Physiological and molecular responses of golden pompano *Trachinotus ovatus* subjected to the acute salinity stress

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Abstract

Golden pompano (*Trachinotus ovatus*) is a commercially important marine fish and is widely cultured in the coastal area of South China. Salinity is one of the most important environmental factors influencing aquatic organisms. In this study, *T. ovatus* was subjected to the acute salinity stress treatments using four salinity levels of 0‰, 10‰, 20‰, and 40‰ to investigate its physiological and molecular responses. Our results revealed that serum osmolality, Na^+, and Cl^- concentrations showed a similar decreasing trend after salinity stress at the first 2 h and then increased after 8 h. In particular, the Na^+/K^+-ATPase alpha 1-isoform (*Tonka α1*) rapidly responded to changes in salinity levels. In the gills, the expression of *Tonka α1* was markedly upregulated at 2 h in all the experimental groups (0‰, 10‰, 20‰, and 40‰). In the kidney, *Tonka α1* expression in these experimental groups tended first to increase but then decreased, with its values peaking at 2 h, 4 h, and 8 h, respectively. In the intestine, *Tonka α1* responded rapidly to salinity changes and then changed dramatically within 4 h under salinity stress. The Na^+/K^+-ATPase activity was also affected by the acute salinity stress, and it was higher in the 10‰ and 40‰ groups than in the control group (0 h). Interestingly, Na^+/K^+-ATPase activity was lowest in the 20‰ group. Our results show the various physiological response of *T. ovatus* under acute salinity stress and demonstrate that under such conditions, Na^+/K^+-ATPase is primarily involved in the osmoregulation required to maintain homeostasis in this species.

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Introduction

Salinity is one of the most important environmental factors influencing aquatic organisms and the drastic salinity changes can cause the adverse effects physiological functions in individuals (Nie et al., 2017). The first impact is the maintenance of fluid balance in aquatic organisms, such as changes in concentrations of sodium (Na^+), chloride (Cl-), potassium (K^+), and serum osmolality (Shui et al., 2018). Salinity changes also has a direct influence on hematological and biochemical variables, such as growth hormone, cortisol and thyroxine, which provides essential information regarding clinical status and energy of fish (Arnason et al., 2013).

Osmoregulation is the main process by which aquatic animals respond to changes in salinity, especially in fish (Yang et al., 2016). Osmolality increases with increased salinity during the adaptation (Schmitz et al., 2017). Euryhaline teleost fishes regulate and maintain their plasma ionic composition and osmotic concentration following changes in the environmental salinity (Yamaguchi et al., 2018). In a hypotonic environment, to maintain ionic homeostasis, teleosts absorb ions through the gills and intestine and excrete excess water, and filter solute to form diluted urine through renal reabsorption. In contrast, in hypertonic environments, teleosts passively lose water and gain excess ions. Therefore, teleosts actively secrete ions and drinking water to maintain homeostasis (Urbina et al., 2013). However, the acclimation to changing environmental salinity is a complex process involved a set of physiological and molecular responses based on the function of several osmoregulatory organs (e.g., gills, kidney and intestine), such as active ion transport of concentration gradients using various molecular pumps and channels (Yang et al., 2016). Hormones and proteins are also involved in osmoregulation, such as cortisol (COR), prolactin (PRL), Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and aquaporins (AQPs) (Choi and An, 2008). NKA location in the osmoregulatory organs' epithelia is essential to ensure effective ion transport to maintain ion homeostasis (Lin et al., 2003). In teleost fishes, the mitochondria-rich cells (MRCs) have been extensively studied, since they are a significant site for osmoregulation (Kang et al., 2012).

Nonetheless, MRCs are also critically involved in both the acid-base balance and $Ca2^+$ uptake, thus playing crucial roles in adjusting to salinity changes (Kang et al., 2012). MRCs form an extensive tubular system in the cytoplasm, connected to the basolateral membrane, thus expanding the surface area available for the placement of ion transporters proteins, including NKA (Kaneko et al., 2008). NKA is a ubiquitous enzyme that energizes transports three Na^+ out of and two K^+ into the cell by the hydrolysis of one molecule ATP. Not surprisingly, NKA is vital for not only sustaining intracellular homeostasis but also for maintaining an electrochemical gradient that provides the driving force for ion transport in vital osmoregulatory organs (Horisberger et al., 1991; Ching et al., 2015).

NKA is a multi-subunit enzyme that contains α and β subunits. A γ subunit has also been identified as NKA regulatory subunits that were predominantly expressed in kidney cells of several species (Horisberger et al., 1991; Blanco and Mercer, 1998; Richards et al., 2003; Ching et al., 2015). The α-subunit is considered as catalytic subunit, containing the Na^+, K^+ and ATP binding sites, while the β-subunit is thought to be a type Ⅱ glycosylated polypeptide that promotes the entry of the α-subunit into the cell membrane (Blanco and Mercer, 1998). The α-subunit exists in four different isoforms (α1, α2, α3 and α4). The four α-isoforms are highly conserved, although their expression patterns within mammalian tissues and cell types are different (Yang et al., 2016). In fish, only the three α subunit isoforms (α1, α2 and α3) are identified, while paralogs nka α1a, nka α1b and nka α1c are also found in several species (Armesto et al., 2014). In *Danio rerio*, eight different nka α subunits isoforms (five α1, one α2 and two α3) have been reported (Rajarao et al., 2001). In *Oncorhynchus mykiss*, seven nka α subunit isoforms (four α1, one α2 and two α3) were identified (Richards et al., 2003). These nka α subunit isoforms show specific expression patterns in salinity transfer and species (Blondeaubidet et al., 2016). After the salinity challenge, the transcription level of nka α1a in *Solea senegalensis* was significantly increased when transferred to high salinity water (60‰). In contrast, nka α3a transcription level increased at low salinity (5‰) (Armesto et al., 2014). When fish were transferred from seawater to freshwater, the expression levels of nka α1a and nka α1b were detected in the osmoregulatory organs. The results showed that the expression of nka α1a in *Dicentrarchus labrax* was higher than that of nka α1b in different osmoregulatory organs (Blondeaubidet et al., 2016).

Golden pompano (*Trachinotus ovatus*) is distributed in tropical and subtropical areas of Southeast Asia and the Mediterranean Sea. *T. ovatus* can be found naturally, or commercially cultured in brackish water ponds, seawater floating sea cages in southern China (Qi et al., 2017). However, heavy rainfall can dramatically reduce the salinity in these ponds and sea bays, which can result in the mass mortality of this species (Ma et al., 2016). However, little is known about the osmotic regulation mechanism of *T. ovatus* in response to acute salinity stress. Hence it is imperative that we comprehensively study the mechanisms underpinning physiological and molecular responses to the acute salinity stress in *T. ovatus*. Therefore, we first investigated its physiological responses and determined the serum osmolality, Na^+ and Cl^- concentrations. Finally, we also studied molecular mechanisms responding to the imposed acute salinity stress by detecting the expression profile of nka α1 mRNAs and examining the NKA enzyme activity.

Materials and Methods

Fish and experimental design

Experimental fish (body weight: 35.08 ± 3.15 g) were obtained from the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Lingshui, Hainan Province. The fish were reared in 2m × 2m ×1m tanks with recirculating seawater (30‰) at 29 ± 1℃ for one week before the experiment. Two air stones were used in each tank to maintain dissolved oxygen close to saturation (6.5-7.0 mg/L). The fish were fed commercial pellets (Hengxing, Guangzhou, China) twice a day at 07:00 and 17:00 to satiation. Experimental fish did not feed on the day prior to the experiment. Spleen, kidney, stomach, muscle, fin, blood, gills, brain, liver and intestine were collected from three fish for spatial expression pattern detection.

