Bulletin of Entomological Research

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Molecular characterisation and developmental expression analysis of the *5-HT7* receptor in *Chrysopa formosa*

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Research Paper

Cite this article: Li F, Wang L, Li Y, Tian X, Wang J, Shen Z, Zhang J, Zhang L, Li Y (2025) Molecular characterisation and developmental expression analysis of the *5-HT7* receptor in *Chrysopa formosa*. *Bulletin of Entomological Research* **115**, 317–328. https://doi.org/10.1017/S0007485325000148

Received: 27 November 2024
Revised: 24 February 2025
Accepted: 25 February 2025
First published online: 18 March 2025

Keywords:
5-HT7 receptor; diapause; expression pattern; lacewing; serotonin

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Abstract

Serotonin (5-hydroxytryptamine, 5-HT) is a key monoamine neurotransmitter in insects, which regulates neural functions and influences various developmental and physiological processes by binding to its receptors. In this study, we investigate the molecular characteristics, phylogenetic relationships, and expression patterns of the *5-HT7* receptor (*Cf5-HT7*) in *Chrysopa formosa*, with a focus on its potential involvement in developmental and diapause regulation. The *Cf5-HT7* gene was identified and cloned from the *C. formosa* transcriptome, revealing an open reading frame of 1788 bp encoding a 596 amino acid protein. Sequence analysis confirmed that *Cf5-HT7* is a typical class A G protein-coupled receptor, characterised by seven transmembrane domains and several post-translational modifications, including palmitoylation and N-glycosylation sites. Phylogenetic analysis revealed that *Cf5-HT7* is most closely related to the *5-HT7* receptor from *Chrysoperla carnea*, with high conservation of key motifs involved in ligand binding and receptor activation. Expression analysis across different developmental stages of *C. formosa* showed that *Cf5-HT7* is highly expressed in the first instar larvae, with significant upregulation observed during the prepupal stage. Under diapause-inducing conditions, *Cf5-HT7* expression is modulated in a stage-specific manner, showing a marked decrease at the onset of diapause, followed by a significant increase during the mid-to-late diapause maintenance phase. These findings suggest that it plays a pivotal role in regulating development and diapause processes in *C. formosa*, offering new insights into the molecular mechanisms governing insect life cycle transitions. This study lays the groundwork for future research into the functional roles of 5-HT7 receptors in insect physiology and their potential applications in manipulating diapause.

Introduction

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a critical monoamine neurotransmitter and modulator in both vertebrates and invertebrates. This neurotransmitter is synthesised from tryptophan through the enzymatic actions of tryptophan hydroxylase and aromatic L-amino acid decarboxylase (Movassaghi and Milasincic, 2024; Wang *et al.*, 2023). Once synthesised, 5-HT is distributed in various systems, such as the central and peripheral nervous systems, digestive system, muscles, and sensory organs in insects (Blenau and Thamm, 2011; Vleugels *et al.*, 2015). 5-HT plays a crucial role in regulating and modulating a wide range of behaviours, developmental, and physiological processes in insects. These include circadian rhythms (Blenau and Thamm, 2011), feeding (Falibene *et al.*, 2012; Gasque *et al.*, 2013), learning and memory (Sitaraman *et al.*, 2012; Zeng *et al.*, 2022). Additionally, 5-HT regulates growth and molting (Sato, 2020), development (Apeksha *et al.*, 2018), and reproduction and energy metabolism (Ling and S, 2018; Luo *et al.*, 2012). The effects of 5-HT are mediated by its binding to specific membrane receptors, which activates various intracellular second messengers such as cyclic adenosine monophosphate (cAMP) and phosphoinositide turnover, thereby trigger a variety of cellular responses and behavioural outcomes (Millan *et al.*, 2008; Vleugels *et al.*, 2015). These receptors are primarily classified into three types (5-HT1, 5-HT2, and 5-HT7) in most insects, based on their conserved amino acid sequence with their vertebrate counterparts. Notably, a novel 5-HT8 receptor was identified in *Pieris rapae* larvae, which differs significantly from other known receptor types and presents a unique pharmacological profile (Qi *et al.*, 2014). These insect 5-HT receptors belong to the superfamily of G protein-coupled receptors (GPCRs),

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characterised by seven transmembrane domains that play a crucial role in signal transduction (Millan *et al.*, 2008; Vleugels *et al.*, 2015).

