

Apelin/APJ Signaling Participates in Ammonia-Induced Gill Hyperplasia in Largemouth Bass (*Micropterus salmoides*)

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Research Article

Keywords: *Micropterus salmoides*, Apelin, Gill hyperplasia, Ammonia-induced toxicity

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10984925/v1>

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Additional Declarations: No competing interests reported.

Abstract

Intensive aquaculture exposes largemouth bass (*Micropterus salmoides*) to chronic ammonia stress, resulting in gill tissue hyperplasia and damage. This study focused on the Apelin/APJ signaling pathway to explore the molecular mechanism underlying ammonia-induced gill hyperplasia. Experiments included a 21-day in vivo exposure to 8 mg/L total ammonia nitrogen expressed on a nitrogen basis (TAN-N), corresponding to an estimated unionized ammonia concentration of 0.336 mg/L $\text{NH}_3\text{-N}$, equivalent to 0.409 mg/L NH_3 , under the mean experimental conditions of pH 7.98 and 22 °C. Gill explant and primary-cell models were exposed to 7.5 μM NH_4Cl , corresponding to an estimated 0.000594 mg/L $\text{NH}_3\text{-N}$, equivalent to 0.000723 mg/L NH_3 , at pH 7.0 and 25 °C. The results demonstrated that ammonia stress induced progressive thickening of the gill epithelium and significantly upregulated the expression of core genes in the Apelin/APJ cell cycle pathway, including *apelin*, *akt*, *pi3k*, and *ccnd*; the relative expression of *akt* was significantly upregulated, reaching 2.93-fold in explants and 4.76-fold in primary cells compared to the control group. Apelin-13 treatment increased the cell proliferation index (PI) by 24.8%, whereas 5 nM *apelin* siRNA partially reversed the TAN-induced increase in PI, and suppressed cell differentiation. In vivo assays verified the regulatory effects of the agonist and inhibitor. In conclusion, our findings demonstrated that ammonia promotes gill hyperplasia in largemouth bass by activating the Apelin/APJ signaling pathway, which may serve as a molecular marker for breeding ammonia-tolerant strains.

1 Introduction

Intensive and high-density aquaculture has promoted the rapid development of the largemouth bass (*Micropterus salmoides*) industry; however, the accumulation of feed residues and metabolic products leads to elevated levels of total ammonia nitrogen (TAN), which has become one of the important environmental factors restricting production yield and fish health (Hou et al., 2023; Yu et al., 2024). Ammonia ions in water mainly co-exist in two forms, NH_4^+ and NH_3 ; among them, non-ionic NH_3 has strong cell membrane penetration capacity and higher toxicity, and its content in water is significantly affected by temperature and pH (Francis-Floyd Ruth, 2022). As a key organ for gas exchange, osmoregulation, and ion homeostasis in fish, the gill is prone to pathological changes such as increased mucus cell production, fusion of gill lamellae, and epithelial cell hyperplasia under total ammonia nitrogen (TAN) stress, thereby reducing ventilation efficiency, disrupting ion homeostasis, and ultimately leading to growth retardation and decreased survival rate (Jin et al., 2024; Sackville et al., 2024; Wang et al., 2023; Zhong et al., 2023).

At the molecular level, TAN stress can induce oxidative stress and inflammatory responses in the body. Studies have shown that in the mudskipper (*Boleophthalmus boddarti*), ammonia nitrogen exposure causes excessive accumulation of reactive oxygen species (ROS) in gill tissues, leading to decreased antioxidant enzyme activity, activation of the NF- κ B pathway, and upregulated expression of *IL-1 β* and *TNF- α* (Ching et al., 2009). In the whiteleg shrimp (*Penaeus vannamei*), ammonia nitrogen exposure

induces endoplasmic reticulum and mitochondrial stress in the intestinal tract, increases ROS levels, and causes DNA damage (Tong et al., 2025). In addition, most studies have indicated that ammonia nitrogen exposure can induce epithelial cell proliferation in fish gill tissues. For example, in the bighead carp (*Aristichthys nobilis*), TAN stress activates the p38 MAPK and NF- κ B signaling pathways and upregulates the expression of Cyclin D1 and PCNA, thereby promoting cell proliferation (Zou, Y. et al., 2023). In the gill tissues of the yellow catfish (*Pelteobagrus fulvidraco*), IL-17 has been confirmed to mediate epithelial remodeling and hyperplasia through the MAPK signaling pathway (Zhong et al., 2023). Collectively, ammonia-triggered oxidative stress and inflammatory signaling act as essential upstream drivers that initiate gill cell proliferation and subsequent tissue remodeling in teleosts.

The Apelin/APJ (APLNR) signaling pathway, composed of the polypeptide ligand Apelin and its G protein-coupled receptor APJ, is an important signaling axis involved in various biological processes in vertebrates, including angiogenesis, fluid balance, immune and inflammatory regulation, as well as cell proliferation and migration (Cox et al., 2006; Hu et al., 2021; Kälén et al., 2007; Park et al., 2024). In the Siberian sturgeon (*Acipenser baerii*), the amino acid sequence of Apelin shares a high homology of up to 95% with that of mammals, indicating its high conservation during evolution (Tang et al., 2018). In fish, studies on Apelin have mainly focused on feeding regulation; for example, *apelin* mRNA is highly expressed in the olfactory bulb, telencephalon, and hypothalamus, suggesting its involvement in the regulation of feeding behavior (Volkoff and Wyatt, 2009). In the Ya-fish (*Schizothorax prenanti*), Apelin/APJ is highly expressed in the hypothalamus, heart, and spleen, and intraperitoneal injection of Apelin-13 can significantly increase food intake (Lin et al., 2014). However, current studies on the changes in Apelin/APJ expression and its immunoregulatory mechanism in fish under TAN stress are still relatively scarce. The largemouth bass, native to freshwater basins in North America, is an eurythermal carnivorous fish. Since its introduction into China in 1983, it has rapidly developed into an important economic aquaculture species (Bai et al., 2008). In recent years, the yield of largemouth bass has continued to grow, reaching 940,000 tons in 2024 (Bureau of Fisheries, 2025). However, the increase in ammonia nitrogen concentration in water under high-density aquaculture conditions poses a major threat to the largemouth bass aquaculture industry. In the acute TAN stress experiment (96 h-LC₅₀=19.61 mg/L), gill tissue cells of largemouth bass showed necrosis, karyorrhexis, and lymphocyte infiltration, accompanied by a decrease in ATP levels and activation of the glycolytic pathway in gill tissues, compensating for energy deficiency through anaerobic metabolism (Wang et al., 2023). In addition, the activity of Na⁺/K⁺-ATPase⁺ (NKA) in the gills decreased, Na⁺/NH₄⁺ exchange was inhibited, and ammonia nitrogen excretion rate was reduced. TAN stress can also induce significant oxidative stress in the liver, characterized by decreased activities of antioxidant enzymes SOD and GSH-Px, increased levels of GSH, GST, and MDA, and enhanced activities of immune-related enzymes LZM, ALP, and ACP (Pu et al., 2024). At the same time, the liver tissue structure was severely damaged, accompanied by increased activities of ALT, AST, and ALP in serum (Zou, J. et al., 2023). However, the molecular mechanism underlying gill hyperplasia in largemouth bass under TAN stress remains poorly understood. Therefore, this study first explored the dynamic pathological characteristics of gill tissue changes under TAN stress, determined the changes in the expression levels of genes related to the Apelin/APJ signaling

pathway obtained from previous transcriptome analysis, then verified the role of the Apelin/APJ signaling pathway in gill hyperplasia using explant and primary gill cell models, and finally validated this pathway at the *in vivo* animal level.

2 Materials and Methods

2.1 Animals

Largemouth bass were purchased from a farm in Sichuan Province, China. A total of 600 fish were used, with an average body weight of 7.54 ± 0.45 g. Random examination confirmed that all fish were free of visible disease and injury. Prior to the experiment, the fish were acclimated for 2 weeks in three plastic tanks (3 m in diameter and 1.6 m in depth). These tanks were used only for acclimation and were not considered experimental units. During the acclimation period, dissolved oxygen was maintained above 6.5 mg/L, pH was 7.98 ± 0.16 , and water temperature was maintained at 22 °C. One-third of the rearing water was replaced daily, and the fish were fed a floating commercial diet at 08:00 and 20:00. Residual feed and excreta were removed within 30 min after each feeding.

2.2 Determination of Total Ammonia Nitrogen and Calculation of Unionized Ammonia

A 10 g/L ammonium chloride (NH₄Cl) stock solution was prepared using analytical grade NH₄Cl and subsequently diluted to the designated ammonia nitrogen concentrations according to the experimental requirements. The ammonia nitrogen concentration in the water was determined using a multi-parameter water quality analyzer (Model W-II, Wuxi Okdan Biotechnology Co., Ltd., China) based on the Nessler's reagent spectrophotometric method. The specific operational procedure was as follows: following instrument calibration, 5 mL of distilled water was added to a cuvette, followed sequentially by 50 µL of Ammonia Nitrogen Reagent Ⅰ (potassium sodium tartrate solution) and 50 µL of Ammonia Nitrogen Reagent Ⅱ (Nessler's reagent). The cuvette was capped, inverted to mix thoroughly, and allowed to stand for 10 min. The cuvette was then placed into the colorimetric chamber for blank control measurement. The ammonia nitrogen concentration in the water samples was subsequently determined following the same operational procedure.

