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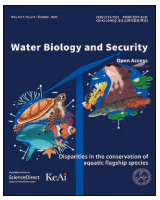

Research Article

Intermittent hypoxia training remodels the hepatic mitochondrial network and upregulates ANT expression to enhance hypoxia tolerance in *Micropterus salmoides*

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ABSTRACT

Oxygen is critical for life, and aquatic organisms are especially susceptible to hypoxic stress caused by environmental fluctuations. However, the mechanisms underpinning their tolerance to hypoxia remain poorly understood. Largemouth bass (*Micropterus salmoides*) is widely distributed across a range of freshwater ecosystems and has significant economic and ecological value. Low oxygen has become a key limiting factor in the aquaculture of this species. This study examined the impact of intermittent hypoxia training (IHT) on hypoxia tolerance of largemouth bass by simulating the daily fluctuations in dissolved oxygen typical of natural aquatic environments. We found that IHT increased the hypoxic tolerance of largemouth bass by activating adenine nucleotide translocase (ANT) which mediated Ca^{2+} influx and cellular resistance to hypoxia. Inhibition of ANT compromised hypoxia tolerance by reducing hypoxia-induced mitochondrial Ca^{2+} accumulation and mitochondrial quality control. Additionally, ANT inhibition upregulated the expression of genes associated with oxidative stress and apoptosis. These findings highlight a key relationship between ANT and mitochondrial Ca^{2+} signaling in response to hypoxia, providing insights into the mechanism that enhances tolerance to hypoxia in largemouth bass.

1. Introduction

Dissolved oxygen (DO), which is essential for aquatic organisms (Chabot and Claireaux, 2008; Zhao et al., 2022a), exhibits large circadian fluctuations. Diurnal DO variation exceeds 60 % in productive rivers (Williams et al., 2000) and 70 % in aquaculture ponds (Alam et al., 2018). Nocturnal DO minima in aquatic ecosystems induce oxidative stress, impairing behavior, growth, and reproduction (Baxa et al., 2020, 2021; Hou et al., 2020; Sun et al., 2020b; Zhao et al., 2023a). On the other hand, fish have a certain degree of low-oxygen tolerance when confronted with hypoxic stress. In both mammals and fishes, intermittent hypoxia training (IHT) enhances the tolerance of individuals to low oxygen conditions. However, the underlying mechanisms remain elusive (Gangwar et al., 2019; Zhao et al., 2024). Largemouth bass (*Micropterus salmoides*), an ecologically and economically important species (Zhao et al., 2021), exhibit enhanced hypoxia tolerance following intermittent hypoxia exposure in both urban and coastal systems (Gaulke et al., 2015; Brown et al., 2015).

An increasing number of hypoxic events driven by anthropogenic nutrient inputs and climate change threaten aquaculture yields (Galic et al., 2019). Investigating the relationship between intermittent hypoxia and enhanced hypoxia tolerance is critical for understanding how fishes copewith aquatic DO fluctuations and can contribute a theoretical framework for aquaculture applications.

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Fish unferhypoxic stress, rapidly activated physiological responses to mitigate low-oxygen damage (Leger et al., 2021; Zhao et al., 2022b). Ca^{2+}, is a key secondary messenger in cells with a central role in their adaptation (Boag et al., 2021; Monteith et al., 2007; Rossi et al., 2019). In previous studies we showed that Ca^{2+} signaling dynamics in largemouth bass contributed to sustain hepatic energy supply during acute hypoxic stress (Zhao et al., 2023). Furthermore, IHT induced, cardiac remodeling is mediated through reprogramming Ca^{2+} signaling and enhancing mitochondrial turnover, serving as a compensatory acclimation mechanism (Zhao et al., 2024). Mitochondria serve as central hubs in Ca^{2+} signaling networks, regulating ATP synthesis, subcellular Ca^{2+} distribution, and cell death (Rossi et al., 2019). Mitochondrial quality control maintains mitochondrial balance and ensures cellular physiological activities (Youle and van der Bliek, 2012). Hypoxia can induce modifications in mitochondrial quality control and Ca^{2+} signaling to allow fish to cope with environmental stress (An et al., 2013; Chitra and Boopathy, 2014; Giorgi et al., 2018; Gutsaeva et al., 2008; Hernansanz-Agustin et al., 2020; Liu et al., 2018). Nevertheless, the role of Ca^{2+} signaling and mitochondrial quality control in enhancing hypoxia tolerance through IHT is not well understood.

The liver is a vital organ for metabolism and detoxification, whose functions critically depend on the orchestration of Ca^{2+} signaling and the maintenance of an intact mitochondrial network (Lagoudakis et al., 2010; Kulkarni et al., 2016; Humbert et al., 2023; Da Dalt et al., 2024). Alterations in Ca^{2+} signaling and mitochondrial network dynamics exert profound effects on hepatic physiology. Furthermore, the liver is a primary target organ for the hypoxic response in both mammals and fishes (Aron-Wisnewsky et al., 2012; Du et al., 2016). Ca^{2+} exchange between mitochondria and the endoplasmic reticulum contributes to sustain normal liver function and confers protection against acute hypoxic stress (Zhao et al., 2023a). However, the specific effects of IHT on the liver remain to be elucidated.

Adenine nucleotide translocase (ANT), the most abundant protein in the inner mitochondrial membrane, is crucial for maintaining the energy supply in cells by exchanging ATP from the mitochondrial matrix with ADP outside the intermembrane space (Bround et al., 2020). Cytoplasmic Ca^{2+} is essential for activating ANT and ANT-dependent functions, including the enhancement of mitochondrial integrity, induction of mitochondrial autophagy, promoting ATP/ADP exchange, and stimulation of respiratory chain complexes (Hoshino et al., 2019). Thus, hypoxia can increase intracellular Ca^{2+} concentrations (Hernansanz-Agustin et al., 2020; Zhao et al., 2023a), and ANT can stabilize intracellular Ca^{2+} signals, maintaining mitochondrial homeostasis and regulating the cellular stress response (Bround et al., 2020; Hoshino et al., 2019). An intimate connection between ANT and mitochondrial Ca^{2+} transport has also been reported in zebrafish (*Danio rerio*) models (Azzolin et al., 2010), although how this occurs requires further exploration. ANT has been identified as a key regulator of macrophage hematopoiesis in goldfish (*Carassius auratus*) (Barreda et al., 2004). However, research on ANT in fishes remains limited, and its role in hypoxia tolerance in fish species is unclear.

Here, we propose that Ca^{2+} signaling and mitochondrial quality control are essential for enhancing hypoxia tolerance to IHT in the largemouth bass. IHT was applied to mimic diurnal fluctuations of DO within aquatic environments, followed by an analysis of the hepatic response. We found that IHT remodeled cellular Ca^{2+} signaling, enhanced mitochondrial quality control, and improved mitochondrial function, ultimately augmenting tolerance to subsequent acute hypoxia exposure by inducing ANT expression. Additionally, in primary liver cell cultures, hypoxia increased cytosolic Ca^{2+}, promoted ANT expression, enhanced mitochondrial Ca^{2+} transport and buffering capacity, activated mitochondrial quality control, and ultimately improved cellular tolerance to hypoxia.

2. Materials and methods

2.1. Experimental largemouth bass

Healthy largemouth bass (100 ± 5.0 g) were purchased from a local farm (Qionglai, Sichuan, China) and acclimated for 14 days in the Laboratory of the College of Animal Science and Technology (SICAU) before intermittent hypoxia training. The water parameters were as follows: temperature 21.0 ± 0.5 °C, DO 7.0 ± 0.7 mg/L, and pH 7.5 ± 0.2. The fish were fed a commercial Tongwei pellet diet (43.0 % protein, 10.69 % lipid, 14.8 % ash, 8.9 % moisture) twice daily at 08:00 and 15:00. Feces were removed at 09:00 each day, and one-fourth of the volume of water was changed daily at 09:30. All experimental data are presented as mean ± SD.

2.2. IHT protocol and tissue collection

A total of 384 largemouth bass were randomly divided into two groups, an intermittent hypoxia training (IHT) group and a control (C) group, with six replicates in each group (32 fish/tank). The IHT experiment was performed according to the protocol described in our previous study (Zhao et al., 2024; Yan et al., 2023). The fish in the IHT group were subjected to intermittent hypoxia for 32 days as follows. Each day between 17:30 and 18:00, the concentration of DO was reduced from 7.0 ± 0.7 mg/L to 2.5 ± 0.5 mg/L using nitrogen gas and maintained at 2.5 ± 0.5 mg/L from 18:00 to 21:00 by placing a plastic film over the water surface. The concentration of DO was raised from 2.5 ± 0.5 mg/L to 7.0 ± 0.7 mg/L by bubbling pure oxygen from 21:00 to 21:30. The remaining experimental conditions were the same as those for acclimation (French and Wahl, 2018). The experimental design is shown in Fig. S1. Under natural conditions, largemouth bass showed avoidance of oxygen concentrations approaching 3.0 mg/L (Hoshino et al., 2019).