Recent studies have further elucidated the molecular and pharmacological characteristics of 5-HT receptors in insects, encompassing various species such as *Drosophila melanogaster* (Blenau and Thamm, 2011), *Aedes aegypti* (Li *et al.*, 2022; Pietrantonio *et al.*, 2001), *Apis mellifera* (Thamm *et al.*, 2010), *Manduca sexta* (Dacks *et al.*, 2006, 2013) and *Rhodnius prolixus* (Paluzzi *et al.*, 2015). These findings highlight the critical role of 5-HT in regulating insect growth, development and reproduction through its receptors. For instance, disrupting the 5-HT7A receptor in *A. aegypti* mutants leads to abnormal larval and pupal development, reduced motility and lower egg hatching rates in offspring (Li *et al.*, 2022). Moreover, 5-HT signaling governs developmental timing and progression by modulating hormonal pathways, particularly those involving insulin-like peptides and ecdysteroids. For example, Ling and Raikhel (2018) demonstrated that the fat-body-specific 5-HT2B receptor is essential for regulating body-size growth, lipid metabolism, and ovarian development in *A. aegypti* through interactions with insulin-like peptides. In addition to regulating development, 5-HT and its receptors also coordinate an insect's physiological responses to environmental cues (e.g. food, light, temperature), ensuring normal development and survival in a way that maximises fitness. For example, serotonergic neurons in *D. melanogaster* can sense nutritional conditions, innervate the prothoracic gland (PG), and activate 5-HT7 receptor in the PG to regulate the timing of ecdysteriod biosynthesis, ultimately influencing the timing of pupariation (Shimada-Niwa and Niwa, 2014). Several studies have also highlighted the role of 5-HT signaling as a key regulator of diapause, a developmental arrest state triggered by adverse conditions. In *Antheraea pernyi*, the 5-HTB receptor may inhibit the synthesis or release of prothoracicotropic hormone (PTTH), thereby maintaining pupal diapause (Wang *et al.*, 2013). Similarly, Andreatta *et al.* (2018) found that 5-HT1A receptor activity is linked to the induction of dormancy through modulation of insulin signaling pathways. Overall, understanding the functions of 5-HT receptors in insects, particularly their signaling transduction mechanisms, not only sheds light on their biological and ecological roles but also provides valuable insights into potential broader applications through targeted manipulation of these receptor-mediated pathways.

The green lacewing *Chrysopa formosa* Brauer (Neuroptera: Chrysopidae) is an effective predator of numerous agricultural and forestry pests, including aphids, thrips, whiteflies, and lepidopteran eggs and larvae (Li *et al.*, 2023). Due to its broad prey spectrum, high predation ability, strong reproductive capacity and wide adaptability, *C. formosa* has been recognised as a potential biocontrol agent. It has been widely used for pest management in field crops, greenhouse vegetables, and orchards in China, where it has demonstrated significant pest control efficacy (Li *et al.*, 2018). Under natural conditions, *C. formosa* enters a prepupal diapause to overwinter, an important seasonal adaptation strategy. Diapause is a state of developmental arrest which is regulated by environmental and hormonal factors (Denlinger, 2022a). For natural enemies, the feature of diapause offers a state that can be exploited to improve their shelf life, shipment and application in biological control (Denlinger, 2022b). Our previous research has demonstrated that the environmental factors, such as photoperiod and temperature, play a significant role in regulating the induction, maintenance and termination of diapause in *C. formosa* (Li *et al.*, 2018). However, the molecular mechanisms by which *C. formosa* integrates these environmental signals into its hormonal pathways to produce the diapause phenotype remain unclear. Previous studies in *D. melanogaster, A. aegypti* and *A. pernyi* have shown that the 5-HT signaling play a bridging role between environmental signal transduction and the endocrine system response during insect diapause and dormancy (Andreatta *et al.*, 2018; Liao *et al.*, 2017; Shimada-Niwa and Niwa, 2014; Takeda and Suzuki, 2022; Wang *et al.*, 2013). Therefore, identifying and characterizing different 5-HT receptor subtypes and investigating their expression patterns and functions will provide valuable insights into the role of 5-HT signaling in regulating insect diapause.

The 5-HT7 receptor is the most recently identified member of the 5-HT receptor family and is characterized by positive coupling to cAMP through binding to G_s proteins (Huang *et al.*, 2022). Since its initial discovery in *D. melanogaster* (Witz *et al.*, 1990), 5-HT7 receptor have been identified and characterised in several insects, including *A. aegypti, A. mellifera, Tribolium castaneum, Mythimna separata* and *Polyrhachis vicina* (Chen *et al.*, 2023; Pietrantonio *et al.*, 2001; Schlenstedt *et al.*, 2006; Vleugels *et al.*, 2014; Yang *et al.*, 2019). Current research suggests that 5-HT7 receptor is involved in the regulation of courtship and mating (Luo *et al.*, 2012), appetitive olfactory learning (Ganguly *et al.*, 2020), growth and developmental metamorphosis (Li *et al.*, 2022; Shimada-Niwa and Niwa, 2014), and stress response (Vesala *et al.*, 2012). However, the molecular and functional characteristics of the 5-HT7 receptor in economically important beneficial insects remain largely unexplored. The aim of this study was to investigate the molecular characteristics and expression pattern of the 5-HT7 receptor in *C. formosa*. We cloned the *5-HT7* receptor gene and analyse its sequence characteristics. Additionally, we determined its expression profiles across various developmental stages and during different phases of diapause in *C. formosa*. These findings may provide a valuable insight into the biological role of *Cf5-HT7* receptor in regulating development and diapause, and could contribute to the identification of novel targets for manipulating the diapause process in the future.

