Cloning and functional characterization of the DA$_2$ receptor gene in Chinese mitten crab (*Eriocheir sinensis*)

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Abstract

Dopamine (DA) plays a modulatory role in numerous physiological processes such as light adaptation and food intake, and exerts these functions through DA receptors (DARs). This study presents, for the first time, isolation and characterization of the dopamine receptor 2 (DA$_2$ receptor) cDNA from the intestinal tissue of *Eriocheir sinensis*, an economically important freshwater aquaculture species in China. The DA$_2$ receptor cDNA sequence, which was obtained by rapid amplification of cDNA ends, is 2369bp long, encode peptide of 589 amino acid, and is highly homologous to related sequences in crustaceans. Analysis of the deduced amino acid sequence and the structure of the DA$_2$ indicated that this receptor is a member of the family of G protein-coupled receptors (GPCRs), as it contains seven transmembrane domains and other common signatures of GPCRs. RT-PCR showed that the expression of the DA$_2$ receptor gene was distributed in various tissues, and high expression levels were observed in the cranial ganglia and the thoracic ganglia. Further study of the effect of photoperiod on DA$_2$ expression showed that constant darkness induced a significant increase in DA$_2$ expression in the cranial ganglia. Finally, analysis of DA$_2$ receptor expression under different feeding statuses showed that there was significantly greater expression in the hepatopancreas and intestines after feeding than before feeding, but there were no differences in expression between the before feeding and during feeding periods in either tissue. Our results indicate that the DA$_2$ receptor structurally belongs to the family of G protein-coupled receptors, and that the cranial ganglia are the main tissues in which the DA$_2$ receptor participates in light adaptation during dark hours. In addition, the DA$_2$ receptor in *E. sinensis* may be involved in the physiological regulation of the hepatopancreas and digestive tract after the ingestion of food. This study provides a foundation for further exploration of the light adaptation and digestive functions of the DA$_2$ receptor in decapods.

Introduction

Dopamine (DA) is a biogenic amine neurotransmitter found in both vertebrates and invertebrates that affects a wide variety of physiological and behavioral functions, including reproduction[1,2], hormone synthesis and release[3], locomotion[4], respiration[5], feeding behavior [6], and the circadian rhythm[7]. The dopamine receptors can be divided into five subtypes (DA_1-DA_5), which all belong to the family of G protein-coupled receptors (GPCRs). According to their conserved structures, signaling mechanisms and pharmacological profiles, these receptors are further classified into two types the D1-like and D2-like receptors[1]. The D1-like receptors include the DA_1 and DA_5 subtypes, which activate adenylyl cyclase, resulting in increased levels of intracellular cyclic adenosine monophosphate (cAMP); regulate cell metabolism, including ion channel function, and desensitize GPCRs, leading to the release of neurotransmitters. D2-like receptors consist of the DA_2, DA_3, and DA_4 subtypes, which inhibit adenylyl cyclase through the coupled signal transduction pathway and thus decrease cAMP; D2-like receptors can be blocked by the pertussis toxin[8]. In mammals, these receptors occur in the brain, peripheral nervous system, cornea of the eye, heart, kidney and lymphocytes[2]. However, only a few studies on the dopamine receptor in crustaceans have been reported. Using RACE technology and a degenerate PCR strategy with conventional library screening, the gene and protein sequences of $DA_{1\alpha}$, $DA_{1\beta}$ and $DA_{2\alpha}$ in *Panulirus interruptus* have been obtained[8,9]. In the sequencing of transcriptomes from the nervous systems of *Cancer borealis* and *Homarus americanus*, $DA_{1\alpha}$, $DA_{1\beta}$ and $DA_{2\alpha}$were found in both decapod crustaceans [10]. In addition, a type 1 dopamine receptor from *Penaeus monodon* has been identified[2]. However, information is still lacking on gene and protein sequences of dopamine receptors in economically valuable decapod crustaceans, such as *E. sinensis*, a richly nutritious species with high market demand that has become economically important in Chinese freshwater aquaculture[11].

Light influences the growth and development of crustaceans[12,13], such as *Macrobrachium rosenbergii* and *Portunus pelagicus*, and daily changes in dopamine synthesis and release depend on the interactions between the photoreceptors and the dopaminergic neurons, where dopamine release is induced by light[14,15]. High levels of dopamine have been detected during light periods and low levels during dark periods[16–18], for this reason, it is believed that dopamine promotes light adaptation. However, constant light results in a dramatic reduction in dopamine levels in chicken retina[19]. Constant light and constant darkness have significant effects on survival and growth of larvae of *P. pelagicus*[12] and *M. rosenbergii*[13], but the effects of different photoperiods on dopamine receptors in crablets remain uncertain.

In addition to promoting light adaptation, dopamine can also participate in feeding regulation. Exogenous injection of DA has been found to significantly decrease food intake compared to that of a control group in neonatal layer-type chickens[6], and cannabinoid-induced feeding behavior may be modulated by dopamine receptor 2[20]. However, by promoting either the initiation or cessation of feeding behavior, increased activity of DA neurons can either increase or reduce food intake[21]; inhibition of D1-type dopamine receptor neurons decreases food intake[22]. Dopamine receptors have also been found to be distributed in the intestinal tract and are considered to be involved in regulating gastrointestinal motility[23,24]. In addition, the presence of specific receptors on the membranes of target cells is essential for dopamine to produce any physiological effects. Inhibitors of dopamine receptors can block the effects of dopamine[25]. Due to the diversity of dopamine receptors, light stimulation has different effects on their expression levels. An understanding of the variation of receptors will help to identify cellular targets of DA and to understand which receptors are activated for particular processes.

The present study describes the molecular cloning and characterization of the dopamine receptor 2 full-length cDNA from *E. sinensis* and its expression profile in various tissues under different photoperiods and feeding statuses.

Materials and methods

Animals and sampling

In this study, the experimental animals (n = 48) were healthy crablets (exhibiting secondary sexual characteristics) with initial masses of 13.43±1.81 g, collected from the Shuxin crab base in Chongming, Shanghai (China). Crabs were housed for one week for acclimatization in clear glass aquaria (length× width× height = 120×60×40cm) with sufficient ambient medium and cyclic water flow. Crabs were fed once a day at 09:00.

Thirty crabs were acclimatized to 26±1˚C and assigned randomly to three groups: a control group (L:D = 12h:12h), a group held in constant darkness (L:D = 0h:24h) and a group held in constant light (L:D = 24h:0h). There were 10 crabs per group, and treatments continued for 14 days[26]. Then crabs from the control group were frozen on ice and dissected, and different tissues, including the gill, heart, muscle, hepatopancreas, intestine, cranial ganglia, thoracic ganglia, eyestalks, and hemolymph were harvested. At the same time, eyestalks, cranial ganglia and thoracic ganglia were harvested on ice from the constant darkness group and constant light group.

For a separate group of 18 crabs, the hepatopancreas and intestine were collected on ice after a week of rearing. Tissues were collected at 08:00 (before feeding, n = 6), 10:00 (feeding period, feeding time was 09:00 to 10:00, n = 6), or 16:00 (after feeding, feces were mostly in the hind gut and the crabs began to evacuate 6h after feeding, n = 6), [27]. All the samples were stored at -80˚C until RNA isolation.

Nucleic acid extraction

Total RNA was extracted from *E. sinensis* using the RNAiso Plus reagent (RNA Extraction Kit, TaKaRa, Japan) according to the manufacturer's instructions. Briefly, tissues were ground in a mortar with liquid nitrogen and collected in 1.5 ml centrifuge tubes. The RNAiso Plus reagent was added (1 ml), and samples were left at room temperature for 5 min. Samples were then centrifuged 5 min at 4˚C and 12000 rpm, and the supernatant was collected in new 1.5 ml tubes. Chloroform (200 μl) was added, and samples were then oscillated and again left at room temperature for 5 min before a 15 min centrifugation at 4˚C and 12000 rpm. The supernatant was collected in new 1.5 ml tubes and 500 μl of isopropyl alcohol was added. Samples were left at room temperature for 10 min and then centrifuged 10 min at 4˚C and 12000 rpm. Pellets were washed with 1 ml of 75% alcohol and centrifuged 10 min at 4˚C and 7500 rpm. The supernatant was removed, and the remaining pellets were dried and dissolved in 30 μl of DEPC-treated water. The concentration and quality of the total RNA were estimated by micro-volume ultraviolet-visible spectrophotometer (Quawell Q5000; Thmorgan, China) and agarose-gel electrophoresis, respectively.

