Elevated CO2 reduces juvenile Scophthalmus maximus growth and liver function in a recirculating aquaculture system

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Abstract

Elevated CO2 negatively affects marine fish. In a recirculating aquaculture systems, we exposed juvenile Scophthalmus maximus, a CO2¬-sensitive, high-value species , to CO2 at 0 mg/L (control), or at 8, 16, 24, or 32 mg/L, for 7, 14, 30, or 60 d. Cumulative survival decreased significantly with increasing CO2 , to 68% at 32 mg/L. Weight gain, specific growth rate, and feed conversion rate differed significantly between the control and maximum concentration. CO2 caused histopathological damage. Plasma glutamate pyruvate transaminase and glutamate oxalate transaminase were significantly and substantially elevated at 24 and 32 mg/L CO¬2, relative to the control. At 32 mg/L, hemoglobin was significantly reduced, and methemoglobin significantly elevated , indicating reduced oxygen-carrying capacity. GHR, IGF-1, IGF-1R, and THR expression were substantially lower at 32 mg/L than in the control. For Scophthalmus maximus , these findings indicate that elevated CO2 retards growth, impairs health, and causes metabolic disorders, possibly by impairing liver function.

1. Introduction

CO_2 regulation is central in aquaculture, and particularly high-density aquaculture, where fish metabolic activities may elevate CO_2 (Hill*et al* . 2004; Summerfelt *et al* . 2000) to levels 10–40 times greater than in the ocean (Fivelstad *et al* . 2003; Foss*et al* . 2003; Steffensen & Lomholt 1988). Elevated CO_2 can significantly affect fish health and growth (Fivelstad *et al* . 2003; Foss *et al* . 2003; Steffensen & Lomholt 1988). Although recirculating aquaculture systems provide efficient environmental control and visibility, allowing optimal production efficiency (Ebeling & Timmons 2012), farmed fish are more susceptible than wild fish to external stressors (Kvamme *et al* . 2013).

In fish, exposure to elevated CO_2 is counteracted by increased respiration amplitude and frequency (Gilmour and Perry 2006). This can, in turn, cause chronic excess O_2 levels, thus indirectly reducing growth. Prolonged exposure of farmed fish to elevated CO_2 leads to hypercarbia and respiratory acidosis, reducing feed intake and growth (Fivelstad *et al* . 2007) and fertility (Ben-Asher *et al* . 2013), and causing renal calcium deposition (Fivelstad *et al* . 2018). In marine fish, elevated CO_2 causes metabolic acidosis (Bernier and Randall 1998), ionic imbalance (Brauner *et al* . 2000), stress hormone activation (Iwama *et al* . 1989), respiratory acidosis, and excess reactive oxygen species (ROS) production, leading to oxidative cellular damage (Cao *et al* . 2010). To prevent oxidative stress, the antioxidant enzyme system must be activated (Song *et al* . 2017). CO_2 can affect liver biosynthesis (Rognstad 1983; Stapp*et al* . 2015), increasing the incidence of lymphocytic portal hepatitis (Good *et al* . 2010) and liver tissue damage (Frommel*et al* . 2012).

Exposure to elevated CO_2 affects fish growth, and tolerance to CO_2 varies among species (Martens *et al* . 2006). *Salmo salar* (Atlantic salmon) growth decreases linearly with increasing CO_2 (Fivelstad *et al* . 2018; Khan *et al* . 2018; Mota *et al* . 2019). In Atlantic cod, exposure to 18 mg/L CO_2 affected growth and cataract

incidence (Moran and Støttrup 2011; Neves and Brown 2015).*Scophthalmus maximus* growth declined by 26% on exposure to 21 mg/L CO_2 (Stiller *et al* . 2015).

The pituitary and thyroid glands secrete growth hormone (GH) and thyroxine, which act downstream of the liver, constituting the hypothalamic–pituitary–thyroid–liver axis. The liver is the primary metabolic organ, with key roles in metabolic homeostasis, metabolism, endocrine regulation, endogenous compound degradation, and detoxification, among other functions (Almroth *et al* . 2019). Chronic stress can lead to hepatic insufficiency. As lipids accumulate in hepatocytes, microscopic changes occur in the liver, eventually leading to macroscopic lesions (Bolla *et al* . 2011). Liver tissue expresses large amounts of various proteins, and contains high levels of antioxidant enzymes and antioxidants. Liver enzymes can be used to assess liver function (Casas-Grajales & Muriel 2015; Kaplowitz 1981). The liver is a major source of endogenous type-1 insulin-like growth factor (IGF-I), which can be used to assess fish growth and health; IGF is associated with growth, metabolism, development, cell differentiation, reproduction, osmoregulation, and immune response in fish (Reinecke 2005). In the hypothalamic–pituitary–thyroid–liver axis, pituitary secretion of GH stimulates hepatic synthesis and the release of IGF-I, which specifically inhibits GH gene transcription and secretion via negative feedback (Wallenius 2001). Pituitary GH/hepatic IGF-I is an important endocrine regulatory axis (Reinecke 2005, 2006) and important signaling pathway in the thyroid–liver axis.