Materials and methods

Insect rearing and sample collection

The colony of *C. formosa* was maintained under conditions of 26 ±1°C, 16 h light/8 h dark (16L:8D) and 70 ±5% relative humidity (RH) as described by Li *et al.* (2018). Both larvae and adults of the lacewings were fed aphids *Megoura japonica* Matsumura. Normally developed lacewings (non-diapausing individuals) were reared in a climate chamber (RXZ-500B, Ningbo Jiangnan, China) at 20 ±1°C, 16L:8D and 70 ±5% RH. To determine relative mRNA expression of *5-HT7* at different developmental life stages, we sampled the newly hatched eggs within 24 hours; 3-day-old first (L1), second (L2) and third (L3) instar larvae; prepupae at 1-day (NDPP1), 3-day (NDPP3), 5-day (NDPP5) and 7-day-old (NDPP7); 1-day-old pupae (NDP1) and adult of non-diapausing *C. formosa*.

The diapausing prepupae of *C. formosa* were produced by continuously rearing eggs and larvae under diapause-inducing conditions of 20 ±1°C, 8L:16D and 70 ±5%RH, until they formed cocoons (Li *et al.*, 2018). Cocoons were collected daily and transferred into a petri dish (9 cm × 1.5 cm) for the purpose of sampling prepupae at different stages of diapause. The diapause in prepupae of *C. formosa* can last from 60 days to 120 days as reported by Li *et al.* (2018). To collect samples for diapause termination, prepupae that had been in diapause for 10 days were transferred to diapause-terminating conditions (5°C, 0L:24D) for 60 days, and then moved

https://doi.org/10.1017/S0007485325000148 Published online by Cambridge University Press

to the normal culturing conditions (20 ±1°C, 16L:8D) to promote their development. The pupae on the first day of pupation are considered as individuals that terminated diapause (DT). Eggs, larvae and prepupae of *C. formosa* reared under diapause-inducing conditions were collected for subsequent qRT-PCR experiments, and the sampling time points were identical to those of the non-diapause group. To characterize the expression pattern of *5-HT7* throughout diapause development, prepupae that had been in diapause for 1 day (D1), 10 days (D10), 30 days (D30), 60 days (D60) and 90 days (D90), as well as diapause terminated pupae (DT) were sampled. The collected *C. formosa* samples were cleaned and frozen with liquid nitrogen, and then stored in a refrigerator (−80°C) until analysis. Six biological replicates were used for each treatment, with each replicate containing three lacewings.

Cloning and sequencing of 5-HT7

Total RNA was extracted from adults of *C. formosa* using the TRIzol reagent method. The adults were ground into a powder in liquid nitrogen and then processed according to the instructions of the TransZol Up Plus RNA Kit (TransGen, Beijing, China). The concentration of isolated RNA was measured using a microvolume spectrophotometer (NanoPhotometer®NP80, Germany). First-strand cDNA was synthesised using the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen, Beijing, China), following the manufacturer's protocol. The cDNA was stored at −80°C for subsequent experiments.

Based on the transcriptome database of *C. formosa* constructed in our laboratory, the nucleotide sequence of one putative *5-HT7* gene was identified for cloning and verification. Specific primers for three sub-fragments of the *5-HT7* were designed using the NCBI web-based Primer-BLAST tool, and synthesised by Tsingke Biotech Co., Ltd. (Beijing, China) (table 1). Using adult cDNA as a template, PCR amplification was performed with 2 × Phanta® Max Master Mix (Dye Plus). The PCR products were purified and recovered by 1% agarose gel electrophoresis using the EasyPure Quick Gel Extraction Kit (TransGen, Beijing, China). Cleaned PCR products were ligated into a *pEASY®*-T&B Zero Cloning vector (TransGen, Beijing, China), transformed into DH5α Chemically Competent Cells, and then sequenced by Tsingke Biotech Co., Ltd. (Beijing, China). The sequences of the three sub-fragments were assembled into a complete sequence using Vector NTI v11.5 software. The assembled complete sequence of *5-HT7* was aligned with the target gene sequence obtained from *C. formosa* genome for further verification. The coding region for *5-HT7* was identified by using the ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder/). Similarity between *Cf5-HT7* and orthologs in other insect species was analysed by using the NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast).