For the acute salinity stress experiment, 600 fish were randomly selected from the tank (30‰, control) and allocated in equal numbers to four treatment groups: 0‰, 10‰, 20‰ and 40‰. Each treatment group hadthree replicates. Waters of different salinities (10‰ and 20‰) were prepared by mixing seawater with an appropriate quantity of freshwater. 40‰ salinity seawater was prepared by mixing seawater salt with normal seawater. Salinity was measured using a ZN17-DMD salinometer (Zhongnuo Far East Technology Co., Ltd. Beijing, China). The filtration system used a circulating water system .

All experimental fish were anesthetized with 100 mg/L Eugenol (Shanghai Medical Instruments Co., Ltd., Shanghai, China). Blood samples were obtained from the caudal vein of nine fish from each group, using a 2.5-mL syringe without heparin at each time point (0, 2, 4, 8, 12, 24, 48 and 96 h) from each group. After centrifugation (3000×g, 4 °C, 10 min), serum was separated from coagulated blood and stored at -80 °C. At each time point (0, 2, 4, 8, 12, 24, 48 and 96 h), tissues (gills, intestine and kidney) were surgically isolated, immediately stored in liquid nitrogen until later use. All the experiments in this study were approved by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (no.SCSFRI96-253) and performed according to the regulations and guidelines established by this committee.

Serum analyses

Blood samples were obtained from the caudal vein of nine fish from each group, using a 2.5 mL syringe without heparin. Following centrifugation (3000×g, 4 °C, 10min), serum was separated for analysis of the Na^+, Cl- concentrations and serum osmolality. Serum osmolality was determined using a BS-100 freezing point osmotic pressure instrument (Shanghai Yida Medical Devices Co., Ltd, China). The remaining serum was stored at -80 °C for analysis of the Na^+ and Cl^- concentrations. Both the Na^+ and Cl^- concentrations were determined using the electrode method with an URIT-910A electrolyte analyzer (URIT, Guilin, China).

Gene cloning and bioinformatic analysis

Total RNA samples were extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. DNase Ⅰ was used to remove the genomic DNA from the extracted total RNA to ensure the purity of the total RNA. The quality and integrity of the total RNA were measured by 1.2% agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) was used to estimate the quantity of total RNA. cDNA was synthesized from total RNA using the PrimeScript™ Reverse Transcriptase kit (TaKaRa, Dalian, China) according to

the manufacturer's instructions and subsequently stored at -80°C. The working solution of the cDNA samples was diluted to 100 ng/μl and stored at -20°C until later use.

The DNA sequences of ToNKA α1 was obtained from the genome database of *T. ovatus* (Accession No. PRJEB22654 under ENA; Sequence Read Archive under BioProject PRJNA406847). DNA specific primers (**Table 1**) were designed with Primer Premier 5.0 software to verify the accuracy of the sequences (Lalitha, 2000). The homology of the full-length cDNA sequences of Tonka α1 was searched using the BLAST program of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast). The N-glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGylc/). The functional domain of Tonka α1 was predicted using ExPASy software (http://smart.embl-heidelberg.de/smart/set_mode). ExPaSy compute tool was also used to compute the molecular weights, amino acid contents and isoelectric points of ToNKA α1 polypeptide (http://web.expasy.org/protparam/). The signal peptide of ToNKAα1 was predicted by the SignaIP 4.1 Server (http://www.cbs.dtu.dk/services/SignaIP.(/

The homologous sequences of the nka were downloaded from GenBank. Multiple nucleotide sequences were aligned by CluatalW (http://www.clustal.org/) with default parameters. The genetic identity was calculated by Bioedit software (Hall, 1999). The evolutionary distances between mammals, amphibians and teleost species were calculated following the Kimura 2-parameter method, and a cladogram was generated using the neighbor-joining (NJ) method in MEGA7, with a bootstrap test performed with 1000 replicates (Kumar et al., 2008) (**Table 2**).

Table 1 Primers used for gene clone and qRT-PCR.

Primers	Sequence(5′→3′)	Primer type
Tonka α1-F-1	ATAACGGGCACATCAAGGA	ORF
Tonka α1-R-1	CGACAGCAGAAAGCACGAC	ORF
Tonka α1-F-2	GGTGGTTGGTGATTTAGTGG	ORF
Tonka α1-R-2	TTTGTTGGTGGAGTTGAAGG	ORF
Tonka α1-F-3	GCCTTGCTGAAGTGTATTGAG	ORF
Tonka α1-R-3	AAGATGCGAGCTGACTGGAC	ORF
qTonka α1-F	CCTATCAACGAAGTCAACCCAA	qPCR
qTonka α1-R	TCCAGCAGGATCATGTCAGC	qPCR
ef-1α-F	CCCCTTGGTCGTTTTGCC	Reference gene
ef-1α-R	GCCTTGGTTGTCTTTCCGCTA	Reference gene

QRT-PCR of ToNKA α1 mRNA

The main primers (qTonka α1-F, qTonka α1-R) and elongation factor 1α (ef-1a) gene primers used in this study were listed in **Table 1**. PCR amplifications were performed in a total volume of 12.5 μL containing 6.25 μL SYBR Pre-mix ExTaq (TaKaRa, Dalian, China), 1 μL of the cDNA template, 0.5 μL of each primer, and 4.25 μL of RNase-free water. The qRT-PCR reaction cycle program started at 94 °C for 30 s, followed by 40 cycles at 94 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. A melt curve analysis from 65 °C to 95 °C was employed to demonstrate the specificity of the obtained PCR products. The relative expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method (Livak and Schmittgen, 2001).

Table 2 Genetic identity of amino acid sequences of NKA α1 between speices.

		1	2	3	4	5	6
1	*T.ovatus*	100					
2	*S.dumerili*	98.0	100				
3	*O.latipes*	95.9	96.3	100			
4	*D.rerio*	93.7	93.6	93.3	100		
5	*H.sapiens*	89.0	88.9	89.0	88.6	100	
6	*X.tropicalis*	89.0	89.3	89.1	89.1	92	100

Gill NKA enzyme activity

Approximately 0.2 g of gill filaments per fish were placed in 1 ml of an ice-cold enzyme extract (0.25 mM sucrose, 6 mM EDTA-Na2, 10 mM Tris-HCl (pH=7.5), 0.1% sodium deoxycholate) and homogenized on ice with a glass homogenizer. The homogenate was centrifuged at 6000×g for 30 min at 4°C, and the supernatant was used to determine gill NKA activity. Total soluble protein was determined using the Coomassie brilliant blue method with bovine serum albumin as the standard, and the amount of total soluble protein was measured at 454 nm (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). NKA activity was determined by measuring the release of inorganic phosphate (Pi) from ATP according to the kit protocol, and the amount of inorganic phosphorus was measured at 636 nm (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). One unit of NKA activity was defined as the ATP enzyme in one-milligram tissue protein that broke down ATP to produce 1 μmol inorganic phosphorus in one hour.

Statistical analysis

All the data were analyzed by one-way analysis of variance (one-way ANOVA) with tests of normality and homogeneity of variance done in SPSS statistics version 23.0 (SPSS Inc., Chicago, USA). Multiple comparisons of means made using Tukey's test. The results were expressed as means ± standard error (SE).

Results

Serum chemistry

As **Figure 1** showed, the serum osmolality, Na^+ and Cl^- concentrations in all experimental groups were lower than the control (0 h). The serum osmolality and Na^+, Cl^- concentrations sharply decreased at 2 h, and the serum osmolality of the 0‰, 20‰ and 40‰ groups reached their lowest value (0.38-fold, 0.82-fold and 0.79-fold higher compared with that of the control (0 h), respectively; $p < 0.01$) (**Figure 1A**), while the serum osmolality of 10‰ group reached its minimum value at 8 h, at which point it was 0.80-fold higher compared with that of the control ($p < 0.01$). Compared with control (0 h), the Na^+ and Cl^- concentrations of 10‰ and 20‰ groups significantly decreased at 4 h ($p < 0.01$). After 8 h, the serum osmolality, Na^+ and Cl^- concentrations showed a similar trend as found in the 10‰, 20‰ and 40‰ groups, as they gradually increased and stabilized (**Figure 1B, C**). In the first 8 h, the survival rate of *T. ovatus* was zero in the 0‰ group.