The measured ammonia concentration was expressed as total ammonia nitrogen on a nitrogen basis (TAN-N, mg/L as N). The fraction of unionized ammonia was calculated according to Emerson *et al.* (1975) using the following equations:

$$pK_a = 0.09018 + \frac{2729.92}{273.2 + T}$$

$$f_{NH_3} = \frac{1}{1 + 10^{(pK_a - pH)}}$$

where T is the water temperature ($^{\circ}\text{C}$), pK_a is the dissociation constant of the $\text{NH}_4^+/\text{NH}_3$ equilibrium, and f_{NH_3} is the fraction of TAN present as unionized NH_3 . The unionized ammonia concentration was calculated as:

$$\text{NH}_3\text{-N} = \text{TAN-N} \times f_{\text{NH}_3}$$

Under the mean in vivo conditions of pH 7.98 and 22°C , 8 mg/L TAN-N corresponded to an estimated 0.336 mg/L $\text{NH}_3\text{-N}$. In the gill explant and primary gill cell experiments, $7.5\text{ }\mu\text{M}$ NH_4Cl was equivalent to 0.105 mg/L TAN-N and corresponded to an estimated 0.000594 mg/L $\text{NH}_3\text{-N}$ at pH 7.0 and 25°C .

Because $\text{NH}_3\text{-N}$ was calculated rather than directly measured, the reported in vitro $\text{NH}_3\text{-N}$ concentration represents an estimated value.

2.3 Total Ammonia Nitrogen Stress

After acclimation, 180 largemouth bass were randomly assigned to two treatments: the control group (Con) and the total ammonia nitrogen group (TAN). Each treatment consisted of three independent replicate tanks, with 30 fish per tank, and each tank was considered an independent experimental unit. The TAN exposure experiment lasted for 21 days, with the TAN concentration maintained at 8 mg/L TAN-N, corresponding to an estimated 0.336 mg/L $\text{NH}_3\text{-N}$, using NH_4Cl as the ammonia source. During the experiment, water TAN concentration was measured every 12 h (09:00 and 21:00) and adjusted with NH_4Cl stock solution to maintain the target concentration. Other rearing conditions were consistent with those used during the acclimation period. Gill tissues were collected on days 0, 3, 6, 9, 15, and 21 of exposure. At each sampling time point, five fish were randomly sampled from each replicate tank (15 fish per treatment), ensuring that all three independent exposure units were represented. The collected gill tissues were used for subsequent hematoxylin-eosin (H&E) staining and qRT-PCR analysis.

2.4 Gill Explant Culture

2.4.1 Isolation of Gill Explant

Three healthy largemouth bass were randomly selected, anesthetized with MS-222, and blood was collected via tail cutting. Under sterile conditions, the whole gill tissues were dissected and rinsed three times sequentially with 75% ethanol, pre-cooled PBS buffer at 4°C , and PBS buffer containing triple antibiotics (penicillin-streptomycin-amphotericin) (Solarbio, Beijing, China). Subsequently, the gill arches were removed, and gill filaments were cut into gill explants with a volume of approximately 2 mm^3 , each containing 10–15 individual gill filaments. The explants were immediately transferred into six-well plates (one explant per well, 5 mL culture medium per well). The gill explants were cultured in L-15 medium (Solarbio, Beijing, China) supplemented with 15% fetal bovine serum (Corning, Shanghai, China) and 1% triple antibiotics (penicillin-streptomycin-amphotericin) at 25°C in an incubator with 5% CO_2 .

2.4.2 Determination of the Application Concentrations of SiRNA

apelin siRNA (Forward: 5'-CGCUGGUGAUUGUGCUCUU/dT//dT/-3'; Reverse: 5'-AAGAGCACAAUCACCAGCG/dT//dT/-3') was synthesized by Sangon Biotech (Shanghai) Co., Ltd. According to the manufacturer's protocol, siRNA transfection was performed to knock down the *apelin* gene. Briefly, siRNA was dissolved in DEPC to prepare a 20 μ M stock solution. Subsequently, the siRNA solution was mixed with RNA TransMate (Order NO. E607402, Sangon Biotech, Shanghai, China) and incubated for 15 min to form transfection complexes at final concentrations of 0, 2.5, 5, 10, and 20 nM. Gill explants were transfected in serum-free medium for 1 h, followed by supplementation with 15% fetal bovine serum and further incubation for 4 h under unchanged culture conditions. The relative expression level of the *apelin* gene was detected by qRT-PCR to determine the optimal siRNA concentration.

2.4.3 Gill Explant Treatments

After determining the working concentration of *apelin* siRNA, the gill explants were divided into four groups: control group (Con), TAN group (TAN), TAN+Apelin-13 group (TAN+A13), and TAN+*apelin* siRNA group (TAN + siA). The Con group was cultured in basal culture medium without any treatment. The TAN group was treated with 7.5 μ M NH_4Cl , equivalent to 0.105 mg/L TAN-N and corresponding to an estimated 0.000594 mg/L $\text{NH}_3\text{-N}$. Based on the medium pH of 7.0 and the incubation temperature of 25 $^{\circ}\text{C}$, the unionized ammonia concentration was estimated to be approximately 0.000594 mg/L $\text{NH}_3\text{-N}$, equivalent to 0.000722 mg/L NH_3 . The TAN+A13 group was exposed to 7.5 μ M [Pyr1]-Apelin-13 (CAS No. 217082-60-5, MCE, Shanghai, China). The TAN + siA group was pretreated with *apelin* siRNA. All groups were initially incubated in serum-free medium for 1 h, followed by replacement with basal culture medium for explants. Except for the Con group, the other groups were subjected to TAN stress induced by 7.5 μ M NH_4Cl for 4 h under consistent culture conditions. Samples were collected immediately after incubation for immunofluorescence staining, qRT-PCR analysis, and flow cytometry detection.

2.5 Primary Gill Cell Culture and Treatment

Primary gill cell culture medium consisted of L-15 medium supplemented with 20% fetal bovine serum and 1% triple antibiotics. Primary gill cells were isolated using the tissue explant outgrowth method. Briefly, 0.1% sodium heparin was injected into the heart of largemouth bass, followed by immediate perfusion of PBS buffer containing 1% triple antibiotics via a peristaltic pump until the gill filaments turned pale. Whole gill tissues were harvested and rinsed with PBS containing 1% triple antibiotics. Mucus was removed by repeated gentle pipetting using a Pasteur pipette. Gill arches were discarded, and gill filaments were minced before repeated rinsing with PBS containing 1% triple antibiotics. The minced gill filaments were placed at the bottom of six-well plates (3 filaments per well), with each well containing 2 mL primary gill cell culture medium. Cells were cultured at 25 $^{\circ}\text{C}$ in an incubator with 5% CO_2 . Cell adherence was observed after 24 h, and the primary cell culture medium was refreshed every 24 h.

When the cells were stably adherent and reached a density of 1.0×10^6 cells per well, six-well plates were randomly divided into four groups: Control group (Con), TAN group (TAN), TAN+Apelin-13 group (TAN+A13), and TAN+*apelin* siRNA group (TAN + siA). The Con group was maintained in basal culture

medium only. The TAN group was treated with 7.5 μM NH_4Cl , the TAN+A13 group with 8 nM [Pyr¹]-Apelin-13, and the TAN + siA group was transfected with *apelin* siRNA. The treatment procedures and culture conditions were consistent with those used for gill explants.

2.6 In Vivo Pharmacological Modulation under TAN Exposure

This experiment was designed to assess whether pharmacological modulation of Apelin/APJ signaling alters gill responses under TAN exposure, rather than to characterize the independent effects of Apelin-13 or ML221 under basal conditions. After acclimation, 120 largemouth bass were randomly assigned to four treatment groups: the control group (Con), TAN group (TAN), TAN+Apelin-13 group (TAN+A13), and TAN+ML221 group (TAN+ML221). Each treatment consisted of three independent replicate tanks, with 10 fish per tank, and each tank was considered an independent experimental unit. Fish in the TAN+A13 group received an intraperitoneal injection of 100 μL of 20 mg/L [Pyr¹]-Apelin-13 per individual, whereas fish in the TAN+ML221 group received an intraperitoneal injection of 100 μL of 20 mg/L ML221, an APJ antagonist (CAS No. 877636-42-5, MCE, Shanghai, China), per individual. Fish in the Con and TAN groups were injected with an equal volume (100 μL) of PBS. Following injection, fish in the TAN, TAN+A13, and TAN+ML221 groups were exposed to 8 mg/L TAN-N, corresponding to an estimated 0.336 mg/L $\text{NH}_3\text{-N}$, for 7 days, whereas the Con group was maintained under the same conditions without TAN exposure. Water TAN concentration was measured every 12 h (09:00 and 21:00) and adjusted to the target concentration using NH_4Cl stock solution. Other rearing conditions were consistent with those used during the acclimation period. After 7 days of exposure, three fish were randomly sampled from each replicate tank for qRT-PCR analysis and another three fish from each replicate tank for immunofluorescence staining, resulting in nine fish per treatment for each assay and ensuring representation of all three independent exposure units.

2.7 Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using a Tissue Total RNA Extraction Kit (Fujing, Chengdu). The quality and concentration of RNA were determined with a Nanodrop 2000 nucleic acid analyzer, followed by reverse transcription of RNA into cDNA using the RT EasyTM II (With gDNase) Kit (Fujing, Chengdu). $\beta\text{-actin}$ of largemouth bass was used as the internal reference gene in the experiment. The real-time quantitative PCR (qRT-PCR) reaction system was as follows: a total volume of 10 μL contained 5 μL of TB Green™ Premix Ex Taq™ II, 0.2 μL of Rox, 1 μL of cDNA, 0.4 μL of each primer (Table 1), and 2 μL of ddH₂O. The reaction program was set as follows: pre-denaturation at 95°C for 3 min, followed by 39 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s; the melting curve was gradually raised to 95°C at an increment of 0.5°C every 5 s. The Ct values of largemouth bass genes were normalized to the Ct value of $\beta\text{-actin}$, and the relative expression levels of genes were calculated using the $2^{-\Delta\Delta\text{CT}}$ method.