Sequence and phylogenetic analysis

The amino acid sequence was deduced using DNAMAN v9 software, and the physical and chemical parameters of the protein were predicted with ExPASy ProtParam (https://web.expasy.org/protparam/). Conserved domain prediction was performed using the NCBI CD-Search program (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Functional analysis of the *Cf5-HT7* receptor was carried out using the InterPro102.0 (https://www.ebi.ac.uk/interpro/), while putative signal peptides were identified using the SignalP 6.0 server (https://services.healthtech.dtu.dk/services/SignalP-6.0/). Phosphorylation and palmitoylation sites were predicted using the GPS 6.0 web server (https://gps.biocuckoo.cn/online.php). The secondary structure of the *Cf5-HT7* receptor was predicted using the SPOMA tool (NPS@: SOPMA secondary structure prediction-NPSA-Lyon-France (inserm.fr)). Intrinsically disordered regions were predicted using the MobiDB-lite tool (https://caid.idpcentral.org/portal). The tertiary structure of *Cf5-HT7* receptor was predicted using AlphaFold 2, which infers the protein's 3D structure based on multiple sequence alignment (MSA) and structural templates. Default parameters were used during the prediction process unless specified otherwise. AlphaFold 2 generates multiple models, each associated with a corresponding confidence score, pLDDT (Predicted Local Distance Difference Test). To ensure the reliability of the structure, the model with the highest pLDDT value was selected for further analysis. The amino acid sequence of *Cf5-HT7*, along with orthologs from other insects, was aligned using ClustalW and MEGA v11. To explore the evolutionary relationships of *Cf5-HT7*, the deduced amino acid sequences of insect 5-HT1, 5-HT2, and 5-HT7 receptors were downloaded from NCBI (table 2), and a phylogenetic tree was constructed using the neighbour-joining method with a bootstrap value of 1000 in MEGA v11.

Table 1. Sequences of primers used for cDNA cloning and quantitative real-time PCR (qRT-PCR)

Primers name	Primer sequences (5′–3′)	Usage
5-HT7-F1	ATGGCGTCCTTTAATCAAAC	cDNA cloning
5-HT7-R1	CGCCGTATTCTAAGGGTTTG	
5-HT7-F2	TGGTGAGTTTGGCAGTCAGT	
5-HT7-R2	CCGCCTGCAGATGATGTAGT	
5-HT7-F3	CAACTCAATCAAATACGACCCA	
5-HT7-R3	TCATAGAAATGATTCACGGGCAGC	
q5-HT7-F	GGTCGAGTTCGGCTAGTACG	qRT-PCR
q5-HT7-R	CCGCCTGCAGATGATGTAGT	
qRP-F	CGAAACAGAAGTAAAACCGAAC	
qRP-R	TGCGTTGTTGTTGTGGAT	

Expression profiles of 5-HT7 determined by qRT-PCR

To investigate the potential functions of *5-HT7* gene in the growth and development of *C. formosa*, the expression profiles of *5-HT7* gene in different life stages (egg, L1, L2, L3, prepupae, pupae, adult) were analysed using qRT-PCR. The expression pattern of *Cf5-HT7* at different diapause stages in *C. formosa,* including D1, D5, D10, D30, D60, D90 and DT, were also determined. Furthermore, to examine the differential expressions of *Cf5-HT7* gene between the diapause-destined and non-diapause individuals of *C. formosa*, we compared the expression profiles across various life stages (egg, L1, L2, L3, prepupae at 1-day (PP1), 3-day (PP3), 5-day (PP5) and 7-day (PP7)) under these two conditions.

Different developmental stages of *C. formosa* were selected for total RNA extraction and the first-strand cDNA was synthesised using TransZol Up Plus RNA Kit and TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit. The primers used for qRT-PCR are listed in table 1, and ribosomal protein gene was explored as the internal references. The qRT-PCR reactions were conducted using TOROGreen 5 G qPCR PreMix. All reactions

https://doi.org/10.1017/S0007485325000148 Published online by Cambridge University Press

Table 2. The protein sequences of 5-HT receptors used for phylogenetic analysis

Species	Protein	GenBank ID
Tribolium castaneum	5-HT1A	AGN56420.1
	5-HT7	XP_966577.2
Drosophila melanogaster	5-HT7A	NP_524599.1
	5-HT7B	NP_001263131.1
Bombyx mori	5-HT1A	NP 001296510.1
	5-HT1B	NP_001037502.1
	5-HT2A	NP_001296483.1
	5-HT2B	XP_012552773.1
	5-HT7	AIZ66402.1
Gryllus bimaculatus	5-HT1A	BAJ83479.1
	5-HT1B	BAJ83480.1
Manduca sexta	5-HT1A	ABI33826.1
	5-HT1B	ABI33827.1
	5-HT7	AGL46976.1
Helicoverpa armigera	5-HT1A	WPQ67116.1
	5-HT1B	WPQ67115.1
	5-HT2A	WPQ67117.1
	5-HT2B	WPQ67118.1
Apis mellifera	5-HT1	CBI75449.1
	5-HT2	CBX90121.1
	5-HT7	NP_001071289.1
Aedes aegypti	5-HT2A	EJY57683.1
	5-HT7	AAG49292
Pieris rapae	5-HT7	AMQ67549.1
Chrysoperla carnea	5-HT7-like	XP_044727064.1
Polyrhachis vicina	5-HT7	AGZ62050.1
Danaus plexippus	5-HT1	EHJ73524.1

were performed in triple in a total volume of 20 μl containing 10 μl Premix, 1 μl of each primer, 1 μl sample cDNA, and 7 μl nuclease-free water. The qPCR reaction conditions were set as follows: 95°C pre-denaturation for 5 minutes; 95°C denaturation for 10 s, and 58°C annealing for 20 s for a total of 40 cycles; and finally, 95°C for 10 seconds, 65°C for 1 min, and 97°C for 1 s (The increment is 0.2°C/s; Add 5 readings /°C). The relative expression level of *5-HT7* was calculated using the $2^{-\Delta\Delta Ct}$ method (Li *et al.*, 2022). For each treatment, 6 independent biological samples were included, and three technical replicates of each biological sample were performed for all reactions.

