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# Identification, expression analysis, and functional characterization of *motilin* and its receptor in spotted sea bass (*Lateolabrax maculatus*)

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ABSTRACT

Motilin (MLN), an interdigestive hormone secreted by endocrine cells of the intestinal mucosa, binds to a G protein-coupled receptor to exert its biological function of regulating gastrointestinal motility. In the present study, we identified the *prepromotilin* and *mln receptor* (*mlnr*) from the spotted sea bass, *Lateolabrax maculatus*. *Mln* consisted of an ORF of 336 nucleotides encoding 111 amino acids. The precursor protein contained a 17-amino-acid mature peptide. *Mlnr* had an ORF of 1089 bp encoding a protein of 362 amino acids. Seven transmembrane domains were predicted with TMHMM analysis. The phylogenetic analysis of *mln* and *mlnr* showed that they fell into the same clade with respective counterpart of selected fishes before clustering with other detected vertebrates. Both *mln* and *mlnr* genes were highly expressed in intestine of spotted sea bass using quantitative real-time PCR. In situ hybridization indicated that *mln* and *mlnr* mRNA were both localized in the lamina propria and the epithelial cell of intestinal villus. The expressions of both genes were regulated under short-term starvation in a time-dependent manner. *In vitro* experiments indicated that the expressions of *ghrelin* (*ghrl*), *gastrin* (*gas*) and *cholecystokinin* (*cck*) were enhanced by MLN after 3-h treatment, but the effect was absent after 6 or 12-h incubation. Taken together, the MLN and its receptor might play important roles in regulating intestinal motility in spotted sea bass.

## 1. Introduction

Motilin (MLN), a 22 amino acid peptide, was first isolated and sequenced from porcine gut in 1971 (Brown et al., 1971). Until now, MLN has been identified in several vertebrates, including human (*Homo sapiens*) (Seino et al., 1987), monkey (*Macaca mulatta tcheliensis*) (Huang et al., 1998), sheep (*Ovis aries*) (De Clercq et al., 1997), rabbit (*Oryctolagus cuniculus*) (Depoortere et al., 1997), and quail (*Coturnix coturnix*) (Apu et al., 2016). It is known to play important roles in the regulation of interdigestive motility, gallbladder contractions, and enzyme secretion in the stomach and pancreas (Layer et al., 1988). For example, physiological experiments of human (You et al., 1980) and dog (Poitras, 1984) showed that MLN mainly regulates gastrointestinal motility in the fasting state. It is noteworthy that MLN can also slightly stimulate the release of growth hormone (Samson et al., 1984). In 1999, an unknown G protein-coupled receptor, GPR38, was identified as the specific receptor for MLN, and was re-named as MLNR. MLNR has seven transmembranes with high affinity for MLN (Samson et al., 1984), suggesting that endogenous MLN exerts all or most of its activity via this receptor. The *mlnr* cDNA sequences have been cloned from several mammalian species, such as human (Feighner et al., 1999), dog (Ohshiro et al., 2008), suncus (Suzuki et al., 2012) and rabbit (Dass et al., 2010). In teleost, *mlnr* was first identified from zebrafish, and it was found to mediate the effects of mammalian MLNs on the contractile activity were examined in zebrafish gastrointestinal tract (Olsson et al., 2008). On the other hand, the functional significance of MLN/MLNR system in the regulation of zebrafish gastrointestinal motility has also been examined (Kitazawa et al., 2017).

In recent decades, gastrointestinal hormones such as *ghrelin* (*ghrl*), *gastrin* (*gas*) and *cholecystokinin* (*cck*) have been a central place in the complex neuroendocrine interactions that became the basis of the regulation of gastrointestinal motility (Gué and Buéno, 1996). *Ghrelin* (*ghrl*), a 28-amino acid peptide, synthesised in the upper gastrointestinal tract, was found to have function on regulating gastrointestinal motility (Ohno et al., 2010). *Gastrin* (*gas*), a peptide hormon, it was also widely presented in the digestive tract and the main biological activity was stimulating gastrointestinal motility, gastrointestinal mucosal growth and delaying gastric emptying (Misiewicz et al., 1969).

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**Table 1**
Primers used for quantitative RT-PCR of hormone genes and their receptors.

| Primer name | Primer sequence 5′–3′ |
|---|---|
| *mln-clone*-F | AGCGTGGTGAGCTTATTGC |
| *mln-clone*-R | TCTTTGGCGTTCTCGTTAT |
| *mlnr-clone*-F | ATGCACTGGGCCAGACCT |
| *mlnr-clone*-R | CCAGTCAGGCTTTCGTTCA |
| *mln*-F | TGCTGATGAAGGAGCGAGAA |
| *mln*-R | TCCACCATGTTCCACCTGAG |
| *mln* probe -F | CGCATTTAGGTGACACTATAGAAGCGAGAGCAGTGGCTGGTTGT |
| *mln* probe-R | CCGTAATACGACTCACTATAGGGAGACACGTTGCTCCACTATTTCG |
| *mlnr* probe -F | CGCATTTAGGTGACACTATAGAAGCGCCGCTTCTATCACTACATCTTT |
| *mlnr* probe-R | CCGTAATACGACTCACTATAGGGAGACATTTCCACAACTTGCACCC |
| *mlnr*-F | ATCATCCAGCACTTCAAGGA |
| *mlnr*-R | CAGGCGGTAGAGGTCAAA |
| *ghrl*-F | ACACCTGTTTGCTGGTCTTTC |
| *ghrl*-R | ATGTGATGTGGTTGGCCTCTG |
| *gas*-F | TGCTAAGAGGGAGAAACTG |
| *gas*-R | TATCTCGCGTTCATCGTC |
| *cck*-F | TGCCAACTACAACCAACCT |
| *cck*-R | GCGTCGTCCAAAGTCCAT |
| *18s*-F | GGGTCCGAAGCGTTTACT |
| *18s*-R | TCACCTCTAGCGGCACAA |

*Cholecystokinin* (*cck*) as a gastrointestinal hormone that took a role of gall bladder contraction was discovered (Ivy and Oldberg, 1928). *Mln* has been suggested as a hormone that stimulated gastrointestinal motility in a manner similar to gastrointestinal peptides, such as *ghrelin*, *gastrin* and *cholesystokinin* (Inui et al., 2004; Poitras and Tomasetto, 2009).

In aquaculture, understanding the energy absorption and consumption is important to develop special feeding program for the culturing species. The spotted sea bass (*Lateolab*rax *maculatus*), an euryhaline fish widely cultured in China, is welcomed by consumers due to its delicious taste and high nutritional value (Wang et al., 2017). The production of spotted sea bass is over 200 thousand tons per year in China. The high production always causes the decrease of prices in the harvest season. As a result, farmers preferred a short-term starvation every couple week to delay the on-market time point for a better price. However, this will lead to another problem, the slow recondition of digestive tract motility, as well as the lower efficiency of feed utilization. In several previous studies, the motilin was described as a peptide, which was able to increase the motility of intestine motility. On the other hand, base on the function of motilin, agonist derived by the pharmological research would be used in the inducing digestive tract motility. Taken together, in the present study, we chose to primarily test the function of this peptide to show whether it has similar function compared with other species. In this study, we cloned cDNA of *mln* and its receptor *mlnr*, determined their location and tested the variation of their expression after short-term starvation in spotted sea bass. To evaluate the function of *mln*, we predicted the docking effect between *mln* and *mlnr*. In addition, we measured mRNA expressions of *ghrelin* (*ghrl*), *gastrin* (*gas*) and *cholecystokinin* (*cck*) after *mln* stimulation to the in vitro cultured intestine fragments.