At 0, 8, 16, 24, and 32 days post-experiment initiation, three fish were sampled from each of the three replicate aquariums per treatment, resulting in a total of nine fish per treatment at each time point. At each sampling time point, fish were sampled prior to the initiation of feeding and hypoxic exposure experiments. The fish were euthanized with an overdose of MS-222 (100 mg/L), followed by liver extraction. Liver tissue from 1 fish per aquarium was dissected into approximately 1 mm^3 cubes. Following dissection, a portion of the cubes was fixed in G1102 (Servicebio, Wuhan, China) electron microscopy fixative for Transmission Electron Microscopy examination. The remaining tissue was stored at −80 °C for subsequent Western blot analysis. A portion of the liver from two fish per aquarium (six fish in total) was rapidly frozen at −80 °C for qRT-PCR analysis, while the remainder was used for mitochondrial isolation.

2.3. Loss of Equilibrium (LOE)

At the end of the experimental hypoxia exposure (Day 32), the 6 fish from the same 3 tanks in each group were fasted 24 h to empty their gut (6 fish/group. The oxygen concentration leading to the loss of equilibrium (LOE) was determined to evaluate the impact of IHT on hypoxia tolerance. In brief, the concentration of DO was reduced using nitrogen gas, and the DO level in the water was recorded using traditional iodimetry (Yang et al., 2017). Swimming activity was observed to determine at what level the LOE occurred (the oxygen concentration at which half of the fish in each tank lose balance; Bergstedt et al., 2021).

2.4. Secondary hypoxic exposure protocol and tissue collection

Following the 32-day IHT, the largemouth bass in the remaining 3 tanks in each group were subjected to more severe hypoxia (1.2 ± 0.2

mg/L) to evaluate further impacts of IHT on hypoxia tolerance. The method of inducing hypoxia was the same as in section 2.2. The DO level was monitored every half hour using traditional iodimetry. The specific experimental design is shown in Fig. S1. Defining this DO concentration as severe hypoxia is based on the studies of French and Wahl (2018) and Sun et al. (2020b). The DO concentration of 1.5 mg/L is lower than the critical oxygen tension of largemouth bass in natural water bodies and is a level that is conspicuously avoided by them (French and Wahl, 2018; Yamanaka et al., 2007).

Samples were taken from the experimental fish after 4 and 8 h of severe hypoxia (7 fish/tank at each time point) when the hypoxic response is most intense (Yang et al., 2017). Fish were euthanized with an overdose of MS-222, a blood sample was taken from the caudal vein into a heparinized sterile syringe, and plasma was stored at −80 °C. Livers were removed, and a portion from one fish per tank was fixed in 4 % paraformaldehyde for TUNEL staining. The remaining tissue from this liver was stored at −80 °C for Western blot analysis. The other 6 livers per tank were stored at −80 °C to assay enzyme activity and mRNA-Seq. All information about the kits used is displayed in Table S2.

2.5. Transmission electron microscopy

Livers fixed in G1102 fixative were dehydrated, embedded, and sliced into ultrathin sections using a Leica ultrathin slicer (UC7) using published methods (Galic et al., 2019). The mitochondria were observed under a 5000x magnification using an electron microscope (Hitachi 7700, Japan). Images were obtained using a Gaten DAT-832 Orius camera and analyzed using ImageJ software. (service provided by Wuhan Seville Biotechnology Co., Ltd.

2.6. Mitochondrial indicators

Mitochondria were isolated using an extraction kit and following the instructions provided by the manufacturer, and mitochondrial protein concentration was measured with an Enhanced BCA Protein Assay Kit. All procedures were performed on ice.

The levels of Ca^{2+} in mitochondria and cytoplasm were quantified using a Calcium Assay Kit.

Mitochondrial membrane potential was assessed using a Mitochondrial Membrane Potential Assay Kit with JC-1 following the manufacturer's instructions. Fluorescence intensity was measured directly with a fluorescence spectrophotometer at 485 nm excitation and 590 nm emission wavelengths.

Functional analysis of mitochondria was conducted following the manufacturer's instructions for manganese superoxide dismutase (Mn-SOD), ATP, ATPase, total reactive oxygen species (ROS), and the activities of the mitochondrial respiratory chain complexes I–IV. Detailed specifications of all commercial kits and reagents used are provided in Supplementary Table S2.

2.7. RNA extraction and quantification

RNA extraction and analysis were carried out as previously described (Sun et al., 2020a). A Total RNA Isolation Kit and Cell Total RNA Isolation Kit were used to extract total RNA from the liver and cell, respectively. RNA quantification and quality assessment were performed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Samples with 260/280 ratios between 1.9 and 2.1 were deemed suitable for subsequent experiments. An RT Easy™ II (with gDNase) kit was used for reverse transcription. A Real-Time PCR EasyTM-SYBR Green I kit was used for quantitative real-time polymerase chain reaction (qRT-PCR). Primer Premier 5.0 was used for primer design (Table S3). *β-actin* and *18s* RNA were used as references genes. The mRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Western blot

Western blotting was carried out as previously described (Zhao et al., 2021). The primary antibodies used were AMPK (1:2000), PGC-1α (1:1000), LC3 (1:1000), MCU (1:1000), NCX (1:1000), caspase3(1:500), pNF-κB (1:2000), Keap1 (1:1000), ANT (1:1000), and β-actin (1:1000). The specific steps for the Western blot analysis can be found in Text S1.

2.9. mRNA-seq

The extracted total RNA was sent to Biomarker Biotechnology Co., Ltd. (Qingdao, China) for transcriptome sequencing ($n = 6$). Each sequencing library used 3 μg total RNA and was sequenced on an Illumina Novaseq 6000 platform. Quality filtering was used to obtain clean reads, which were mapped to the reference genome of largemouth bass (GenBank: JAKUMD000000. 1). Reads per kilobase per million reads (RPKM) of each gene were calculated. DESeq2 was used for differential expression analysis of gene transcripts between groups. A fold change ≥ 1 and P-value <0.05 were used as the cut-off for identification of genes with significant differential expression between experimental groups. The specific steps for mRNA-seq can be found in Text S2.

2.10. Biochemistry

Three plasma biochemical parameters were measured by spectrophotometricy using commercial kits (see Supplementary Table S2) and following the manufacturer's instructions: alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH).

A 10 % liver homogenate in 0.9 % cold physiological saline was prepared and centrifuged at 2500 g at 4 °C for 10–15 min and the supernatant collected. The following analysis was carried out using the supernatant and the respective kits (see Supplementary Table S2): protein using an Enhanced BCA Protein Assay Kit, ALT, AST, LDH, carnitine O-acetyltransferase (CAT), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-PX), and malonaldehyde (MDA).

2.11. TUNEL staining

Apoptosis was measured using a TMR (red) TUNEL kit on 3 μm paraffin slices of 4 % paraformaldehyde-fixed livers. In brief, proteinase K was added to the deparaffinized and rehydrated sections, followed by recombinant TdT enzyme (TMR-12-dUTP:labeling mix:equilibration buffer 1:5:50) to label cells that were apoptotic. After washing and drying the glass slide, a DAPI was added to stain the cell nucleus, followed by an anti-fluorescence quenching sealing agent before observation by fluorescence microscopy.

2.12. Cell culture and transfection

The primary liver cells were obtained from healthy largemouth bass, adjusted to a density of 10^4–10^5 cells/mL, and added to a 12-well plate. They were cultured under 5 % CO_2 at 25 °C until the convergence rate exceeded 80 %. Then small interfering RNA for siANT, the control (siNC), or the Ca^{2+} channel inhibitors, (SKF96365) was used to transfectthe cells.The; siANT and siNC were synthesized by Guangzhou Ribobio Biotechnology Co., Ltd. (Guangzhou, China). After 24 h of transfection, 100 μmol/L $CoCl_2$ was added for a further 24 h to induce the expression of HIF-1α by simulating a hypoxic environment. The specific steps for isolating and cultivating primary liver cells are reported in Text S3. The quantity and timing of $CoCl_2$ addition was based on Livak et al. (Livak and Schmittgen, 2001), as well as our own investigations.

HEK 293T cells (from the cell bank of the Chinese Academy of Sciences) were cultured in DEME medium and supplemented with 10 % heat-inactivated fetal bovine serum to a density of 10^4–10^5 cells/mL and incubated in 12-well plates at 37 °C with 5 % CO_2 until the confluence

was greater than 80 %. The pcDNA3.1-ANT (4.0 μg) or its negative control (pcDNA3.1) were transfected into the HEK 293T cells using TransEasyTM Transfer Agent. The approach for hypoxia treatment was the same as outlined for the primary liver cells. The specific steps for synthesizing the pcDNA3.1-ANT plasmid vector are shown in Text S4. Details of the plasmids used for transfection, including their constructs, can be found in Supplementary Fig. S8.

2.13. Assessment of cellular and mitochondrial parameters

Cell viability was detected using an MTT assay kit following the manufacturer's instructions, and absorbance measured in a spectrophotometer at 570 nm.

Ca^{2+} was quantified in the mitochondria, endoplasmic reticulum, and cytoplasm as previously described (Wang et al., 2015), using the fluorescent dyes Mag-Fluo-4-AM, Rhod-2 AM, and Fluo-4-AM.

The mitochondrial membrane potential (MMP) was determined using a JC-1 Mitochondrial Membrane Potential Assay Kit and a fluorescent microscope.

The cell total ROS was detected using DCFH-DA dye according to the manufacturer's instructions, then measured with a fluorescence microscope (200 ×) and fluorescence microplate reader (Ex/Em = 504/529 nm).

CAT, Mn-SOD, and ATP in the cells were measured as described for the primary liver cell cultures.