Statistical analysis of data

Statistical data were analysed using GraphPad prism (GraphPad Software, San Diego, CA, United States). One-way analysis of variance followed by Tukey's multiple tests was performed to identify significant differences of gene expression data among different developmental stages. The differences in gene expression between diapause and non-diapause sample were evaluated using Student's *t*-test. All data are expressed as mean ± standard error (SE), and statistical significance was considered at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Results

Molecular feature of Cf5-HT7

The candidate *5-HT7* gene identified from the *C. formosa* transcriptome was further validated through gene cloning. Sequence analysis revealed that the open reading frame of the *C. formosa 5-HT7* gene (*Cf5-HT7*) was 1788 bp, encoding a protein of 596 amino acid residues in length (fig. 1). The predicted molecular weight of *Cf5-HT7* protein was 66.08 kD, and its isoelectric point (pI) was 8.54. The *Cf5-HT7* receptor comprises seven transmembrane domains (TM1-TM7), interspersed with three intracellular and three extracellular loops, supporting its classification within the typical class A family of GPCRs (fig. 2). There is no signal peptide present in *Cf5-HT7*. The *Cf5-HT7* protein contains a disordered region spanning residues 329-412, which is located in intracellular loop 3 (fig. 2a). It is speculated that the 5-HT7 receptor contains 28 putative ligand-binding sites. Secondary structure analysis revealed that the *Cf5-HT7* protein predominantly consists of alpha helices (29.70%), extended strands (25%), random coils (37.42%), and a smaller proportion of beta turns (7.89%). The disulfide bond between Cysteine residues118 (in extracellular loop 1) and Cysteine 204 (in extracellular loop 2) contributes to the structural stability of the protein, suggesting functional relevance (fig. 2b). The tertiary structure of the *Cf5-HT7* receptor was modelled using AlphaFold 2, yielding an average pLDDT score of 64.662. The regions corresponding to the seven transmembrane domains were predicted with high pLDDT scores (>70), indicating high model confidence. However, the extracellular region at the N-terminus, the cytoplasmic domain between TM5 and TM6, and the extracellular region at the C-terminus, which all exhibit low pLDDT scores, suggest that these regions may be unstructured in isolation (fig. 2c).

MSA and phylogenetic analysis of Cf5-HT7

A comparison of the amino acid sequence of *Cf5-HT7* with other insect 5-HT7 receptor orthologues revealed high degree of conservation (fig. 3). The *Cf5-HT7* receptor shared the highest similarity to the *Cc5-HT7* (*Chrysoperla carnea*), with the identity is 89.26%. The similarity between *Cf5-HT7* and *Tc5-HT7* (*Tribolium castaneum*) is 58.84%, followed by a 53.01% identity with the *Bm5-HT7* (*Bombyx mori*), and 52.59% with the *Aae5-HT7* (*Aedes aegypti*). MSA of *Cf5-HT7* revealed two conserved motifs: 'D-R-Y' are located at positions Asp_{142}-Arg_{143}-Tyr_{144} in TM3, and 'N-P-x-x-Y' are located at positions Asp_{479}-Pro_{480} -Tyr_{483} in TM7. The conserved consensus sequence 'F-x-x-x-W-x-P' is located at Phe_{437}-Trp_{441}-Pro_{443} within TM6, followed by two phenylalanine residues. The *Cf5-HT7* receptor also has four palmitoylation sites, located at Cys_{67}, Cys_{71}, Cys_{80} and Cys_{329}, as well as 7 N-glycosylation sites at Asn_5, Asn_8, Asn_{22}, Asn_{336}, Asn_{401}, Asn_{405}, and Asn_{529}. Four consensus sites for phosphorylation by protein kinase C are located at positions Thr_{331}, Ser_{427}, Ser_{590}, and Ser_{594} (fig. 3).