Thyroid hormones regulate GH expression in the pituitary gland, and thyroid hormone response elements are present in the GH gene promoter in mammals (Forhead 2000) and in scleractinian fish (Eppler 2011). In mammals, GH controls growth and development by mediating IGF-I secretion (Daughaday & Rotwein 1989; Eppler 2011; Schmid 2000). Thyroid hormone, GH, IGF-1, IGF-1 receptor (IFG-1R), and other hormones affect growth, development, differentiation, metabolism, immunity, and other processes (Omer 2011). Although studies have addressed liver responses to toxicants such as ammonia (Cheng *et al* . 2015), zinc oxide (Horie*et al* . 2020), and nitrate (Yu *et al* . 2021), responses to CO_2 have not previously been studied.

Scophthalmus maximus (turbot; Pleuronectiformes [flounders], Scophthalmidae), a key aquaculture species in China (particularly northern China), has high economic value and market demand, and is farmed mainly in recirculating aquaculture systems. Using this species, we applied a CO_2 gradient to evaluate responses in terms of growth, health, and oxygen-carrying capacity. Further, we evaluated the role of endocrine function (in terms of GHR and IGF expression) in CO_2-induced growth retardation. Elevated CO_2 (32 mg/L) negatively affected juvenile*Scophthalmus maximus* growth and health. Growth can be hampered even at CO_2 concentrations below 8 mg/L. This study provides valuable insights into the growth of juvenile*Scophthalmus maximus* under CO_2 stress.

2. Materials and Methods

2.1. Experimental conditions

The experimental fish used in this experiment were provided by Shandong Oriental Ocean Sci-Tech Co., Ltd. (Shandong, China). The experimental area was equipped with 15 RAS, each comprising an aquaculture tank (3 m^3), foam separator, waste collector, storage tank (1.5 m^3), biological filter, dissolved O_2 source, circulating pump, and an integrated control box. The fish were domesticated for two weeks before the experiment. After acclimation, the fish were divided into five groups (three replicates in each group, each containing 3 samples). Following our experimental scheme and previous studies (Fivelstad *et al* . 2017; Khan 2018; Mota *et al* . 2019; Stiller *et al* . 2015), we used the following CO_2 concentrations: 0 mg/L (control), and 8, 16, 24, and 32 mg/L. Fish treatment was approved by the Animal Protection and Utilization Committee of the Institute of Oceanography, Chinese Academy of Sciences.

Throughout the experiment, dissolved O_2 saturation was 90% to 100%, temperature was 14.6 +- 0.1 degC, and salinity was 33 +- 1.0 g. Fish were initially stocked at a density of 2.35 kg/m^3. Natural light was provided for 10–14 h per day. Fish were fed twice daily at 0.8% of body mass with a commercial fish feed (52% crude protein, 12% crude fat, 16.0% crude ash, 3.0% crude fiber, 12% water, 5% Ca, [?]2.3% P, [?]2.3% lysine, and [?]3.8% NaCl).

2.2 Fish Sampling

The experimental period was 60 d. After being fasted for 24 h, and being placed under anesthesia in buffered tricaine methanesulfonate (35–40 mg/L; MS-222, Sigma-Aldrich, St. Louis, MO), nine fish were randomly selected from each experimental tank on days 0, 7, 14, 30, and 60. Caudal vein blood, sampled with a syringe, was stored in lithium heparin anticoagulant tubes at 4 degC, centrifuged within 1 h at 3000 rpm for 10 min, then stored at -80 degC. The liver, kidney, and spleen samples were stored in liquid nitrogen for genetic analysis.

Histological samples were stored overnight in 4% paraformaldehyde, then in 70% ethanol for long-term storage. For histological analysis, the livers were dehydrated using an increasing alcohol gradient, and embedded in paraffin. Embedded samples were sectioned to 5 μm using a microtome (RM 2235, Leica, Wetzlar, Germany) and stained with hematoxylin and eosin (Suvarna 2012).

2.3 Growth parameters

Nine samples per sink were used for each sampling period. At the end of the experiment, body length (cm^3), body weight (g), and total feed weight (g) were used to calculate final mean body weight (g), weight gain rate (WGR, %), specific growth rate (SGR, %), feed conversion ratio (FCR, %), and condition factor (CF, g/cm^3), as follows:

SGR = (ln(Wt) - ln(W0)) / t × 100,

where W0 is initial biomass of the fish, Wt is the total biomass of the fish at the end of the test cycle, and t is the experimental cycle;

FCR = total feed quantity / (Wt - W0);

CF = Wt / body length; and

WGR= (Wt - W0) / W0 x 100.