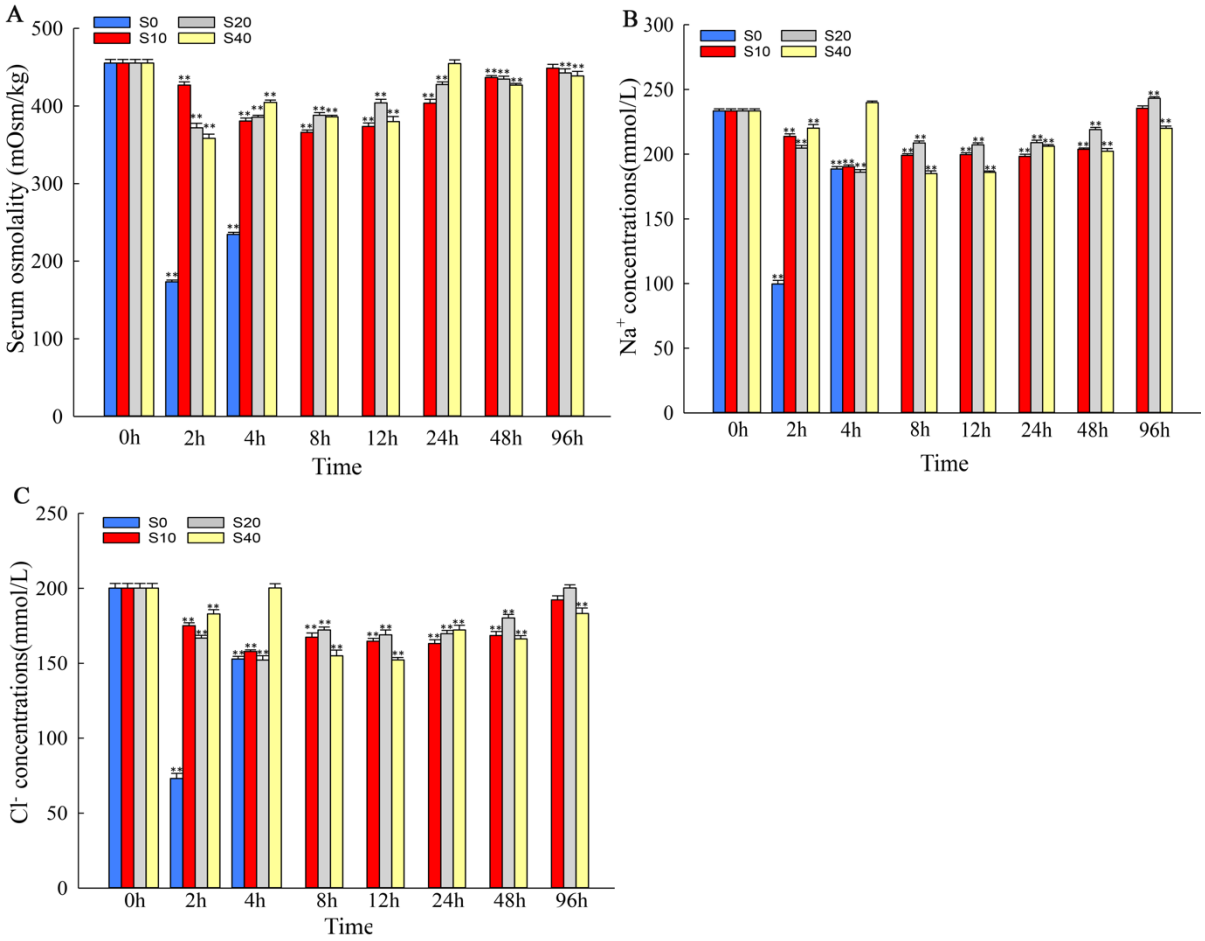


Figure 1 Serum osmolality, Na^+, and Cl^- concentrations of golden pompano *T. ovatus* after acute salinity stress. Relation between osmolality and salinity of the serum osmolality (A), Na^+ concentrations (B) and Cl^- concentrations (C) were analyzed with Tukey's multiple-comparison test following one-way ANOVA ($P<0.05$, n=3). Significant differences at $P<0.05$ were labeled with *, significant differences at $P<0.01$ were labeled with **, mean ± SE of each quantity was showed for each group detected. The untreated (0h) group was used as a control.

Sequence characteristics

The full length of ToNKA α1 cDNA (Accession number: MG843838) was 3631 bp. It contained an open reading frame (ORF) with 3072 bp that encoding 1024 amino acids, a 5'-untranslated region (UTR) of 210 bp and a 3'-UTR of 349 bp with the putative polyadenylation consensus signal (aataaa). The predicted protein molecular mass was 112.4 kDa and the theoretical pI value was 5.27. The SignalP 4.1 analysis showed that ToNKA α1 lacked a signal peptide.

ToNKA α1 showed the typical topology features of the P-ATPase with 10 transmembrane domains (TM) at N- and C-termini, which were associated with alternating hydrophilic intracellular and extracellular domains. The protein structure analysis reveale that ToNKA α1 protein contained two cation transporting ATPase domains (residue 43-117, residue 800-1009), an E1-E2-ATPase domain (residue 135-366) and a haloacid dehalogenase-like hydrolase domain (residue 371-730) (**Figure 2**). Moreover, the threonine-glycine-aspartic acid-glycine-valine-asparagine-aspartic acid (TGDGVND) motif, the threonine-glycine-aspartic acid (TGD) motif, the threonine-glycine-glutamic acid-serine (TGES) motif, and the aspartic acid-lysine-threonine-glycine-threonine (DKTGTLT) motif, were also all identified. Phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC), and binding sites for Na^+ and K^+ (for short, respectively: Na^+ I, II, III and K^+ I, II), were also identified and labeled accordingly. There were five predicted N-glycosylation sites in ToNKA α1 (namely N256, N406, N484, N639 and N721) (**Figure 3**).