To investigate the phylogenetic relationships among 5-HTR subtypes, 5-HT1, 5-HT2 and 5-HT7 proteins from insects belonging to the orders Neuroptera, Lepidoptera, Coleoptera, Hymenoptera, Orthoptera and Diptera were downloaded from

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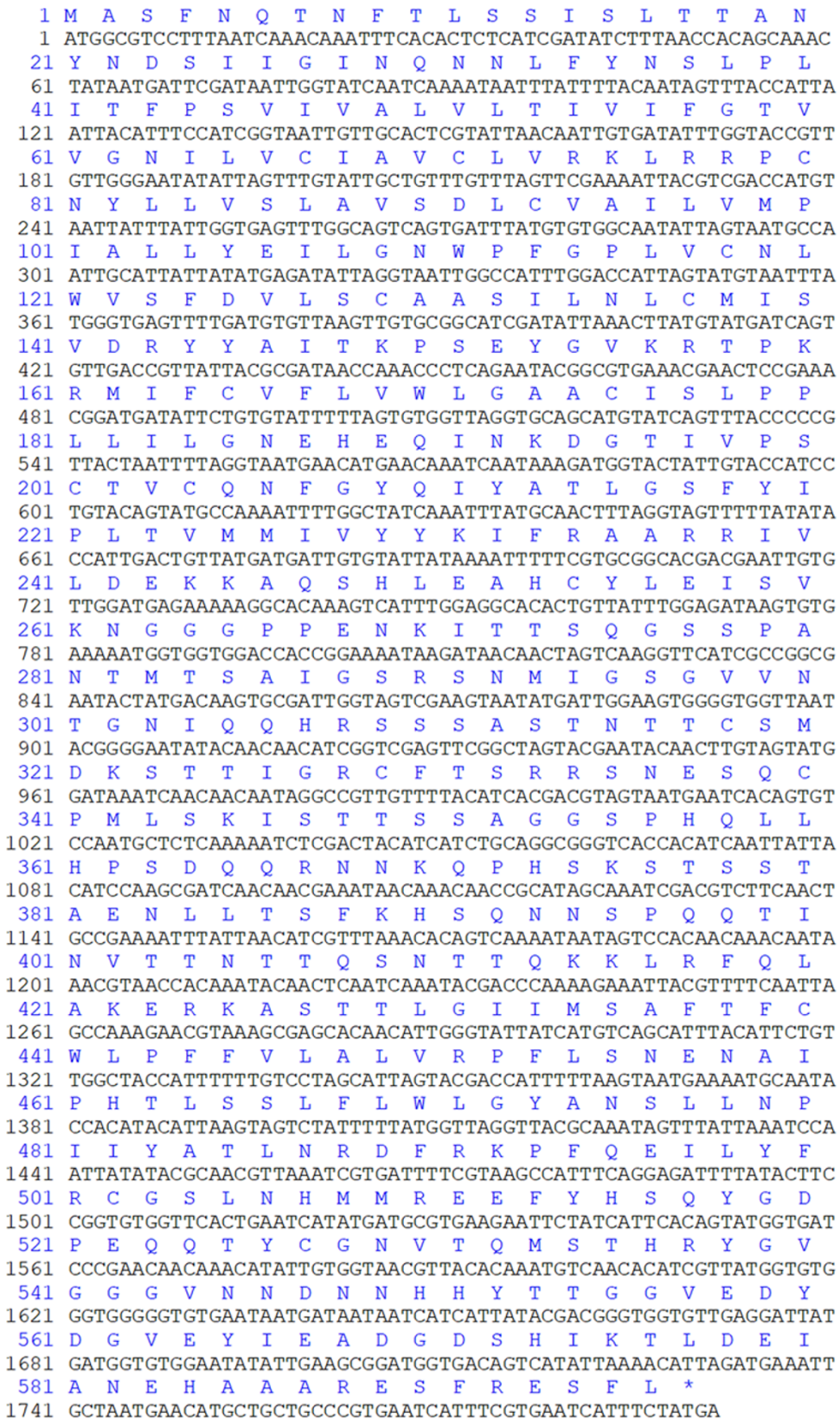


Figure 1. Nucleotide and deduced amino acid sequence of *Chrysopa formosa 5-HT7*.

NCBI to construct a phylogenetic tree (fig. 4). These 5-HT receptors, belonging to various subtypes, each formed a distinct branch, leading to three main evolutionary family branches. The insects 5-HT7 family is more closely related to 5-HT1A family in