2.14. Statistical analysis

All the data were recorded are presented as mean ± standard deviation. Normality and homogeneity of variances were assessed using the Kolmogorov-Smirnov test and Levene's test, respectively. An unpaired, two-tailed Student's t-test was employed to compare the mean levels of LOE and the relative area of CaP precipitation between the two groups. The effects of various interventions—including IHT on mitochondrial gene and protein expression, a second exposure to hypoxia on liver and serum injury markers and antioxidant indices, and the addition of siRNA

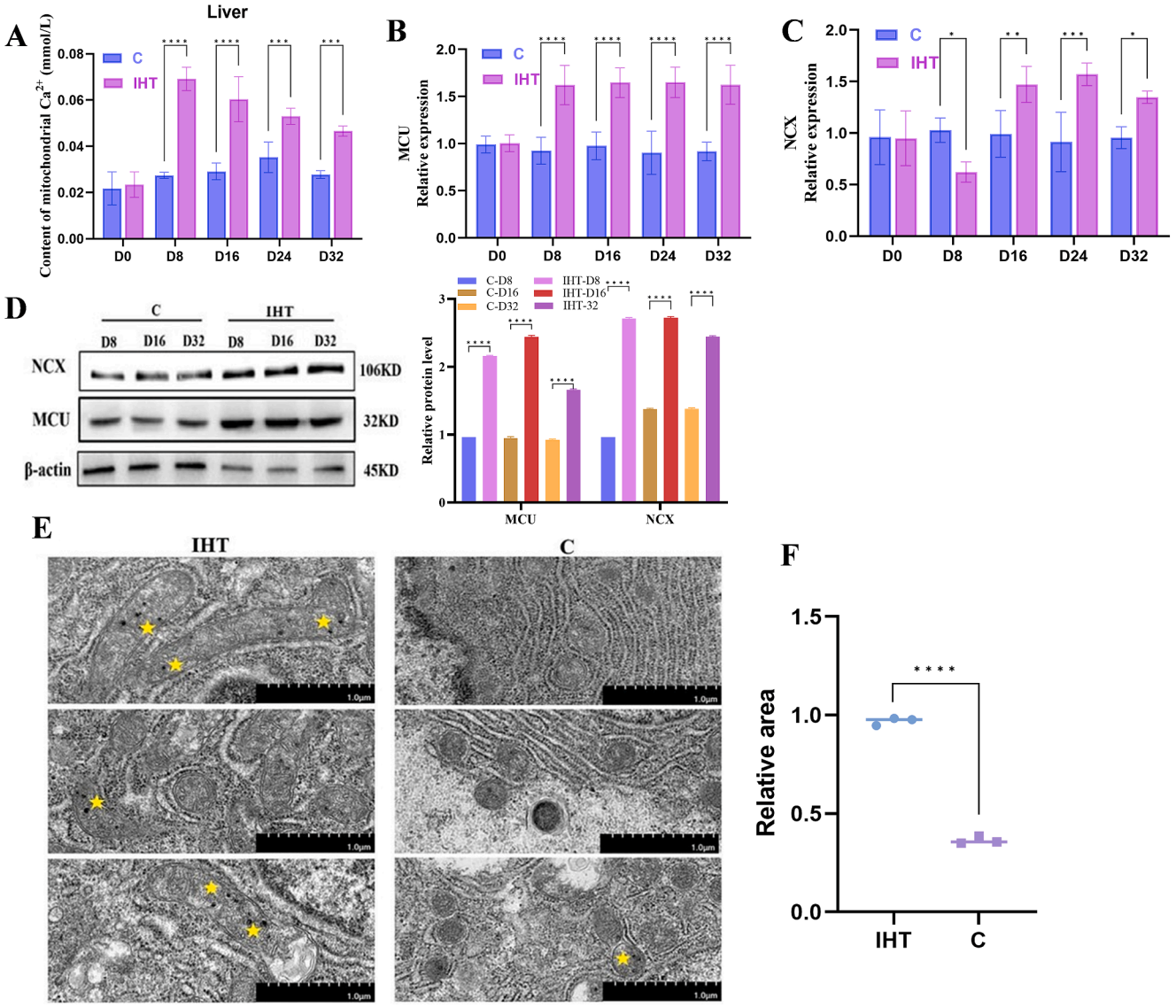


Fig. 1. Intermittent hypoxia training (IHT) enhances mitochondrial Ca^{2+} transport and buffering capacity.
(**A**) Mitochondrial [Ca^{2+}] in the liver during IHT (n = 6/group). (**B**–**C**) The effects of IHT on the liver mRNA levels of MCU and NCX (n = 6/group). (**D**) The effects of IHT on the liver protein levels of MCU and NCX (n = 3/group). (**E**–**F**) Representative transmission electron microscopy images and the relative area of mitochondrial CaP precipitates in the liver; in **E** the yellow stars mark the mitochondrial CaP precipitates (n = 3/group). D = days, C = control, IHT= Intermittent hypoxia training. Scale bar in **E**: 1 μm. All data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

or PCDNA3.1-ANT on gene/protein expression, ROS production, and calcium transport in cells exposed to simulated hypoxia—were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The value of $P < 0.05$ was considered statistically significant. T All statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, USA).

3. Results

3.1. Intermittent hypoxia training enhances mitochondrial Ca^{2+} transport and buffering capacity

Intermittent hypoxic training resulted in a significant increase in mitochondrial matrix calcium concentration (Ca^{2+}_{mito}) compared to the control group. (Fig. 1A), and this was accompanied by increases in the mRNA (Fig. 1B) and protein (Fig. 1D) of the mitochondrial Ca^{2+} uniporter (MCU) ($P < 0.05$). Interestingly, the mRNA of the Na^{+}/Ca^{2+} exchange (NCX) was lower in the treatment than the control on day 8 and higher than the control on days 16, 24, and 32 ($P < 0.05$, Fig. 1C). At the same timepoints, the protein level in the experimental groups was significantly higher than the control groups ($P < 0.05$, Fig. 1D). Transmission electron microscopy analysis showed that the frequency of CaP precipitation in the IHT group was higher than the control group ($P < 0.05$, Fig. 1E and F). In addition, we observed that PMCA expression was significantly decreased at all time points compared to the control group. ($P < 0.05$, Fig. S2A). However, the expression levels of SERCA, ORAI1, STIM1, and IP3R (with the exception of IP3R at D32) were significantly increased at all time points compared to the control group. ($P < 0.05$, Fig. S2B–E). This suggests that IHT enhances cellular Ca^{2+} uptake and modulates its distribution among subcellular compartments, potentially establishing a distinct calcium signaling signature to tolerate DO fluctuations.