## 2. Materials and methods

### 2.1. Animals and short-term starvation

Spotted sea bass were collected from Shuangying Aquatic Breeding Factory of Lijin, Shandong. Before the experiment, 100 healthy spotted sea bass (weighing 100.0 ± 5.00 g) were kept in the indoor cement pool for 2 weeks to adapt to the environment. They were fed twice per day to satiety at 9:00am and 5:00 pm, with 1/3 of water exchanged every day. A short-term fasting experiment included sampling at 0, 1, 6, 12, 24, 48 and 72 h following a meal, with 0 h serving as the control group. Three fish were randomly sampled at different times of fasting and quickly anesthetized with MS-222 (200 mg/L). All animal experiments were conducted in accordance with the national guideline and approved by Animal Research and Ethics Committee of Ocean University of China (Permit Number: 20141201).

### 2.2. Cloning and sequence analysis of mln and mlnr

To identify *mln* and *mlnr* in spotted sea bass from the transcriptomic database (GFDU00000000) (Zhang et al., 2017) and the whole genome database (unpublished data), the amino acid sequences of MLN and MLNR in human and zebrafish were used as query sequences for TBLASTN analysis, all with a cutoff E-value of 1e-5. Human, zebrafish, rat, rabbit, chicken, damselfish, large yellow croaker, gorilla, common mallard, sheep, cow, dolphin, wild boar, dog, domestic cat, African elephant, small-eared bushbabies, thirteen-lined ground squirrels, goat, fugu, medaka sequences were download from NCBI (http://www.ncbi.nlm.nih.gov/) and Ensembl genome databases (http://www.ensembl.org). Then, ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict coding sequences. The signal peptide and the neuropeptide prohormone cleavage sites were predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and Neuropred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py), respectively. Putative transmembrane domains were predicted with the program TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Phylogenetic analysis was conducted using amino acid sequences of MLN and MLNR in spotted sea bass and selected vertebrate species retrieved from NCBI or Ensembl genome databases. Multiple sequences alignment was performed using DNAMAN. The phylogenetic trees were constructed using MEGA 6 software (Tamura et al., 2013).

### 2.3. Molecular docking of mln peptide to its receptor

The docking of MLN and its receptor were carried out using Auto Dock (Guedes et al., 2014). First, the optimal protein structure models of MLN and MLNR were acquired by SWISS-MODEL (https://www.swissmodel.expasy.org/). All the torsion angles in the small-molecules were set free to execute flexible docking. Polar hydrogen was added using the Hydrogen module in AutoDock Tools for MLNR. Then, the empirical free energy function and Lamarckian genetic algorithm (LGA) were used to model MLN binding to its receptor MLNR. All LGA settings were kept to their default values, apart from the number of energy evaluations and the number of generations which were set to 250,000 and 27000, respectively. Results were clustered according to the root-

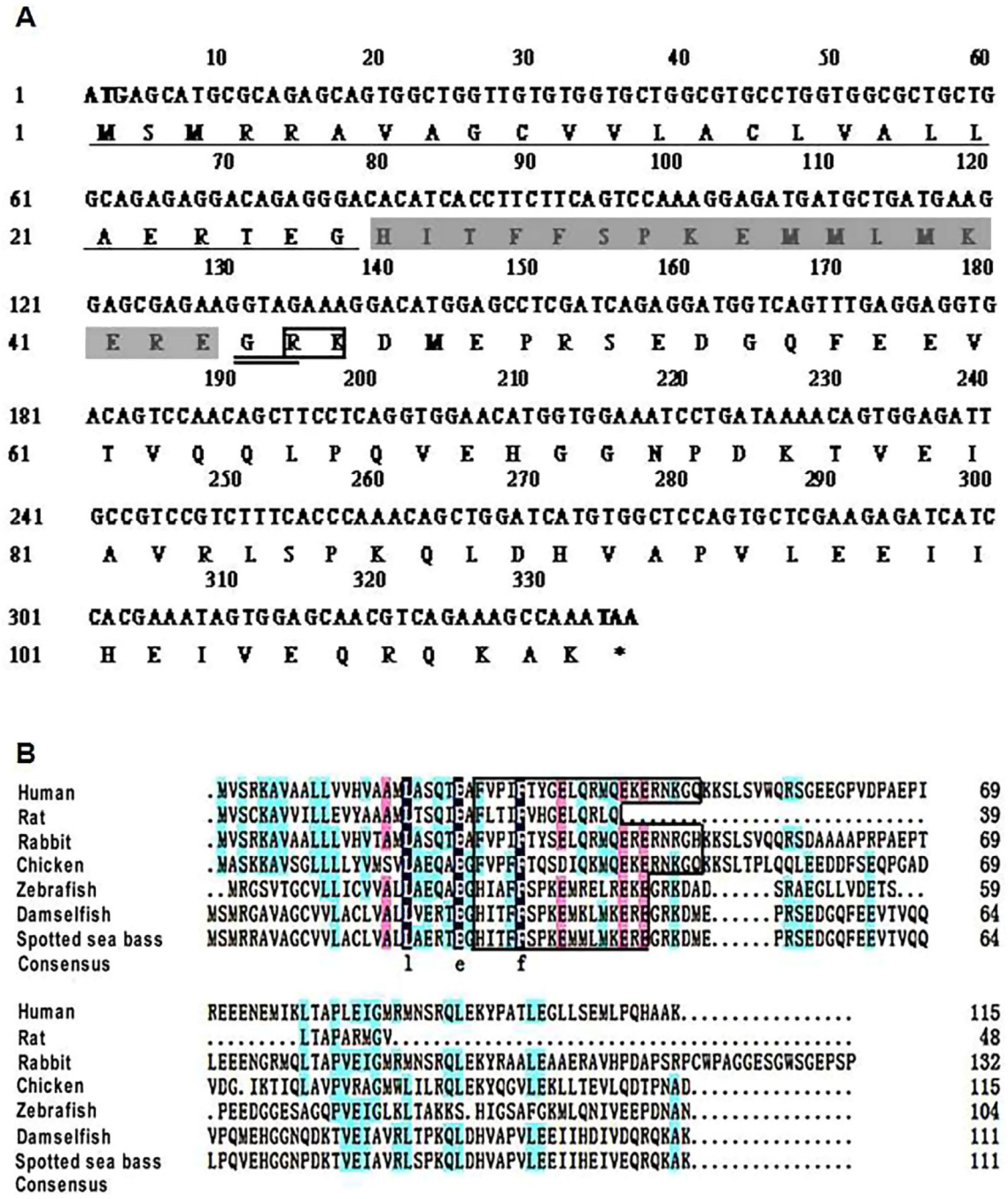


**Fig. 1.** Sequence analysis of *mln* in spotted sea bass. (A) The nucleotide sequences and the deduced amino acid sequences of *mln* in spotted sea bass. The start codon and stop codon are shown in bold front. Signal peptide amino acid sequences are underlined. The 17 amino acids of the mature *mln* peptide are shaded. Typical amidation signal (GR) is double-underlined and the putative cleavage sites are boxed. (B) Comparison of amino acid sequences of spotted sea bass with some vertebrates including human (AAA59860.1), rat (BAF85821.1), rabbit (CAA45342.1), chicken (NP_001292058.1), zebrafish (XP_002665930.1), damselfish (XP_008278729.1) and spotted sea bass (MH046054). The box letters indicated the sequence of mature *mln* peptide in detected species.

mean square deviation (RMSD) criterion. The structure figures were prepared with PyMol.