2.4 Physiological and biochemical analysis

Within 1 h of collection, total blood hemoglobin (Hb) and methemoglobin were directly measured with using a kit (#A102-1, Nanjing Jiancheng Bioengineering Research Institute, China), according to the manufacturer's instructions.

Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) levels were determined using the corresponding kits (A007-1-1, visible light method; A001-3-2, WST-1 method; A005-1-2, colorimetric method; Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China), according to the relevant instructions. Plasma lysozyme was determined using the turbidimetric method (A050-1-1 kit, Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

2.5 Health performance

The cumulative survival (CS, %), hepatosomatic index (HSI, %), spleen somatic index (SSI, %), and kidney somatic index (KSI, %) were calculated using the number of experimental fish, as follows (weights given in grams):

CS = (N1 / N0) x 100,

where N1 and N0 are the final and initial numbers of fish, respectively;

HSI = WH / WA x 100,

where WH and WA are the liver and final body weights of individuals, respectively;

SSI = WS / WA x 100,

where WS is the spleen weight; and

KSI = WK / WA x 100,

where WK is the kidney weight.

2.6 Gene molecular analysis

Total RNA was extracted from tissue according to the operating instructions of SPARKeasy Tissue/Cell RNA Rapid Extraction Kit with Genomic DNA Clean Column (Sparkjade, Shandong, China), followed by qPCR using the Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (AG, China). The reaction system (20 μL) was reverse transcribed to cDNA, and total RNA concentration was determined using a Nanodrop 2000 spectrophotometer(GENE COMPANY LIMITED,Hong Kong, China). Sample purity was assessed by determining the 260 nm / 280 nm optical density ratio.

Primers (Table 1) were designed using Primer Premier 6.2, using a SYBR Green Premix Pro Taq HS qPCR Kit (AG, China), with a 20 μL reaction solution. qRT-PCR was conducted using a CFX Connet Real-Time PCR System (Bio-Rad, China), as follows: 95 °C for 15 min, followed by 35 cycles of 15 s at 95 °C and 60 s at 60 °C.

2.7 Statistical analysis

Results are expressed as the mean ± standard deviation (SE). Statistical analysis using IBM SPSS included one-way ANOVA, followed by the Tukey test to check for significant differences between groups. A significance level of $P < 0.05$ was used.

3. Results

3.1. Elevated CO_2 significantly inhibited growth.

CO_2 concentration affected the weight gain rate (WGR), specific growth rate (SGR), feed coefficient (FCR), and condition factor (CF) (Fig. 1). At the initial stage of culture (at 7 d), there was no significant difference among the groups. Over time, differences in growth among the treatments became evident. WGR and SGR, which characterize juvenile growth, declined linearly with increasing CO_2 exposure (Fig. 1A, B). At 14 d, growth stratification appeared between the groups, increasing with concentration and time (Fig. 1). At 60 d, the differences between the 32 mg/L treatment and control were almost three-fold for WGR (27.86% ± 3.32% and 118.39% ± 6.89%, respectively) and SGR (0.41% ± 0.04% and 1.30% ± 0.05%, respectively). In contrast, FCR was positively associated with CO_2 concentration (Fig. 1C), being highest at 32 mg/L. CF, which varied slightly among the treatments and over time, was generally poor at high CO_2 levels.

Elevated CO_2 caused stress that reduced fish health. CSR declined with increasing CO_2 concentration (Fig. 2A), with significant differences between the control and treatments. CSR was lowest at 32 mg/L, at only 68.42%, which was 29.83% lower ($P < 0.05$) than in the control group (Fig. 2A).

In the fish sampled on day 30, HSI and KSI decreased with increasing CO_2, and were significantly lower at 32 mg/L than at 0 and 8 mg/L (Fig. 2B, C). In contrast, SSI was significantly higher at 16, 24, and 32 mg/L than at 0 and 8 mg/L (Fig. 2D).

3.2. CO_2 concentration affected Hb oxygen-carrying capacity.

Plasma Hb (in g/L) decreased significantly with increasing CO_2 (Fig. 3A), from 76.69 ± 3.81 in the control, to 74.23 ± 3.80 at 8 mg/L CO_2, 71.42 ± 2.21 at 16 mg/L, 70.72 ± 2.90 at 24 mg/L, and 70.15 ± 3.78 at 32 mg/L, which had the most highly significant change (Fig. 3). Total methemoglobin (in g/L) increased significantly with increasing CO_2concentration: it was 0.29 ± 0.05 at 32 mg/L CO_2, the most significant difference relative to the control (0.15 ± 0.01).