3.2. Intermittent hypoxia training promotes mitochondrial quality control

Ca^{2+} signaling serves as a central regulator of mitochondrial

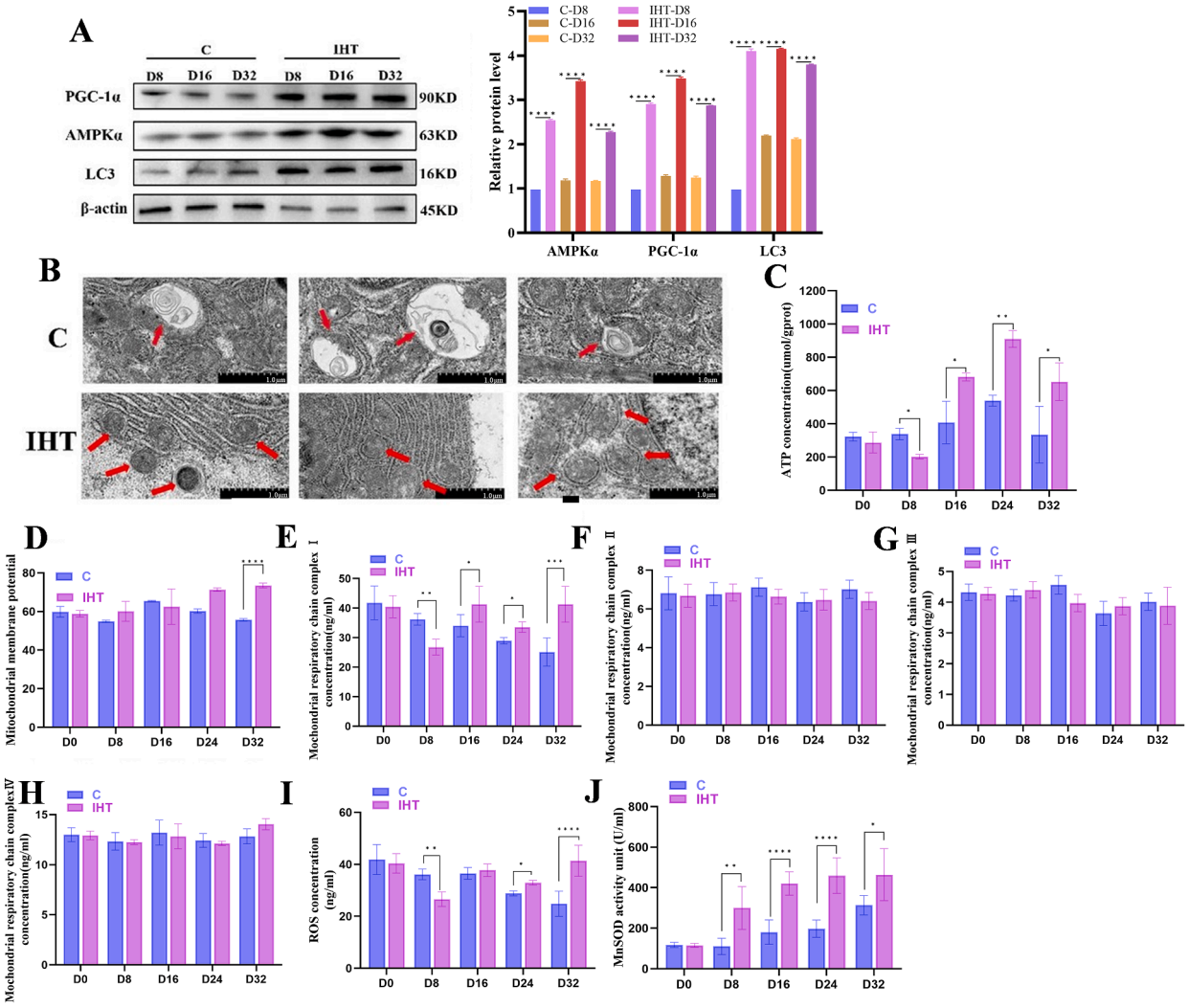


Fig. 2. Intermittent hypoxia training promotes mitochondrial quality control.
(**A**) The effects of IHT on the liver protein levels of AMPKα, PGC-1α, and LC3 ($n = 3$). (**B**) Representative transmission electron microscopy images in the liver ($n = 3$); the red arrows mark the mitochondrial autophagy. Scale bar: 1 μm. (**C**) The concentration of ATP in the liver ($n = 6$). (**D**) The mitochondrial membrane potential in the liver ($n = 6$). (**E–H**) The concentration of mitochondria respiratory chain complexes I, II, III, and IV in the liver ($n = 6$). (**I**) The concentration of ROS in the liver ($n = 6$). (**J**) The activity of MnSOD in the liver ($n = 6$). D = days, C = control, IHT = intermittent hypoxia training. All data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

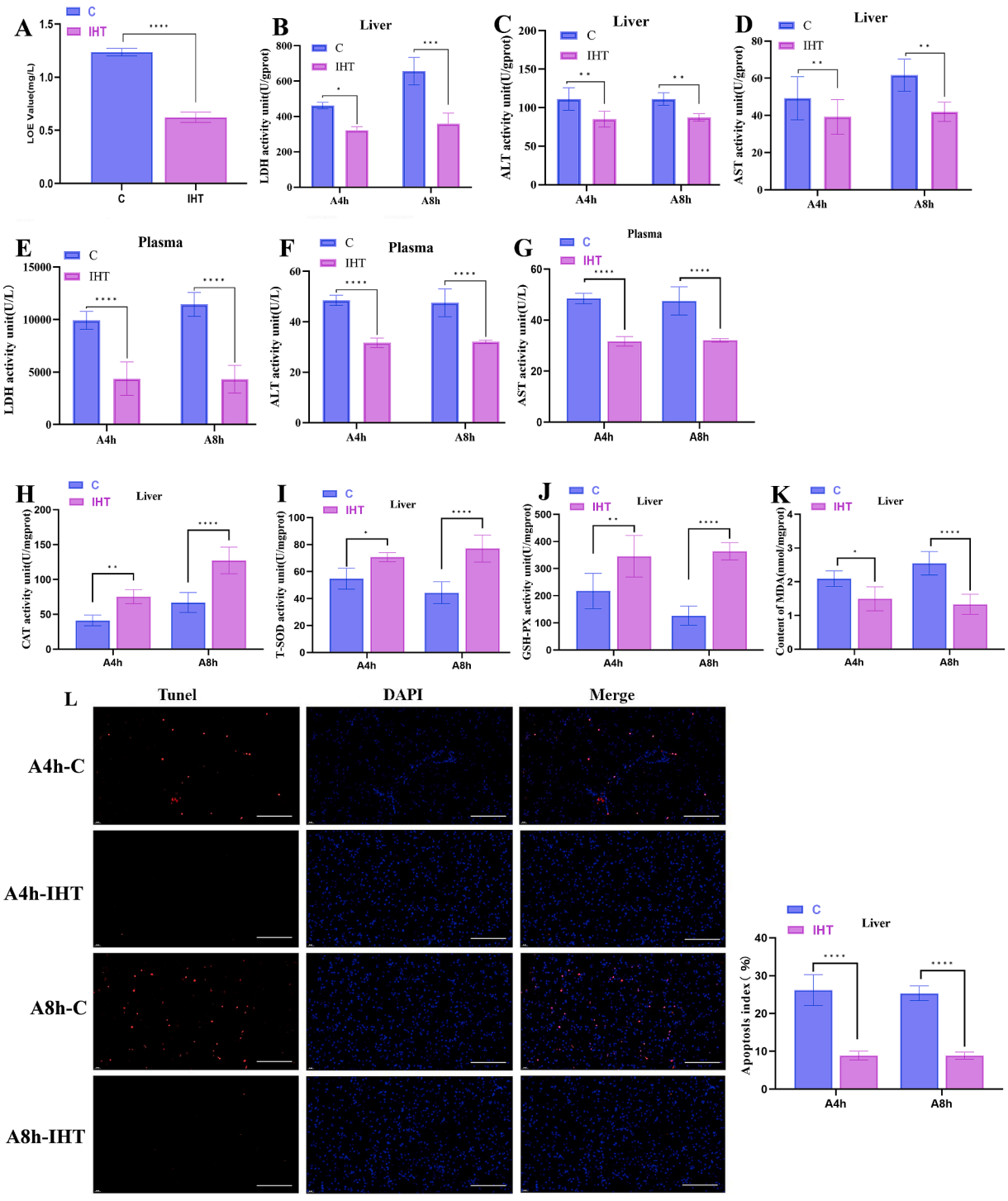


Fig. 3. Intermittent hypoxia training promotes acute hypoxia tolerance.
(**A**) The loss of equilibrium (LOE) oxygen concentration of largemouth bass ($n = 6$). For A-K, A4h-C = control group and A4h = IH = IHT group in acute hypoxia for 4 h; A8h-C = control group and A8h-IH = IHT group in acute hypoxia for 8 h ($n = 6$). B-G) The activity of LDH, ALT, and AST in the liver (**B**–**D**) and plasma (**E**–**G**) during acute hypoxia. (**H**–**J**) CAT, T-SOD, and GSH-PX activity in the liver during acute hypoxia ($n = 6$). (**K**) The content of MDA in the liver during acute hypoxia ($n = 6$). (**L**) Representative microscopic images with TUNEL staining (red fluorescence marks apoptotic cells) and the apoptosis index in the liver ($n = 3$). Scale bar: 50 μm. D = days, C = control, IHT = intermittent hypoxia training. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

function. Transmission electron microscopy revealed that the mitochondria in the experimental group exhibited an elongated morphology (Fig. 1E), which may be associated with activation of mitochondrial quality control. We observed upregulated expression of genes regulating mitochondrial fusion (*OPA1* and *MFN1*) and fission (*MFF* and *DRP1*) in the IHT group compared to the control group ($P < 0.05$; Fig. S3A–D), while FIS1 expression did not change (Fig. S3E). Additionally, elevated expression of mitochondrial biogenesis-related genes (*AMPK*, *PGC-1α*, *SIRT1*, *NRF-1*) and autophagy-related genes (*LC3*, *BNIP3*, *P62*) in the IHT compared to the control groups suggests enhanced mitochondrial biogenesis and mitophagy ($P < 0.05$; Fig. S3F–M). Western blotting confirmed these results; IHT also upregulated protein levels of PGC1α,