### 2.4. Quantitative real-time PCR

Samples including gonad, intestine, liver, brain, spleen, skin, gill, kidney, head kidney, stomach, heart, pituitary, muslce and fin, were collected and placed into DEPC-treated centrifuge tube, snap frozen and stored at −80 °C. RNA was extracted using RNAiso Plus reagent (Takara, Otsu, Japan) according to the manufacturer's instructions. The quantification and purity of total RNA were assessed using a Biodropsis BD-1000 Nucleic Acid Analyzer (OSTC, Beijing) as well as by 1.5% agarose electrophoresis. After extraction, RNA was reverse transcribed into cDNA by using the PrimeScript™ RT reagent kit (Takara) for the quantitative real-time PCR reaction following manufacturer's guidelines.

StepOne Plus Real-Time PCR system (Applied Bio Systems) was used to detect *mln* and *mlnr* mRNA expression by quantitative real-time PCR used the to detect mRNA expression. We also analyzed the mRNA expression of gastrointestinal feeding related genes (*ghrl*, *gas*, *cck*) after different times (3, 6, 12 h) and concentrations ($10^{-6}$, $10^{-7}$, $10^{-8}$ M) of MLN treatment. The specific primers used in quantitative real-time RT-

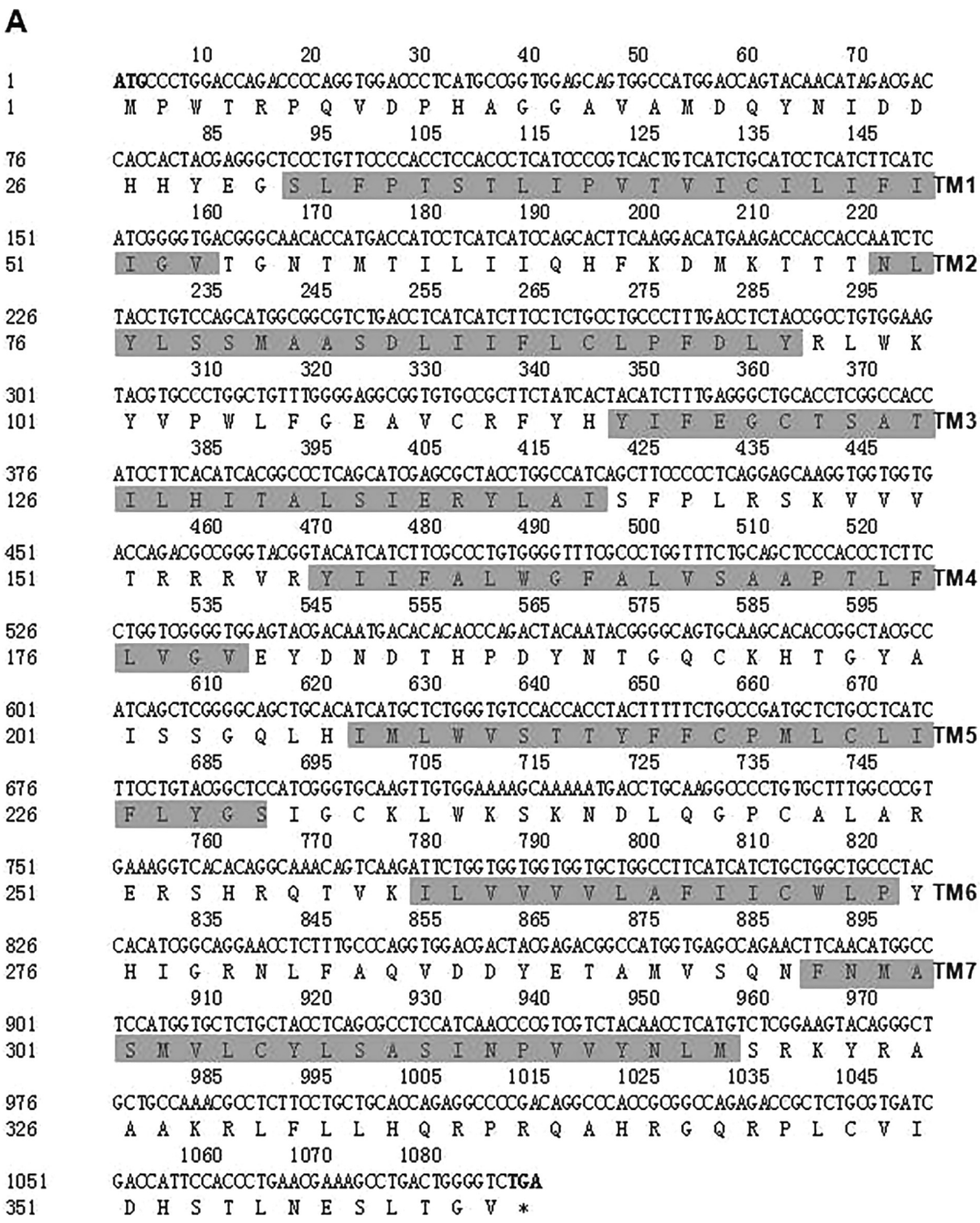


**Fig. 2.** Sequence analysis of *mlnr* in spotted sea bass. (A) The nucleotide sequence and the deduced amino acid sequences of spotted sea bass *mlnr*. The transmembrane domains of *mlnr* are numbered on the right side of figure and the sequence composition of them are shaped. (B) Comparison of amino acid sequences of *mlnr* from different species, including human (AAC26081.1), rat (NP_852029.3), rabbit (NP_001093437.1), chicken (NP_001120785.1), zebrafish (ENSDARP00000106018), damselfish (XP_008288581.1), large yellow croaker (XP_010741665.1) and spotted sea bass (MH046057).

PCR were listed in Table 1. The 18S rRNA was used as the reference gene to normalize the gene expression based on the previous studies of spotted sea bass gene expression (Wang et al., 2018). Three biological replicate RNA samples in gene expression profiles. Each qRT-PCR reaction consisted of a total volume of 20 μl containing 10 μl SYBR®FAST qPCR Master Mix (2×), 2 μl sample cDNA, 6.8 μl of nuclease-free water, 0.4 μl of each positive/negative primer and 0.4 μl Rox. Melting curve analysis for the genes qPCR assay showed a single peak, confirming specificity of PCRs. For the expression analysis of spotted sea bass tissues, Ct values of each gene in various tissues were measured, and 18S rRNA was used as reference for normalization of the relative expression of these genes. The relative mRNA expression levels of genes were calculated using the comparative $2^{-\Delta\Delta CT}$ method.