Cloning of full-length *E. sinensis* DA₂ cDNA

Transcriptomes sequences were obtained from the Y-organ of *E. sinensis*. The amino acid sequence of the EST (length: 637bp) was verified to be highly homologous to the *C. borealis* dopamine receptor 2 (AOG14374.1) using BlastX analysis. A pair of gene primers, DA₂-F and DA₂-R (Table 1), was designed to amplify the full-length DA₂ cDNA from *E. sinensis* for sequence verification.

Four gene-specific primers, DA₂-3'Outer, DA₂-3'Inner, DA₂-5'Outer and DA₂-5'Inner (Table 1), were designed based on the 637bp singlet to clone the 3'- and 5'-ends of the DA₂

Table 1. Primers used in cloning and characterizing the DA₂ gene.

Primers	Sequences (5'-3')	Usage
DA₂-F	CTAGCCATAGTTCTGGCGGCG	RT-PCR
DA₂-R	TCCTTACCGGACCACAGAACG	RT-PCR
DA₂-3'Outer	TGAACTCCTTCCTCAACCCCG	3'RACE
DA₂-3'Inner	GTATGCCAGAGCTAGCCGGG	3'RACE
DA₂-5'Outer	ATCTTCACTTTCTTCTGCTTCACGA	5'RACE
DA₂-5'Inner	CTCGTCTGGCTTACGTTCTCGATCAC	5'RACE
qRT-DA₂-F	TGCTATTATCTGGGTGGTGT	q-RT-PCR
qRT-DA₂-R	ATGATGAAGTCTGCGTTGTG	q-RT-PCR
18S-F	TCCAGTTCGCAGCTTCTTCTT	q-RT-PCR
18S-R	AACATCTAAGGGCATCACAGA	q-RT-PCR

cDNA by rapid amplification of cDNA ends (RACE) using the SMARTer RACE 5'/3' Kit (Clontech, USA). The 3'- and 5'-end cDNA templates were synthesized according to the manufacturer's instructions. Specific products were obtained via touchdown PCR and nested PCR. Touchdown PCR was carried out as follows: 94˚C for 5 min; 5 cycles of 94˚C for 30 s and 72˚C for 3 min; 5 cycles of 94˚C for 30 s, 70˚C for 30 s, and 72˚C for 3 min; 30 cycles of 94˚C for 30 s, 68˚C for 30 s, and 72˚C for 3 min; a final extension for 10 min at 72˚C; and a cooling hold at 4˚C. Nested PCR amplification conditions were as follows: 94˚C for 3 min; 34 cycles of 94˚C for 30 s, 66˚C for 30 s, and 72˚C for 2 min; 72˚C for 7 min, and a cooling hold at 4˚C. Amplification products were run on a 1.5% agarose gel and purified with a TIANgel Midi Purification Kit (TIANGEN, China). The DNA fragments were cloned into a pMD18-T vector (TaKaRa, Japan) and transformed into TOP10 chemically competent *E. coli* cells (TIANGEN, China). Bacteria were grown according to the manufacturer's instructions. The positive clones containing the inserts of the expected size were sequenced using M13±primers by Sangon Biotech (Shanghai).

Sequence analysis

The generated sequences were verified for similarity by using the BLAST programs (http://blast.ncbi.nlm.nih.gov/). Then, to obtain the full-length DA₂ cDNA, the partial fragment, and the 3'- and 5'-end sequences were assembled. After the open reading frame (ORF) was obtained using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), the coding region sequences were translated into amino acid sequences by using the sequence manipulation suite (SMS) tool (http://www.bio-soft.net/sms/index.html). The molecular mass and the theoretical isoelectric point of the DA₂ protein were predicted using the Compute pI/Mw tool (http://cn.expasy.org/tools/pi_tool.html). The trans-membrane domains of the protein sequence were predicted by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM). Protein phosphorylation sites were predicted using DISPHOS 1.3 (http://www.dabi.temple.edu/disphos/). N-glycosylation sites were predicted using the NetNGly 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). An amino acid multiple sequence alignment was performed with the ClustalX program, and phylogenetic tree was constructed using the neighbor-joining (NJ) method with1000 bootstraps in the MEGA 5.0 program[28].

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

The mRNA expression pattern of the DA₂ transcript in various tissues (gill, heart, muscle, hepatopancreas, intestine, cranial ganglia, thoracic ganglia, eyestalks, and hemolymph) were measured by qRT-PCR using 18S ribosome RNA (18S) as a reference gene. We confirmed that

18S expression was stable. Total RNA was extracted from each sample, and reverse transcription was performed with equal quantities of total RNA (1μg). Relative quantification was performed using the ABI 7500 Real-Time PCR System (Life Technology, USA). Gene-specific primers, qRT-DA$_2$-F and qRT-DA$_2$-R (Table 1), were designed based on the cloned DA$_2$ cDNA to produce a 126bp amplicon. Real-time qPCR amplification reactions were performed in a final volume of 10 μl, which contained 5 μl of 2×SYBR Premix Ex Taq^{TM} (TaKaRa, Japan), 1 μl of diluted cDNA template, 3.4 μl of PCR-grade water, 0.2 μl of ROX Reference Dye II, and 0.2 μl each of the forward and reverse primers. The PCR conditions used were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, and 60 0078C for 34 s; and generation of a melting curve at 95°C for 15 s, 60°C for 1min, and 95°C for 15 s. Samples were run in triplicate, and results were normalized to the expression of the reference gene 18S. The DA$_2$ cDNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method (where $^{\Delta}Ct = {}^{\Delta}Ct$ sample - $^{\Delta}Ct$ reference). Data were analyzed and presented as triplicate means± SE (standard error) and as n-fold differences relative to the control data.

Statistical analysis

Statistical analysis of relative gene expression was performed using SPSS software (Chicago, USA; Version 17.0). Data are presented as the means± SE. Statistical significance was determined using one-way analysis of variance and post-hoc Duncan multiple range tests. $P < 0.05$ indicated statistical significance.

Results

Cloning and identification of the DA$_2$ cDNA

The full-length DA$_2$ cDNA was isolated from the intestine of the Chinese mitten crab. The full-length cDNA (2369bp) contained a 1770bp open reading frame (ORF), which encodes a putative DA$_2$ protein with 589 amino acids, a 192bp 5'-untranslated region (UTR), and a 407bp 3'-UTR with a 27bp poly (A) tail. Sequence analysis revealed that the DA$_2$ protein has a theoretical isoelectric point of 8.37 and a molecular weight of 64.81kDa. The deduced DA$_2$ protein has three major domains, four extracellular domains, seven transmembrane domains (TM-I through TM-VII) and four cytoplasmic domains. The transmembrane domains consist of seven hydrophobic regions, which are highly conserved compared with those of other dopamine receptors. In contrast, the amino-terminal region, the second extracellular loop between TM-IV and TM-V, and most of the third cytoplasmic loop between TM-V and TM-VI display a low degree of sequence identity. Despite the low homology between the extracellular N-terminal and the third cytoplamic loops of the *E. sinensis* DA$_2$ receptor and those of other DA$_2$ receptors, consensus motifs for N-linked glycosylation sites (N-x-[S/T]) and consensus sites for phosphorylation by protein kinase C (PKC) (S/T-x-[R/K]) are found in these domains (Fig 1). As in the DA$_2$ receptors of other species, there is a conserved DRY motif in the second intracellular loop. Sequence analysis of the DA$_2$ cDNA with BLASTn and BLASTp revealed a significant sequence similarity to *C. borealis* and *P. interruptus* DA$_2$ sequences found in the National Center for Biotechnology Information database.