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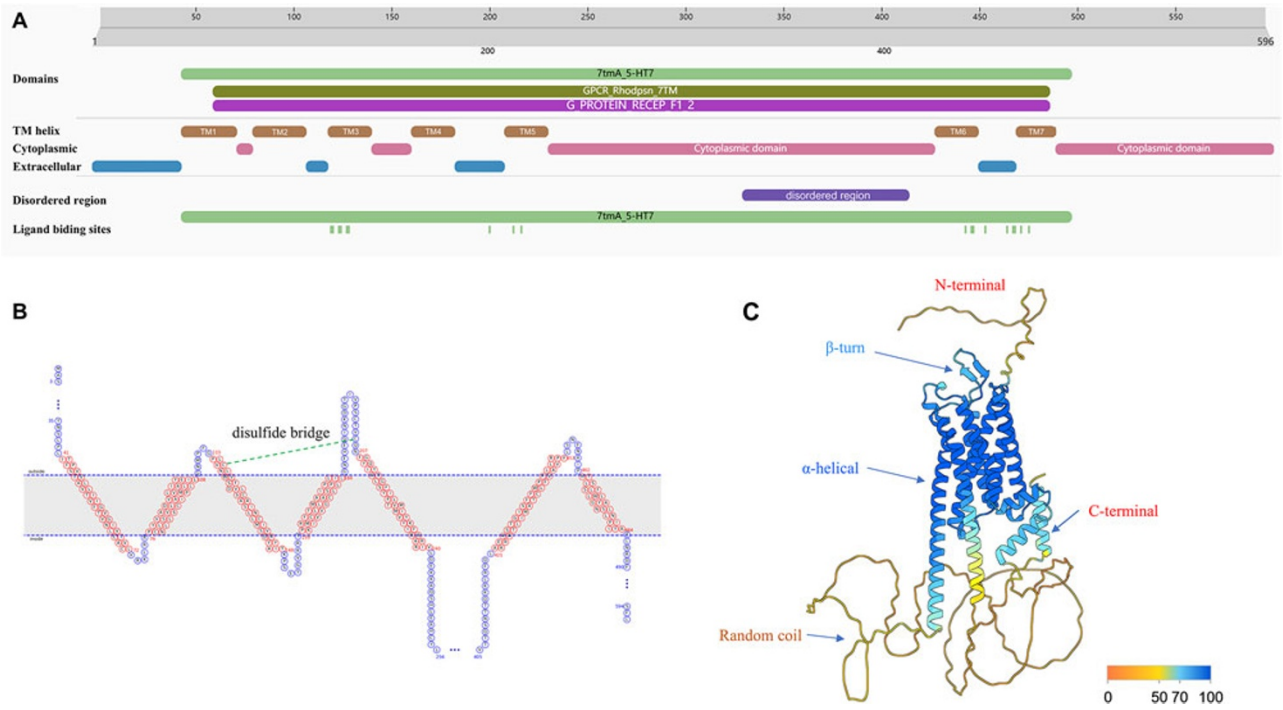


Figure 2. Molecular features and predicted structure of the *Cf5-HT7* protein. (A) Predicted domains, TM helix, extracellular and cytoplasmic regions, disordered region, and ligand binding sites in the *Cf5-HT7* protein. (B) Topology model of the secondary structure of the *Cf5-HT7* receptor, and the disulfide bridge is labelled with dotted green line. (C) Tertiary structure model of the *Cf5-HT7* receptor. The different colours indicate the pLDDT scores.

evolutionary terms. Phylogenetic analysis indicated that *Cf5-HT7* was classified within the 5-HT7 family clade, first clustering with 5-HT7 from *Ch. carnea*, in a shared branch, and subsequently clustering with 5-HT7 from *T. castaneum*. This is consistent with biological evolutionary relationships.

Expression profile of Cf5-HT7 *at different developmental stages of* C. formosa

The transcript levels of *Cf5-HT7* among different developmental stages of *C. formosa* are significantly different ($F_{(9, 29)} = 6.062$, $p < 0.0001$) (fig. 5). *Cf5-HT7* expression level in eggs was low, but drastically increased in the 1st instar larvae, 26 times higher than that in the eggs. As the larvae progressed from the first to the third instar, the expression level of *Cf5-HT7* was consistently and significantly downregulated. Interestingly, the expression of *Cf5-HT7* upregulated dramatically during the prepupal stage, and it remained at a high level from the first to the fifth day of the prepupal period, comparable to that of the first instar larvae. However, the mRNA abundance of *Cf5-HT7* rapidly declined to a lower level in 7-day-old prepupae, showing no significant difference compared to that in newly emerged pupae and adults (fig. 5).

Expression pattern of Cf5-HT7 *throughout diapause development*

To investigate the transcriptional expression of *Cf5-HT7* in response to stimuli inducing diapause, we determined the relative mRNA abundance of *Cf5-HT7* in various developmental stages of *C. formosa*, including eggs, larvae, and prepupae, that were reared under both non-diapause and diapause-inducing conditions. In the preparation stage for diapause, the expression level of *Cf5-HT7* in both the first instar and third instar larvae was significantly higher than that in their counterparts reared under non-diapause conditions. However, the expression of *Cf5-HT7* was lower in the second instar larvae under diapausing conditions, suggesting stage-specific regulation (fig. 6a). Once diapause was initiated, the mRNA abundance of *Cf5-HT7* in diapausing prepupae decreased dramatically, with the expression levels of *Cf5-HT7* in prepupae from day 1 to day 5 all being significantly lower than those of non-diapause prepupae (fig. 6a). The expression of *Cf5-HT7* in eggs and prepupae at day 7, reared under the two conditions, did not exhibit any significant difference (fig. 6a).

During the diapause maintenance phase, the expression level of *Cf5-HT7* remained at a low level for the first 10 days, showing no significant difference from the non-diapause group (fig. 6b). Notably, *Cf5-HT7* expression increased significantly from day 30 to 60, up to 4–8 times higher than at day 10, before dropping again at day 90, and stabilising post-diapause termination (fig. 6b).