Table 1
qRT-PCR primers for the expression analysis of genes related to the
Apelin/APJ signaling pathway

Gene	Forward (5'-3')	Reverse (5'-3')
<i>β-actin</i>	AAAGGGAAATCGTGCGTGAC	AAGGAAGGCTGGAAGAGGG
<i>apelin</i>	GGATGGAAGTTCAAAGCCGC	TTGCTCGGCTCCGTAACAAT
<i>ccnd</i>	TGATGGAAACCAGCGAGTCC	TGGCTTACCGAGCAAGACAC
<i>akt</i>	TCTGGAGCATGTCTGCCAAT	TTTTTGCCAAAGGACAGCCG
<i>pi3k</i>	AAGACCTTCCTCATCACGAC	CCTTCCACTACAACACTGCA

2.8 Flow Cytometry Analysis

Flow cytometry analysis was performed using a Cell Cycle Detection Kit (Fujing, Chengdu). Briefly, 1 mL of pre-chilled 70% ethanol was added to the cell pellets collected from explant and primary gill cell cultures, followed by gentle pipetting to ensure thorough contact. The cells were fixed at 4°C for 24 h, then centrifuged at 200 × g for 3–5 min to remove the ethanol fixative, with the cell pellets retained. The bottom of the centrifuge tube was tapped to disperse the cells, which were then resuspended and washed with PBS buffer, centrifuged at 200 × g for 3–5 min, and the supernatant was discarded to collect the cell pellets. A 500 µL staining working solution was prepared per sample, consisting of 400 µL staining buffer, 10 µL PI staining solution 50×, and 10 µL RNase A reagent 50×. The cell pellets were dispersed by tapping the bottom of the tube, 500 µL of the prepared staining working solution was added, and gentle pipetting was performed to disperse the cells and mix thoroughly with the staining solution. The samples were incubated at 37°C in the dark for 30 min, after which analysis was conducted using a NovoCyte 2060R flow cytometer Agilent, USA. $PI = (S + G2/M) / (G0/G1 + S + G2/M) \times 100\%$.

2.9 Histological observation

The gills of largemouth bass were fixed in 10% neutral buffered formalin, and dehydrated in ethanol. After two days of fixation, the tissues were trimmed into cassettes, dehydrated in graded ethanol solutions, cleared in xylene, and embedded in the paraffin wax. Sections (4 µm) were prepared for H&E staining before a microscopic analysis (Nikon, Tokyo, Japan).

2.10 Immunofluorescence

Gill tissues and cell climbing slices fixed with 4% paraformaldehyde were permeabilized with 0.5% Triton X-100 at room temperature for 15 min, then rinsed 3 times with PBS and gently blotted dry to remove residual surface liquid. Subsequently, an appropriate amount of diluted primary antibody solution was added dropwise to each glass slide, followed by incubation at 4°C overnight. The slides were then rinsed 3 times with PBS (3 min each time), excess liquid was blotted dry, and diluted fluorescently labeled secondary antibody was added dropwise; incubation was performed at 37°C for 1 h in a humidified

chamber, followed by another 3 washes with PBS. Finally, DAPI staining solution was added for nuclear counterstaining with incubation in the dark for 5 min, and the slides were then rinsed 4 times with PBS to remove excess DAPI. Detailed information of primary and secondary antibodies, including catalog number, manufacturer and working dilution, is summarized in Table 2. After mounting with mounting medium, observation and photography were conducted using a fluorescence microscope.

Table 2
Details of primary and secondary antibodies used in immunofluorescence staining

Antibody type	Target protein	Catalog No.	Manufacturer	Dilution ratio
Primary antibody	Na ⁺ /K ⁺ -ATPase	A11683	ABclonal, China	1:200
Primary antibody	MUC2	ab272692	Abcam, UK	1:1000
Primary antibody	PCNA	BM0104	Boster Biological Technology, China	1:3200
Secondary antibody	Donkey anti-Rabbit IgG (H + L) Alexa Fluor 594	A21207	Thermo Fisher Scientific, USA	1:400
Secondary antibody	Donkey anti-Mouse IgG (H + L) Alexa Fluor 488	A21202	Thermo Fisher Scientific, USA	1:400

2.11 Statistical Analysis

Quantitative data are presented as the mean ± standard error of the mean (SEM). Relative gene expression was calculated using the 2^{-ΔΔCt} method with *β-actin* as the reference gene. Statistical analyses were performed using GraphPad Prism. For the in vivo experiments, the tank was considered the independent experimental unit. Measurements obtained from multiple fish within the same tank were treated as subsamples and were averaged to generate one value for each tank; therefore, treatment-level statistical analyses were based on three independent replicate tanks per treatment (n = 3). For comparisons between two independent treatment groups, Student’s t-test was used. For experiments involving three or more treatment groups, one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparisons test was performed. For the gill explant and primary gill cell experiments, independently prepared experimental replicates were used as the statistical units. Differences were considered statistically significant at P < 0.05. Significance levels are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.

3 Results

3.1 Dynamic Pathological Observation of Gill Tissues Under TAN Stress

To investigate the dynamic pathological changes in gill tissues of largemouth bass under TAN stress, a 21-day ammonia nitrogen exposure experiment was conducted. Proliferation of interlamellar cell masses between adjacent gill lamellae and narrowed inter-lamellar spaces were observed on day 3, accompanied by slight shortening and blunting of gill lamellae (Fig. 1D). On day 6, interlamellar proliferation was aggravated, resulting in a lamellar-like appearance (black arrows in Fig. 1F); gill lamellae were further shortened and blunted, with increased mucous cells (red arrows in Fig. 1F). On day 9, partial gill lamellae exhibited continuous elongation (black arrows in Fig. 1H), mucous masses were visible at the lamellar base, and gill lamellae were shortened with uneven spacing (yellow arrows in Fig. 1H). Progressive gill hyperplasia was observed on day 15 (Fig. 1J). Severe inter-lamellar fusion accompanied by further hyperplasia occurred on day 21 (Fig. 1L).

3.2 Effects of TAN Stress Duration on the Expression Levels of Related Genes

qRT-PCR analysis showed that the relative mRNA levels of *apelin* and *ccnd* remained stable in the early stage of 21-day TAN stress, followed by significant upregulation at day 15 ($P < 0.05$) and further elevation at day 21 relative to the control group (Fig. 2A,B). In contrast, *akt* and *pi3k* were markedly induced at the early stress stage ($P < 0.05$), with their expression returning to control levels at later time points ($P > 0.05$) (Fig. 2C,D). These results reveal distinct temporal transcriptional patterns among *apelin*, *ccnd*, *akt*, and *pi3k* during chronic TAN exposure.

3.3 Mechanistic Insights from Gill Explant and Primary Cell Models

3.3.1 Effects of TAN Stress on Gene Expression Levels of Gill Explants and Primary Gill Cells

To determine the optimal interference concentration of *apelin* siRNA, gill explants were treated with siRNA at concentrations of 0, 2.5, 5, 10, and 20 nM, and the expression level of *apelin* was detected by qRT-PCR. The results showed that *apelin* expression was significantly downregulated in the 5, 10, and 20 nM treatment groups, among which the 5 nM group exhibited the most obvious inhibitory effect (Fig. 3A); therefore, 5 nM was selected as the concentration for subsequent experiments. On this basis, the expression of genes related to the Apelin/APJ signaling pathway in gill explants and primary gill cells was detected. The results showed that in explants and primary cells, the relative expression levels of *akt* in the TAN group were 2.93 and 4.76, which were increased by 1.57 and 3.67 compared with the Con group, respectively (Fig. 3B, D); in the Apelin-13 group, the expression levels of *apelin* and *akt* were both extremely significantly upregulated; in the TAN + siA group, *apelin* expression was significantly lower than that in the Con group, whereas *akt* expression showed no significant difference compared with the Con group (Fig. 3C-E).

3.3.2 Effects of *Apelin* on Gill Cell Proliferation and Differentiation of Largemouth Bass Under TAN Stress

To explore the effect of *apelin* on the proliferation and differentiation of largemouth bass gill cells under TAN stress, immunofluorescence staining was performed on paraffin sections of gill explants and cell climbing slices. MUC2 staining results showed that MUC2-positive cells in gill explants of the Con group were mainly distributed at the base of gill filaments with a moderate number (Fig. 4A); gill lamellae in the TAN group showed adhesion and fusion, forming interlamellar cell masses with obvious hyperplasia (Fig. 4A). Statistical analysis of relative fluorescence intensity indicated that the relative fluorescence intensity of MUC2 in the TAN group and TAN+A13 group was extremely significantly higher than that in the Con group and TAN + siA group in explants, and the same trend was observed in primary cells (Fig. 4A, Fig. 5A, B). These results suggested that *apelin* siRNA treatment could inhibit TAN stress-induced gill cell proliferation and their differentiation into mucus cells to a certain extent.

The results of PCNA⁺ relative fluorescence intensity showed that compared with the Con group, the number of undifferentiated cells (PCNA⁺) increased in the TAN group of explants, further increased in the TAN+A13 group, and significantly decreased in the TAN + siA group compared with the TAN group. In primary cells, although there was no significant difference between the TAN + siA group and the TAN group, it was significantly lower than that in the TAN+A13 group (Fig. 4A, C, Fig. 5A, C). Mitochondrion-rich cells (Na⁺/K⁺-ATPase⁺) in the Con group of explants were mainly distributed in the gill filament epithelium, and the change trend of relative fluorescence intensity among the four groups was consistent with that of MUC2: the number of positive cells increased in the TAN group and TAN+A13 group, which were mainly concentrated in interlamellar cell masses. In primary cells, the relative intensity of Na⁺/K⁺-ATPase⁺ cells in the TAN+A13 group was significantly lower than that in the TAN group (Fig. 4A, D, Fig. 5A, D). Notably, the Na⁺/K⁺-ATPase response differed between the two experimental models. In gill explants, the relative fluorescence intensity was higher in the TAN+A13 group than in the TAN group, whereas in primary gill cells, Apelin-13 treatment significantly reduced Na⁺/K⁺-ATPase fluorescence relative to TAN alone. These results indicate a model-dependent response of Na⁺/K⁺-ATPase-positive cells to Apelin-13.