Homology analysis of the DA$_2$ gene

The degree of homology of the DA$_2$ gene with other representative vertebrate and invertebrate DA$_2$ amino acid sequences was investigated via multiple sequence alignment in ClustalX (Fig 2). The alignment indicated that the *E. sinensis* DA$_2$ sequence shares high amino acid identity with the DA$_2$ sequence of *C. borealis* (84%) and *P. interruptus* (72%). The alignment also

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   1  GGGGAGTCAGGTTGTGAGCGTTGCACCGCCACCACCGTCCGCCAGCACCTCGCCCGCGTCTCGCTGCTCAGTGGT
  76  GTACAGAGAGCCCAGCGTCCTCGTCTCCCACAGCCCCAGTGACCCGCTCCCTTGGCCCCGCTGCCCTCGGGCACA
 151  GACAGGTCTGAAGGGGAGCCTCGGTGGTGTGGGTGACCAGCCATGTCACCCACATTGTACCCGCCGTCGTCCGCG
   1                                              M  S  P  T  L  Y  P  P  S  S  A
 226  CCCTCTTCAATCGCGGGGGCGGTGTCCTCGGGCGTGCCCATGGGCCTTAAGATTCTGGCCCAAACGCTTCCTGAG
  12  P  S  S  I  A  G  A  V  S  S  G  V  P  M  G  L  K  I  L  A  Q  T  L  P  E
 301  CTTCAGGTGCCCGCCGTGACCGACAAGGAGGAGGATTCCTTGGAGGCACAGTACAACGGTGGGATAGTGAACGGA
  37  L  Q  V  P  A  V  T  D  K  E  E  D  S  L  E  A  Q  Y  N  G  G  I  V  N  G
 376  AACGACGAGCTGGCCACGGTCGCCCCAAGTCCTCGAGTTACTTCCATCATCGCCGACGTGATCAGCATGTGTGAT
  62  N  D  E  L  A  T  V  A  P  S  P  R  V  T  S  I  I  A  D  V  I  S  M  C  D
 451  AACTGCACCGCCAACGAGACCGAGAATGCTGTACTCTAAACTTCCCCGAGGACGACCCCGCTAACACGGAGAGAAAC
  87  N  C  T  A  N  E  T  E  N  C  T  L  N  F  P  E  D  D  P  A  N  T  E  R  N
 526  TACTGGACTCTGCTGCTCCTGCTGTTCCCAGTGTTCACCGTGTTTGGCAACATTCTGGTGCTCATGTCCGTGTAC
 112  Y  W  T  L  L  L  L  F  P  V  F  T  V  F  G  N  I  L  V  L  M  S  V  Y
 601  AAGGAACGTTCGCTGCAGACCGTCACCAACTACTTCATAGTGTCCTTGGCGGTCGCCGACCTGCTCCTCGCCTCC
 137  K  E  R  S  L  Q  T  V  T  N  Y  F  I  V  S  L  A  V  A  D  L  L  L  A  S
 676  ATCGTCATGCCCTTCGCCGTCTACTATCTGCTGTGCGGCTACTGGGGCTTGGGACCCTTCGTGTGCGACGTGTAT
 162  I  V  M  P  F  A  V  Y  Y  L  L  C  G  Y  W  G  L  G  P  F  V  C  D  V  Y
 751  ATAGCCATGGACGTGGTCTGCTCCACTTCCTCCATCTTCAACTTGGTGGCCATCAGTATAGACAGGTACATCGCG
 187  I  A  M  D  V  V  C  S  T  S  S  I  F  N  L  V  A  I  S  I  D  R  Y  I  A
 826  GTGACTCAGCCCATCAAGTACGCCCAGTCCAAGAACAACAAGCGTATTGTCTTCACCATTGCTATTATCTGGGTG
 212  V  T  Q  P  I  K  Y  A  Q  S  K  N  N  K  R  I  V  F  T  I  A  I  I  W  V
 901  GTGTCGACAGCCATCGGCATCCCTCTGTTCTTCGTCAACCAGTGGAACGCGTCGAAGAGGAAATCTGACCCTGAT
 237  V  S  T  A  I  G  I  P  L  F  F  V  N  Q  W  N  A  S  K  R  K  S  D  P  D
 976  ACTGAATGTACATTCCACAACGCAGACTTCATCATCTGCTCCTCACTCTCCTCCTTTTACATTCCCTGCATTGCG
 262  T  E  C  T  F  H  N  A  D  F  I  I  C  S  S  L  S  S  F  Y  I  P  C  I  A
1051  ATGATCTACCTCTATTGCAGGATATTCAAGGCTTTGAAAGAACGTGCCCGAAAAAAGAAGCCGAAAGTGAACGAG
 287  M  I  Y  L  Y  C  R  I  F  K  A  L  K  E  R  A  R  K  K  K  P  K  V  N  E
1126  ATCAAGGCGGGGAGCGTGATCGAGAACGTAAGCCAGACGAGAATGCTGGAAGAGACGGCCCTGAGTTCAGAGACA
 312  I  K  A  G  S  V  I  E  N  V  S  Q  T  R  M  L  E  E  T  A  L  S  S  E  T
1201  CCACGTGCTGCGCATGCAGGCGCTAAGGGCCCGCCCGTGCCGCTCATAGAGGAGGACAAGAACACCAACAACACC
 337  P  R  A  A  H  A  G  A  K  G  P  P  V  P  L  I  E  D  K  N  T  N  N  T
1276  AGCGCCAGCCAGGACGAAGAAGACGATGAGGTAGAAGAGGCCGGAGTGGTTCTGGCAGGCGCTGAGAACTGCCAT
 362  S  A  S  Q  D  E  E  D  D  E  V  E  E  A  G  V  V  L  A  G  A  E  N  C  H
1351  ATTACCAAGAACCCTAGACCGGAGGAGCTGAACTTGGGGCCCCTCAGGGAGGAAGAGGCAATGGAAGAGGGGGCG
 387  I  T  K  N  P  R  P  E  E  L  N  L  G  P  L  R  E  E  A  M  E  R  G  A
1426  ACGGCTACTGTACAGTCCACGCGGAATGGGGCGGCGGAGGTGGTGCGGAGCGTGGAAGGGCGAGGCGGGGCACTG
 412  T  A  T  V  Q  S  T  R  N  G  A  A  E  V  V  R  S  V  E  G  R  G  G  A  L
1501  GCAGACCCCTCCCCAAGAAAGCACTTCTCGTGAAGCAGGAAAGTGAAGATCCAGTCTAAGAGAACGGATCT
 437  A  D  P  S  P  R  K  A  L  L  V  K  Q  K  K  V  K  I  Q  S  K  K  N  G  S
1576  GCGGGGGGAAGGATGCTGGAGGGCGATGAGCTAATGGAAAAAAAGGACAAGAAGGCTTCCTCTGCCCGCTTTACT
 462  A  G  G  R  M  L  E  G  D  E  L  M  E  K  K  D  K  K  A  S  S  A  R  F  T
1651  ATCTACAAAGTCAACAAAGCCAGCAAAGAAAAAGAGAGAAGTCCTCCGCCAAGAAGGAGAGAAAAGCGACGAAA
 487  I  Y  K  V  N  K  A  S  K  K  K  R  E  K  S  S  A  K  K  E  R  K  A  T  K
1726  ACTCTAGCCCATAGTTCTGGCGGCGTTTCTGGTGTGCTGGGTCCCATTTTTTTACCTGCAATATTGTGGACGCCATC
 512  T  L  A  I  V  L  A  A  F  L  V  C  W  V  P  F  F  T  C  N  I  V  D  A  I
1801  AGCAAAAAGACGAATAACGACAGCATCGCACCAAGCATGACCCTCTTCTGGCTCACCACCTGGATCGGCTACATG
 537  S  K  K  T  N  N  D  S  I  A  P  S  M  T  L  F  W  L  T  T  W  I  G  Y  M
1876  AACTCCTTCCTCAACCCCGTCATCTACACGATCTTCAATCCAGAGTTTAGAAAAGCTTTCAAGAAGATCCTCGTA
 562  N  S  F  L  N  P  V  I  Y  T  I  F  N  P  E  F  R  K  A  F  K  K  I  L  V
1951  TGCCAGAGCTAGCCGGGAAGGGCAGGCGCTGTTTCTTTATGAGATTAATTTCCTCATCATTTTCTCCTCCTCATC
 587  C  Q  S  *
2026  TCTATTGAAAGCTTTACCTCGTAGGTCACAAATATAGTAGTAAGGAACCTTTTTTTTTCTTGCATAATTTATAAAA
2101  AAAACGGACCAGGATGTTGTGTTTCTCTGTAGTTCTCAGTGTTAAGCTAATATCTAAGGTCACGTATTCGTCTGA
2176  GAGAAGATATGAGGCGGTCTATATCTGTGTCCCGTCCTTCGTGTCTGTTGTCAGTGGCGTTCTGTGTGTGTGTGT
2251  GCGTGTGTGTGTGTGTGTGTGCGTGTGTGTGTGTGTGTGTGAGACAATAGATCTTGTATAGAGAATTGCAGTTAAATGT
2326  ACTATTCTGGTATGACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig 1. Nucleotide and deduced amino acid sequences of the DA₂ gene. The nucleotide sequence is enumerated from the 192bp end and the single-letter amino acid code is shown above each corresponding codon. The start codon (ATG) and the stop codon (TAG) are indicated in the black boxes. The putative phosphorylation sites are indicated in the red boxes. The putative N-glycosylation sites are indicated in the green boxes. The seven transmembrane regions are underlined. Dashed boxes indicate the poly (A) tail.

revealed that the amino acid sequence in the transmembrane domains is highly conserved among these three species.