CAT, SOD, and GPX are important antioxidant enzymes that protect cells from oxidative damage caused by ROS. On day 60, SOD, GPX, and CAT levels did not differ significantly between 0 and 8 mg/L CO_2; GPX levels did not differ significantly between 0 (or 8) mg/L and 16 mg/L, but did differ significantly between 24 and 32 mg/L (Table 2). SOD activity was significantly lower at 16 mg/L than at 24 and 32 mg/L. CAT

activity was significantly lower at 16 and 24 mg/L than at 32 mg/L. Overall, antioxidant enzyme activity increased with CO_2concentration. Lysozyme levels increased significantly with increasing CO_2: they were not significantly different between 0 and 8 mg/L CO_2, but were significantly higher in the other groups.

Blood pH first declined with increasing CO_2concentration, then increased significantly, with the most significant increase at 32 mg/L (Table 3).

3.3. Liver injury became more pronounced with age.

Relative to the control (Fig. 4A), elevated CO_2 caused varying degrees of liver damage (Fig. 4B, C, D, E), predominantly involving hepatocyte vacuolization, nuclear atrophy and deformation, and vascular congestion in the sinus or portal vein. Relative to the control, plasma glutamate pyruvate transaminase (GPT) and glutamate oxalate transaminase (GOT) were significantly higher following CO_2 treatment; GPT was highest at 32 mg/L (Fig. 5A, B), consistent with the liver injury results (Fig. 4).

3.4. CO_2 stress reduced fish growth.

To assess whether elevated CO_2 affects GH/IGF-1 levels, we measured the expression of four genes, GHR, IGF-1, IGF-1R, and thyroid hormone receptor (THR). GHR expression was significantly lower following CO_2 treatment than in the control, and was lowest at 32 mg/L (Fig. 6C). IGF-1 and IGF-1R expression decreased similarly with increasing CO_2 (Fig. 6A, B), and was lowest at 60 d at 32 mg/L CO_2. THR expression declined substantially with CO_2 concentration: at 32 mg/L, it was significantly lower than in the other groups (Fig. 6D). These results indicate that CO_2 stress substantially inhibited fish growth.

4. Discussion

These findings suggest that elevated CO_2 affects*Scophthalmus maximus* more than previously reported. The effects of CO_2 on aquatic organisms are diverse, and depend greatly on biotic factors such as species and life stage, and on abiotic factors such as exposure time and concentration (Fivelstad *et al* . 2018; Khan *et al* . 2018; Moran & Støttrup 2011; Mota *et al* . 2020; Noor *et al* . 2019; Pan *et al* . 2020).

At 7 d, relative to the control, plasma pH was significantly lower following CO_2 treatment, whereas it increased over time, especially at 32 mg/L (Table 3). Under short-term exposure to elevated CO_2, blood pH initially decreases; plasma HCO^{3-} then increases to regulate acidity, returning pH to the initial level, or even higher (Portner *et al* . 2004). For example, in rainbow trout, elevated CO_2 raised blood pH (Eddy *et al* . 1977). Throughout our experiment, blood pH was higher at 32 mg/L than at 8 mg/L. We speculate that compensatory regulation occurs in fish, and will study this further.

Here, fish growth responded negatively to elevated CO_2, with the maximum CO_2 level associated with the lowest SGR, WGR, and CF, and highest FCR. This is consistent with reports for other fish species, where long-term exposure to CO_2concentrations of 5–40 mg/L negatively affected growth (Hhpa *et al* . 2020; Moran and Stottrup 2011; Mota *et al* . 2020; Neves and Brown 2015; Nmn *et al* . 2019; Stiller *et al* . 2015).

Elevated CO_2 negatively impacted fish health. Cumulative survival declined significantly with increasing CO_2, consistent with reports for other fish species (Huong *et al* . 2020; Hwang *et al* . 2009; Noor *et al* . 2021). Hb is the most important blood parameter for O_2 delivery (Segner*et al* . 2012), and stressors can lead to insufficient Hb mobilization in the spleen and other hematopoietic organs, reducing Hb content (Harikrishnan *et al* . 2012). CO_2 hampers oxygen-carrying capacity, via the "Bohr effect" (Bohr *et al* . 1904), whereby it causes H^+ to bind to Hb, reducing its affinity for O_2 and leading to inadequate O_2 transport. In our study, at 32 mg/L, elevated plasma CO_2 caused plasma Hb to be significantly lower than in the control. Similarly, elevated CO_2 caused Hb to decrease in *Salmo salar* (Good *et al* . 2018).

Among the body's molecules, erythrocyte Hb contains the most iron (Stein*et al* . 2010). The effects of CO_2 on methemoglobinemia have not been reported. Here, methemoglobin levels were significantly higher at 32 mg/L than in the control. Elevated CO_2 caused plasma Hb to increase slowly (Fig. 3A), reflecting a decline in Fe^{2+} levels. Iron homeostasis is regulated entirely by iron absorption via the digestive system. Fe^{2+} solubility decreases rapidly at pH $>$ 6 (Stein *et al* . 2010). The blood pH levels that we observed reflect

alkaline conditions (Table 3). We speculate that Fe^{2+} absorption declines rapidly in an alkaline intestinal environment. Reduced Hb, together with insufficient Fe^{2+} supplementation due to the alkaline intestinal environment, can lead to anemia, which may affect growth. Our results are consistent with this.