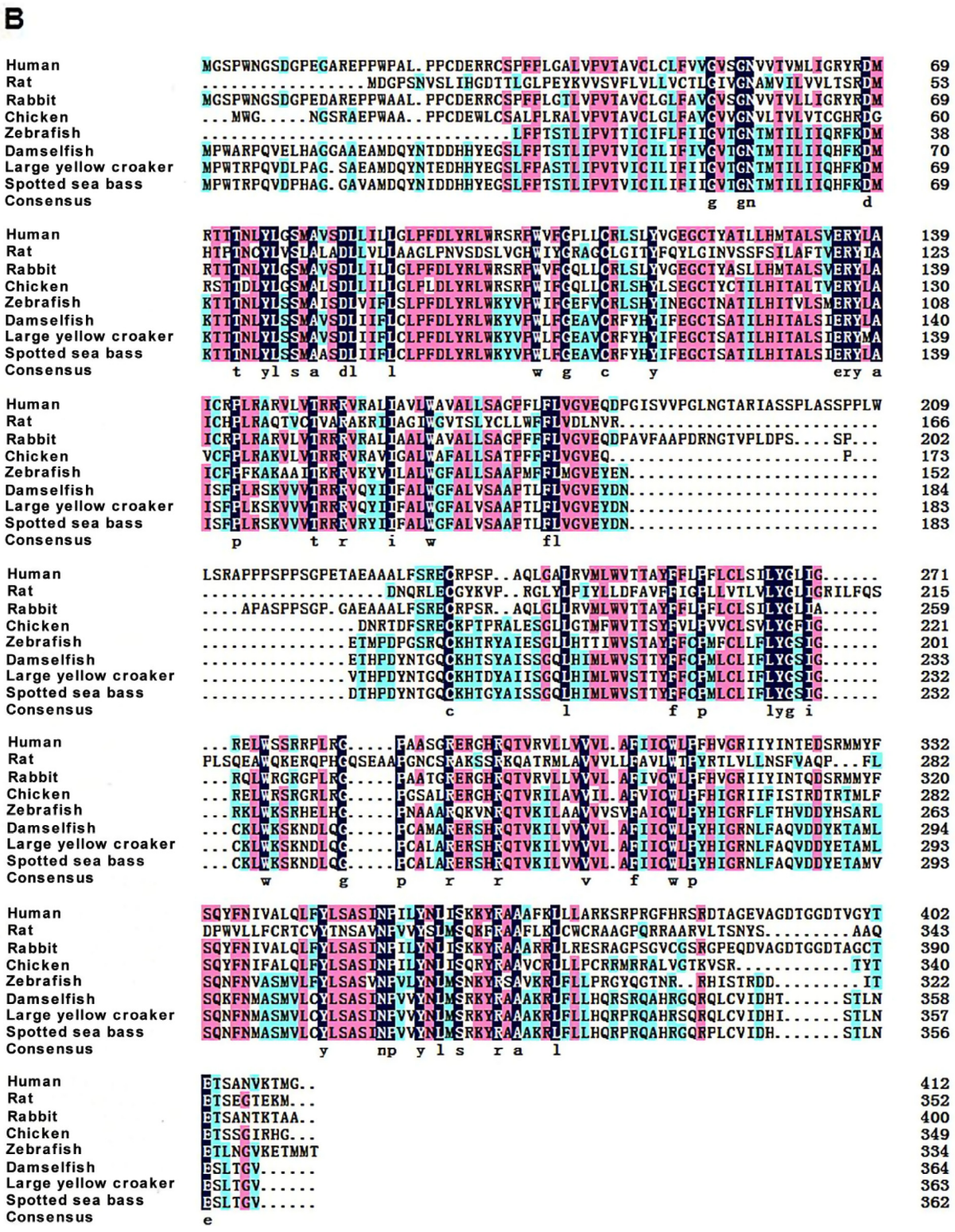


**Fig. 2.** (*continued*)

### 2.5. *In situ hybridization (ISH) of mln and mlnr in spotted sea bass intestine*

Intestine of a spotted sea bass was fixed in buffered 4% paraformaldehyde for 12 h and then dehydrated using a series of graded ethanol solutions (70–100%) and cleared in xylene and embed in paraffin. Seven micron intestine sections were cut for ISH. The primers for probe preparation were listed in Table 1. Sense and antisense digoxigenin (DIG)-labeled riboprobes were synthesized from ORF sequence of sea bass *mln* and *mlnr* using DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). DIG-labeled in situ hybridization was performed as described previously with slight modification (Parhar et al., 2004). Briefly, sections were cleared in xylenere and hydrated using a series of graded ethanol solution (100–70%), permeabilized with 0.1 M HCl for 10 min followed by proteinase K (10 ng/ul) digestion for 20 min at 37 °C, washed in 2 × standard saline citrate (SSC) for 10 min and prehybridized at 55 °C for 1 h. Hybridization was carried