A neighbor-joining phylogenetic tree was constructed based on the reported DA₂ amino acid sequences using MEGA 5.0 software (Fig 3), with confidence in the resulting tree branch topology measured by bootstrapping through 1000 pseudo replicates. The tree provides evidence that the *E. sinensis* DA₂ gene is grouped with DA₂ genes of other species.
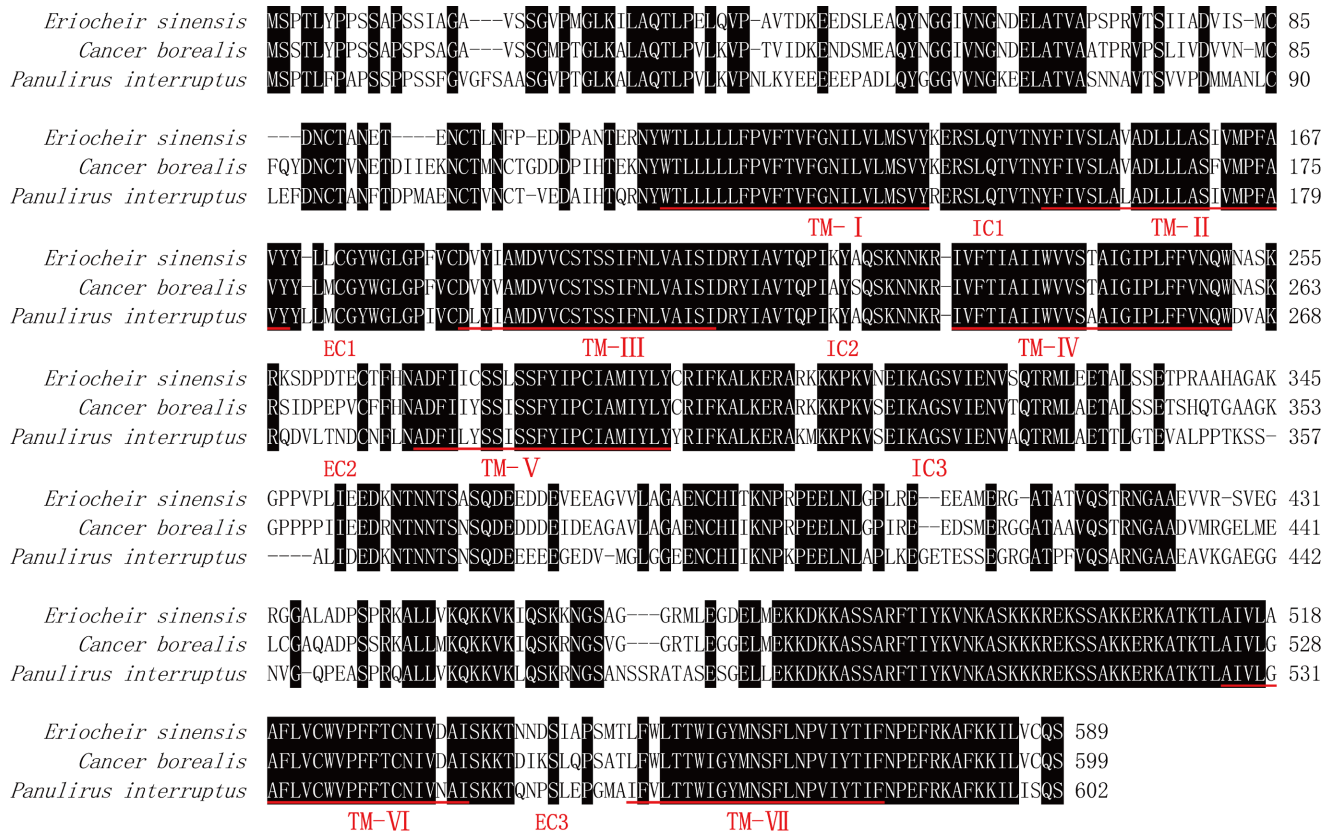
Fig 2. Alignment of amino acid sequences of the DA₂ receptor from *E. sinensis* and other invertebrates. The deduced amino acid sequence of the DA₂ receptor from *E. sinensis* was compared with the DA₂ receptor sequences from *C. borealis* (AOG14374.1) and *P. interruptus* (ABI64137.1) with the ClustalX program. Conserved of residues are highlighted in black. The seven transmembrane domains (TM-I –TM-VII), three extracellular loops (EC) and three intracellular loops (IC) are marked.

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Tissue distribution of expressed DA₂ mRNA

To determine mRNA expression patterns of DA₂ in *E. sinensis*, the total RNA extracted from various tissues including gill, heart, muscle, hepatopancreas, intestine, cranial ganglia, thoracic ganglia, eyestalks, and hemolymph was reverse transcribed and subjected to quantitative real-time PCR with qRT-DA₂-F and qRT-DA₂-R primers (Table 1). The results (Fig 4) show that DA₂ mRNA was expressed in all of these tissues, but the expression levels varied. DA₂ mRNA expression levels were highest in the cranial ganglia and thoracic ganglia (*P*<0.05); only low levels of expression were detected in other tissues (*P*<0.05).

Effect of photoperiod on DA₂ mRNA expression in the cranial ganglia and thoracic ganglia

We determined the effect of photoperiod on DA₂ mRNA expression in the cranial ganglia and thoracic ganglia after the crabs were cultured in different photoperiods for 14 days (Fig 5). The relative expression of DA₂ in the cranial ganglia was significantly induced by constant darkness compared with the control treatment (*P*<0.05), while there was no effect of constant light in this tissue. Although the DA₂ mRNA expression in the thoracic ganglia appeared higher in crabs exposed to constant darkness and constant light than in crabs under control condition, there was no statistically significant difference between treatment (*P*>0.05). As in the cranial
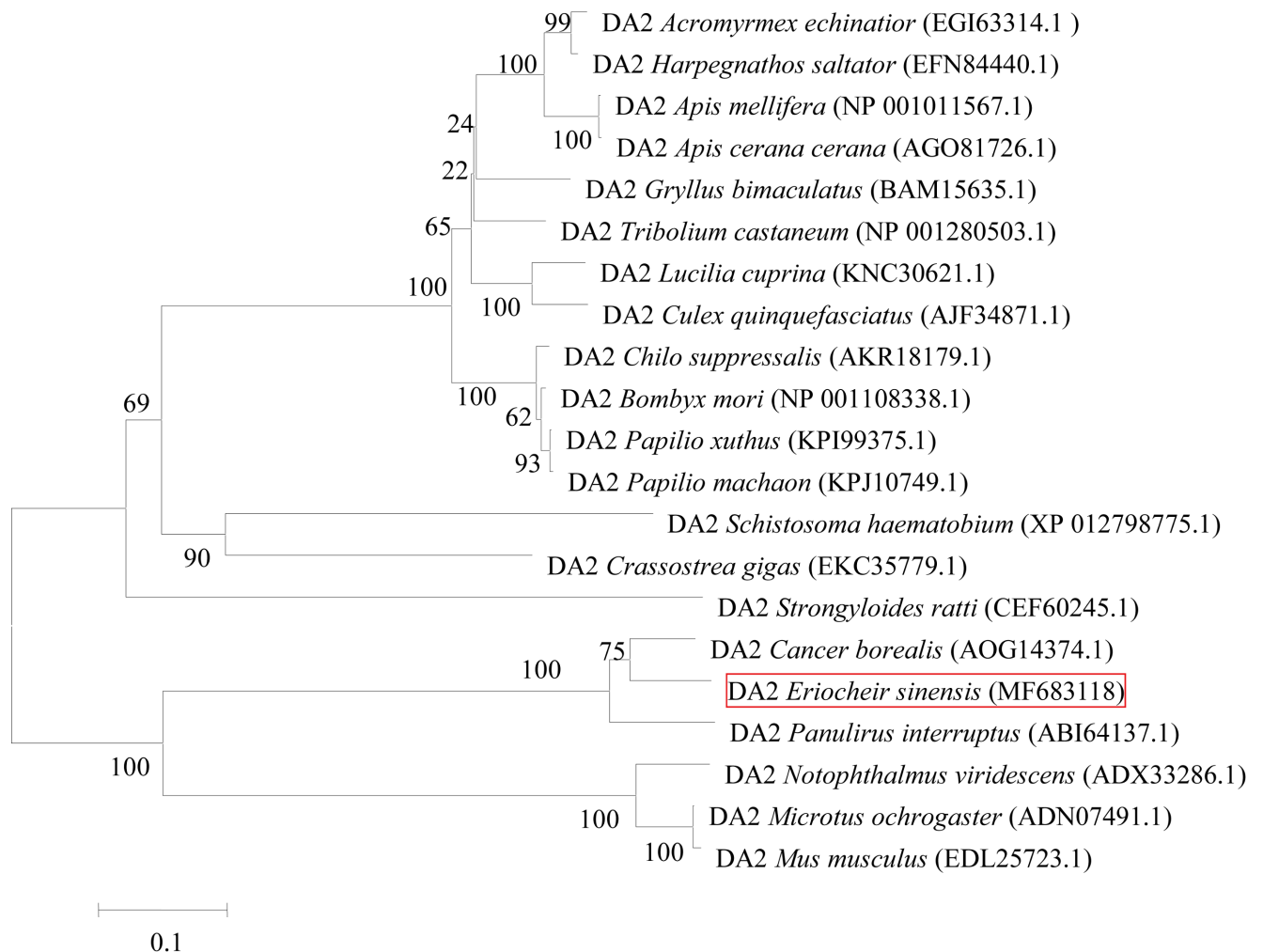
Fig 3. Phylogenetic relationships of the DA$_2$ receptor from *E. sinensis* and other invertebrates were analyzed with the MEGA 5 program using the neighbor-joining distance analysis. Bootstrap values from 1000 replicates are indicated at the nodes.