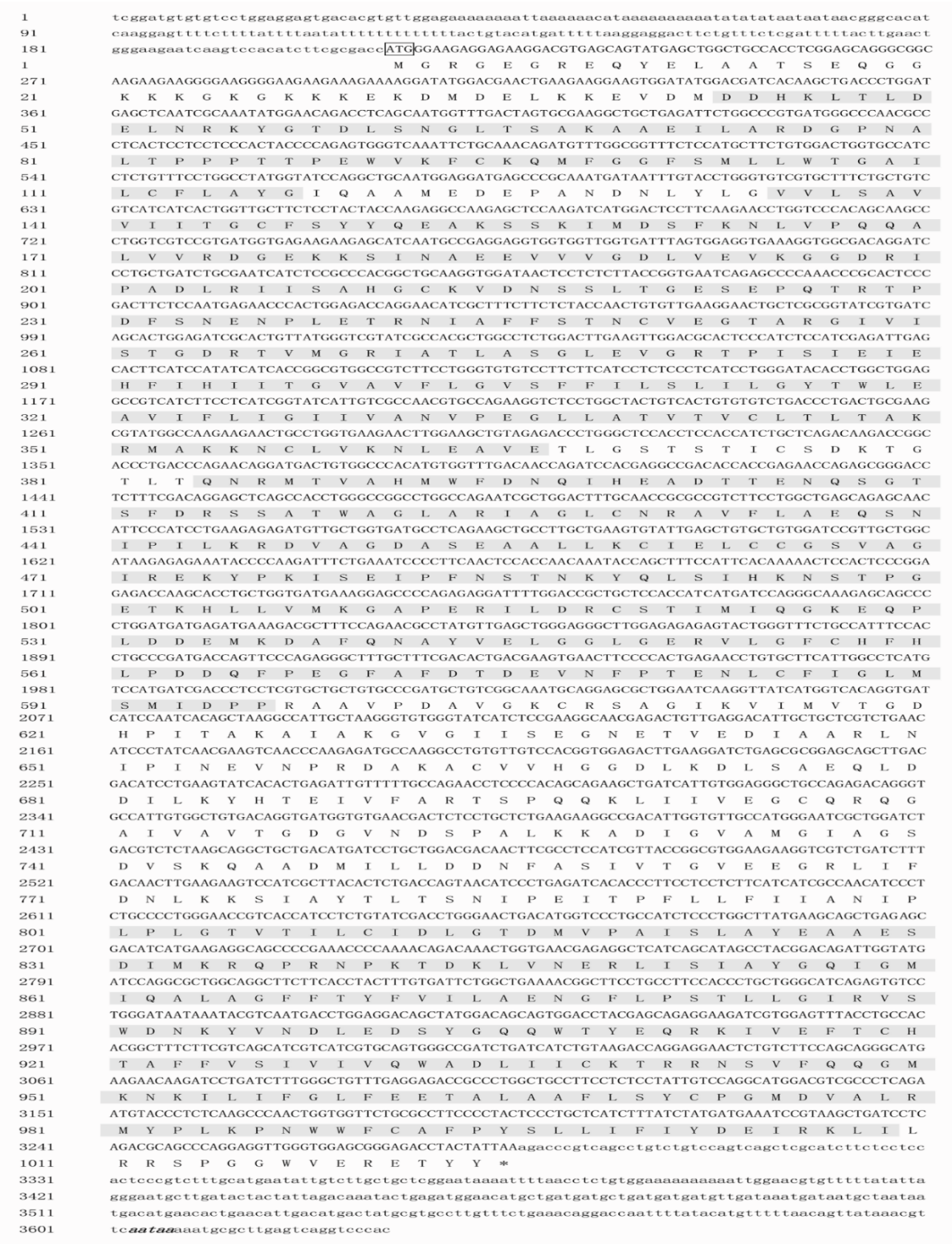
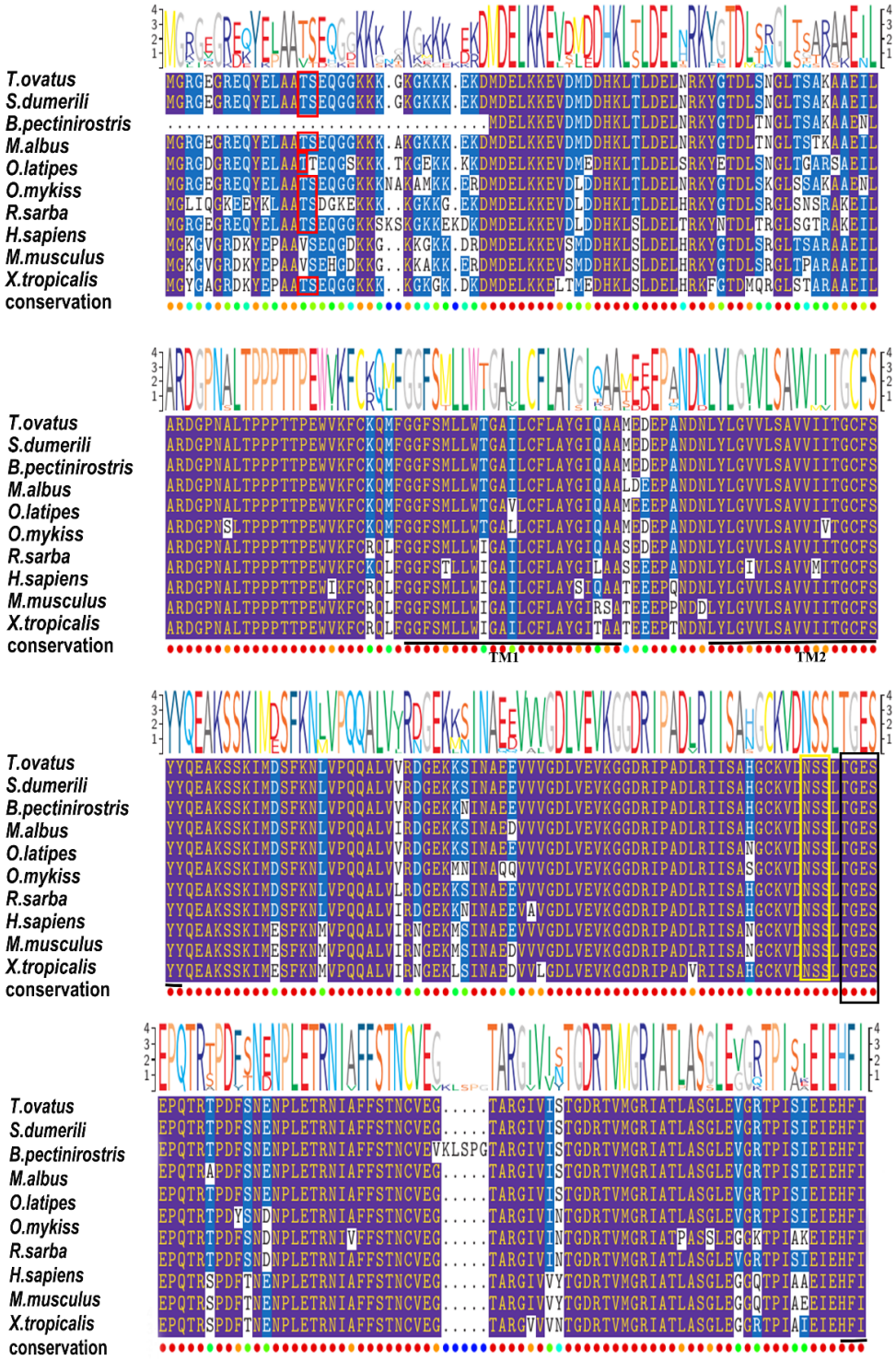