3.3.3 Effects of TAN Stress on Cell Cycle of Gill Explants and Primary Gill Cells

To further analyze the changes in the gill cell cycle, flow cytometry was used to detect the cell cycle distribution. The results of the explant cell cycle showed that compared with the Con group (Fig. 6A, E), the percentages of cells in the DNA synthesis phase (S phase) were extremely significantly increased, while those in the pre-DNA synthesis phase (G₀/G₁ phase) were significantly decreased to 39.2% and 14.3% in the TAN group and Apelin-13 group (Fig. 6B, E), respectively. The proliferation index (PI) showed that the PI value in the explant Apelin-13 group was significantly higher than that in the TAN group and

was increased by 24.8% relative to the Con group (Fig. 6F). These findings indicated that TAN stress promoted DNA synthesis and induced proliferation in gill cells, and Apelin-13 further enhanced this effect. In contrast, the PI value in the TAN + siA group (Fig. 6F) was 49.5%, which was significantly lower than the 85.8% observed in the TAN group (Fig. 6F), suggesting that *apelin* siRNA blocked TAN stress-induced cell proliferation. In primary cell cycle distribution, the changing trends of G₀/G₁ and S phases were consistent with those in explants (Fig. 7E), and the PI value of 35.0% in the TAN + siA group was extremely significantly lower than the 73.7% in the TAN+A13 group (Fig. 7F). These results demonstrated that siRNA effectively inhibited TAN stress-induced gill cell proliferation by targeting *apelin*.

3.4 In Vivo Pharmacological Modulation of TAN-Induced Gill Responses

3.4.1 Effects of in vivo pharmacological modulation on the expression of *apelin*, *ccnd*, *akt*, and *pi3k*

The relative expression levels of *apelin*, *ccnd*, *akt*, and *pi3k* in gill tissues are shown in Fig. 8A. For *apelin*, no significant difference was observed between the Con and TAN groups, whereas expression was significantly increased in the TAN+A13 group compared with the Con and TAN groups ($P < 0.001$). In addition, *apelin* expression in the TAN+ML221 group was significantly lower than that in the TAN+A13 group ($P < 0.001$). For *ccnd*, expression was significantly decreased in the TAN and TAN+A13 groups compared with the Con group ($P < 0.001$), and was also lower in the TAN+ML221 group than in the Con group ($P < 0.01$). For *akt*, expression in the TAN+ML221 group was significantly higher than that in the Con and TAN groups ($P < 0.05$) and the TAN+A13 group ($P < 0.01$), whereas no significant differences were observed among the Con, TAN, and TAN+A13 groups. Similarly, *pi3k* expression was significantly higher in the TAN+ML221 group than in the Con, TAN, and TAN+A13 groups ($P < 0.001$). Collectively, these results indicate that pharmacological modulation of Apelin/APJ signaling under TAN exposure was accompanied by distinct transcriptional responses of *apelin*, *ccnd*, *akt*, and *pi3k*.

3.4.2 In Vivo Validation of Gill Immunofluorescence under TAN Stress

According to the results of MUC2 fluorescent staining, the number of mucus cells (MUC2⁺) was significantly increased in the TAN+A13 group, and the relative fluorescence intensity was significantly higher than that in the Con group and TAN+ML221 group (Fig. 8B, C). These findings indicated that the Apelin-13 could effectively induce gill cell proliferation and exacerbate their differentiation into mucus cells. The staining results of PCNA⁺ undifferentiated cells showed that compared with the Con group, the relative fluorescence intensity of PCNA⁺ cells was significantly increased in the TAN group, and further extremely significantly increased in the TAN+A13 group; in contrast, the relative fluorescence intensity in the TAN+ML221 group was significantly lower than that in the TAN+A13 group (Fig. 8B, D). The results of Na⁺/K⁺-ATPase⁺ immunofluorescence showed that the number of mitochondrion-rich cells (Na⁺/K⁺-

ATPase⁺) in the Con group was significantly less than that in the TAN group (Fig. 8B, D). Analysis of relative fluorescence intensity indicated that the fluorescence intensity was extremely significantly increased in the TAN group and further enhanced in the TAN+A13 group (Fig. 8B, D); compared with the TAN group, the relative fluorescence intensity of mitochondrion-rich cells in the TAN+ML221 group was extremely significantly decreased. In summary, TAN exposure was associated with enhanced gill cell proliferation, Apelin-13 further increased several proliferation- and differentiation-related responses, whereas ML221 attenuated these responses under TAN exposure.

4 Discussion

The success of aquaculture relies on a healthy and stable aquatic environment. Among water quality parameters in aquaculture systems, ammonia represents the most critical concern second only to dissolved oxygen (Francis-Floyd Ruth, 2022). The intensification of aquaculture practices increases ammonia production in culture systems, and inappropriate management further elevates ambient ammonia levels, thereby exerting adverse effects on cultured fish (Parvathy et al., 2022). Previous studies have well documented the histopathological lesions induced by ammonia stress in various fish species, such as gill hyperplasia, lamellar fusion and epithelial damage in large yellow croaker, yellowfin tuna and yellow catfish (Guo et al., 2023; Sun et al., 2024; Zhong et al., 2023). Consistent with these findings, our pathological observations confirmed that total ammonia nitrogen (TAN) stress induced progressive gill damage in largemouth bass, characterized by interlamellar cell mass proliferation, lamellar shortening and fusion, which deteriorated with prolonged stress duration. However, the molecular mechanisms driving these adaptive pathological changes remain poorly understood, especially the role of regulatory signaling pathways in this process. In the present study, we revealed that Apelin/APJ signaling acts as a core regulator mediating ammonia-induced gill remodeling, via modulating cell cycle progression, proliferation and differentiation of gill cells, which addresses the core molecular mechanism of Apelin in this process.

Apelin is an endogenous ligand of the G protein-coupled receptor APJ and is widely distributed in animal tissues (Cayabyab et al., 2000; Hosoya et al., 2000). In mammals, *apelin* has been confirmed to regulate cell migration, proliferation, and apoptosis (Han et al., 2007; Kasai et al., 2004; Xie et al., 2007). In a rat model of intestinal inflammation, hypoxic and inflammatory stimuli upregulate *apelin* expression in the gastrointestinal tract, thereby promoting epithelial cell proliferation (Han et al., 2008). Moreover, siRNA-mediated knockdown of APJ suppresses hypoxia-induced endothelial cell proliferation in vitro and inhibits hypoxia-driven angiogenesis during fin regeneration in Fli-1 transgenic zebrafish (Eyries et al., 2008). The downstream signaling cascade of Apelin/APJ, mainly the PI3K/Akt-Cyclin D1 axis, is closely associated with the regulation of cell proliferation (Ebner et al., 2000; Pibiri et al., 2001; Zhang, 2011). In this study, Apelin/APJ pathway-related genes in largemouth bass exhibited a time-dependent response to ammonia stress, *akt* and *pi3k* were upregulated during the early stage, whereas *apelin* and *ccnd* were significantly increased at the later stage. The upregulation of *apelin* coincided with the enhanced

proliferation of gill cells, which was consistent with the morphological observations of hyperplasia in paraffin sections.

The temporal expression patterns observed in this study indicate that genes associated with the Apelin/APJ–PI3K/Akt axis respond differently during chronic TAN exposure. Specifically, *akt* and *pi3k* were upregulated at the early stage, whereas *apelin* and *ccnd* increased at the later stage. However, these temporal changes alone do not demonstrate that they represent two sequential phases of the same signaling mechanism. The early transcriptional response of *akt* and *pi3k* may reflect a general cellular stress response to ammonia exposure, whereas the later increase in *apelin* may be more closely associated with proliferative remodeling of the gill. The present data cannot distinguish between these possibilities. Therefore, the observed temporal patterns should be interpreted as associative rather than causal, and further studies combining phosphoprotein analysis and pathway-specific interventions at different time points are required to determine whether a sequential regulatory relationship exists.

To further investigate the involvement of Apelin/APJ signaling in TAN-associated gill remodeling at the organ and cellular levels, this study established gill explant (ex vivo) and primary gill cell models of largemouth bass. The results showed that TAN exposure and Apelin-13 altered the expression of *akt* and *apelin*, increased PCNA-positive cells and cell proliferation indices, and affected differentiation-related markers in both gill explants and primary gill cells, although the Na^+/K^+ -ATPase response differed between the two models; on the contrary, *apelin* siRNA intervention could effectively downregulate *apelin* expression, reduce the proportion of S-phase cells, weaken the proliferation ability of gill cells, and decrease the number of mucus cells. These results indicated that Apelin/APJ signal activation is not only a key link in ammonia nitrogen-induced gill cell proliferation but also involved in the differentiation process of undifferentiated cells into epithelial cell types such as mucus cells, which is consistent with the research conclusions that *apelin* promotes the proliferation of vascular endothelium and mucosal epithelium in mammals (Eyries et al., 2008; Han et al., 2008; Han et al., 2007; Kasai et al., 2004; Xie et al., 2007). In this study, the largemouth bass gill ex vivo model successfully identified the Apelin/APJ signaling pathway as a crucial mediator of ammonia-induced gill hyperplasia.

The Na^+/K^+ -ATPase response to Apelin-13 differed between gill explants and primary gill cells. This discrepancy may reflect intrinsic differences between the two experimental models. Gill explants retain the native tissue architecture, cell-cell interactions, and multiple gill cell populations, whereas primary cultures represent a dissociated and compositionally altered cell population. In addition, the Apelin-13 concentration was independently optimized for each model in preliminary experiments, resulting in different working concentrations. Therefore, the two systems should not be directly compared on the basis of absolute drug concentration, and the divergent Na^+/K^+ -ATPase responses are more appropriately interpreted as model-dependent effects.