ganglia, the expression of DA$_2$ in eyestalks was significantly increased by constant darkness ($P<0.05$), but there was no effect of constant light ($P>0.05$).

DA$_2$ mRNA expression levels during different feeding statuses

To research the relationship between DA$_2$ receptors and feeding/digestion, we determined the DA$_2$ expression levels in the hepatopancreas and intestines of crabs during three feeding status (Fig 6). In both tissues, the levels of DA$_2$ expression were significantly higher after feeding than before feeding ($P<0.05$), but there were no significant differences between the before feeding and during feeding periods ($P>0.05$). In the hepatopancreas, the DA$_2$ expression level after feeding was also significantly higher than during the feeding period ($P<0.05$), while in the intestines, the DA$_2$ expression level after feeding was not significantly different from expression during with feeding period ($P>0.05$).

Discussion

In the present study, we have characterized a DA$_2$ receptor of the Chinese mitten crab, *E. sinensis*. The obtained sequence has considerable similarity with orthologous receptors from
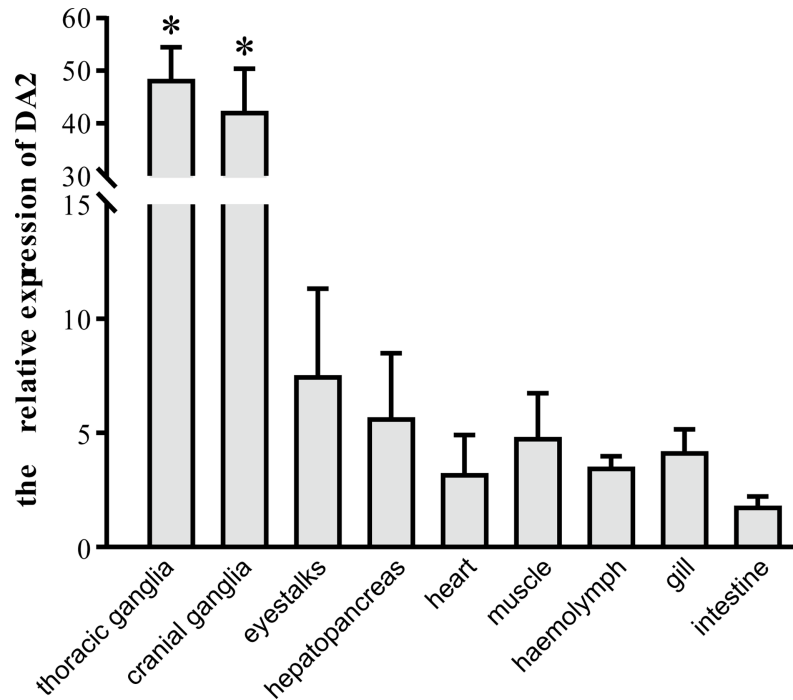
Fig 4. Expression level of the DA₂ gene normalized to 18S expression in the thoracic ganglia, cranial ganglia, eyestalks, gill, heart, muscle, hepatopancreas, intestine, and haemolymph of the crab. Values are the mean ± SE (n = 4–6). *indicates that expression in the corresponding tissues is significantly different from expression in the control organ (intestine) at $P<0.05$.

other invertebrates[2,8–10,29] and vertebrates[30,31]. The encoded 589 amino acids sequence contains typical characteristics of DA₂ receptors, such as a large third intracellular loop, a
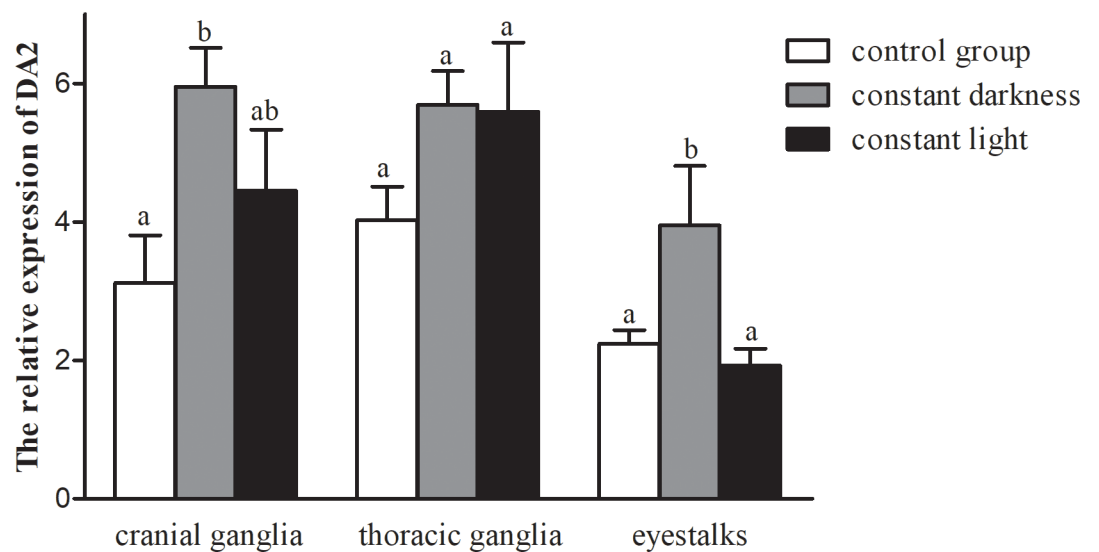

Fig 5. Effect of photoperiod on DA₂ mRNA expression (normalized to 18S expression) in the cranial ganglia, thoracic ganglia and eyestalks of crabs cultured in different light conditions compared with the control treatment (L: D = 12h:12h). Values are the means± SE (n = 8–10). Bars with different letters indicate statistically significant differences ($P<0.05$).
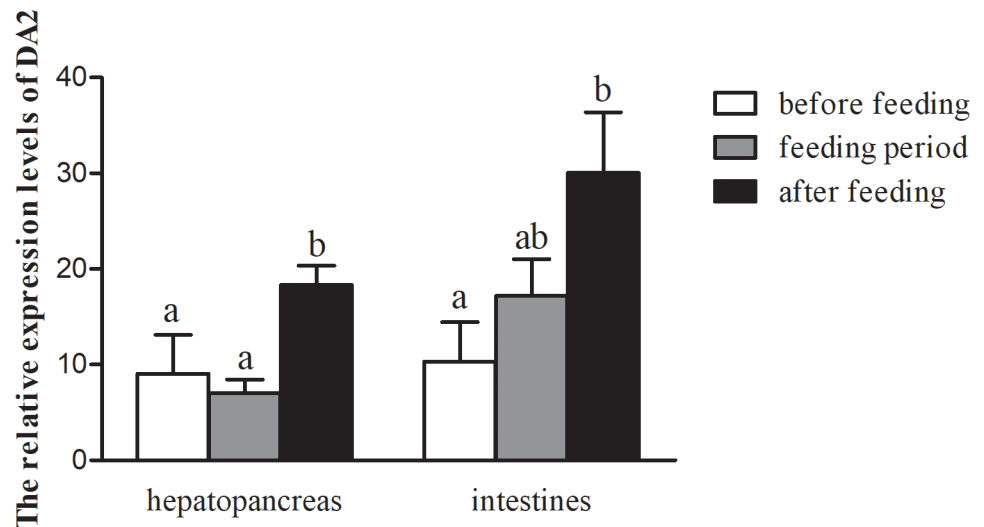
Fig 6. DA$_2$ mRNA expression levels in the hepatopancreas and intestines during different feeding statuses. Values are the means± SE (n = 6). Bars with different letters indicate statistical significant differences ($P<0.05$).