Figure 2 Nucleotide and deduced amino acid sequence of *ToNKA α1*. Start and termination codon were marked with box and "*", respectively. ATPase domain, E1-E2-ATPase domain and haloacid dehalogenase-like hydrolase domain were shadowed.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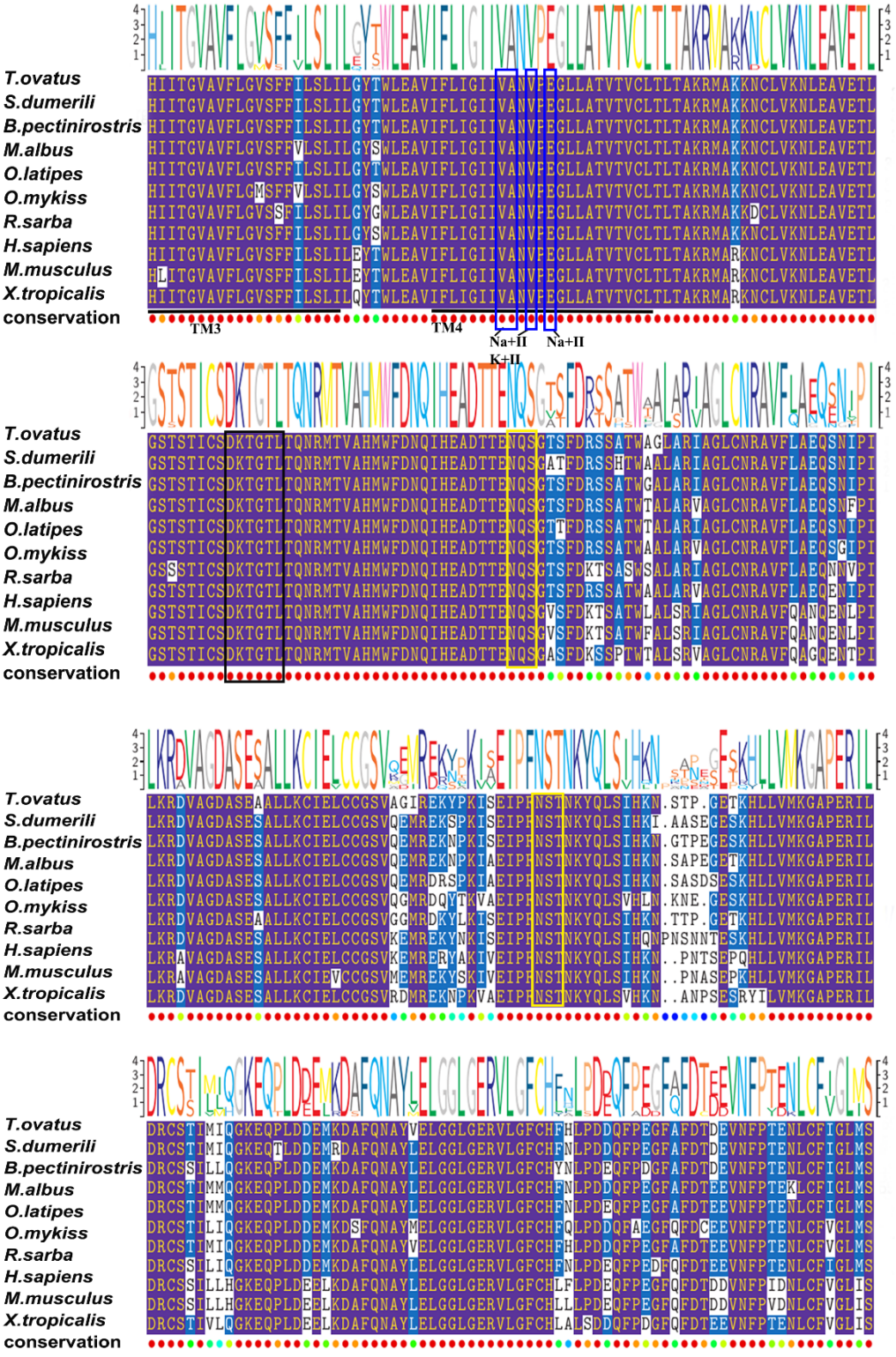
S.dumerili
B.pectinirostris
M.albus
O.latipes
O.mykiss
R.sarba
H.sapiens
M.musculus
X.tropicalis
conservation
TM1
TM2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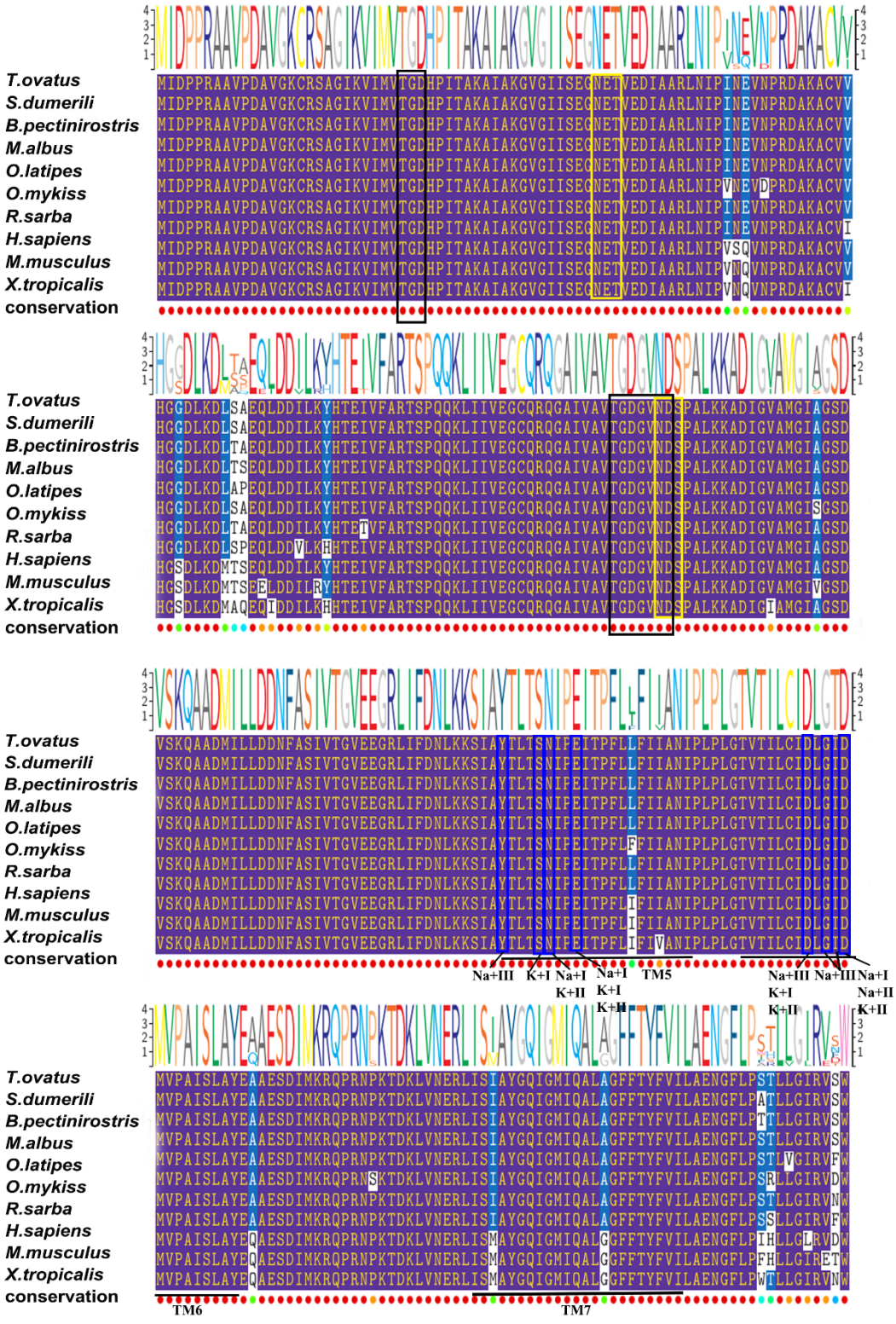
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conservation
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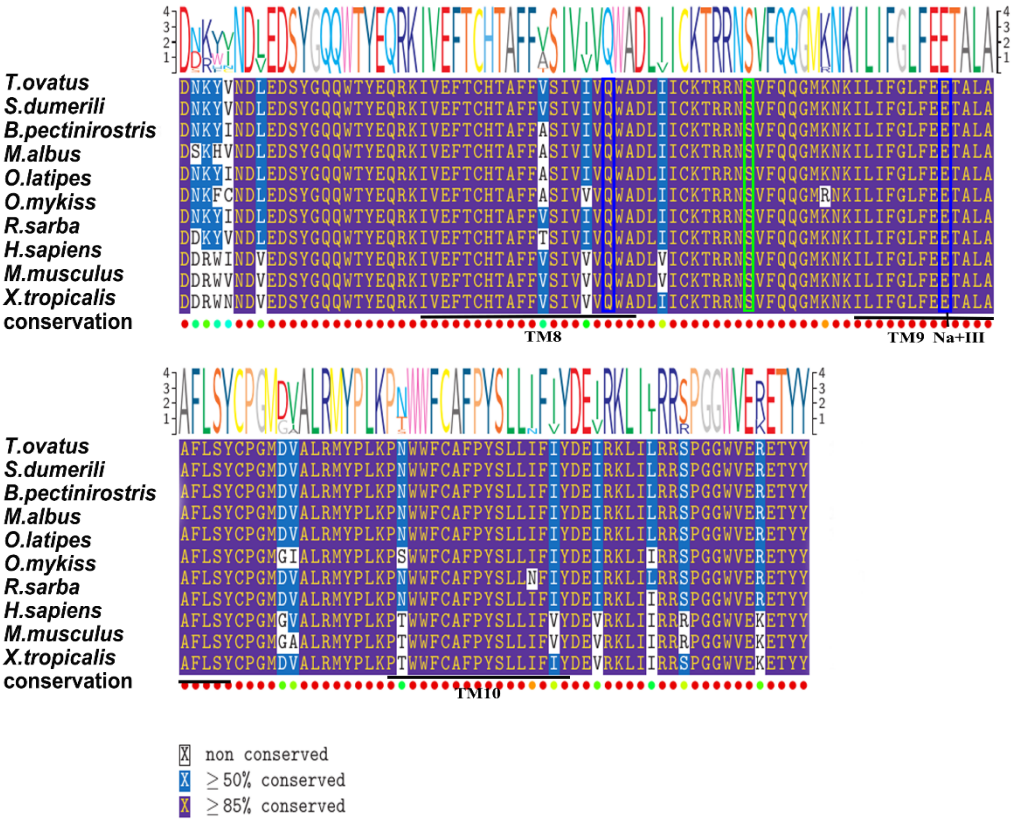