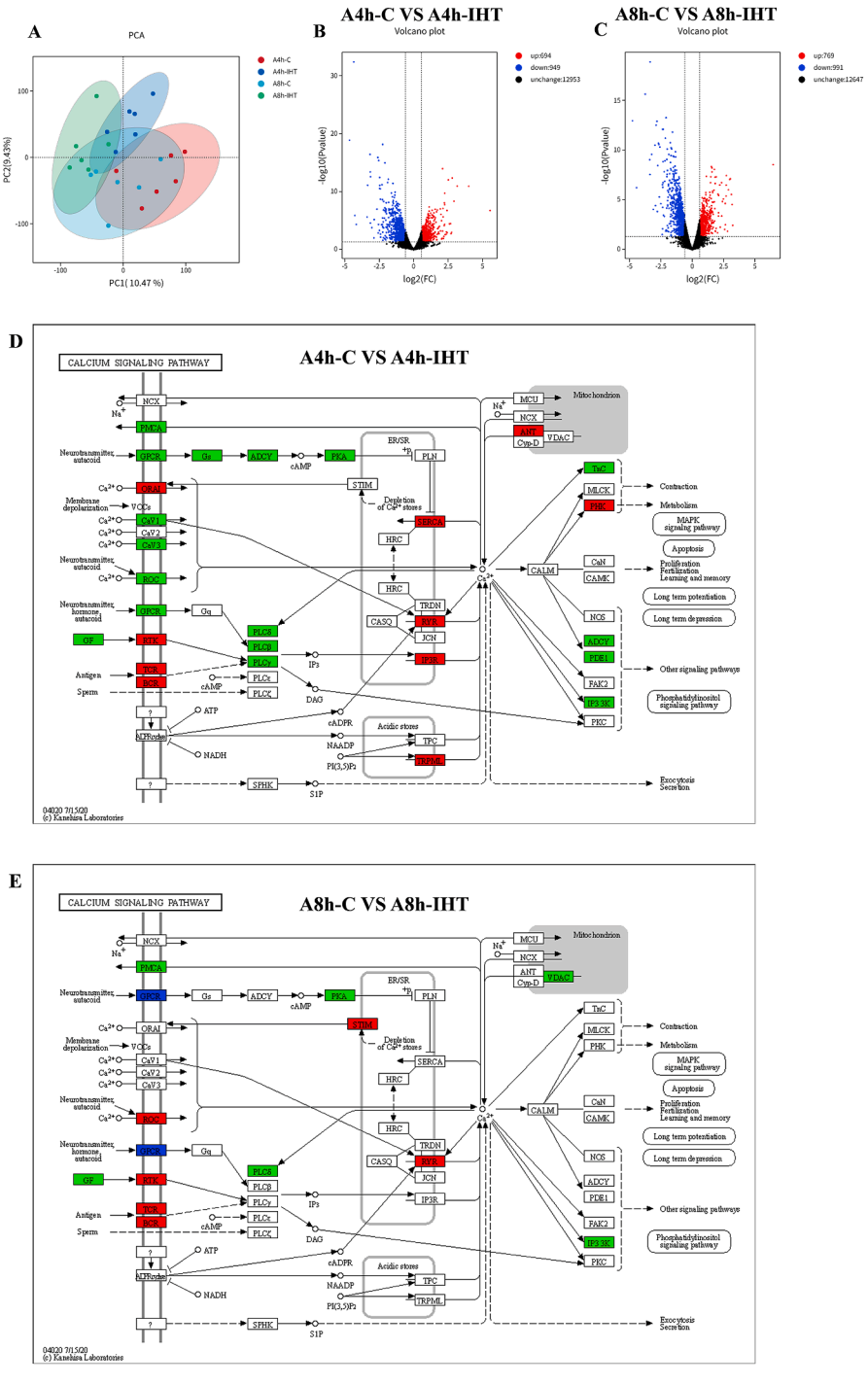


Fig. 4. Intermittent hypoxia training activates calcium signaling pathways.

A4h-C = control group and A4h = IH = IHT group in acute hypoxia for 4 h; A8h-C = control group and A8h-IH = IHT group in acute hypoxia for 8 h. (**A**) Principal component analysis of the liver transcriptomes of the control and IHT group at 4 and 8 h (n = 6). (**B–C**) Volcano plot of gene expression levels between (**B**) the control and IHT group at 4 h and (**C**) the control and IHT group at 8 h. The green dots represent downregulated differentially expressed genes, the red dots represent upregulated differentially expressed genes, and the black dots represent non-differentially expressed genes (n = 6). (**D–E**) KEGG calcium signaling pathways of differentially expressed genes. Red box = upregulated, green box = downregulated, blue box = upregulated and downregulated (n = 6). (**F**) The liver protein levels of Keap1, pNF-κB, Caspase3, and ANT during acute hypoxia (n = 3). Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Table 1
Differentially expressed genes.

DEG comparison	DEG Number	Up-regulated	Down-regulated
A4h-C vs A4h-IH	1643	694	949
A8h-C vs A8h-IH	1760	769	991

AMPK, and LC3 compared to the control group ($P < 0.05$, Fig. 2A). The ultrastructure of liver mitochondria at day 32 was examined via transmission electron microscopy at 6000 × magnification; no swelling or damage to cristae was observed in either group (Fig. 2B). In the IHT group, clear formation of mitochondrial autophagosomes was observed (Fig. 2B). ATP, a key component of mitochondrial bioenergetics, significantly increased in the IHT group compared to the control group starting at day 16 ($P < 0.05$, Fig. 2C), indicating enhanced hepatic energy supply capacity in largemouth bass after 16 days of IHT. Mitochondrial membrane potential was higher in the IHT than control group at day 32 ($P < 0.05$; Fig. 2D), indicating augmented accumulation of the proton gradient across the mitochondrial membrane, which is commonly associated with enhanced electron transport chain (ETC) complex activity. Consistent with this observation, complex I activity of the mitochondrial ETC was lower in the IHT group than the control group at day 8, then was higher in the IHT than the control group at days 16, 24, and 32 ($P < 0.05$; Fig. 2E), and total mitochondrial ROS levels varied in a similar way ($P < 0.05$, Fig. 2I). Complexes I and III are primary sources of ROS under hypoxic conditions (Schieber and Chandel, 2014). Regulated ROS production activates transcription factors that enhance the expression of endogenous antioxidants and exogenous detoxifying enzymes, thereby improving antioxidant capacity (Xirouchaki et al., 2021). There was higher antioxidant gene expression in the IHT compared to the control group starting on day 8 ($P < 0.05$, Fig. 2J). This demonstrates that IHT promotes mitochondrial quality control mechanisms, thereby stimulating mitochondrial network remodeling and enhancing both bioenergetic capacity and antioxidant competence.

3.3. Intermittent hypoxia training activates calcium signaling pathways and promotes acute hypoxia tolerance

Following IHT, the level at which the LOE of largemouth bass occurred decreased from an average of 1.17 mg/L (control group) to 0.66 mg/L (treatment group) ($P < 0.05$, Fig. 3A). Furthermore, IHT significantly attenuated hepatic damage during subsequent acute hypoxia exposure, as evidenced by reduced activities of LDH, ALT, and AST in both serum and hepatic tissues compared to the control group ($P < 0.05$; Fig. 3B–G). This protective effect was accompanied by enhanced activities of CAT, total T-SOD, and GSH-Px, alongside decreased MDA content in liver tissues compared to the control group ($P < 0.05$; Fig. 3H–K). The TUNEL staining showed that the control group experienced apoptosis during severe hypoxia, which was reduced by IHT ($P < 0.05$, Fig. 3L).

RNA sequencing was performed on the liver of largemouth bass that were subjected to acute hypoxia with or without IHT. Transcriptome analysis was completed for 24 samples, and 149.50 Gb of clean data were obtained. The Q30 base percentage was at least 92.37 % (Table S4). Sequence alignment was performed between the clean reads of each sample and the designated reference genome, with alignment efficiency ranging from 92.48 % to 95.33 % (Table S5). Principal component analysis (PCA) revealed distinct gene expression patterns among the different groups. (Fig. 4A). There were 694 genes highly expressed in the IHT group compared to the control group at 4 h (Fig. 4B), while 769 genes were highly expressed in the IHT group compared to the control group at 8 h ($P < 0.05$, Fig. 4C; Table 1). Most of the genes related to signal transduction and calcium signaling pathway were enriched by IHT (Fig. S4A–D), and the most upregulated pathways involved the exchange of Ca^{2+} among the cytoplasm, endoplasmic reticulum, lysosome, and mitochondria (Fig. 4D and E). The 15 genes related to calcium signaling and apoptosis selected for qRT-PCR showed consistency of expression compared with the RNA-seq results (Fig. S4E–S). Western blotting further confirmed that IHT suppressed acute hypoxia-induced expression of caspase-3, Keap1, and p-NF-κB except for Keap1 at 8 h ($P < 0.05$; Fig. 4F). Notably, upon acute hypoxia exposure, the IHT group exhibited significant upregulation of ANT mRNA and protein levels compared to the control group ($P < 0.05$; Supplementary Fig. S4E, Fig. 4F), suggesting that ANT may play a pivotal role in IHT-mediated hypoxia tolerance.

3.4. ANT contributes to hypoxia-induced transport activity of $Ca^{2+}{}_{mito}$

Using Rhod-2 AM, Mag-Fluo 4 a.m., and Fluo 4 a.m., we detected hypoxia-induced decreases in mitochondrial and endoplasmic reticulum Ca^{2+} levels and an increase in cytosolic Ca^{2+} levels. These effects were enhanced by siANT ($P < 0.05$, Fig. 5A and B) and the Ca^{2+} channel blocker SKF-96365 ($P < 0.05$,Fig. S5A and B). Overexpressing ANT reversed these changes ($P < 0.05$, Fig. 5C and D) and normalized hypoxia-induced decreases in MCU and NCX expression ($P < 0.05$, Fig. 5E–H; Fig. S5C and D). This indicates that under hypoxic conditions, ANT orchestrates calcium signaling reprogramming in cells by modulating Ca^{2+} channel activity.

3.5. ANT promotes mitochondrial quality control

Hypoxia reduced mRNA (AMPK, PGC-1α, LC3, Mfn1, TFAM7, OPA1, and DRP1) and protein (AMPK and LC3) expression related to mitochondrial biogenesis, autophagy, and fusion in liver cells and 293T cells compared to the control group ($P < 0.05$, Fig. 6A and B, D). This effect was exacerbated by ANT siRNA and SKF-96365 ($P < 0.05$, Fig. 6A and B; Fig. S6A and B) but partially reversed by ANT overexpression ($P < 0.05$, Fig. 6C and D). This demonstrates that under hypoxic conditions, ANT upregulates mitochondrial quality control mechanisms to drive mitochondrial network remodeling, thereby facilitating hypoxia tolerance. Elevated ROS levels correlate with enhanced mitochondrial quality control mechanisms (Kasai et al., 2020). Our experiments revealed that inhibiting ANT with siRNA or the Ca^{2+} channel blocker SKF-96365 reduced mitochondrial ROS production under hypoxia ($P < 0.05$ Fig. 6E; Fig. S6C), while ANT overexpression increased it ($P < 0.05$, Fig. 6F). These ROS dynamics coincided with changes in mitochondrial quality control, further suggesting that ANT-mediated regulation of mitochondrial quality control is linked to ROS production.