short C-terminal region, a DRY motif in the second intracellular loop and other conserved consensus sequences[32]. Sequence comparison and phylogenetic analysis suggest that DA$_2$ is a member of the D-2 like subfamily of dopamine receptors. The DA$_2$ in *E. sinensis* is most related to the DA$_2$ of *P. interruptus*, while lower but still remarkable degrees of homology are detected with other arthropods' DA$_2$ receptors; this suggests that the *E. sinensis* DA$_2$ possesses a highly conserved structure typical of type 2 dopamine receptors.

All residues involved in dopamine receptor activation were present in the *E. sinensis* DA$_2$ sequence. These include several putative phosphorylation sites and 5 putative N-glycosylation sites, such as those found in mammalian DA$_2$ receptors, which are highly phosphorylated and glycosylated neural receptors[31]. In addition, the *E. sinensis* DA$_2$ contains the conserved DRY (Asp-Arg-Tyr) motif at the interface between TM-III and the second intracellular loop; the DRY tripeptide is the key to the conformational changes necessary for receptor activation[33]. The Phe528 in TM-VI of the DA$_2$ is analogous to the conserved phenylalanine in other dopamine receptors that interacts with the aromatic biogenic amine ligand. Two serine residues in TM-V (Ser275 and Ser276) are known to be involved in the formation of hydrogen bonds with the catechol hydroxyl group of the dopamine agonist[1]. These interactions aid in the proper positioning of dopamine in the binding pocket of the receptor[34]. On the basis of our analysis, we conclude that the patterns of conservation and divergence observed in the DA$_2$ will help to describe the parts of the receptor molecule that are important for proper receptor function[30].

A fundamental aspect of dopamine function in the whole organism is the localization of its receptors in the various areas of the nervous system or in the periphery. When studying DA$_2$ gene transcript levels with qRT-PCR, the highest expression was observed in the central nervous tissues, including the cranial ganglia and the thoracic ganglia, followed by the periphery tissues. This result has been supported by several studies showing that the DA$_2$ receptor is expressed in the central nervous system and in peripheral tissues. In *Oreochromis niloticus*, higher expression of DA$_2$ is found in the anterior part of the brain than in other parts of the brain, and DA$_2$ is also expressed in the pituitary gland, liver and gills[31]. high levels of DA$_2$ have also been found in the pituitary gland of *Xenopus*[30]. These studies were thus somewhat

similar to our results; however, in contrast to our results, the DA$_2$ receptor is not expressed in the heart and muscle of the tilapia, *Oreochromis niloticus*. DA$_2$ receptors are also expressed by amacrine, bipolar, and ganglia cells[35,36] and possibly by the intrinsically photosensitive retinal ganglion cells[37], functioning as both postsynaptic receptors and autoreceptors that inhibit dopamine release[38]. In addition, the DA$_2$ receptor is expressed at high levels in the caudate nucleus, putamen, olfactory bulb, substantia nigra, nucleus accumbens, and ventral tegmental area and is found at low levels in the hypothalamus, kidney, blood vessels, heart, septum, cortex, gastrointestinal tract and sympathetic ganglia in mammals such as rats and humans[23], where it is involved in reward-motivation functions, blood pressure regulation, working memory, and gastrointestinal motility. Thus, overall, DA$_2$ receptor primarily present in the nervous system, but also present in peripheral tissues. In the eyestalks and intestines of *E. sinensis*, which contain neurons, the expression levels of DA$_2$ are similar to levels in the heart, gill and hemolymph; this may be caused by spatial and temporal differences in DA$_2$ gene expression. Further research on expression under different environmental conditions is needed to investigate this possibility.

It is well known that DA mediates a various functions via different DA receptors. DA$_1$ receptors are involved in coordinating metamorphosis in *Drosophila*[39], and DA$_1$ and DA$_2$ receptors regulate the phase change of migratory locust in two different directions[40]. In the inner retina, the DA$_2$ receptor also plays a role in regulating the development of light responses[41], and in *Daphnia magna*, the DA$_2$ receptor is involved in swimming behavior [42]. In the mushroom body of the silkworm, DA$_2$ plays a role in the release of the diapauses hormone[29]. In the rat, the DA$_3$ receptor may reinforce the effects of cocaine and may be a useful target for treating cocaine abuse[43]. Recent research has shown that DA$_4$ activation induces the hippocampal neuronal calcium response[44], and activation of DA$_5$ inhibits gastric cancer cell growth[45]. In this study, we researched, for the first time, the effect of photoperiod on DA$_2$ mRNA expression.

Light is one of the important environmental factors affecting crustaceans' survival, directly or indirectly influencing their growth, feeding and reproduction[13,46,47], and light environment is the major factor regulating the synthesis and metabolism of dopamine[16]. As a benthic animal, the Chinese mitten crab prefers a dark environment, and planting waterweed can have a shading and cooling effect that is beneficial to growth and survival[48]. Boosting dopamine can also promote biological growth and survival[49]. In this study, the effect of photoperiod on DA$_2$ mRNA expression in the cranial ganglia, thoracic ganglia and eyestalks was analyzed by qRT-PCR. We found that the expression level of the DA$_2$ receptor was significantly induced by constant darkness in the cranial ganglia and eyestalks but not affected by constant light in any tissues. Therefore, we speculate that a prolonged dark period is good for the growth of these crabs. Our results are in conformity with Dubocovich's finding that constant light activates the dopamine-containing retinal neurons, leading to elevated dopamine release and DA$_2$ receptor down-regulation[50]. Furthermore, other studies have shown that in the retina, exposure to constant light induces dopamine release and dopamine receptor 2 down-regulation[19,51], and under different light:dark cycles, retinal levels of dopamine are high during light phases and low during dark phases[16]. We believe that the main causes of this difference are species differences and tissue differences. However, there are few studies on the effects of constant darkness on dopamine and dopamine receptors. Therefore, this aspect remains to be thoroughly investigated.

Previous studies have found that feeding status affects glucose metabolism[52], insulin secretion[53] and hypothalamic neuronal activity[54] in vertebrates and that food intake is accompanied by a significant decrease in DA levels in rats compared with levels before food was provided[55]. However, the effect of food intake on DA receptors is not clear. In a

previous study, we divided feeding status into 3 categories: before feeding, during the feeding period and after feeding[27]. In this study, we studied the variation of DA$_2$ receptor expression in tissues from crabs of different feeding statuses, and the results showed that the expression levels of the DA$_2$ receptor were significantly higher after feeding than before feeding or during the feeding period, indicating that the DA$_2$ receptor plays an important digestive role in the hepatopancreas and intestines[56]. Although, dopamine hinders the absorption rate of glucose, it seems to play a role in regulating the digestion and transport function of the enterocyte membrane in rats[57]. At the same time, in the hepatopancreas of *Cyrtograpsus angulatus*, DA significantly decreases lipase activity and the digestive capacity was reduced[58]. These findings suggest that there are differential and specific mechanisms by which DA modulates the activity of digestive enzymes in such tissues. Our research on the effects of DA on digestion is of great significance. There have been few studies investigating which types of dopamine receptors play roles in digestion, and therefore these pathway have not yet been elucidated.

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Author Contributions

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References

1. Sukthaworn S, Panyim S, Udomkit A. Molecular and functional characterization of a dopamine receptor type1 from *Penaeus monodon*. Aquaclture. 2013; 380-383(1):99–105. https://doi.org/10.1016/j.aquaculture.2012.12.001

2. Yang BY, Ni JB, Zeng Z, Shi B, You WW, Ke CH. Cloning and characterization of the dopamine like receptor in the oyster *Crassostrea angulata*: Expression during the ovarian cycle. Comparative Biochemistry and Physiology Part B. 2013; 164(3):168–175. https://doi.org/10.1016/j.cbpb.2012.12.006 PMID: 23274282.