Figure 3 Comparison of deduced amino acid sequences of golden pompano *T. ovatus nka α1* with other published *NKA α1*. Identical or strongly similar amino acids were indicated by shaded residues. The ten predicted transmembrane regions (TM1-TM10) were underlined and in bold. Vertical blue boxes represent coordinating residues for Na^+ or K^+ binding. The conserved regions containing the TGES, DKTGTLT, TGD and TGDGVND sequence motifs were indicated with black boxes. The red marks denoted the amino acid residues phosphorylated by PKA. The green mark denoted the amino acid residues phosphorylated by PKC. Glycosylation sites were yellow boxes.

The deduced amino acid sequences of *Tonka α1* were particularly conserved compared with mammals, amphibians and teleost species (**Table 2**), and showed 98% identity with Seriola dumerili. To better discern the phylogenetic relationships, a phylogenetic tree was constructed

based on the deduced amino acid sequence of *Tonka α1* and those of other species using the NJ method (**Table 3**). The phylogenetic tree could be divided into three clusters (**Figure 4**). The NKA α1, NKA α2, and NKA α3 proteins clustered into one group, *Tonka α1* clustered into the NKA α1 group.

Table 3 The sequences used for reconstruction of phylogenetic relationship.

No	Class	Species	Gene	Accession number
1	Osteichthyes	*Seriola dumerili*	*NKA α1*	XP_022617258.1
2	Osteichthyes	*Seriola dumerili*	*NKA α2*	XP_022624971.1
3	Osteichthyes	*Seriola dumerili*	*NKA α3*	XP_022599779.1
4	Osteichthyes	*Monopterus albus*	*NKA α1*	XP_020476198.1
5	Osteichthyes	*Monopterus albus*	*NKA α2*	XP_020480349.1
6	Osteichthyes	*Boleophthalmus pectinirostris*	*NKA α1*	XP_020792240.1
7	Osteichthyes	*Boleophthalmus pectinirostris*	*NKA α3*	XP_020796248.1
8	Osteichthyes	*Rhabdosargus sarba*	*NKA α1*	AAT48993.1
9	Osteichthyes	*Fundulus heteroclitus*	*NKA α2*	NP_001296928.1
10	Osteichthyes	*Oncorhynchus mykiss*	*NKA α1*	XP_021426673.1
11	Osteichthyes	*Oncorhynchus mykiss*	*NKA α2*	NP_001117930.1
12	Osteichthyes	*Oryzias latipes*	*NKA α2*	XP_004078621.1
13	Osteichthyes	*Oryzias latipes*	*NKA α3*	XP_004074116.1
14	Osteichthyes	*Astyanax mexicanus*	*NKA α3*	XP_015461719.2
15	Osteichthyes	*Danio rerio*	*NKA α1*	NP_571761.1
16	Osteichthyes	*Danio rerio*	*NKA α2*	NP_571758.1
17	Osteichthyes	*Danio rerio*	*NKA α3*	NP_571760.2
18	Amphibian	*Xenopus tropicalis*	*NKA α1*	NP_989407.1
19	Amphibian	*Xenopus tropicalis*	*NKA α2*	NP_001083112.1
20	Amphibian	*Xenopus tropicalis*	*NKA α3*	NP_001120366.1
21	Mammalia	*Homo sapiens*	*NKA α1*	NP_000692.2
22	Mammalia	*Homo sapiens*	*NKA α2*	NP_000693.1
23	Mammalia	*Mus musculus*	*NKA α1*	NP_659149.1
24	Mammalia	*Mus musculus*	*NKA α2*	NP_848492.1
25	Mammalia	*Mus musculus*	*NKA α3*	NP_001277398.1

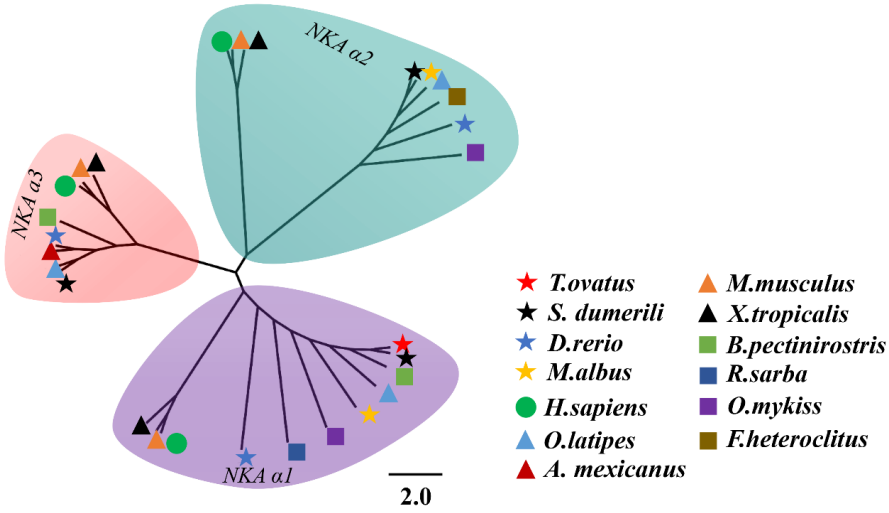

Figure 4 The Neighbor-Joining phylogenetic tree of *NKA* alpha isoform based on their amino acid sequences. The confidence in each node was assessed by 1000 bootstrap replicates with MEGA 7.0. Red star represented golden pompano.

Spatial and temporal expression patterns of Tonka α1 mRNA

The spatial expression patterns of *Tonka α1* mRNA were determined by qRT-PCR with ef-1α as the internal control (**Figure 5**). *Tonka α1* was ubiquitously distributed in all examined tissues and the expression levels varied among the different tissues. The expression level in liver (lowest) was defined as control; the higher expression levels of *Tonka α1* mRNA were detected in the brain, gills, kidney, stomach and intestine. The lower expression levels were detected in spleen and muscle.