Fish and mammals have similar hepatic function, in which the liver accumulates, detoxifies, and metabolizes organic and inorganic pollutants (Kohler 1991). Because the liver responds rapidly to the external environment, hepatic pathological changes can be predictive biomarkers of aquatic toxicology (Frommel *et al* . 2012; Kohler 1990). Here, elevated CO_2 led to hepatic damage, including the presence of fat vacuoles, atrophied nuclei, and unusual or necrotic hepatocytes. Under normal conditions, hepatocytes contain GOT and GPT. Hepatosteatosis causes transaminase levels to increase. Here, plasma transaminase levels were substantially elevated at 16, 24, and 32 mg/L CO_2, reflecting liver damage (Gutierrez & Solis 2009; Rezaeisaber & Nazer 2011; Zheng *et al* . 2006), consistent with our histology findings. Liver damage caused by high CO_2 concentration has been reported for freshwater fish, and in isolated Antarctic fish hepatocytes (Good *et al* . 2010; Langenbuch & Portner 2004). In salmon, chronic stress caused by long-term elevated CO_2 affects growth by modifying digestive capacity (Khan Jr. *et al* . 2006).

In fish, exposure to foreign substances (such as toxicants) causes fat to accumulate in the liver (White *et al* . 1973), which in turn raises HSI levels (Parikh *et al* . 2010). Here, fish condition initially improved, then declined over time under elevated CO_2. We speculate that fat accumulates during early CO_2 stress, leading to liver injury and causing nuclear atrophy, thereby reducing HSI levels.

Lysosomal dysfunction and destruction of the lysosomal vascular system causes fat vacuoles to appear in hepatocytes (Kohler 1990). We speculate that elevated CO_2 disrupts and damages the lysosomes. Damage to the lysosomal system can impair normal hepatocyte function, leading to metabolic disorders, cell transformation, and cell death. The elevated GOT and GPT that we observed via histology and plasma analysis is consistent with this.

Fish blood biochemistry, including changes in plasma enzymes, can provide effective indicators of environmental stress, as well as an overview of the physiological state (Cole *et al* . 2001; Li*et al* . 2010; Noor *et al* . 2019). Under oxidative stress, antioxidant defense enzymes, such as SOD, CAT, and GPX, participate in clearing high-level ROS (Kochhann *et al* . 2009). SOD and CAT protect against oxidative damage by removing partially reduced oxygen species (Di Giulio *et al* .) Here, SOD, CAT, and GPX activity was significantly elevated at 32 mg/L CO_2relative to the control, thereby alleviating hypoxia-induced stress. This proves that elevated CO_2 affects the oxygen-carrying capacity of this species.

Lysozymes, important immune molecules in fish, are well known for their bactericidal effects (Panase 2017; Whang *et al* . 2011). They are also considered opsonins, activating both the complementary system and circulating phagocytes (Grinde 1989; Jolles & Jolles 1984). Further, some lysozymes have antiviral and anti-inflammatory activity (Ibrahim*et al* . 2001; Jolles & Jolles 1984; Lee *et al* . 1999; Samaranayake *et al* . 1997; Zhang *et al* . 2008). Here, exposure to 32 mg/L CO_2 significantly enhanced lysozyme activity relative to the control, revealing that this defense mechanism was activated in in response to CO_2 stress.

Stiller *et al* . (2015) have previously reported that CO_2 negatively affects *Scophthalmus maximus*production and health, addressing two key parameters, WGR and SGR. Here, we have expanded this approach to include FCR, CF, HSI, plasma indices, histology, and quantitative gene expression. Even at CO_2concentrations as low as 1–2 mg/L, O_2 uptake, anti-predation behavior, and growth are reduced (Ou *et al* . 2015), and the olfactory system and central brain function are impaired (Porteus *et al* . 2018). However, dissolved CO_2concentrations of 10–20 mg/L are commonly observed in commercial aquaculture systems (Gorle *et al* . 2018). For Atlantic salmon, based on simulations, CO_2 levels <12 mg/L, with low stocking densities, did not negatively affect fish growth, physiology, or welfare (Mota *et al* . 2019). The effects of multiple stressors (such as temperature) and initial health status tend to be interactive, increasing the negative effects of exogenous substances on organisms (Almroth *et al* . 2019; Fivelstad *et al* . 2007; Park *et al* . 2020; Wendelaar 1997). Future studies of CO_2 stress factors should therefore consider the combined effects of various biological variables.