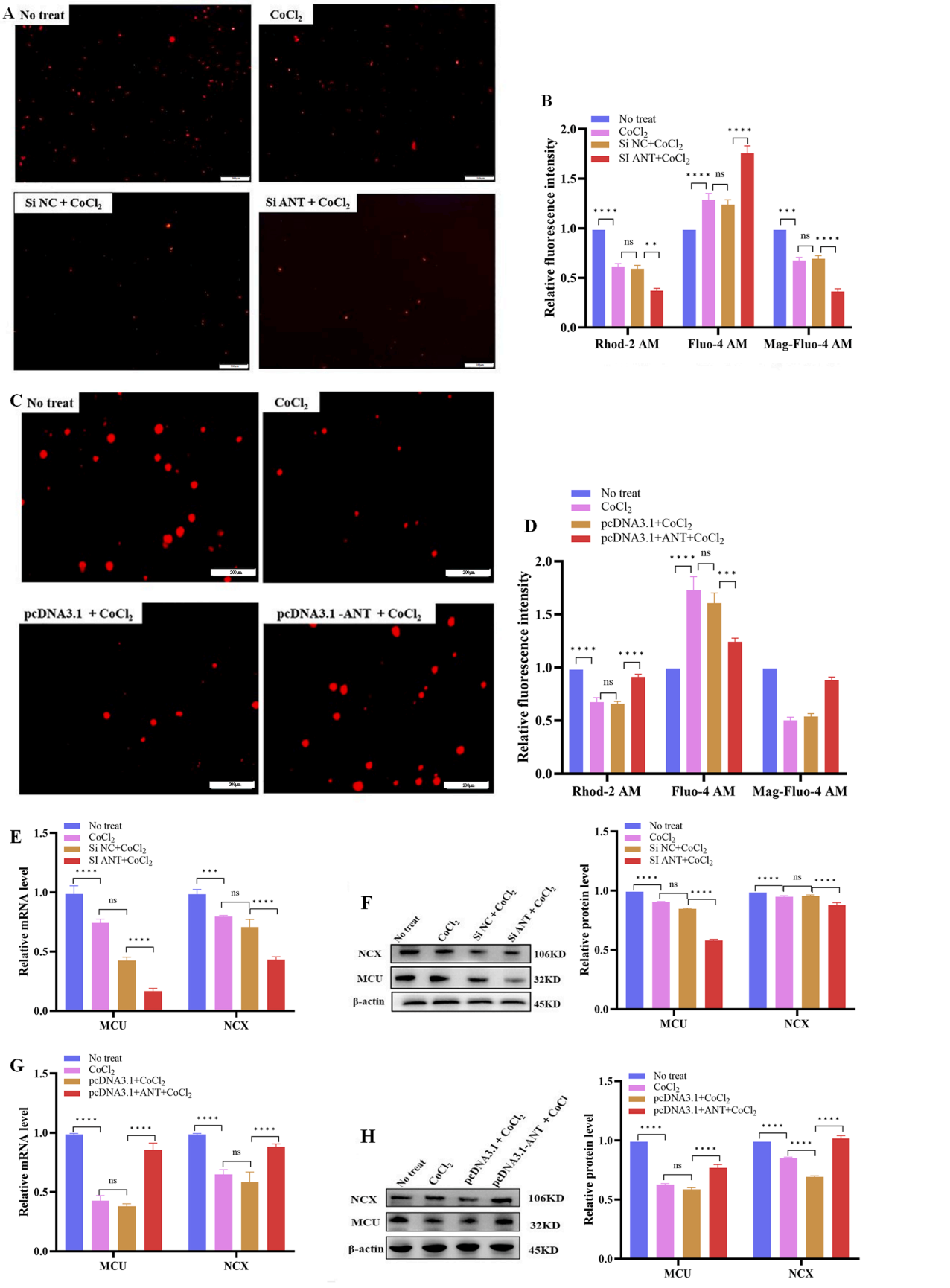
3.6. ANT positively regulates cell hypoxia tolerance

Hypoxia-induced mitochondrial changes enhanced hypoxia tolerance. ANT siRNA and SKF-96365 decreased cell viability ($P < 0.05$, Fig. 7A, Fig. S7A) and reduced the expression of antioxidant genes (CAT, SOD1, SOD2, and GPX) while increasing apoptosis gene expression (Caspase3, Caspase8, and Cyt C) in primary liver cells and 293T cells under hypoxia ($P < 0.05$, Fig. 7C, D, Fig. S7B and C). ANT overexpression reversed these effects, improving cell viability and modulating gene expression ($P < 0.05$, Fig. 7B and E, F), indicating ANT's role in promoting hypoxia tolerance by regulating oxidative stress and apoptosis responses.

4. Discussion

4.1. Intermittent hypoxia training enhances mitochondrial Ca^{2+} transport and buffering capacity and promotes mitochondrial quality control

This study evaluated the effects of IHT on the quality control of mitochondria in largemouth bass liver, and found that IHT regulates



(caption on next page)

Fig. 5. ANT contributes to hypoxia-induced transport activity of $Ca^{2+}{}_{mito}$.
(**A**) Representative Rhod-2 AM staining (red signal) of primary hepatocytes transfected with siANT for 24 h, followed by hypoxia for another 24 h ($n = 6$). Scale bar: 200 μm. (**B**) The mitochondrial, cytoplasmic, and endoplasmic reticulum Ca^{2+} levels in largemouth bass primary hepatocytes transfected with siANT and detected by Rhod-2 AM, Fluo-4 AM, and Mag-Fluo4 AM ($n = 6$). (**C**) Representative Rhod-2 AM staining (red signal) of 293T cells transfected with pcDNA3.1-ANT for 24 h, followed by hypoxia for another 24 h ($n = 6$). Scale bar: 200 μm. (**D**) Mitochondrial, cytoplasmic, and endoplasmic reticulum Ca^{2+} levels of 293T cells after transfection with pcDNA3.1-ANT detected by Rhod-2 AM, Fluo-4 AM and Mag-Fluo4 AM ($n = 6$). (**E–F**) The mRNA and protein levels of MCU and NCX of largemouth bass primary hepatocytes transfected with siANT ($n = 6$). (**G–H**) The mRNA and protein levels of MCU and NCX of 293T cells transfected with pcDNA3.1-ANT ($n = 6$). Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, ns. $P > 0.05$.

mitochondrial fusion, division, biogenesis, autophagy, and bioenergetics, which is consistent with other studies (An et al., 2013; Chitra and Boopathy, 2014; Gutsaeva et al., 2008; Liu et al., 2018; Zhao et al., 2022b). These processes collectively contribute to maintaining the homeostasis of hepatic mitochondrial networks while partially enhancing their functionality in largemouth bass under hypoxic stress. This regulatory cascade may represent one of the key mechanisms by which IHT improves tolerance to subsequent acute hypoxia exposure. Importantly, mitochondrial quality control is regulated by IHT through mitochondrial matrix Ca^{2+} concentrations. As a second messenger, Ca^{2+} participates in various cellular responses to hypoxia, serving as a signaling molecule for ion channels, pumps, and downstream effectors like kinases and transcription factors (Filadi and Greotti, 2021; Griffiths, 1999; Hernansanz-Agustin et al., 2020; Kanatous et al., 2009; Liang et al., 2017; Yeung et al., 2007; Zheng et al., 2015). However, mitochondrial Ca^{2+} uptake induced by IHT has not been previously considered in mitochondrial regulation. Here, we demonstrate that IHT induces cellular calcium signaling reprogramming mediated by mitochondria, endoplasmic reticulum, and plasma membrane calcium channels. Notably, mitochondrial Ca^{2+} influx plays a pivotal role in driving mitochondrial network remodeling through enhanced mitochondrial biogenesis, electron transport chain activity, ATP synthesis, and ROS generation—a regulatory axis analogous to mechanisms observed in cancer cells (Patra et al., 2021). The mitochondria can absorb Ca^{2+} released by the endoplasmic reticulum (Wang et al., 2019), and subsequently, the mitochondrial electron transport chain complexes can maintain ATP levels during hypoxia, thus promoting hypoxia tolerance (Bell et al., 2006; Unitt et al., 2010). Notably, an effect was observed exclusively in the activity of mitochondrial respiratory chain complex I. This indicates that complex I may serve as a target for IHT tolerance and is subject to effective regulation by mitochondrial Ca^{2+}. Similarly, the influx of mitochondrial Ca^{2+} increased ROS generation and ATP synthesis (Venditti and Di Meo, 2020). The production of ROS can stimulate mitochondrial quality control and promote the activation of transcription factors, resulting in the expression of antioxidant genes and detoxifying enzymes, thereby enhancing cellular antioxidant capacity and hypoxia tolerance (Xirouchaki et al., 2021). This enhanced antioxidant capacity may mitigate excessive ROS production and sustain ROS levels within safe thresholds (Xirouchaki et al., 2021; Yang et al., 2020). Consistent with these findings, we observed no significant mitochondrial damage following IHT. In ANT-overexpressing cells, the increase in ROS levels was accompanied by activation of the antioxidant enzyme system and restoration of cellular viability.