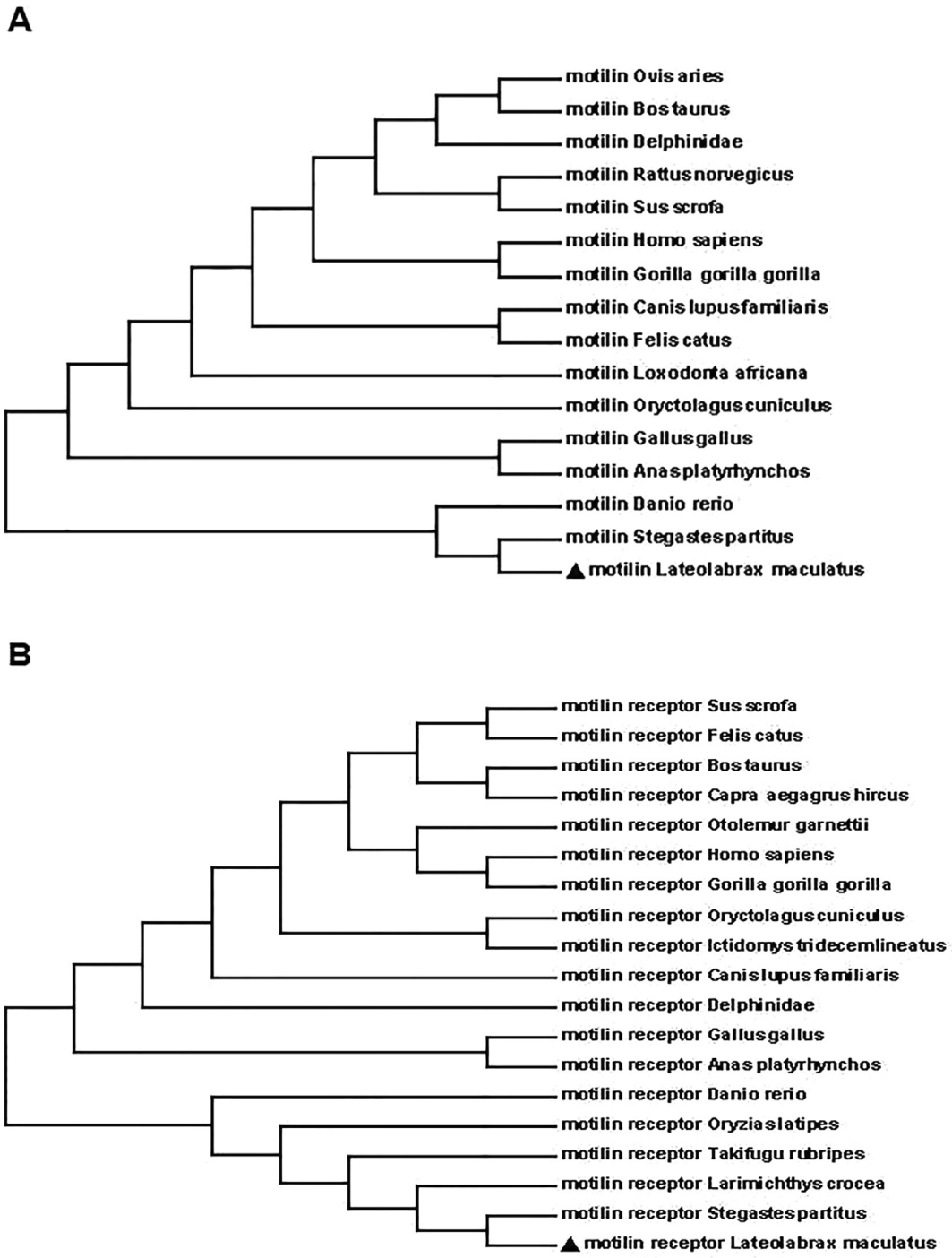


**Fig. 3.** Phylogenetic analisis of *mln*(A) and *mlnr*(B) in spotted sea bass. The phylogenetic tree was constructed by MEGA 6 software using the maximum likelihood method. Bootstrap tests with 1,000 replicates were performed to evaluate the phylogenetic trees. The accession numbers of each sequence are *Homo sapiens* (AAA59860.1), *Rattus norvegicus* (BAF85821.1), *Oryctolagus cuniculus* (CAA45342.1), *Gallus gallus* (NP_001292058.1), *Danio rerio* (XP_002665930.1), *Stegastes partitus* (XP_008278729.1), *Gorilla gorilla gorilla* (G3QYK9), *Anas platyrhynchos* (U3IC45), *Ovis aries* (W5PLB3), *Bos taurus* (C3W8S1), *Delphinidae* (ENSTTRP00000006569), *Sus scrofa* (A0A140TAJ7), *Canis lupus familiaris* (F1PR00), *Felis catus* (A0A0A0MPY7), *Loxodonta africana* (G3T8W6), *Lateolabrax maculatus* (MH046054) and *mlnr* amino acid sequences of *Homo sapiens* (AAC26081.1), *Rattus norvegicus* (NP_852029.3), *Oryctolagus cuniculus* (NP_001093437.1), *Gallus gallus* (NP_001120785.1), *Danio rerio* (ENSDARP00000106018), *Stegastes partitus* (XP_008288581.1), *Larimichthys crocea* (XP_010741665.1), *Gorilla gorilla gorilla* (G3QMK7), *Otolemur garnettii* (H0XJB1), *Ictidomys tridecemlineatus* (I3MIA5), *Sus scrofa* (3LHJ5), *Felis catus* (M3WKE3), *Canis lupus familiaris* (B2NIZ6), *Bos taurus* (F1MPN9), *Delphinidae* (ENSTTRP00000003807), *Capra aegagrus hircus* (ENSCHIP00000003788), *Anas platyrhynchos* (U3IPS6), *Takifugu rubripes* (ENSTRUG00000013958), *Oryzias latipes* (ENSORLT00000003605), *Lateolabrax maculatus* (MH046057). And black triangles indicated the *mln* and its receptors gene in spotted sea bass.

out overnight at 55 °C in sealed moisturizing chambers using a DIG-labeled riboprobes at 400 ng/250ul. After hybridization, sections were followed by graded washing in 2–0.1 × SSC and blocked with Blocking Buffer. DIG was detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics; diluted 1:2000) and chromogenic development with NBT/BCIP Stock Solution (Roche Diagnostics). Sections were examined by light microscopy.

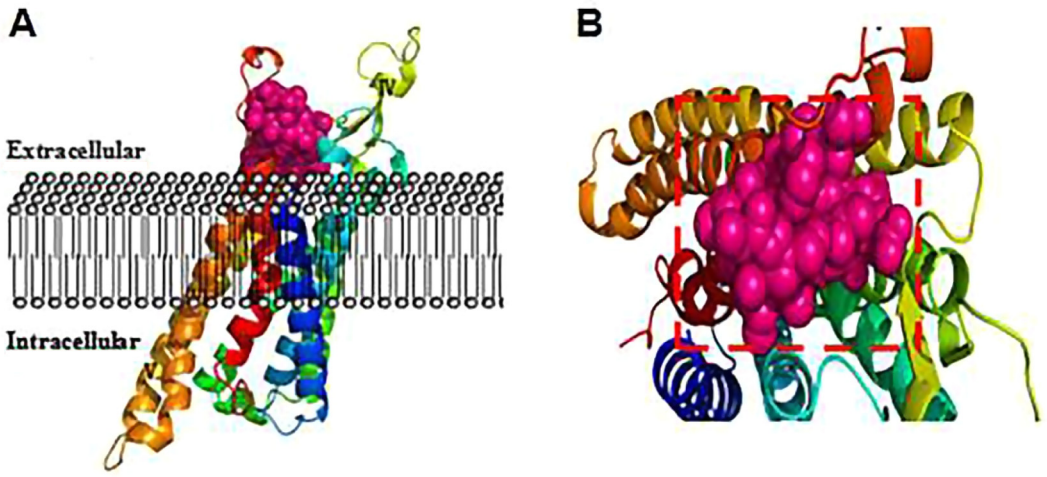


**Fig. 4.** The complex of *mln* and its receptor. The red lumps structure was ligand *mln* and red box was ligand area.

### 2.6. In vitro actions of the spotted sea bass MLN on expression of ghrelin, gastrin and cholecystokinin mRNAs from spotted sea bass intestine fragments

Spotted sea bass MLN was synthesized by GL Biochem, Shanghai, China. The purity was > 97% as determined by analytical HPLC. The peptide was dissolved to the desired concentration in dimethyl sulphoxide (DMSO) (cell culture grade) and diluted with culture media for in vitro experiments.

Spotted sea bass was anesthetized with MS-222 before decapitation. Intestine was removed and washed three times with phosphate buffered saline (PBS), then diced into small pieces (1 $mm^3$), and cultured in a 24-well plate containing 1 mL medium 199 (M199) with 100 U/mL penicillin, 100 mg/mL streptomycin, and 20% fetal bovine serum (FBS). After preincubation at 28 °C for 6 h, the medium was aspirated and fresh culture medium containing MLN ($10^{-6}$, $10^{-7}$ and $10^{-8}$ M) added or media alone. The intestine fragments were harvested after incubation for 3, 6, and 12 h, and were stored at −80 °C for subsequent RNA extraction and real-time PCR.

### 2.7. Statistics

All data were presented as mean ± SEM (n = 3). Data analysis was conducted using One-way ANOVA and the Duncan's method for multiple comparisons with software SPSS 17.0 to determine statistical significance. Significance was considered at $P < 0.05$.

## 3. Results

### 3.1. Cloning and sequence analysis of mln and mlnr

In this study, a putative *mln* gene (MH046054) encoding in spotted sea bass was identified a *mln* precursor. The *mln* ORF was consisted of 336 nucleotides encoding 111 amino acids with a putative signal peptide of 26 amino acids. The deduced mature motilin sequence in spotted sea bass was 17 amino acids (Fig. 1A). As shown in Fig. 1B, the predicted MLN amino acid sequence was relatively conserved among fish sequences and displayed variability compared to other vertebrate sequences. The spotted sea bass *mln* mature peptide showed high sequence homology with other fish species, including 72.73% to damselfish (*Stegastes partitus*) and 50% to zebrafish (*Danio rerio*), and low homology with mammalian and poultry species, including 22.73% to human (*Homo sapiens*), 13.64% to rat (*Rattus norvegicus*), 27.27% to rabbit (*Oryctolagus cuniculus*) and 22.73% to chicken (*Gallus gallus*).

The *mlnr* (MH046057) was also identified from spotted sea bass, with an ORF of 1089 bp encoding a protein of 362 amino acids. The results of topology prediction by TMHMM showed that *mlnr* had seven putative transmembrane domains (Fig. 2A). The MLNR in spotted sea bass shared 79.68% identities to damselfish and large yellow croaker, 53.81% to zebrafish (*Danio rerio*), 40.88% to chicken (*Gallus gallus*), 40.65% to human (*Homo sapiens*), 39.95% to rabbit (*Oryctolagus cuniculus*), and 21.02% to rat (*Rattus norvegicus*). (Fig. 2B).

To analyze the evolutionary relationship of MLN and MLNR in spotted sea bass, phylogenetic trees were constructed using the amino acid sequences of the two genes in several species (Fig. 3). Phylogenetic analysis showed that the spotted sea bass MLN and MLNR were clustered into distinct branches respectively and had the closest relationship with damselfish (*Stegastes partitus*).