The liver is the primary target organ of GH, which is synthesized in the pituitary gland, and is the primary source of IGF-1. In fish, the GH/IGF-1 axis is a key endocrine pathway that regulates somatic-cell growth and development (Beckman 2011). In fish, GH regulates hepatic IGF-I release by binding to GHR (Bertucci *et al* . 2017; Di Prinzio*et al* . 2010; Reinecke *et al* . 2005). GH and IGF are easily disturbed by external factors, thereby affecting fish growth and development (Dang *et al* . 2018; De Las Heras *et al* . 2015; Yu *et al* . 2017). Using qRT-PCR, we therefore examined the expression of several key genes in the GH–IGF-1 axis, to evaluate how this species responds to elevated CO_2 in aquaculture. GHR was significantly downregulated at 24 and 32 mg/L CO_2. At the same time, elevated CO_2significantly reduced IGF-1 and IGF-1R levels. We observed hepatic lesions and elevated GOT and GPT, which reflect CO_2-induced liver damage that caused IGF-1 and IGF-1R levels to decrease. This is consistent with our growth analysis, providing further evidence that elevated CO_2 inhibited growth, particularly at 32 mg/L.

Thyroid hormone, an important regulator of differentiation, growth, metabolism, and adaption to salinity (Crane*et al* . 2004; Orozco *et al* . 2002), binds to a receptor protein to regulate growth and metabolism. Here, this receptor gene was significantly downregulated. Although various toxicants and chemicals are thought to interfere with thyroid hormone and THR expression, reducing growth (Hu *et al* . 2020; Li *et al* . 2014; Wang*et al* . 2012), the effects of CO_2 on THR have not previously been examined. We speculate that CO_2 affects the liver and causes liver damage, which, in turn, inhibits growth.

In conclusion, 32 mg/L CO_2 negatively affected juvenile*Scophthalmus maximus* growth and health. Growth can be hampered even at CO_2 concentrations below 8 mg/L. This provides insight into how juveniles of this commercially important marine fish respond to elevated CO_2.

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Table 1:Quantitative real-time PCR primers list.

Genes	**Primer sequence (5'-3')**	**Annealing temperature (°C)**	**Anne**
GHR	F: ACACGTCCATTTGGATCCCC	F: ACACGTCCATTTGGATCCCC	58
	R: GCTCCCAGTTGACCATGACA	R: GCTCCCAGTTGACCATGACA	
IGF-1	F: CTGAGAGAGCGGAACCATCC	F: CTGAGAGAGCGGAACCATCC	58
	R: CAGATAATGAGCCGCCTGGT	R: CAGATAATGAGCCGCCTGGT	
IGF-1R(Jia, Yudong, et al.,2018)	F: CCTGATGTCACAGTGGGTGT	F: CCTGATGTCACAGTGGGTGT	58
	R: GTCTTCCCACTCACTTTGCTG	R: GTCTTCCCACTCACTTTGCTG	
Ef1α (Wu, Lele, et al.,2021)	F: CTGGGTGCTGGACAAACTG	F: CTGGGTGCTGGACAAACTG	58
	R: GTTCTTGGAAATACCTGCCTC	R: GTTCTTGGAAATACCTGCCTC	

Index	CO2 concentration	CO2 concentration	CO2 concentration	CO2 concentration	
	CG	LC	MC1	MC2	HC
SOD (U/ml)	387.54 ± 5.52^{a}	382.33 ± 3.54^{a}	431.15 ± 5.86^{b}	496.11 ± 9.05^{c}	511.38 ± 20.12^{c}
GPx (U/ml)	135.62 ± 4.38^{a}	135.47 ± 8.34^{a}	143.52 ± 4.65^{a}	171.35 ± 4.02^{b}	184.55 ± 3.58^{c}
CAT (U/ml)	2.77 ± 0.47^{a}	2.90 ± 0.27^{a}	3.46 ± 0.13^{b}	4.26 ± 0.87^{b}	5.37 ± 0.41^{c}
LZM (μg/ml)	40.47 ± 0.89^{a}	42.48 ± 3.43^{a}	49.35 ± 2.56^{b}	57.81 ± 4.58^{c}	64.33 ± 1.36^{d}

Table .2. After 60 days of CO_2 exposure, the contents of antioxidants in turbot plasma: superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) in oxidative stress reaction and lysozyme after emergency response. The data are mean ± S.E. (n = 9). The values of different superscripts were significantly different among groups ($P < 0.05$).

pH Concentration	Time	Time	Time	Time
	7D	14D	30D	60D
CG	7.52 ± 0.17^{a}	7.69 ± 0.14^{ab}	7.64 ± 0.09^{a}	7.56 ± 0.23^{a}
LC	7.4 ± 0.13^{a}	7.44 ± 0.17^{a}	7.54 ± 0.19^{a}	7.47 ± 0.19^{a}
MC1	7.35 ± 0.13^{a}	7.39 ± 0.13^{a}	7.78 ± 0.29^{ab}	7.82 ± 0.26^{ab}
MC2	7.69 ± 0.15^{ab}	7.72 ± 0.19^{ab}	7.85 ± 0.08^{ab}	8.01 ± 0.23^{ab}
HC	8.15 ± 0.11^{b}	8.24 ± 0.17^{b}	8.41 ± 0.17^{b}	8.56 ± 0.12^{b}

Table.3. After 60 days of CO_2 exposure, the content of Ph in turbot plasma. The data are mean ± S.E. (n = 9). The values of different superscripts were significantly different among groups ($P < 0.05$).