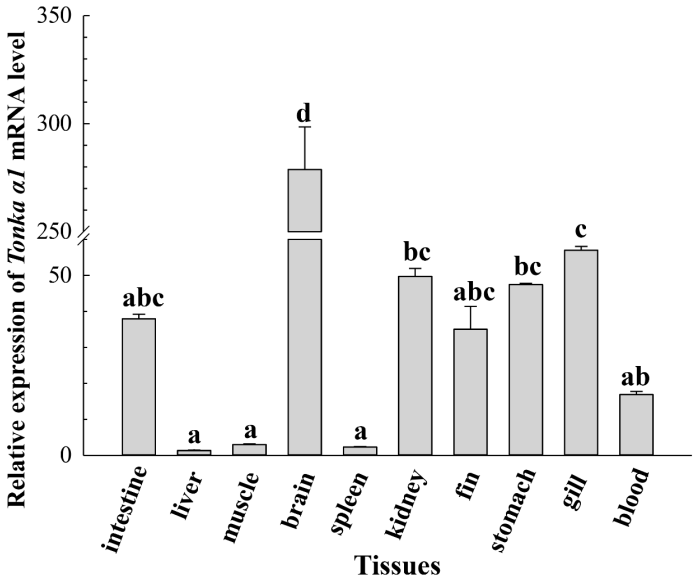

Figure 5 *Tonka α1* transcriptions in various tissues of golden pompano *T. ovatus*. Significant differences at $P<0.05$ are labeled with different letters, mean ± SE (n=3) of each mRNA quantity was shown for each detected tissue.

Temporal profiles of *Tonka α1* mRNA were detected in the osmoregulatory organs (gills, kidney and intestine) of *T. ovatus* under the acute salinity stress (**Figure 6**). *Tonka α1* mRNA first

increased and then decreased in gills and kidney of *T. ovatus* transferred to 0‰. In the gills, the expression of *Tonka a1* mRNA was significantly upregulated at 2 h in three salinity treatment groups of 10‰, 20‰ and 40‰. After the 10‰ challenge, *Tonka a1* mRNA expression was dramatically changed compared with that of the control (0 h) except at 96 h (*T. ovatus*). By contrast, when transferred to 20‰, the expression of *Tonka a1* mRNA was similar to that of the control (0 h), except at 2 and 48 h. After the 40‰ challenge, the highest expression level of *Tonka a1* mRNA was observed at 8 h. In the kidney, the expression of *Tonka a1* mRNA showed a tendency to first increase and then decrease in the three experimental groups of 10‰, 20‰ and 40‰, with corresponding peak values observed at 4 and 8 h. In the intestine, a significant upregulation in the expression of *Tonka a1* mRNA was detected at 2 and 4 h in those fish transferred to 0‰. In those transferred to 10‰, *Tonka a1* mRNA expression peaked at 4 h and was rather stable at 48 and 96 h. The expression levels of *Tonka a1* mRNA in 20‰ were not unlike those of 10‰, with peak values observed at 4 h. Interestingly, relative to the control, *Tonka a1* expression was not significantly changed in fish in 40‰, except at 2 and 12 h.

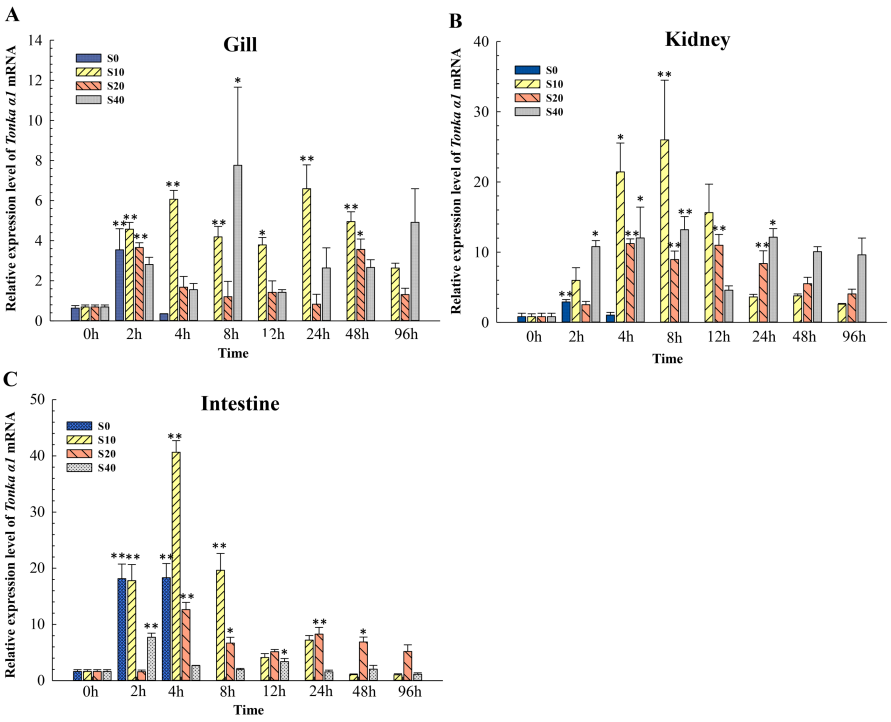

Figure 6 Temporal expression of *Tonka a1* in gill (A), kidney (B) and intestine (C) after acute salinity stress for the indicated time points. *ef-1a* expression was used as an internal control for qRT-PCR. Significant differences at $P<0.05$ were labeled with "*", $P<0.01$ were labeled with "**", means ± SE of (n=3) each mRNA quantity was showed for each detected tissue. The untreated (0 h) group was used as a control.

NKA enzyme activity

Branchial NKA enzyme activity was significantly affected by acute salinity stress. The NKA enzyme activities of fish in four treatment salinity groups were evidently higher at 2 and 4 h than at other time points. After 8 h, the NKA enzyme activity decreased in the 10‰, 20‰ and 40‰ groups, though in the 10‰ and 40‰ groups, it still was higher than that of the control (0 h). Finally, compared with the 10‰ and 40‰ groups, the NKA enzyme activity of 20‰ group was lower (**Figure 7**).

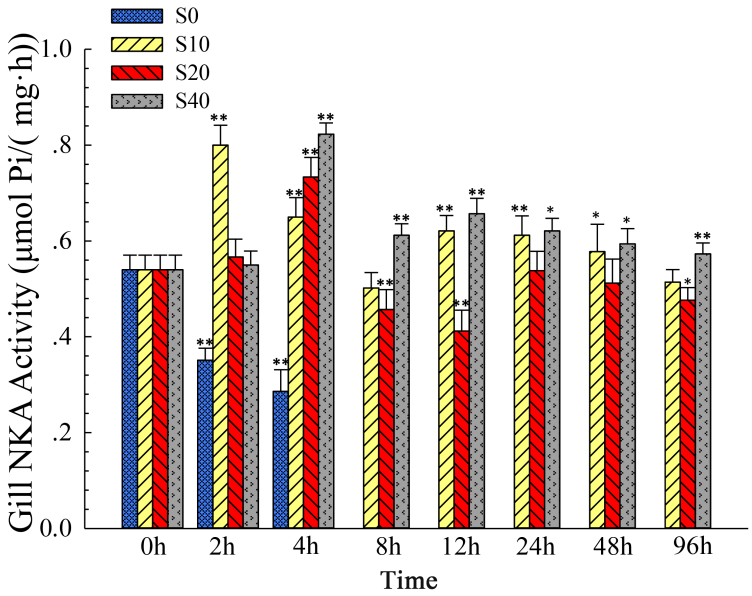

Figure 7 NKA activity. Significant differences at $P<0.05$ were labeled with *, Significant differences at $P<0.01$ were labeled with **, mean ± SE (n=3) of each quantity was showed for each group detected. The untreated (0 h) group was used as a control.