Several limitations should be considered when interpreting the present findings. First, only the mRNA expression levels of genes associated with the PI3K/Akt pathway were examined, whereas phosphorylated PI3K and Akt proteins were not measured. Therefore, the temporal expression patterns

of *akt/pi3k* and *apelin/ccnd* should be interpreted as transcriptional associations rather than direct evidence of a defined two-phase activation mechanism. Second, the siRNA experiments lacked a negative-control siRNA group, and the in vivo pharmacological experiment lacked Apelin-13-only and ML221-only groups under non-TAN conditions. Therefore, potential cytotoxic effects associated with the transfection procedure, siRNA off-target effects, and independent drug effects cannot be completely excluded. Third, the primary gill cell cultures contained heterogeneous cell populations, and the present data therefore mainly reflect population-level responses rather than cell-type-specific effects. Consistent with this limitation, the opposite Na^+/K^+ -ATPase responses observed between gill explants and primary cells further suggest that tissue context and cell composition may influence the response to Apelin-13. Fourth, functional endpoints such as respiratory performance, ion regulation, and ammonia excretion were not measured; therefore, the physiological significance of the observed gill remodeling cannot be conclusively defined. Finally, unionized ammonia was estimated rather than directly measured, and the calculated in vitro $\text{NH}_3\text{-N}$ values should therefore be regarded as estimates. Further studies incorporating phosphoprotein analysis, appropriate control groups, cell-type-specific approaches, and functional measurements are needed to clarify the role of Apelin/APJ signaling in ammonia-induced gill remodeling.

5 Conclusion

In conclusion, chronic TAN exposure induced progressive gill hyperplasia in largemouth bass and was accompanied by distinct temporal changes in *apelin*, *ccnd*, *akt*, and *pi3k* transcription. Functional assays using Apelin-13, *apelin* siRNA, and ML221 further suggest that Apelin/APJ signaling participates in the regulation of TAN-associated gill cell proliferation and remodeling. However, the present data do not establish the phosphorylation-dependent activation state of the PI3K/Akt pathway, and the temporal relationship among these genes should therefore be interpreted as associative rather than as evidence of a defined sequential activation mechanism.

Furthermore, *apelin*, *pi3k*, *akt*, and *ccnd* may represent potential candidate biomarkers associated with the gill response to ammonia stress. However, their applicability for evaluating ammonia tolerance or assisting selective breeding requires further validation at the population and breeding levels. These findings provide a basis for further investigation of Apelin/APJ signaling in ammonia-associated gill remodeling and ammonia-stress responses in largemouth bass.

Declarations

CRedit authorship contribution statement

Yanhao Cao: Conceptualization, Software, Formal analysis, Data curation, Validation, Writing-original draft. **Weiming Liu:** Software, Data curation, Resources. **Xiaolong Bo:** Conceptualization, Formal analysis, Resources. **Hongli Liu:** Formal analysis, Data curation. **Run Zhang:** Supervision, Resources. **Defang Chen:** Supervision, Resources. **Yi Geng:** Supervision, Resources. **Ping Ouyang:** Validation, Methodology,

Investigation. **Wenlong Cai**: Writing-Review & Editing. **Xiaoli Huang**: Supervision, Conceptualization, Methodology, Writing-Review & Editing. All authors read and approved the final manuscript.

Ethics statement

All animal handling procedures were approved by the Animal Care and Use Committee of Sichuan Agricultural University (Chengdu, China) (Approval No: 20240003), and followed the guidelines for animal experiments of Sichuan Agricultural University.

Clinical trial number

Clinical trial number: not applicable.

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.

Funding

This study was funded by the Sichuan Province International Science and Technology Innovation Cooperation/Hong Kong, Macao and Taiwan science and Technology Innovation cooperation project Assignment (No. 2024YFHZ0346).

Author Contribution

Yanhao Cao: Conceptualization, Software, Formal analysis, Data curation, Validation, Writing-original draft. Weiming Liu: Software, Data curation, Resources. Xiaolong Bo: Conceptualization, Formal analysis, Resources. Hongli Liu: Formal analysis, Data curation. Run Zhang: Supervision, Resources. Defang Chen: Supervision, Resources. Yi Geng: Supervision, Resources. Ping Ouyang: Validation, Methodology, Investigation. Wenlong Cai: Writing-Review & Editing. Xiaoli Huang: Supervision, Conceptualization, Methodology, Writing-Review & Editing. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to the Sichuan Conservation and Utilization Center for Rare and Endemic Fishes for providing experimental aquaculture facilities to support this study. We also thank all staff members of the center for their assistance in fish culture and sample collection.

Data availability

No data was used for the research described in the article.

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Figures

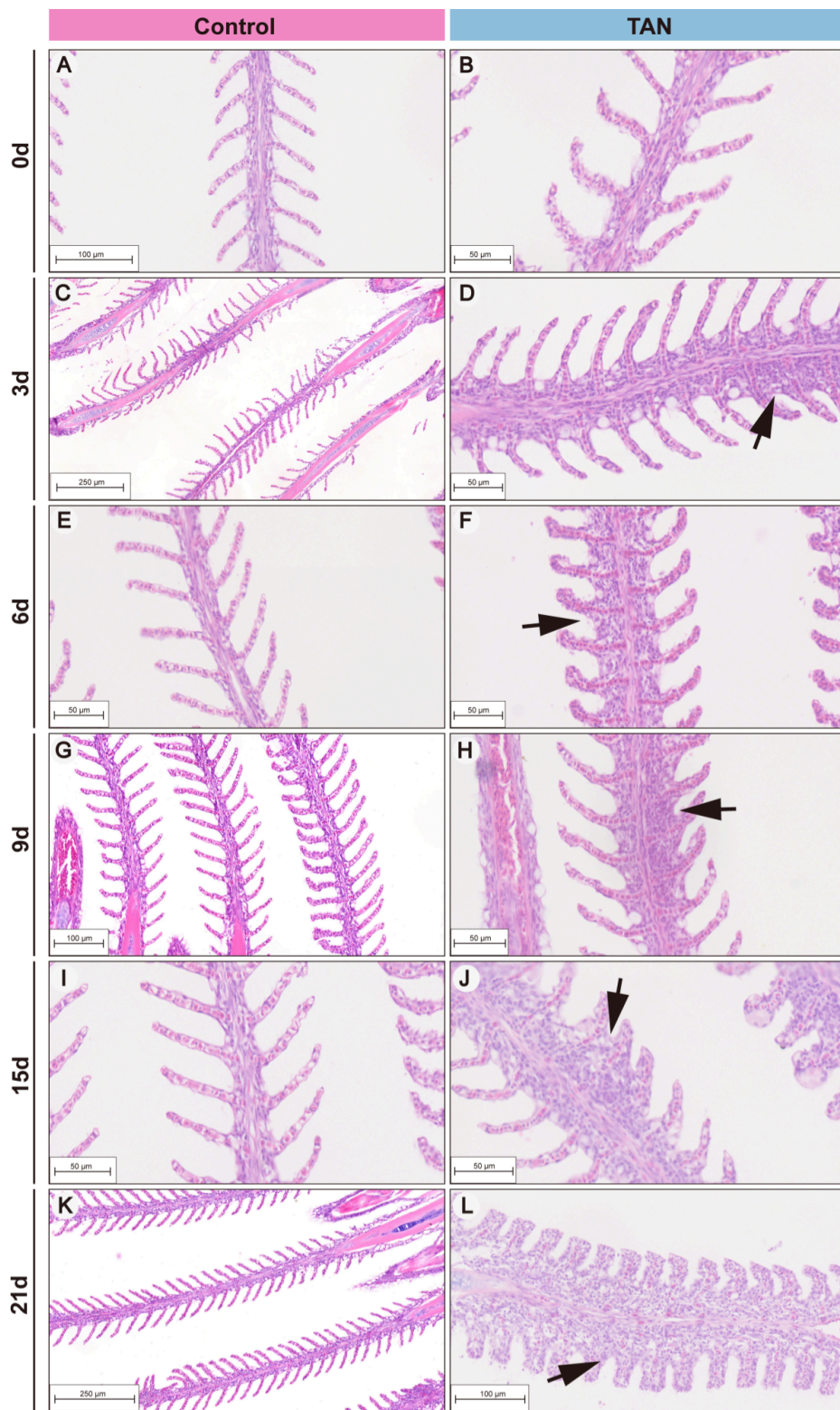


Figure 1

Histopathological changes in the gills of largemouth bass after 21 days of TAN exposure. A-B, C-D, E-F, G-H, I-J, and K-L show representative H&E-stained gill sections from the control and TAN-exposed groups on days 0, 3, 6, 9, 15, and 21, respectively. Black arrows indicate hyperplasia and fusion of adjacent gill lamellae.