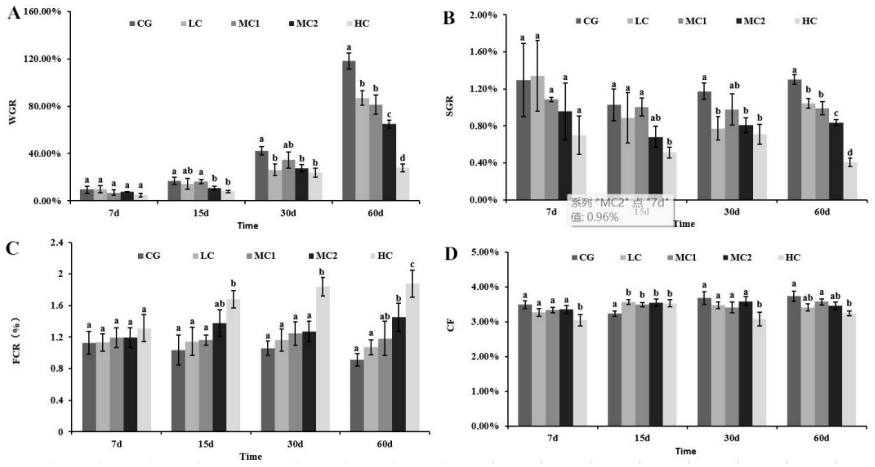

Figure 1.

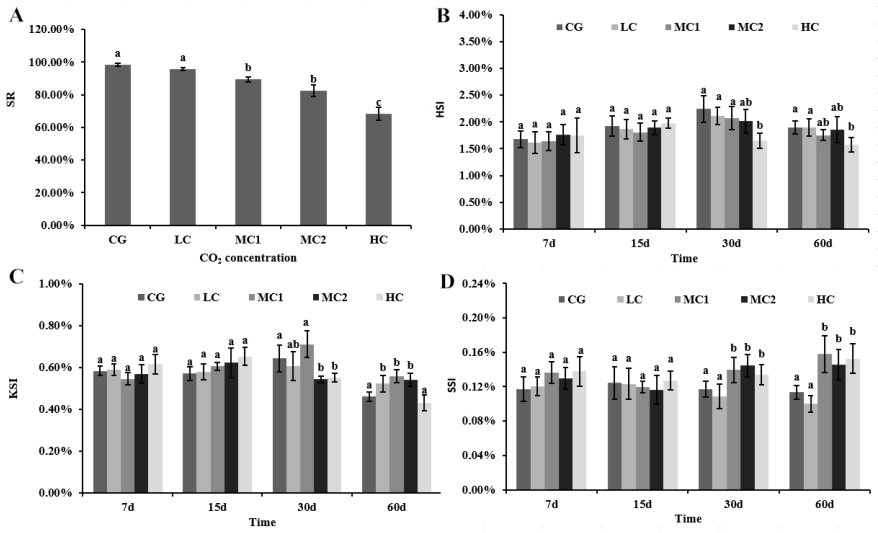

Figure 2.

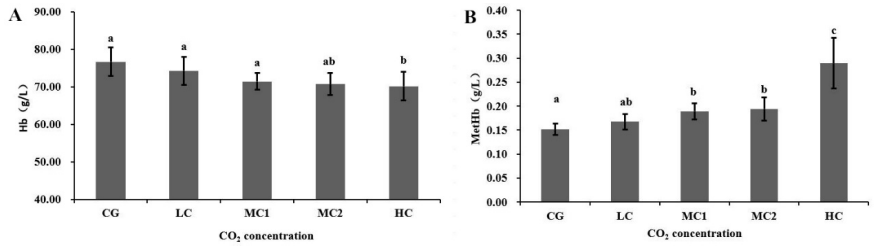

Figure 3.

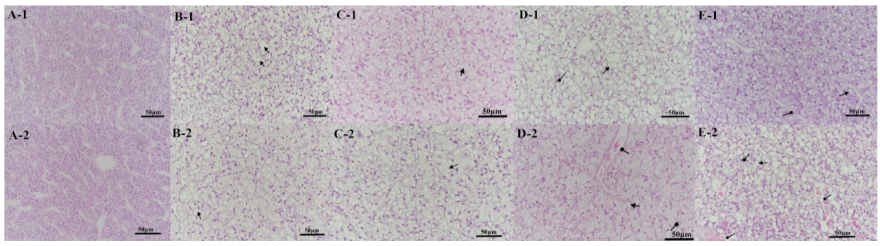

Figure 4.