### 3.2. Molecular docking of mln peptide to its receptor

The ligand acted its functions by binding to receptors. In order to know the interaction of the *mln* with its receptor, a docking of them were carried out using Auto Dock software, and the optimal binding conformation was obtained by cluster analysis and binding energy. Then the structure figures were prepared with PyMol. As shown in Fig. 4A, TM segments were represented by the colored ribbons and labeled with roman numerals according to the software of TMHMM (v.2.0). The red lumps structure was ligand *mln* which was wound with one side of the transmembrane domain and it was the partial display form of molecular docking, red box was the ligand area in Fig. 4B.

### 3.3. Tissue expression of mln and mlnr in spotted sea bass

The tissue expression profile analysis of the *mln* and *mlnr* genes in spotted sea bass were detected in normal feeding conditions. The result indicated that *mln* gene was widely expressed in normal tissues, but it was predominately expressed in spleen and intestine (Fig. 5A), suggesting that *mln* gene probably manifests as a gut hormone. *Mlnr* gene has the high expression level in intestine and head kidney (Fig. 5B).

### 3.4. ISH of mln and mlnr in the spotted sea bass intestine

In situ hybridization of the *mln* and *mlnr* mRNA was examined in the intestine using RNA probe with sequences complementary to the precursor. Sense probe was used as the appropriate controls. The sea bass intestine was consisting of muscular layer (ML), intestinal gland (IG) and intestinal villus (IV). Meanwhile, intestinal villus (IV) was a leaf-like structure formed by the lamina propria (LP) and the epithelial cell (EC) convex toward the intestinal lumen. An intense expression of *mln*

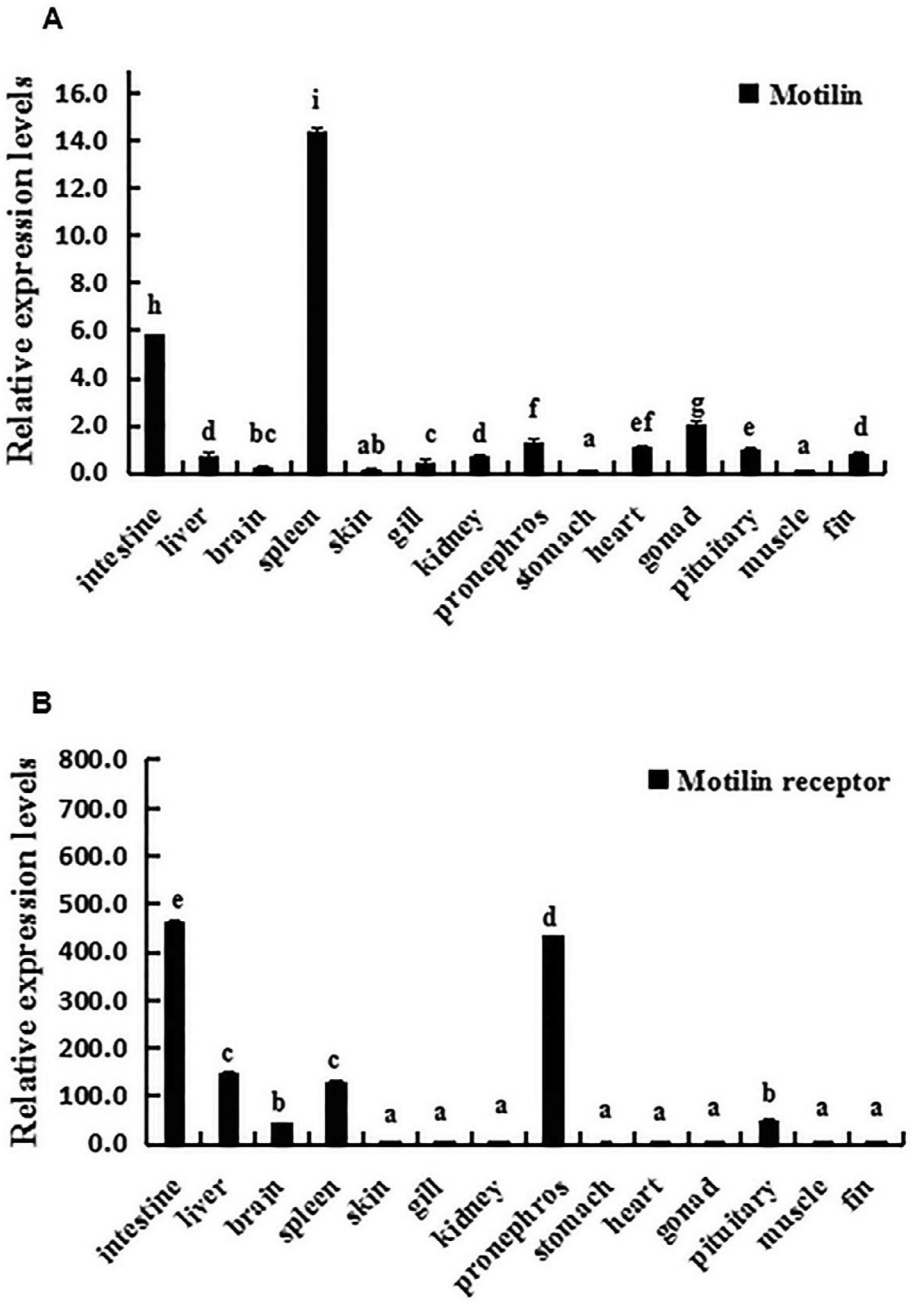


**Fig. 5.** Relative expression level of (A) *mln* and (B) *mlnr* genes in different tissues of spotted sea bass. Fold change compared with controls is indicated on the Y-axis, and tissues displayed on the X-axis. Expression levels were quantified by real-time PCR and normalized by 18S rRNA.

and *mlnr* mRNA were both observed in the lamina propria (LP) and the epithelial cell (EC), while no expression of muscular layer (ML) and intestinal gland (IG) (Fig. 6).

### 3.5. Changes in gene expression induced by short-term fasting in spotted sea bass intestine

In order to gain insight to the potential functions of MLN and its receptor in intestine, the expression of the genes after fasting for different times were systematically established in spotted sea bass. The expression level of *mln* had the highest expression level at 1 h and the expression level was significantly lower than the control group ($P < 0.05$) at 6 h. However, the relative expression of *mlnr* was significantly higher than the control group ($P < 0.05$) at 6 h. Both were not significantly different to the control group after 6 h (Fig. 7).

### 3.6. In vitro actions of spotted sea bass MLN on mRNA expression of ghrelin, gastrin and cholecystokinin from spotted sea bass intestine fragments

To further evaluate the effect of MLN on spotted sea bass, in vitro studies of MLN on mRNA expression of *ghrelin* (*ghrl*), *gastrin* (*gas*) and *cholecystokinin* (*cck*) were proformed in intestine fragments. The sequence of *18 s, mln, ghrl, gas* and *cck* were identified in spotted sea bass genome and transcriptome library. And gel electrophoresis of PCR products of these genes in intestine was conducted, all of them were detected in intestine.