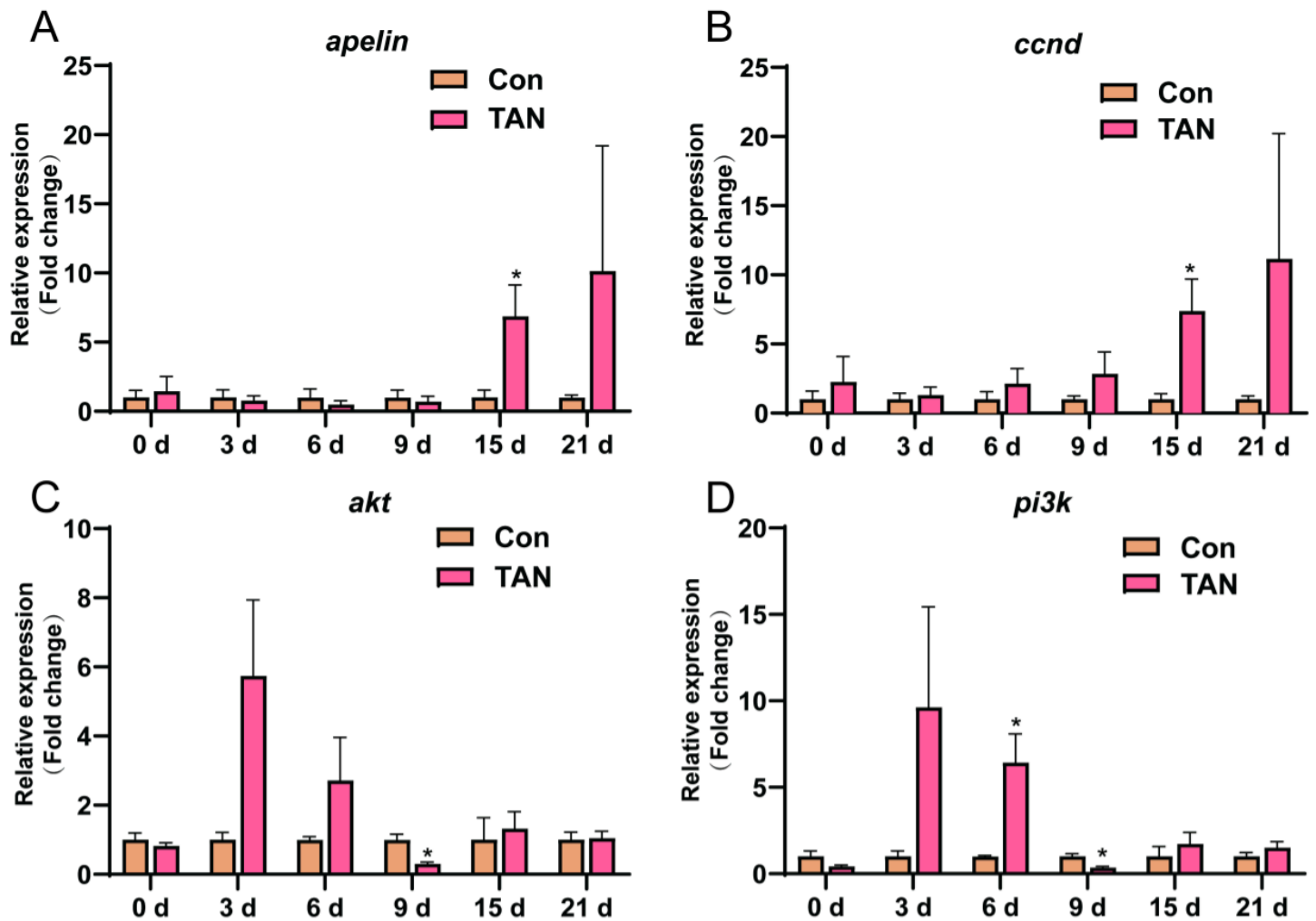


Figure 2

Relative expression levels of genes associated with Apelin/APJ signaling during TAN exposure. A-D: Relative expression levels of *apelin*, *ccnd*, *akt*, and *pi3k*, respectively, at different exposure time points. Data are presented as mean $\pm$ SEM ($n = 3$ independent tanks per treatment). Measurements obtained from fish within the same tank were treated as subsamples and averaged to generate one tank-level value. At each time point, differences between the control and TAN groups were analyzed using an unpaired two-tailed Student's t-test based on tank-level values. $P < 0.05$ versus the corresponding control group.

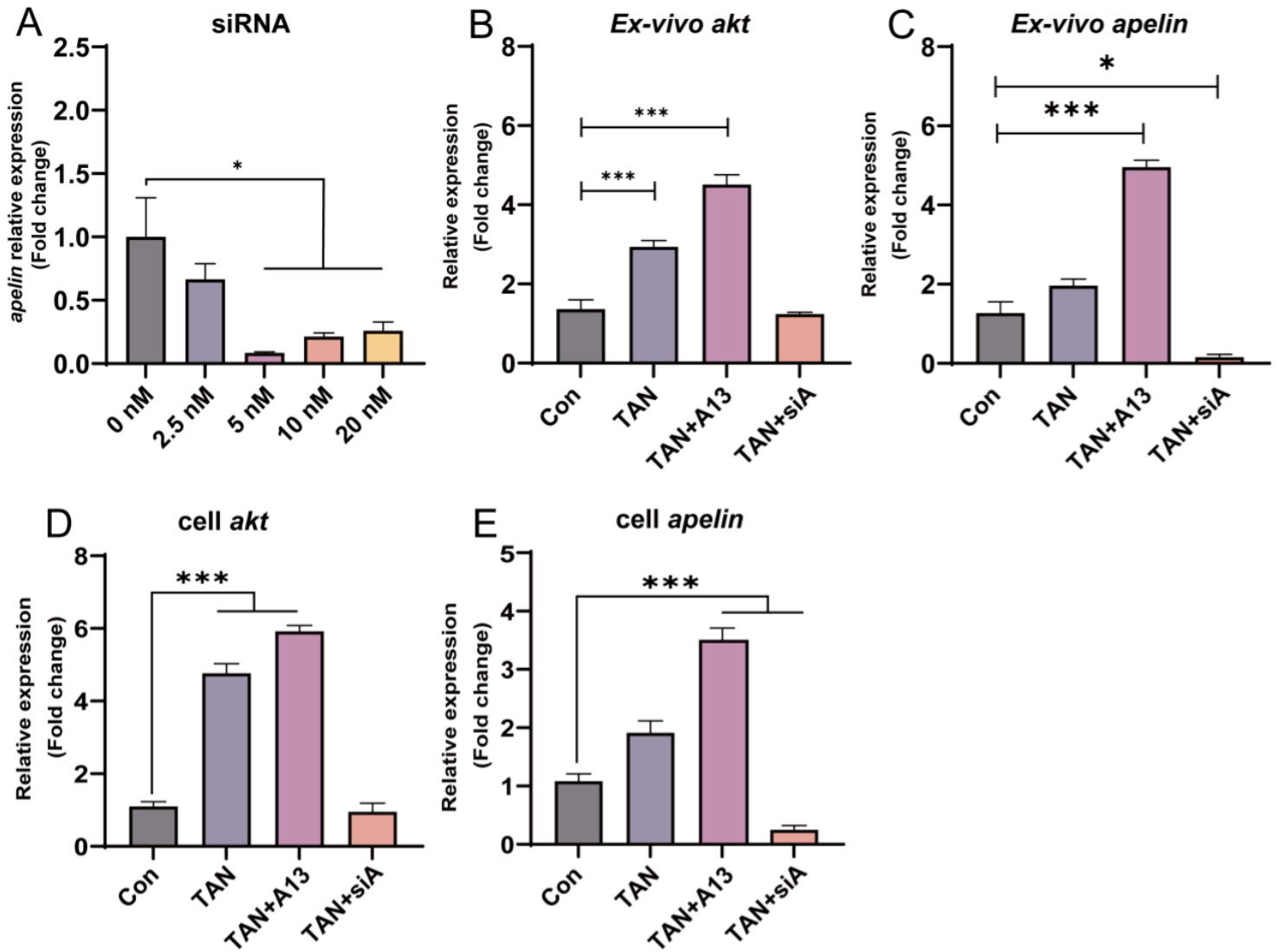


Figure 3

Screening of the optimal siRNA concentration and effects of Apelin/APJ modulation on gene expression in gill explants and primary gill cells. A: Screening of the optimal *apelin* siRNA concentration. B-C: Relative expression levels of *akt* and *apelin* in gill explants, respectively. D-E: Relative expression levels of *akt* and *apelin* in primary gill cells, respectively. Data are presented as mean $\pm$ SEM ($n \geq 3$ independent replicates). Differences among groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. *P < 0.05 and ***P < 0.001 for the indicated pairwise comparisons.