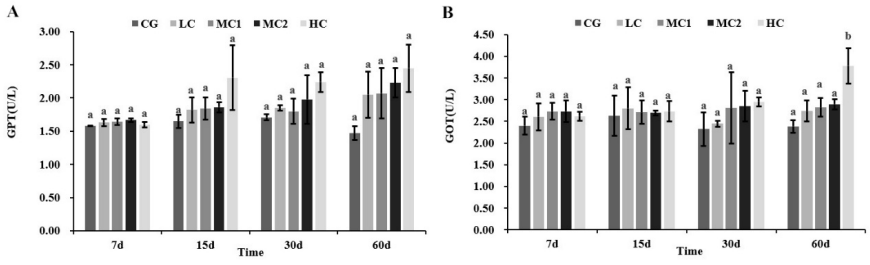

Figure 5.

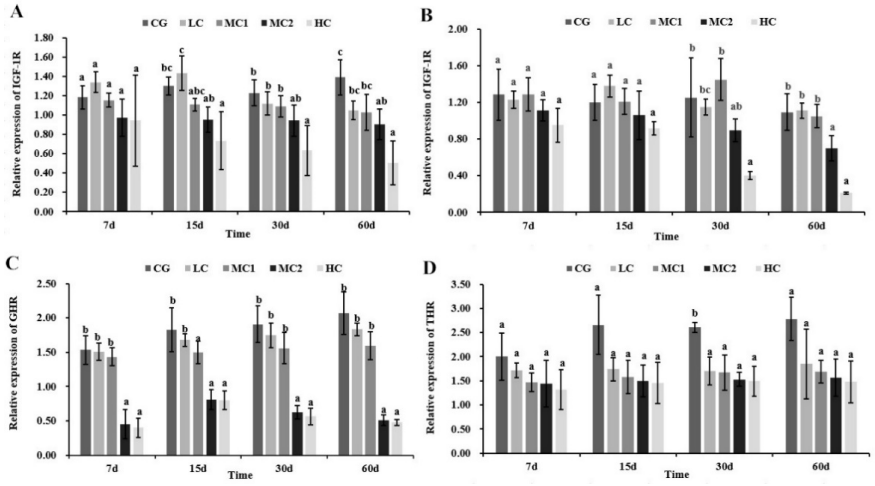

Figure 6.

Figure legends

Fig.1. (A) Weight gain rate (WGR), (B) specific growth rate (SGR), (C) feed conversion rate (FCR) and (D) condition factor(CF) - concentration (CK, LN, Mn and HN) of turbot larvae with different CO2 concentrations (CG, LC, MC1, MC2 and HC) (9 samples for all parameters). The parameter differences between CG, LC, MC1, MC2 and HC treatments were discovered by Tukey test using SPSS software. Different letters indicate a significant difference ($P < 0.05$)

Fig.2. (A) Survival Rate (SR), (B) Hepatosomatic Index (HSI), (C) Kidney Somatic Index (KSI) and (D) Spleen-Somatic Index (SSI) - concentration (CK, LN, Mn and HN) (all parameters have 9 samples). The parameter differences between CG, LC, MC1, MC2 and HC treatments were recognized by Tukey test using SPSS software. Different letters indicate a significant difference ($P < 0.05$)

Fig.3. Turbot larvae were exposed to different concentrations of CO2 (CG, LC, MC1, MC2, HC). After 60 days, the concentrations of (a) hemoglobin (HB) and (b) methemoglobin (MetHb) were expressed as mean ± S.E. (9 samples in each group).

Fig 4. Photographs of liver tissue sections of juvenile turbot exposed to different CO2 levels. A-1. CG, 1-month-old normal liver; A-2. CG, 2-month-old normal liver; B-1、B-2. LC, and C-1, C-2 MC1. There was only slight violation of hepatocytes at the age of 1 month and 2 months (arrow); D-1、E-1. MC2、HC. Acute vacuolization of hepatocytes (arrow) and nuclear atrophy or deformation (square) occurred at the age of 1 month; D-2、E-2. MC2、HC. At 2 months of age, there was vascular congestion (round) in the venous sinus or portal area (hematoxylin eosin staining).

Fig.5. In 60 days of CO_2 exposure, (A) is glutamic oxaloacetic transaminase (GOT) in turbot plasma; (B) It is alanine aminotransferase (GPT). The data are mean ± S.E. (n = 9). The values of different superscripts were significantly different among groups ($P < 0.05$)

Fig.6. Effects of different concentrations of CO_2 on growth related genes in GH-IGF axis during 60 days of CO_2 exposure. The data are expressed as mean ± S.E. (n = 9). There were significant differences in different superscript letters among the groups ($P < 0.05$).