3. Kuo C, Hsu C, Lin C. Hyperglycaemic effects of dopamine in tiger shrimp, *Penaeus monodon*. Aquaclture. 1995; 135(1–3):161–172. https://doi.org/10.1016/0044-8486(95)01011-4

4. Sakharov DA, Salxnki J. Effects of dopamine antagonist on snail locomotion. Experientia. 1982; 38 (9):1090–1091.

5. Syed NI, Winlow W. Respiratory behavior in the pond snail *Lymnaea stagnalis*. II. Neural elements of the central pattern generator (CPG). Journal of Comparative Physiology A. 1991; 169(5):557–568. https://doi.org/10.1007/BF00193546

6. Zendehdel M, Moosadoost Y, Masoumi R, Rostami B, Shahir MH, Hassanpour S. Endogenous Nitric oxide and Dopamine regulates feeding behavior in neonatal layer-type chicken. Annals of Animal Science. 2017. https://doi.org/10.1515/aoas-2016-0094

7. Korshunov KS, Blakemore LJ, Trombley PQ. Dopamine: A Modulator of Circadian Rhythms in the Central Nervous System. Frontiers in Cellular Neuroscience. 2017; 11(91):1–17. https://doi.org/10.3389/fncel.2017.00091 PMID: 28420965

8. Clark MC, Baro DJ. Molecular cloning and characterization of crustacean type-one dopamine receptors: D$_{1\alpha Pan}$ and D$_{1\beta Pan}$. Comparative Biochemistry and Physiology Part B. 2006; 143(3):294–301. https://doi.org/10.1016/j.cbpb.2005.11.017 PMID: 16426885.

9. Clark MC, Baro DJ. Arthropod D$_2$ receptors positively couple with cAMP through the Gi/o protein family. Comparative Biochemistry and Physiology Part B 2007; 146(1):9–19. https://doi.org/10.1016/j.cbpb. 2006.08.018 PMID: 17134931.

10. Northcutt AJ, Lett KM, Garcia VB, Diester CM, Lane BJ, Marder E, et al. Deep sequencing of transcriptomes from the nervous systems of two decapod crustaceans to characterize genes important for neural circuit function and modulation. BMC Genomics. 2016; 17(1):868–889. https://doi.org/10.1186/s12864-016-3215-z PMID: 27809760.

11. Yang XZ, Zhang C, Huang GY, Xu MJ, Cheng YX, Yang ZG, et al. Cellular and biochemical parameters following autotomy and ablation-mediated cheliped loss in the Chinese mitten crab, *Eriocheir sinensis*. Developmental and Comparative Immunology. 2018; 81:33–43. https://doi.org/10.1016/j.dci.2017.11. 003 PMID: 29146453

12. Andrés M, Rotllant G, Zeng CS. Survival, development and growth of larvae of the blue swimmer crab, *Portunus pelagicus*, cultured under different photoperiod conditions. Aquaculture. 2010; 300(1–4):218–222. https://doi.org/10.1016/j.aquaculture.2009.12.026

13. Lin XT. Influence of phtotperiod on food consumption and development of *Macrobrachium Rosenbergii* larvae. Oceanologia et limnologia sinica. 1997; 28(1):13–20.

14. Nir I, Haque R, Iuvone PM. Diurnal metabolism of dopamine in dystrophic retinas of homozygous and heterozygous *retinal degeneration slow (rds)* mice. Brain Research. 2000; 884(1–2):13–22. https://doi. org/10.1016/S0006-8993(00)02855-9 PMID: 11082482.

15. Iuvone PM, Galli CL, Garrison-Gund CK, Neff NH. Light stimulates tyrosine hydroxylase activity and dopamine synthesis in retinal amacrine neurons. Science. 1978; 202(4370):901–902. https://doi.org/10.1126/science.30997 PMID: 30997.

16. Lorenc-Duda A, Berezińska M, Urbańska A, Gołembiowska K, Zawilska JB. Dopamine in the Turkey Retina—An Impact of Environmental Light, Circadian Clock, and Melatonin. Journal of Molecular Neuroscience. 2009; 38(1):12–18. https://doi.org/10.1007/s12031-008-9153-8 PMID: 18953673.

17. Shelke RRJ, Lakshmana MK, Ramamohan Y, Raju TR. Levels of dopamine and noradrenaline in the developing retina—effect of light deprivation. International Journal of Developmental Neuroscience. 1997; 15(1):139–143. https://doi.org/10.1016/s0736-5748(96)00080-9 PMID: 9099624.

18. Doyle SE, McIvor WE, Menaker M. Circadian rhythmicity in dopamine content of mammalian retina: role of the photoreceptors. Journal of Neurochemistry. 2002; 83(1):211–219. https://doi.org/10.1046/j. 1471-4159.2002.01149.x PMID: 12358745.

19. Bartmann M, Schaeffel F, Hagel G, Zrenner E. Constant light affects retinal dopamine levels and blocks deprivation myopia but not lens-induced refractive errors in chickens. Visual Neuroscience. 1994; 11 (2):199–208. https://doi.org/10.1017/S0952523800001565 PMID: 8003448.

20. Khodadadi M, Zendehdel M, Baghbanzadeh A, Babapour V. Consequence of dopamine D2 receptor blockade on the hyperphagic effect induced by cannabinoid CB1 and CB2 receptors in layers. British poultry science. 2017; 58(5):585–593. https://doi.org/10.1080/00071668.2017.1357799 PMID: 28728428.

21. Boekhoudt L, Roelofs TJM, de Jong JW, de Leeuw AE, Luijendijk MCM, Wolterink-Donselaar IG, et al. Does activation of midbrain dopamine neurons promote or reduce feeding? International Journal of Obesity. 2017; 41(7):1131–1140. https://doi.org/10.1038/ijo.2017.74 PMID: 28321131.

22. Land BB, Narayanan NS, Liu RJ, Gianessi CA, Brayton CE, Grimaldi DM, et al. Medial prefrontal D1 dopamine neurons control food intake. Nature Neuroscience. 2014; 17(2):248–253. https://doi.org/10. 1038/nn.3625 PMID: 24441680.

23. Ayano G. Dopamine: Receptors, Functions, Synthesis, Pathways, Locations and Mental Disorders: Review of Literatures. Journal of Mental Disorders and Treatment. 2016; 2(2). https://doi.org/10.4172/2471-271x.1000120

24. Li ZS, Schmauss C, Cuenca A, Ratcliffe E, Gershon MD. Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D2 receptor: analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. Journal of Neuroscience the Official Journal of the Society for Neuroscience. 2006; 26(10):2798–2807. https://doi.org/10.1523/JNEUROSCI.4720-05.2006 PMID: 16525059.

25. Khotimchenko YS, Deridovich II. The effect of dopamine and galoperidol on cyclic amp in the gonad of the bivalve mollusc *Mizuhopecten yessoensis* and the sea urchin *Strongylocentrotus intermedius*. Comparative Biochemistry and Physiology Part C 1989; 92(1):23–26. https://doi.org/10.1016/0742-8413 (89)90196-5

26. Tilden AR, Alt J, Brummer K, Groth R, Herwig K, Wilson A, et al. Influence of photoperiod on N-acetyltransferase activity and melatonin in the fiddler crab *Uca pugilator*. General and Comparative Endocrinology. 2001; 122(3):233–237. https://doi.org/10.1006/gcen.2001.7641 PMID: 11356035.

27. Li T. Effect of 5-HT on the intestinal motility of Eriocheir sinensis. 2016.

28. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution. 2011; 28(10):2731–2739. https://doi.org/10.1093/molbev/msr121 PMID: 21546353.

29. Mitsumasu K, Ohta H, Tsuchihara K, Asaoka K, Ozoe Y, Niimi T, et al. Molecular cloning and characterization of cDNAs encoding dopamine receptor-1 and -2 from brainsuboesophageal ganglion of the silkworm, *Bombyx mori*. Insect Molecular Biology 2008; 17(2):185–195. https://doi.org/10.1111/j.1365-2583.2008.00792.x PMID: 18353107.

30. Martens GJ, Molhuizen HO, Gröneveld D, Roubos EW. Cloning and sequence analysis of brain cDNA encoding a *Xenopus* D2 dopamine receptor. Febs Letters. 1991; 281(1–2):85–89. https://doi.org/10.1016/0014-5793(91)80364-9 PMID: 1826663.