Interestingly, we also found an increase in mitochondrial calcium phosphate precipitation. As is well known, the formation of mitochondrial Ca^{2+}-Pi precipitates is the main pathway in mitochondrial Ca^{2+} buffering (Hernansanz-Agustin et al., 2020; Nicholls and Chalmers, 2004; Traba et al., 2012). In various cell death models entirely mediated by Ca^{2+} overload, many protective effects of matrix Ca^{2+}-Pi precipitation have been observed (Pivovarova and Andrews, 2010). The finding of increased mitochondrial Ca^{2+}-Pi precipitation suggests that IHT enhances mitochondrial Ca^{2+} uptake and retention, preventing Ca^{2+} overload. This implies that improved mitochondrial Ca^{2+} buffering could be a novel strategy to mitigate hypoxia-induced damage.

4.2. Intermittent hypoxia training activates calcium signaling pathways and promotes severe hypoxia tolerance

Largemouth bass inhabiting urban water systems and coastal environments frequently encounter intermittent hypoxia due to the regular fluctuations in DO, yet they appear to possess a heightened capacity compared to other studied teleosts for low-oxygen tolerance (Brown et al., 2015; Gaulke et al., 2015). Our results demonstrated that IHT significantly enhances hypoxia tolerance in largemouth bass upon re-exposure to hypoxic conditions. This is consistent with studies on southern catfish (*Silurus meridionalis*) and rainbow trout (*Oncorhynchus mykiss*), where the LOE was lower after intermittent hypoxia, demonstrating increased hypoxia tolerance (Williams et al., 2019; Yang et al., 2013). The enhanced hypoxia tolerance observed in largemouth bass may be attributed to IHT-mediated mitigation of damage induced by subsequent acute hypoxia exposure. This protective effect could be explained by increased antioxidant enzyme activity during re-exposure, which reduces oxidative stress and suppresses apoptotic and inflammatory injury. Similar studies have shown that humans directly exposed to elevations above 3400 m exhibit higher blood circulating inflammatory markers (Hartmann et al., 2000). However, intermittent hypoxia can promote altitude acclimation by reducing inflammation caused by high-altitude oxygen depletion (Gangwar et al., 2019). Studies in rats (*Rattus norvegicus*) have also found that intermittent hypoxia can reduce oxidative stress damage, promote skeletal muscle repair, and prevent induced cell apoptosis (Bardallo et al., 2021), in addition to hindering cell apoptosis which helps to increase the hypoxic tolerance of brainstem neurons (Simakajornboon et al., 2001). Our research supports and builds on previous findings that intermittent hypoxic training boosts hypoxia tolerance, shedding light on how largemouth bass cope with low dissolved oxygen in the wild and presenting new approaches to counter hypoxia-related losses in aquaculture.

Notably, our transcriptomic analysis revealed that the group subjected to IHT exhibited an enrichment of the Ca^{2+} signaling pathway in response to more severe hypoxic conditions. Such results have also been observed in rice (*Oryza sativa*) (Yemelyanov et al., 2011). In many vertebrates, the increase in intracellular Ca^{2+} signaling is related to the tolerance of neurons to hypoxia by activating the cellular signaling cascade that promotes cell survival (Bickler, 2004). Enhancing intracellular Ca^{2+} signaling promotes hypoxic vasoconstriction of cyclostome aortas, thereby preserving oxygen for the heart and neural tissues (Russell et al., 2001). The Pacific hagfish (*Eptatretus stoutii*) experiences rapid tissue Ca^{2+} accumulation after hypoxic stress, indicating that Ca^{2+} plays a role in hypoxia tolerance (Glover and Goss, 2021). In juvenile rainbow trout, environmental hypoxia induces the upregulation of slc25a24 (SCaMC-1), which plays an essential role in buffering calcium in the mitochondrial matrix and protecting cells from oxidative stress and cell apoptosis caused by high intracellular calcium levels (Hou et al., 2020a). Hypoxia-induced calcium signaling acts as a double-edged sword; while calcium signaling within the physiological range is crucial for initiating downstream adaptive responses, excessive calcium signaling induces structural damage and apoptosis. However, our study demonstrates that within the context of IHT-mediated enhancement of hypoxia tolerance, this signaling pathway contributes to mitigating

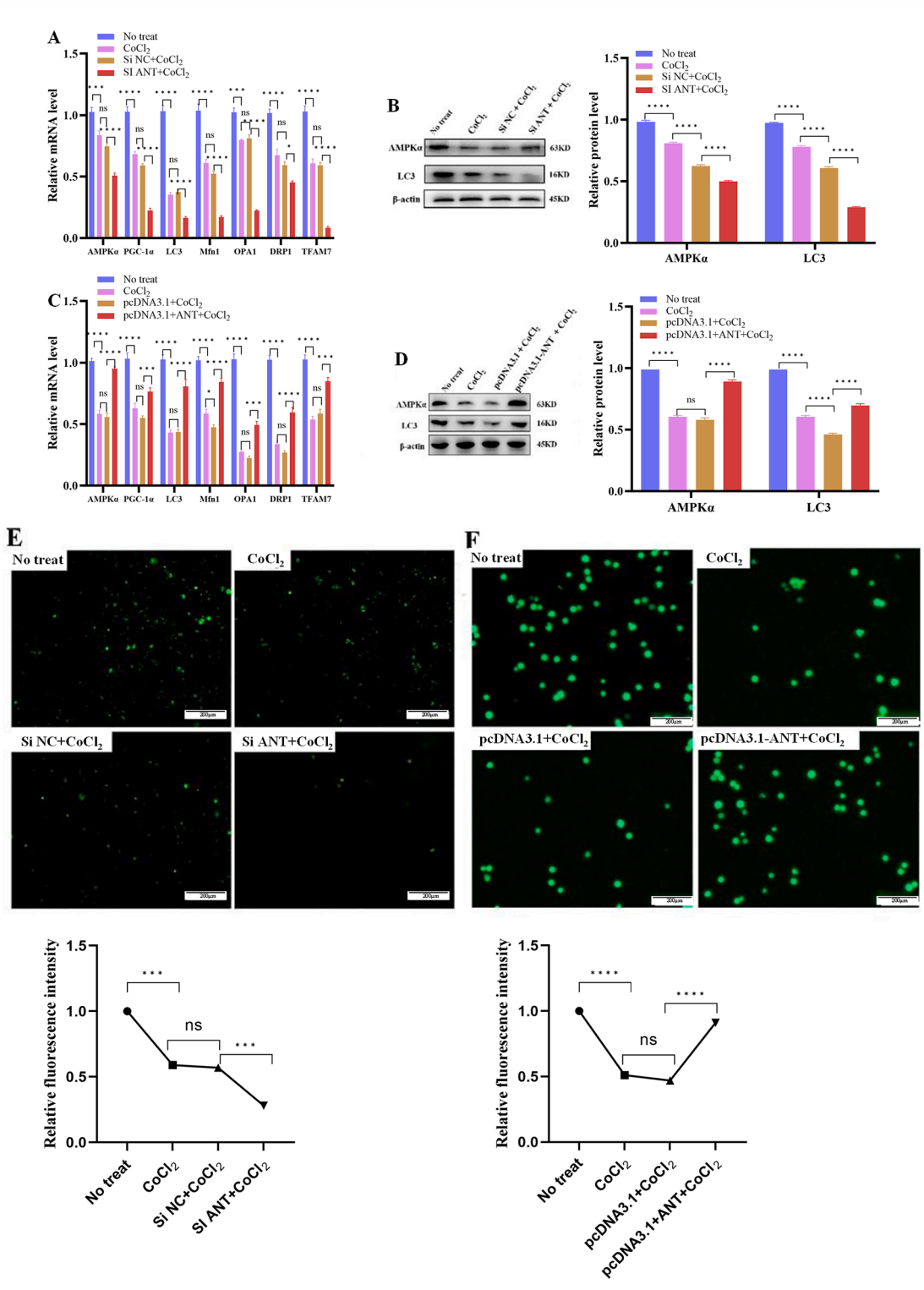


Fig. 6. ANT promotes mitochondrial quality control.
(**A**) The mRNA levels of genes controlling mitochondrial quality (AMPKα, PGC-1α, LC3, Mfn1, OPA1, DRP1, and TFAM7) in primary hepatocytes transfected with siANT ($n = 6$). (**B**) The protein levels of AMPKα and LC3 in primary hepatocytes transfected with siANT ($n = 6$). (**C**) The mRNA levels of genes controlling mitochondrial quality (AMPKα, PGC-1α, LC3, Mfn1, OPA1, DRP1, and TFAM7) in 293T cells transfected with pcDNA3.1-ANT ($n = 6$). (**D**) The protein levels of AMPKα and LC3 in 293T cells transfected with pcDNA3.1-ANT ($n = 6$). (**E**) Representative staining of ROS levels (green signal) detected by DCFH-DA and relative fluorescence intensity of primary hepatocytes transfected with siANT ($n = 6$). (**F**) Representative staining of ROS levels (green signal) detected by DCFH-DA and relative fluorescence intensity of 293T cells transfected with pcDNA3.1-ANT ($n = 6$). Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, ns $P > 0.05$. Scale bar: 100 μm.

