery to the tissues. Furthermore, Ni et al. (2014) found significant increases of serum cortisol and glucose levels during the period of hypoxia stress similar to observations in Siberian sturgeon (*Acipenser baeri*; Maxime et al. 1995) and spotted wolffish (*Anarhichas minor*; Lays et al. 2009). This may indicate a mobilization of these metabolites to overcome the hypoxia disturbance. Guo et al. (2018) evaluated the effects of feeding frequency on growth performance of juvenile

Dolly Varden char *Salvelinus malma* (9.40 ± 0.30 g) and its challenge to hypoxia stress challenge. They assigned fish randomly to one of six feeding frequencies (1, 2, 3, 4, 5, and 6 times/day) for 8 weeks; after that, fish were exposed to hypoxia stress (1.5 mg/L) and fish mortality was recorded on 0, 15, 30, and 45 min. They confirmed that, at the hypoxia challenge test, cumulative fish mortality increased significantly as feeding frequency or hypoxia time increased. The results of Wang et al. (2018) suggest fish were not getting the expected level of protection from vaccination when vaccinated fish kept at intermittent hypoxic under different temperature conditions.

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