31. Levavi-Sivan B, Aizen J, Avitan A. Cloning, characterization and expression of the D$_2$ dopamine receptor from the tilapia pituitary. Molecular and cellular endocrinology. 2005; 236(1–2):17–30. https://doi.org/10.1016/j.mce.2005.03.010 PMID: 15876479.

32. Vleugels R, Lenaerts C, Baumann A, Vanden Broeck J, Verlinden H. Pharmacological characterization of a 5-HT$_1$-type serotonin receptor in the red flour beetle, *Tribolium castaneum*. PLoS One. 2013; 8(5): e65052. https://doi.org/10.1371/journal.pone.0065052 PMID: 23741451.

33. Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocrine Reviews. 2000; 21(1):90–113. https://doi.org/10.1210/edrv.21.1.0390 PMID: 10696571.

34. Strader CD, Fong TM, Graziano MP, Tota MR. The family of G-protein-coupled receptors. Faseb Journal Official Publication of the Federation of American Societies for Experimental Biology. 1995; 9 (9):745–754. PMID: 7601339.

35. Derouiche A, Asan E. The dopamine D$_2$ receptor subfamily in rat retina: ultrastructural immunogold and *in situ* hybridization studies. European Journal of Neuroscience. 1999; 11(4):1391–1402. https://doi.org/10.1046/j.1460-9568.1999.00557.x PMID: 10103134.

36. Nguyen-Legros J, Versaux-Botteri C, Vernier P. Dopamine receptor localization in the mammalian retina. Molecular Neurobiology. 1999; 19(3):181–204. https://doi.org/10.1007/BF02821713 PMID: 10495103.

37. Sakamoto K, Liu C, Kasamatsu M, Pozdeyev NV, Iuvone PM, Tosini G. Dopamine regulates melanopsin mRNA expression in intrinsically photosensitive retinal ganglion cells. European Journal of Neuroscience. 2005; 22(12):3129–3136. https://doi.org/10.1111/j.1460-9568.2005.04512.x PMID: 16367779.

38. Tosini G, Iuvone PM. Role of Melatonin and Dopamine in the Regulation of Retinal Circadian Rhythms. In: Tosini G, Iuvone PM, Mcmahon DG, Collin SP, editors. Retina and Circadian Rhythms. Springer New York; 2014. p. 49–68.

39. Regna K, Kurshan PT, Harwood BN, Jenkins AM, Lai CQ, Muskavitch MA, et al. A critical role for the Drosophila dopamine D1-like receptor Dop1R2 at the onset of metamorphosis. BMC Developmental Biology. 2016; 16(1):15. https://doi.org/10.1186/s12861-016-0115-z PMID: PMC4868058. PMID: 27184815

40. Guo XJ, Ma ZY, Kang L. Two dopamine receptors play different roles in phase change of the migratory locust. Frontiers in Behavioral Neuroscience. 2015; 9:1–13. https://doi.org/10.3389/fnbeh.2015.00001 PMC4379914.

41. Tian N, Xu HP, Wang P. Dopamine D2 receptors preferentially regulate the development of light responses of the inner retina. European Journal of Neuroscience. 2015; 41(1):17–30. https://doi.org/10.1111/ejn.12783 PMID: PMC4331351. PMID: 25393815

42. Barrozo ER, Fowler DA, Beckman ML. Exposure to D2-like dopamine receptor agonists inhibits swimming in *Daphnia magna*. Pharmacology Biochemistry and Behavior. 2015; 137(101–109. https://doi.org/10.1016/j.pbb.2015.08.010 PMID: 26296938

43. Caine SB, Koob GF. Modulation of cocaine self-administration in the rat through D-3 dopamine receptors. Science. 1993; 260(5115):1814–1816. https://doi.org/10.1126/science.8099761 PMID: 8099761

44. Wang YL, Wang JG, Guo FL, Gao XH, Zhao DD, Zhang L, et al. Selective dopamine receptor 4 activation mediates the hippocampal neuronal calcium response via IP3 and ryanodine receptors. Brain Research. 2017; 1670:1–5. https://doi.org/10.1016/j.brainres.2017.05.012 PMID: 28506554

45. Leng ZG, Lin SJ, Wu ZR, Guo YH, Cai L, Shang HB, et al. Activation of DRD5 (dopamine receptor D5) inhibits tumor growth by autophagic cell death. Autophagy. 2017; 13(8):1404–1419. https://doi.org/10.1080/15548627.2017.1328347 PMID: PMC5584849. PMID: 28613975

46. Jiang QC, Zhang WY, Tan HY, Zhang YT, Yang JX, Li F. A study on the feeding rhythm of juvenile Australia red claw crayfish (*Cherax quadricarinatus*) under different photoperiods. Freshwater Fisheries. 2012; 42(5):89–91.

47. Huang XX, Chen MK, Liu B. The effect of photoperiod on reproduction of the brine shrimp, *Artemia SP*. Acta Hydrobiologica Sinica. 2001; 25(3):297–300.

48. Zhang Q, Wang ZB, Yang XB, Zhu SH. Effects of the Coverage of Waterweed in Pond on the Growth of *Eriocheir sinensis*. Animal Husbandry and Feed Science. 2014; 35(12):66–67. https://doi.org/10. 16003/j.cnki.issn1672-5190.2014.12.029

49. Van Alstyne KL, Anderson KJ, van Hees DH, Gifford SA. Dopamine release by *Ulvaria obscura* (Chlorophyta): environmental triggers and impacts on photosynthesis, growth, and survival of the releaser. Journal of Phycology. 2013; 49(4):719–727. https://doi.org/10.1111/jpy.12081 PMID: 27007204.

50. Dubocovich ML, Lucas RC, Takahashi JS. Light-dependent regulation of dopamine receptors in mammalian retina. Brain Research. 1985; 335(2):321–325. https://doi.org/10.1016/0006-8993(85)90485-8 PMID: 2988696.

51. De Mello MC, Ventura AL, Paes de Carvalho R, Klein WL, De Mello FG. Regulation of dopamine- and adenosine-dependent adenylate cyclase systems of chicken embryo retina cells in culture. Proceedings of the National Academy of Sciences of the United States of America. 1982; 79(18):5708–5712. https:// doi.org/10.1073/pnas.79.18.5708 PMID: 6291061.

52. Lawrence L, Soderholm LV, Roberts A, Williams J, Hintz H. Feeding status affects glucose metabolism in exercising horses. Journal of Nutrition. 1993; 123(12):2152–2157. PMID: 8263610.

53. Takahashi H, Kurose Y, Sakaida M, Suzuki Y, Kobayashi S, Sugino T, et al. Ghrelin differentially modulates glucose-induced insulin secretion according to feeding status in sheep. Journal of Endocrinology. 2007; 194(3):621–625. https://doi.org/10.1677/JOE-07-0206 PMID: 17761901.

54. Gautron L, Mingam R, Moranis A, Combe C, Layé S. Influence of feeding status on neuronal activity in the hypothalamus during lipopolysaccharide-induced anorexia in rats. Neuroscience. 2005; 134 (3):933–946. https://doi.org/10.1016/j.neuroscience.2005.03.063 PMID: 16039792.

55. Meguid MM, Fetissov SO, Blaha V, Yang ZJ. Dopamine and serotonin VMN release is related to feeding status in obese and lean Zucker rats. Neuroreport. 2000; 11(10):2069–2072. https://doi.org/10.1097/ 00001756-200007140-00002 PMID: 10923645.

56. Shi OW, Li RH, Mu CK, Song WW, Liu CJ, Wang CL. Optimal conditions for Portunus trituberculatus "Keyong 1" molting. Oceanologia et limnologia sinica. 2015; 46(4):870–878.

57. Guska NI, Sheptitskiĭ VA, Razlovan TA. The role of dopamine in the mechanism of the regulation of the digestive-transport functions of the enterocyte membrane during stress. Fiziologicheskiĭ Zhurnal Imeni Imsechenova. 1993; 79(6):40–47. PMID: 8401653

58. Michiels MS, Del Valle JC, Mananes AAL. Effect of environmental salinity and dopamine injections on key digestive enzymes in hepatopancreas of the euryhaline crab *Cyrtograpsus angulatus* (Decapoda: Brachyura: Varunidae). Scientia Marina. 2013; 77(1):129–136. https://doi.org/10.3989/scimar.03687. 09D