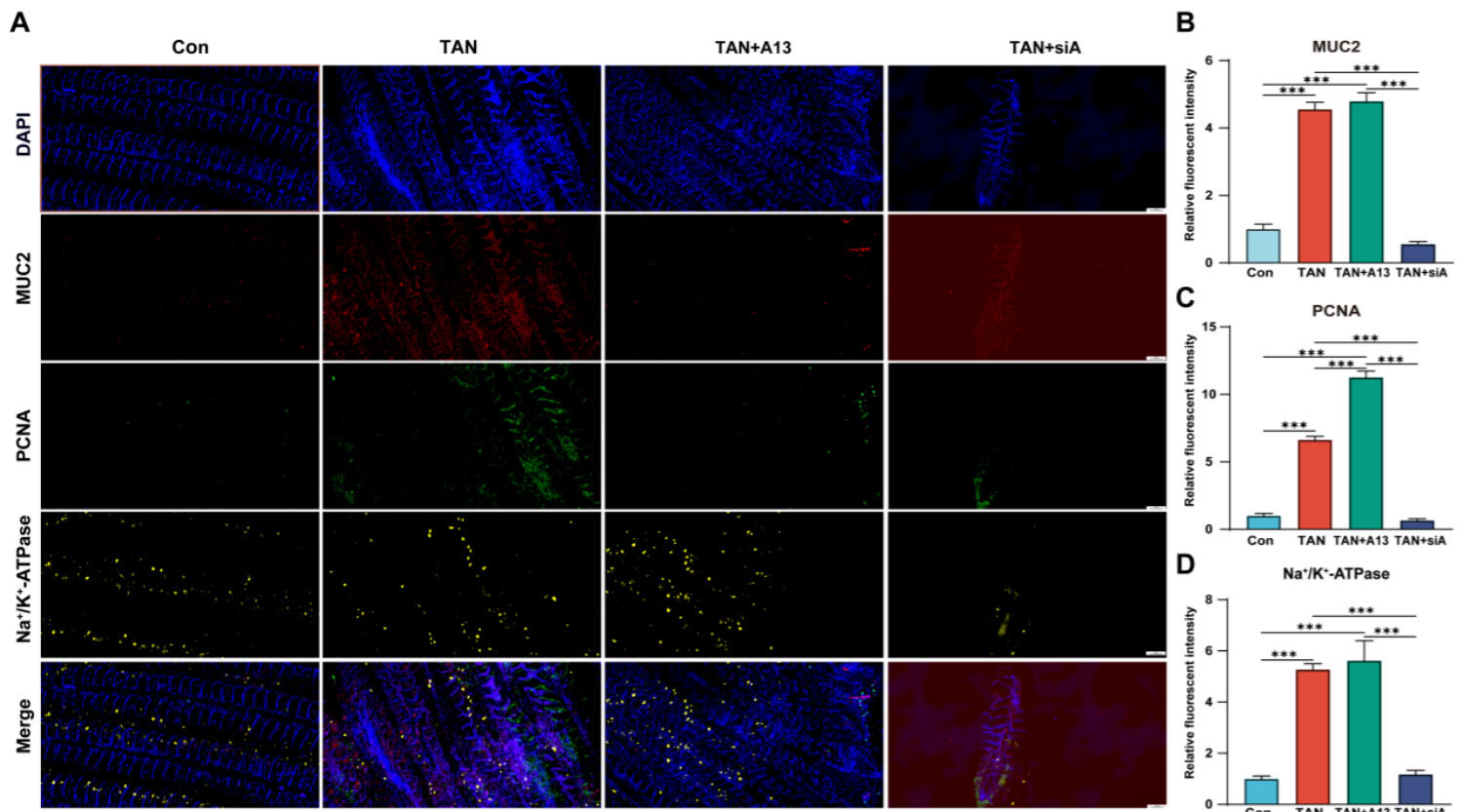


Figure 4

Immunofluorescence analysis of MUC2, PCNA, and Na⁺/K⁺-ATPase in gill explants. A: Representative immunofluorescence images. Cell nuclei were stained blue with DAPI, MUC2-positive mucus cells were stained red, PCNA-positive cells were stained green, and Na⁺/K⁺-ATPase-positive cells were stained yellow. Scale bar = 50 μ m. B-D: Quantification of the relative fluorescence intensity of MUC2, PCNA, and Na⁺/K⁺-ATPase, respectively. Quantitative data are presented as mean $\pm$ SEM (n $\geq$ 3 independent replicates). Differences among groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. ***P < 0.001 for the indicated pairwise comparisons..

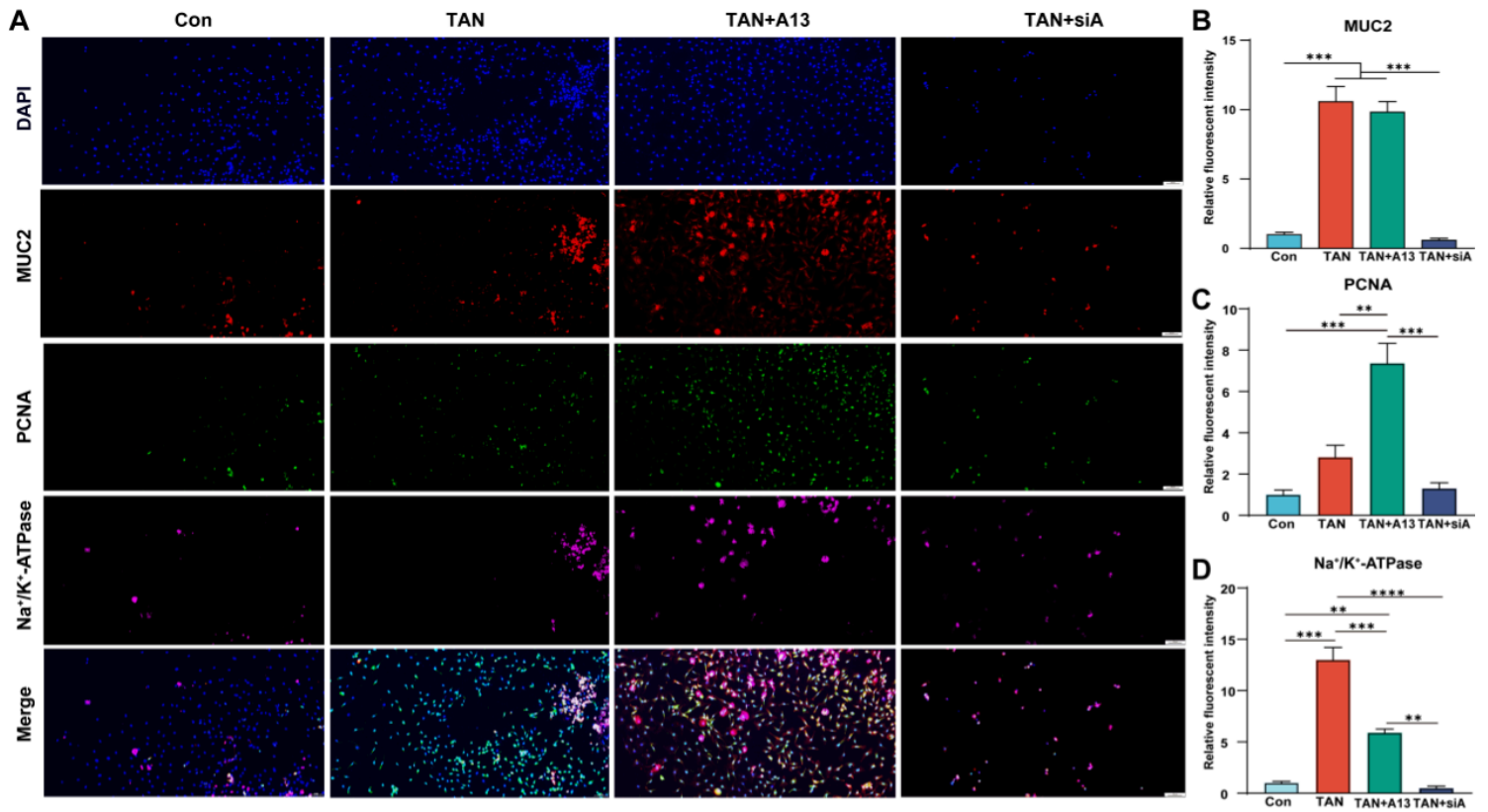


Figure 5

Immunofluorescence analysis of MUC2, PCNA, and Na⁺/K⁺-ATPase in primary gill cells. **A**: Representative immunofluorescence images. Cell nuclei were stained blue with DAPI, MUC2-positive mucus cells were stained red, PCNA-positive cells were stained green, and Na⁺/K⁺-ATPase-positive cells were stained purple. Scale bar = 50 μ m. **B-D**: Quantification of the relative fluorescence intensity of MUC2, PCNA, and Na⁺/K⁺-ATPase, respectively. Quantitative data are presented as mean $\pm$ SEM ($n \geq 3$ independent replicates). Differences among groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. ** $P < 0.01$ and *** $P < 0.001$ for the indicated pairwise comparisons.

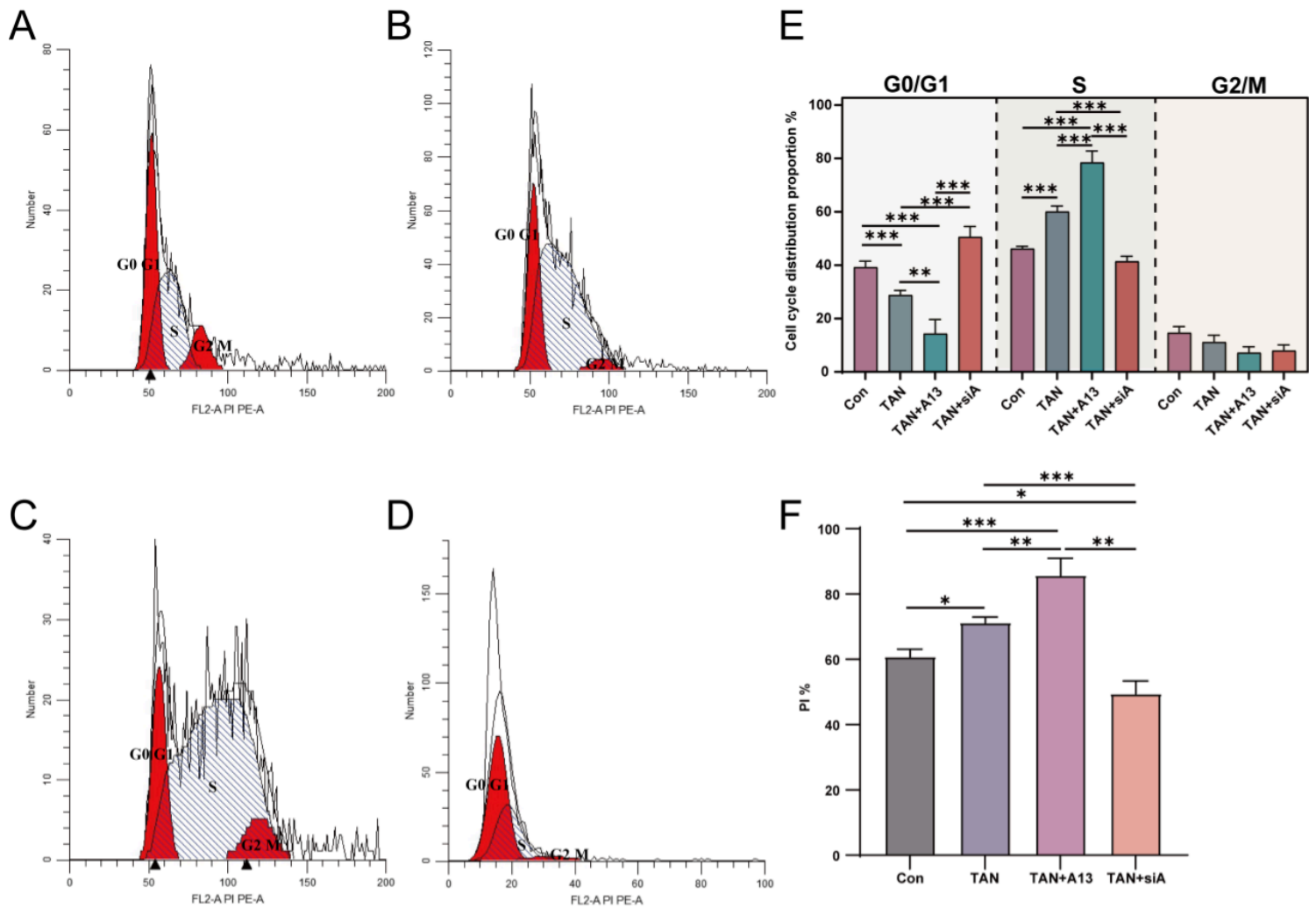


Figure 6

Cell-cycle analysis of gill explants. A-D: DNA-content distributions of cells in the Con, TAN, TAN+A13, and TAN+siA groups, respectively. E: Quantification of the proportions of cells in different cell-cycle phases. F: Cell proliferation index (PI) of gill explants. Quantitative data are presented as mean $\pm$ SEM ($n \geq 3$ independent replicates). For each cell-cycle phase and PI, differences among groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for the indicated pairwise comparisons.