Discussion

Na^+ and Cl− are the two important blood electrolytes that play a crucial role in osmoregulation (Kaneko et al., 2008). When fish were transferred from seawater (30‰) to hypoosmotic salinity stress, both the serum osmolality and Na^+ and Cl^- concentrations of *T. ovatus* showed a similar decreasing trend that began at 2 h. But after 8 h, this trend reversed and eventually stabilized. This result has been documented for many species, such as *Acipenser medirostris* (Sardella and K ültz, 2009), *Salmo salar* (Bystriansky and Schulte, 2011), *Galaxias maculatus (*Urbina et al., 2013*)* and *Sciaenops ocellatus* (Watson et al., 2014). When suddenly transferred to hypoosmotic environments, teleosts experience both crisis and regulatory phases (Yang et al., 2016). In the first 8 h, *T. ovatus* was clearly in crisis, especially in the 0‰ group, with zero survival rate. Upon incurring hypertonic salinity stress, the serum osmolality and Na^+ and Cl^- concentrations of *T. ovatus* did not increase as previously reported (Allen et al., 2015; Lin and Lee, 2016; Shirangi et al., 2016). Instead, they followed a pattern similar to that when transferred to hypotonic stress. This result might be related to the stress and salinity tolerances of different fish species (Chandrasekar et al., 2014). Our results suggest that *T. ovatus* might maintains its osmotic balance in water with a salt content ranging from 10‰ to 40‰ through effective osmoregulatory mechanisms, but such capacity could not overcome the physiological impact of 0‰ .

To further verify the molecular responses to salinity changes in *T. ovatus*, its complete cDNA sequences of NKA α1 were characterized here for the first time. The ToNKA α1 gene-encoded 1024 amino acids and had high homology with other teleosts, thus suggesting that it might have a conserved function in influencing osmoregulatory endurance in fish. From the protein structure analysis, we found several conserved domains and classical motifs features: an E1-E2-ATPase domain that is the cleavage domain of ToNKA α1; it utilizes the energy of ATP hydrolysis to transport ions and phospholipids across the membrane (Cutler et al., 1995). It also contains the TGES-loop critical for metal ion binding, allowing the A and P (phosphorylation) domains to bind tighter to stabilize the E2P form (Bublitz et al., 2010). Furthermore, this domain had a regulatory effect on the phosphoenzyme processing steps by its binding to nucleotides. Highly conserved motifs were also identified, including those of DKTGTLT, TGD and TGDGVND. The phosphorylation site was located in the DKTGTLT motif, which characterizes all P-type ATPases. This conservative motif played an important role in the conformation of K^+ binding (Horisberger et al., 1991). In addition, the TGD and TGDGVND motifs, which were involved in the $Mg2^+$ coordination, were associated with ATP binding at the phosphorylation site and stabilization of the phosphoenzyme intermediates (Horisberger et al., 1991). Both Na^+ and K^+ binding sites were confirmed in the transmembrane domains, thus enabling the release of one type of cation to be coordinated with

the binding of another cation (Ip et al., 2013). Our results show that these coordinating residues were conserved in NKA α1 compared to other teleosts (Ching et al., 2015). Several studies demonstrate that phosphorylation and dephosphorylation lead to changes in the conformation of NKA, releasing Na^+ ions and absorbing K^+ ions (Ip et al., 2013). Both cAMP-dependent PKA and PKC participated in the phosphorylation of NKA. The predicted site of cAMP-dependent PKA phosphorylation was the Ser residue at position 944 (**Figure 3**) and it was conservative in all NKA α1. Thr15 and Ser16, which were PKC phosphorylation sites, were present in NKA α1 polypeptides yet have different affinities for Na^+ and K^+ because of their different phosphorylation abilities. Therefore, these conserved domains and motifs play a crucial role in osmoregulation by altering the conformation of the ToNKA α1, via phosphorylation and dephosphorylation, to control the uptake and excretion of ions.

In this study, our qRT-PCR analyses revealed that ToNKA α1 was ubiquitously distributed in all the examined fish tissues of *T. ovatus*. In addition, high transcription levels were detected in key osmoregulatory organs: gills, kidney and intestine. The expression pattern was similar to *S. senegalensis* (Armesto et al., 2014) and *D. labrax* (Blondeaubidet et al., 2016), and indicated that ToNKA α1 might have an important function in these organs. Gills, being osmoregulatory organs with direct contact with the aquatic environment, are highly sensitive to changes in salinity. Similarly, the kidney organ performs dual functions of mitigating stress and osmoregulation. In hypotonic conditions, the primary kidney function is to excrete excess water and reabsorb the filtered solutes. Conversely, in a hypertonic environment, teleosts would lose water and their ion concentration rises, so an intake of seawater was needed to prevent dehydration (Katoh et al., 2006). The intestine was the main site for digestion and absorption of nutrients, water and ion exchange, and was a vital organ for maintaining the balance between water and salt (Li et al., 2014). The functional realization of these three osmoregulatory organs required the participation of NKA. The results of our experiment showed that *Tonka α1* in the osmoregulatory organ (gills, kidney and intestine) responded rapidly to the imposed salinity changes, its expression in the four salinity treatment groups markedly upregulated, peaking, and then declining. Similar expression patterns were also reported in *S. salar* (Bystriansky and Schulte, 2011) and *D. labrax* (Blondeaubidet et al., 2016). Combined with physiological parameters and molecular responses analysis, when the salinity of the aquatic environment changes abruptly, there is a transient imbalance in the osmotic balance of the fish. Therefore, the activity of NKA α1 was initiated and its expression level was accordingly increased to maintain the cells' morphology and ion balance. The differing expression levels of *Tonka α1* in gill, intestine and kidney organs of *T. ovatus* might be related to their characteristics and response times, and might also be affected by hormones.

NKA is an omnipresent membrane-bound enzyme that can via the hydrolysis ATP to obtain the power to creating ionic and electronic gradients and providing the primary driving force for ion (Na^+/K^+) secretion and absorption in mitochondria-rich cells. Changes in environmental salinity usually changed the NKA activity as well; hence, NKA activity had often been used as a critical indicator to gauge and infer fish osmoregulation ability (Yang et al., 2016). In teleosts, two different patterns of NKA activities were reported in response to changes in salinity: linear and "U-shaped" relationships. For the "U-shaped" relationship, the lowest activity of the gill enzyme occured when the salinity of the medium was close to or slightly above that of the blood (Liu et al., 2019). Some teleosts species showed upregulation in NKA activity when acclimated to hypoosmotic conditions (Lin et al., 2003; Hu et al., 2017). In contrast to these cases, several teleost fishes responded to salinity challenges with higher NKA activities under hypertonic conditions, such as *G. maculatus* (Urbina et al., 2013), *Etroplus suratensis* (Chandrasekar et al., 2014), *A. persicus* (Shirangi et al., 2016) and *Tetraodon nigroviridis* (Lin and Lee, 2016). In our study, gill NKA activity of *T. ovatus* was affected after acute stress. In the 10‰ and 40‰ groups, their NKA activity was greater than in the control group. Interestingly, the 20‰ group had a lower NKA activity than the 10‰ and 40‰ groups. This result, which was consistent with the findings of previous studies (Freire et al., 2008), indicated that when the salinity suddenly changes, *T. ovatus* responded with stress and compensatory processes. In our study, *T. ovatus* was obviously stimulated by such changes, as their NKA activity was increased, thus enabling them to accommodate their new salinity environment better. However, osmoregulation is a complex physiological process, one that also consumes much energy. Hence, teleost fish generally have the lowest activity of NKA near the isosmotic site, because osmotic adjustments consume less energy, which can be used for growth (Yang et al., 2016). Therefore, more accurately reflect physiological changes under the attendant conditions of changing salinity.

This study examined the physiological and molecular responses to acute salinity changes in the marine fish *T. ovatus*. We determined its serum osmolality, Na^+ and Cl^- concentrations, NKA activity and the gene expression of *nka α1*. The results suggeste *T. ovatus* have a great ability to tolerate salinity and could efficiently alter its osmoregulatory mechanism to maintain homeostasis.

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