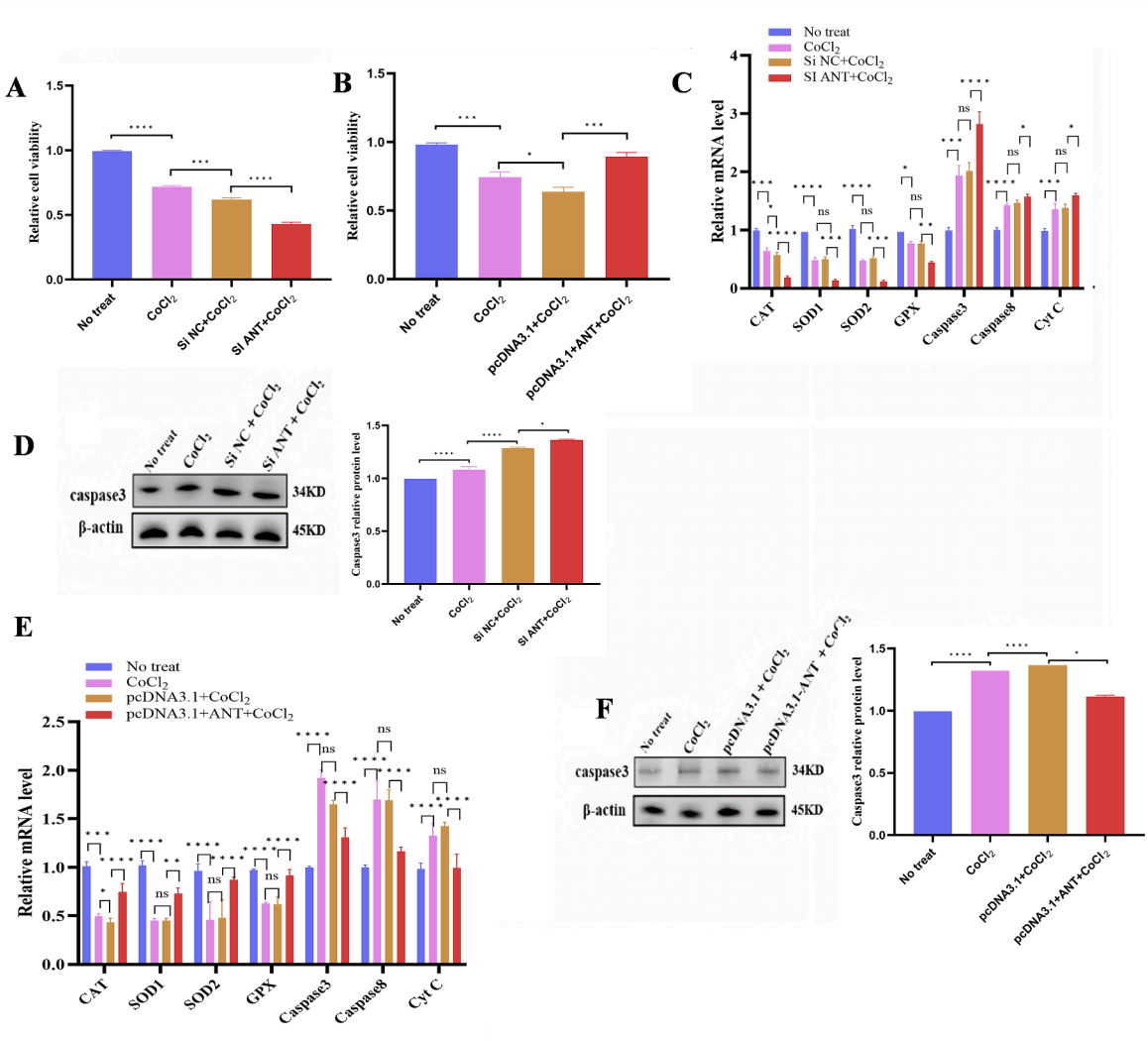


Fig. 7. ANT positively regulates cell hypoxia tolerance.
(**A**) Viability of primary hepatocytes transfected with siANT ($n = 6$). (**B**) Viability of 293T cells transfected with pcDNA3.1-ANT ($n = 6$). (**C**) The mRNA levels of oxidative stress and apoptosis genes CAT, SOD1, SOD2, GPX, Caspase3, Caspase8, and Cyt C in primary hepatocytes transfected with siANT ($n = 6$). (**D**) The protein levels of Caspase3 in primary hepatocytes transfected with siANT ($n = 6$). (**E**) The mRNA levels of oxidative stress and apoptosis genes CAT, SOD1, SOD2, GPX, Caspase3, Caspase8, and Cyt C in 293T cells transfected with pcDNA3.1-ANT ($n = 6$). (**F**) The protein levels of Caspase3 in 293T cells transfected with pcDNA3.1-ANT ($n = 6$). Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, ns $P > 0.05$.

hepatic injury during subsequent acute hypoxic exposure and augments hypoxic acclimation. Overall, our research highlights a significant link between calcium signaling and the hypoxic response.

4.3. ANT contributes to the transport activity of $Ca^{2+}{}_{mito}$, promotes mitochondrial quality control, and regulates cell hypoxia tolerance

Previous research has revealed the unexpected roles of Ca^{2+} homeostasis in mitochondrial quality control and hypoxia acclimation (Zhao et al., 2023a). Surprisingly, following IHT, the transcriptome of largemouth bass liver exhibited a significant upregulation of ANT. The primary function of ANT is to facilitate the exchange of cytosolic ADP with matrix ATP, thereby promoting the export of newly synthesized ATP to the cell and supplying new ADP substrates for mitochondria (Bround et al., 2020). The entire exchange of ATP and ADP by ANT in the mitochondrial inner membrane is crucial for ensuring the cellular energy supply of cells (Bround et al., 2020). Controlling the mitochondrial free Ca^{2+} through ATP/ADP-dependent Ca^{2+} buffering can regulate mitochondrial energy metabolism (Dash et al., 2009), and the increase in the expression of ANT mediated by the mitochondrial fusion factor can positively regulate mitochondrial Ca^{2+} signaling and mitochondrial function (Inagaki et al., 2023; Walther et al., 2007). However, whether ANT promotes Ca^{2+}-associated enhancement of hypoxia tolerance remains unclear. Our study demonstrates that IHT enhances ANT expression during subsequent acute hypoxia exposure. Notably, ANT overexpression significantly augments mitochondrial Ca^{2+} transport capacity in hepatocytes. Therefore, the mitochondrial Ca^{2+} transport mediated by ANT should be considered a driving factor in hypoxia tolerance.

Furthermore, we found that ANT positively regulates cellular hypoxia tolerance, a phenomenon that may involve mitochondrial ROS-mediated activation of defensive gene transcription and concomitant suppression of apoptotic pathways. The production of mitochondrial ROS in cells and tissues is a fundamental feature of several adaptive responses, including the response to hypoxia (Hernansanz-Agustin et al., 2020). Mitochondria, as a source of ROS and sensor of oxidative stress, play a crucial role in the processes of transduction and amplification of cellular apoptosis during oxidative damage (Dedkova and Blatter, 2012; Franklin, 2011). Consistent with our findings, ANT overexpression can drive mitochondrial autophagy, induce protective cellular processes that

are effective against different pathophysiological stimuli, and be used to treat various diseases caused by mitochondrial quality control dysfunction (Fontanesi et al., 2004; Hoshino et al., 2019; Kaukonen et al., 2000; Kunji et al., 2020). The cyto-protection afforded by ANT overexpression is manifested by inhibiting the release of apoptosis factors that activate Caspase from the intermembrane space, thereby preventing DNA degradation and cell death (Halestrap, 2010; Winter et al., 2016). In cardiomyocytes, ANT overexpression can also stabilize the mitochondrial membrane potential under hypoxia and protect the cells from apoptosis, thus increasing cell survival (Heger et al., 2012; Winter et al., 2016). These findings demonstrate ANT's importance in the hypoxia response by regulating mitochondrial ROS and apoptosis, possibly linking it to cell survival under hypoxia. Future studies should explore these mechanisms.

5. Conclusion

IHT enhances hepatic function in largemouth bass and induces ANT expression during subsequent acute hypoxia exposure. This leads to cellular calcium signaling reprogramming and mitochondrial quality control remodeling, thereby improving hypoxia tolerance. Distinct from previous studies, this work establishes a mechanistic link between IHT and tolerance, elucidating how ANT, calcium signaling, and mitochondrial surveillance synergistically drive this adaptive response. Our findings provide novel insights into piscine acclimation to environmental fluctuations.

CRediT authorship contribution statement

Hao Liu: Writing – original draft, Data curation, Conceptualization. **Dongmei Zhang:** Writing – original draft, Data curation, Conceptualization. **Yifan Hu:** Data curation. **Haoxiao Yan:** Formal analysis. **Weizhe Luo:** Conceptualization. **Kuo He:** Investigation. **Zhenghui Zhang:** Investigation. **Hangyu Yang:** Methodology. **Deborah M. Power:** Writing – review & editing. **Adelino V.M. Canario:** Writing – review & editing. **Qiao Liu:** Writing – review & editing. **Song Yang:** Writing – review & editing, Funding acquisition. **Liulan Zhao:** Writing – review & editing, Funding acquisition.

Ethical statement

All experimental fish were kept per the Guidelines for the Care and Use of Laboratory Animals in China. The Institutional Animal Care and Use Committee of the College of Animal Science and Technology of Sichuan Agricultural University (SICAU), Sichuan, China, approved the experimental protocols.

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Declaration of competing interest

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watbs.2025.100513.

Data availability

mRNA-seq data have been deposited in the SRA under the accession code PRJNA1005038.

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