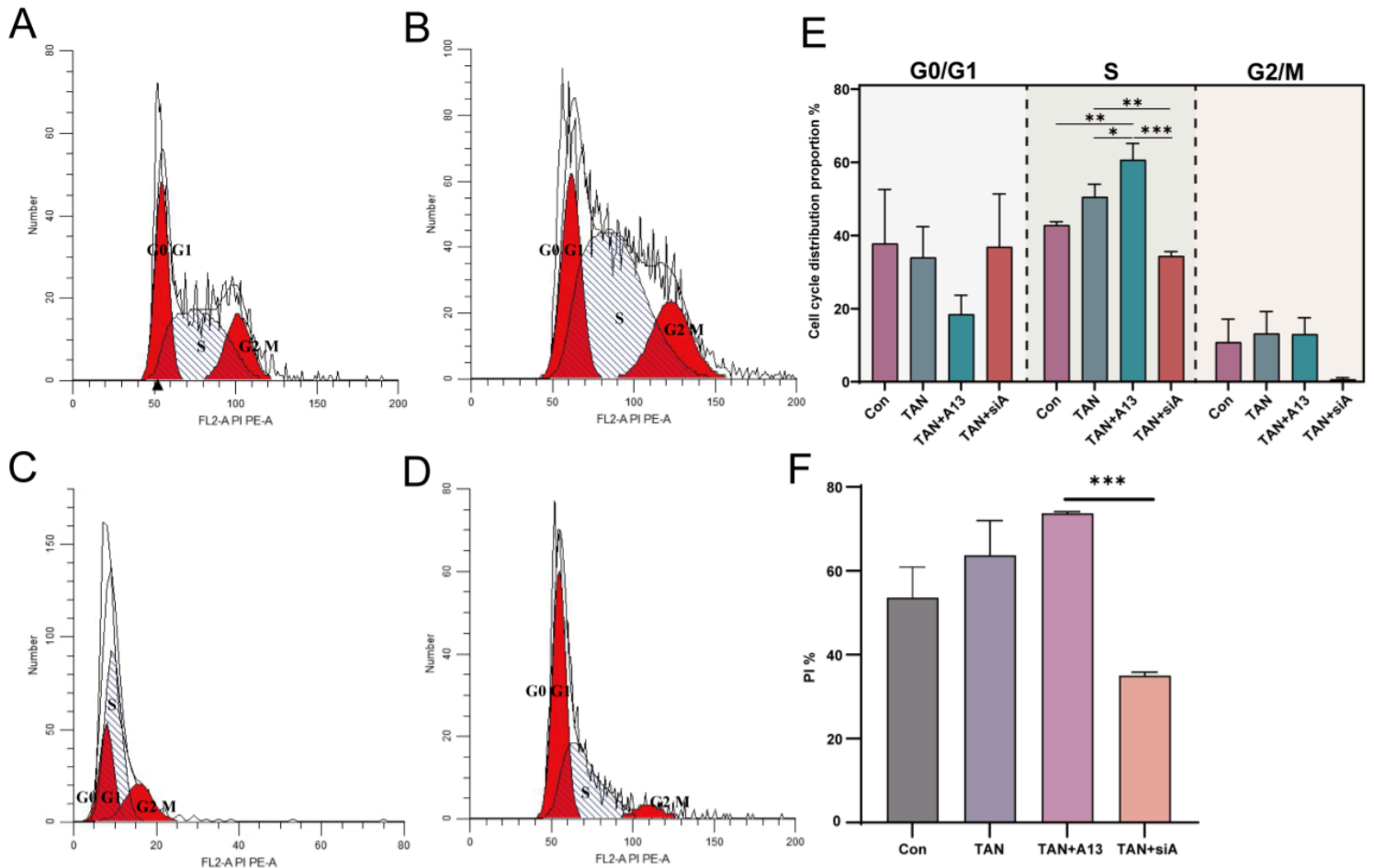


Figure 7

Cell-cycle analysis of primary gill cells. A-D: DNA-content distributions of cells in the Con, TAN, TAN+A13, and TAN+siA groups, respectively. E: Quantification of the proportions of cells in different cell-cycle phases. F: Cell proliferation index (PI) of primary gill cells. Quantitative data are presented as mean $\pm$ SEM ($n \geq 3$ independent replicates). For each cell-cycle phase and PI, differences among groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for the indicated pairwise comparisons.

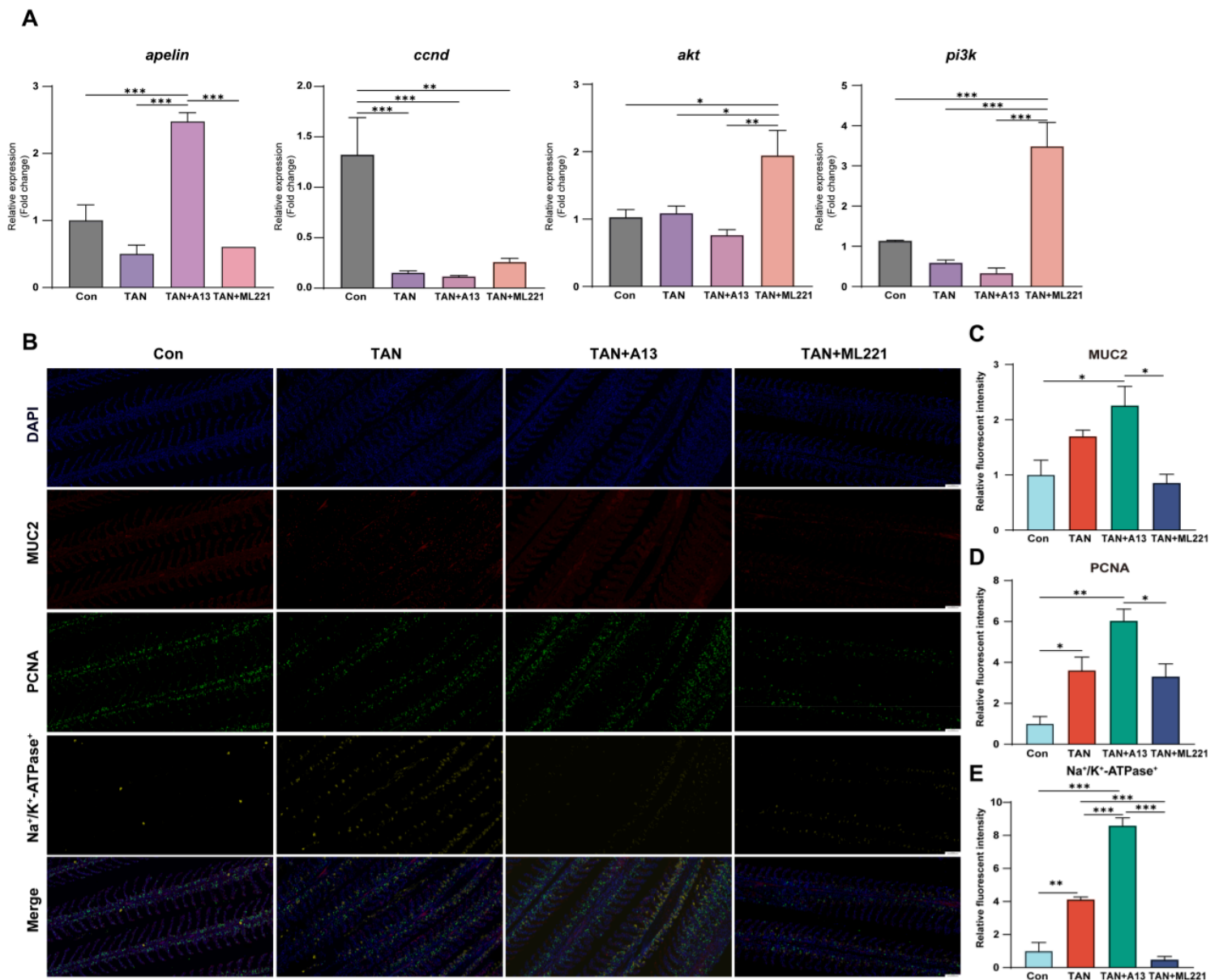


Figure 8

Effects of in vivo pharmacological modulation of Apelin/APJ signaling on TAN-induced gill responses. A: Relative expression levels of apelin, ccnd, akt, and pi3k in gill tissues. B: Representative immunofluorescence images of MUC2, PCNA, and Na⁺/K⁺-ATPase in gill tissues. Cell nuclei were stained blue with DAPI, MUC2-positive mucus cells were stained red, PCNA-positive cells were stained green, and Na⁺/K⁺-ATPase-positive cells were stained yellow. Scale bar = 50 μm. C-E: Quantification of the relative fluorescence intensity of MUC2, PCNA, and Na⁺/K⁺-ATPase, respectively. For qRT-PCR and immunofluorescence analyses, three fish were sampled from each of three independent replicate tanks per treatment. Measurements obtained from fish within the same tank were treated as subsamples and averaged to generate one tank-level value. Quantitative data are presented as mean ± SEM (n = 3 independent tanks per treatment). Differences among groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparisons test based on tank-level values. *P < 0.05, **P < 0.01, and ***P < 0.001 for the indicated pairwise comparisons.