As shown in Fig. 8 shown, the expressions of *ghrl* showed obvious increased after incubated for 3 h, especially, had the hightest levels in $10^{-6}$ ml/L. It indicated that *ghrl* was sensitive and dose-dependent increased to the MLN but then it had no significant variation in 6 and 12 h. Similarly, *gas* and *cck* were also significantly higher than the

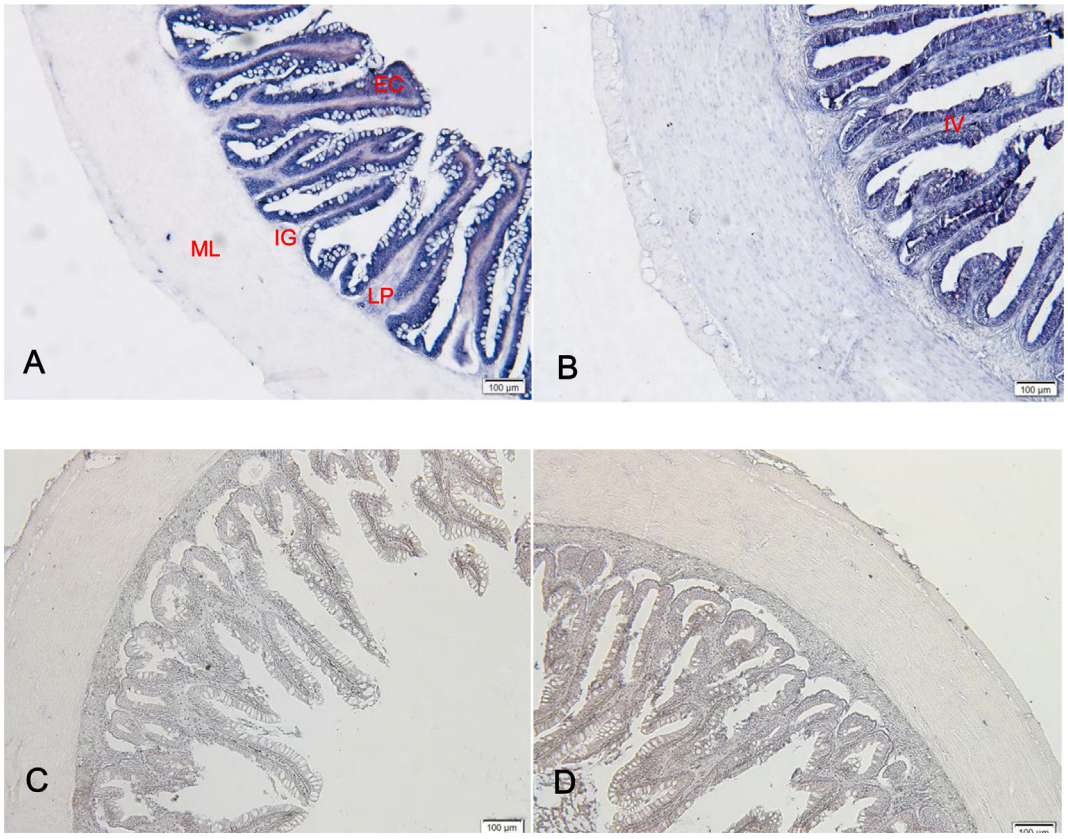


**Fig. 6.** Cellular localization of *mln* and *mlnr* genes in spotted sea bass intestine by in situ hybridization. Adjacent sections hybridized with the *mln* anti-sense (A), *mlnr* anti-sense (B) and sense (C and D) probes. Scale bar: 100 μm.

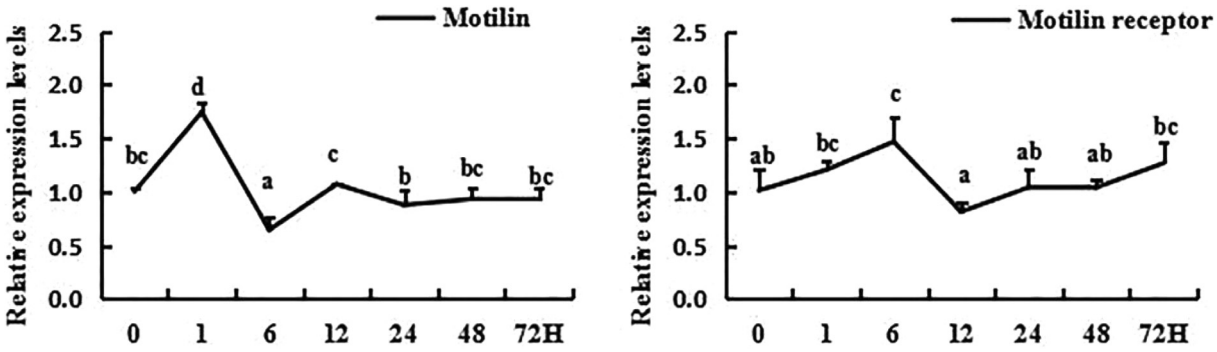


**Fig. 7.** Effect of different fasting time on *mln* with its receptor expressions in the intestine. The y-axis expressed the scale of relative expression levels, and the x-Axis provided the period of fasting time. Significant differences were noted by different letters in each genes ($P < 0.05$).

control group ($P < 0.05$) but had no significant difference between the concentrations after incubated for 3 h , besides, they had no significant variation in 6 and 12 h as well. MLN played a role in regulating the expression of other gastrointestinal related genes after incubated 3 h which was the best effective time in vitro but had no function after 3 h. Besides, the concentration of MLN at $10^{-6}$, $10^{-7}$, $10^{-8}$M had no significant variation for these genes.

## 4. Discussion

Increasing evidences support an important role of MLN/MLNR system in maintaining energy balance, increasing appetite, inhibiting nausea (Sanger and Furness, 2016). However, the functional data of MLN/MLNR system remains largely unexplored in teleost. In the present study, we cloned both *mln* and its receptor *mlnr*, determined their expression pattern and regulation in the intestine and examined the effects of in vitro administration of MLN on the gene expressions of *ghrl, gas* and *cck*. Our findings supported that MLN/MLNR system act as regulator of *ghrl, gas* and *cck* in fish.

Sequence and structure analysis showed that the N-terminal of teleost MLN mature peptide was highly conserved, indicating the importance of this region for its biological activities. However, the mature peptides of teleost were differed from mammal peptides, not only in length, but also exhibited rather low sequence similarity with mammal MLN. In the rabbit, it showed that bioactivity was mostly determined by the N-terminal end, especially residues 1, 4, and 7 (Macielag et al., 1992; Peeters et al., 1992), and assumed that the C-terminal region of *mln* formed an α-helix which stabilized the interaction of the critical N-terminal residues at the active site (Miller et al., 1995).

Recently, bioinformatics has come up to the level that can allow almost accurate prediction of molecular interactions that combined a protein with a ligand in the bound state. The program Auto Dock has

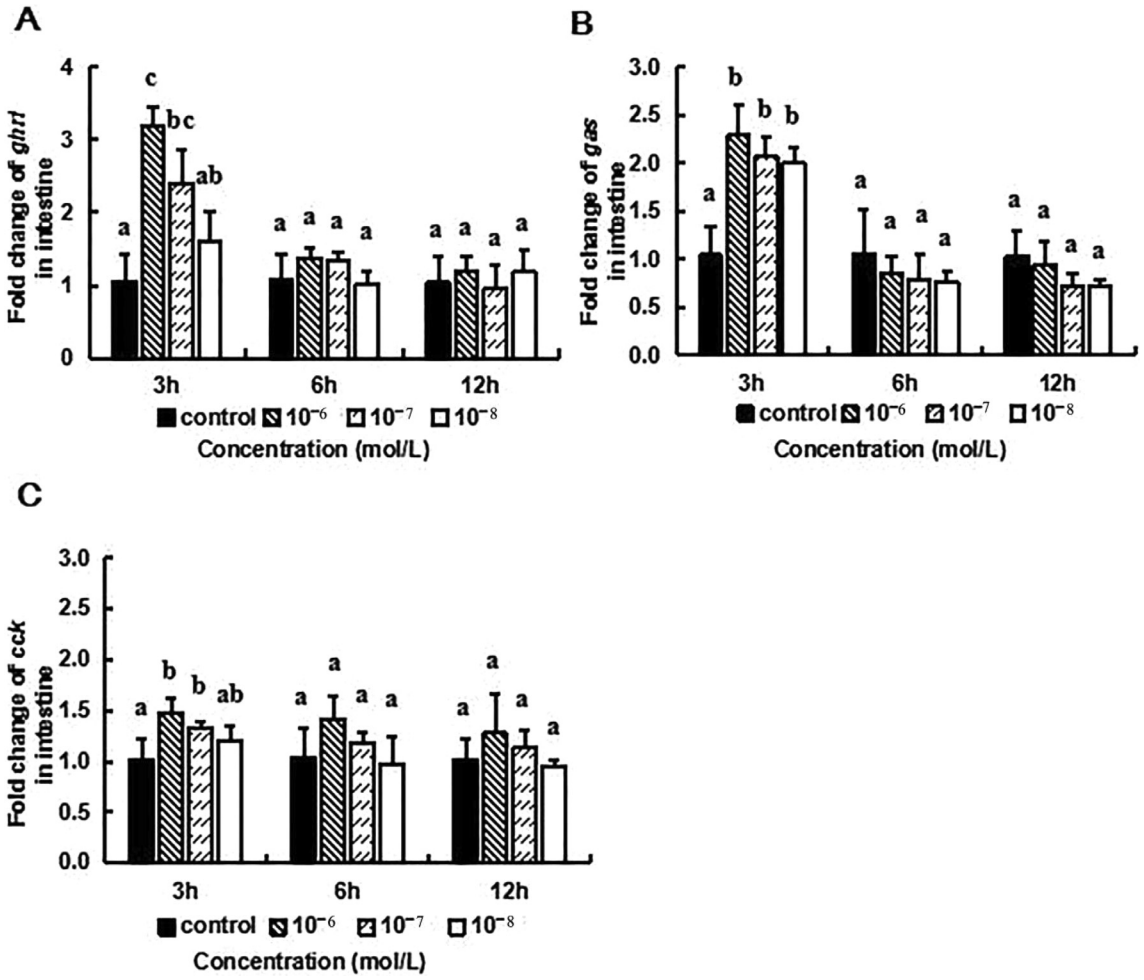


**Fig. 8.** Effect of *mln* peptide treatment on *ghrl* (A), *gas* (B) and *cck* (C) mRNA expression in spotted sea bass intestine. The results were represented as the mean ± SEM and expressed as fold change relative to the mRNA expression in the normal incubated without *mln* peptide. Significant differences were noted by different letters in each concentrations ($P < 0.05$).

been provided a procedure for predicting the interaction of small molecules with macromolecular targets. It could offer the advantage of delivering new drug candidates more quickly and at a lower cost (Gilbert, 2004; Warren et al., 2006). In this study, the molecular docking of MLN peptide to MLNR shown that a mainly binding in the extracellular IV and V transmembrane regions. MLNR has a long second extracellular loop that was a characteristic of MLNR between IV and V transmembrane domain (Matsuura et al., 2002). Of note, both ends of this loop, representing domains that were conserved and found to be functionally vital for binding and action of the natural peptide ligand, MLN, while the non-conserved residues in the mid-region of the loop were not necessary (Matsuura et al., 2002).

For further study the function of MLN/MLNR system, in situ hybridization was employed for the detection the *mln* and *mlnr* mRNA in intestine of spotted sea bass. In intestine, both *mln* and *mlnr* were highly expressed in the lamina propria (LP) and the epithelial cell (EC) of intestinal villus (IV). The intestine epithelium formed one of the largest exposed surfaces of the animal body, representing a unique interface for integrating environmental information with physiologic signals from nervous, immune, and vascular systems (Furness et al., 2013; Öhman et al., 2015). A variety of enteroendocrine cells within the intestine epithelium responded to stimulations by releasing hormones to mediate physiologic responses (Gribble and Reimann, 2016). For example, the enterochromaffin (EC) cell, an enteroendocrine cell subtype that represented one of the major proposed epithelial chemosensory (Bellono et al., 2017), which synthesized, stored and secreted MLN so that realized a significant effect on gastric motility (Itoh, 1997; Pearse et al., 1974). Zebrafish MLN was able to induce contraction in the rabbit duodenum and chicken ileum. in vitro study also showed that zebrafish MLN (3–100 nM) increased the intracellular $Ca^{2+}$ concentration in zebrafish *mln receptor* expressing HEK293 cells (Kitazawa et al., 2017). These results indicated that in fish, MLN binds to its receptor MLNR, activated the $Ca^{2+}$ signaling pathway and played its normal role in the gut.

Based on the expression pattern and localization, intestine was chosen as the target organ to examine the expression profile after a short-term starvation. During the fasting state an intricate interaction occured between gut hormones and gastrointestinal motility generating a specific contractility period named the migrating motor complex (MMC) (Deloose et al., 2012). Although most of the gastrointestinal hormones were released after taking a meal, MLN has a specific feature that it was released at about 100 min intervals during the interdigestive state when no nutrient was present. In human, this cycle repeated every 90–120 min (Shea-Donohue and Urban, 2016), in addition, the release of MLN was inhibited after food intake in human (Achemkaram et al., 1985; Boivin et al., 2010). However, in spotted sea bass, MLN was increased significantly at 1 h, the difference might be the simple structure of intestines in fish compare to mammals.

In order to further study the function of MLN in the gastrointestinal tract, we detected the MLN effect on the expression of gastrointestinal related genes (*ghrelin*, *gastrin* and *cholecystokin*) in the intestine fragments of spotted sea bass by in vitro incubation experiment. The result

showed that the physiological effects of MLN increased the expression of *ghrl, gas* and *cck* after 3 h incubation, and the effect did not last to 6 h or 12 h. *Ghrl* and *mln*, are both gastrointestinal peptides, sharing several common characteristics, including high sequence similarity, same synthesis position and prokinetic activity on gastrointestinal motility (Ohno et al., 2010). *Ghrl* was sensitive to *mln* which increased significantly after 3 h incubation might because of their similar structure and function. *Gas* was located in the gastric antrum and stimulated gastrointestinal motility and gastric emptyingin in rat (Misiewicz et al., 1969; Zhang et al., 2011). The increase expression of *gas* confirmed that *gas* was involved in promoting the activity of gastrointestinal motility. The expression of *cck* was in a dose dependent manner at 3 h after MLN stimulating, which was coincident with previous study in human that injection of MLN would cause the gall bladder volume reduction (Luiking et al., 1998). All these secretory and motor events were considered to be the common phenomena associated with the gastrointestinal activity, especially seen in the interdigestive state in the dog and human.

In conclusion, we identified and characterized MLN/MLNR system in spotted sea bass. We also showed that *mln* and *mlnr* expressions in intestine and both localized in the lamina propria (LP) as well as the epithelial cell (EC) of intestine villus (IV) by ISH. Short-term starvation indicated that *mln* as a hunger hormone the expression level was increased at 1 h but decreased at 6 h. In addition, MLN peptide stimulated the expression of *ghrl, gas* and *cck* verified that incubating for 3 h was the best effective time in a dose-dependent manner in vitro. Over all, the present study provides novel evidences for the function of MLN/MLNR system in regulating gastrointestinal motility in spotted sea bass and offers more reference data for exploring the molecular mechanism during the feeding in aquaculture.

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## Conflicts of interest.

The authors declare no conflicts of interest.

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