Discussion

The molecular characterization of the 5-HT7 receptor in *C. formosa* (*Cf5-HT7*) reveals key structural features that align with the known properties of GPCRs belonging to the class A family. This family of GPCRs include the typical seven transmembrane domains, with intracellular and extracellular loops interspersed between them, a hallmark of GPCRs involved in signal transduction across the cell membrane, as seen in other insect species (Chen *et al.*, 2023; Schlenstedt *et al.*, 2006). GPCRs are known to be the largest and most diverse family of signal transduction receptors in humans (Mykytyn and Askwith, 2017), playing essential roles as signaling molecules that regulate a wide range of physiological processes (Mykytyn and Askwith, 2017; Fried *et al.*, 2022).

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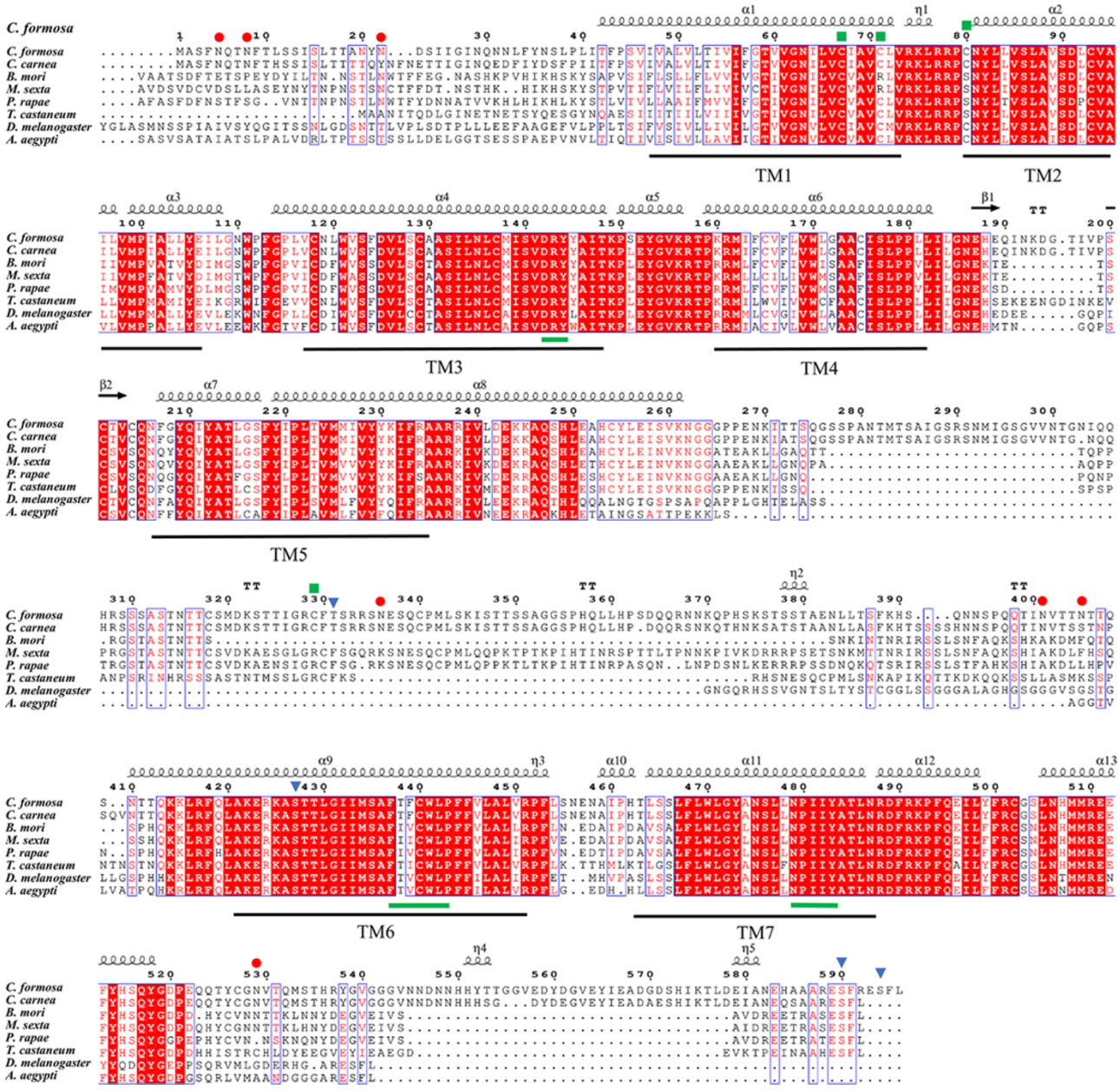


Figure 3. Multiple-sequence alignment of *Cf5-HT7* with the corresponding proteins from other insect species. Alpha-helices, beta-helices, beta strands and beta turns are marked by α, η, β, and TT, respectively. The predicted protein of *Cf5-HT7* contains seven transmembrane domains (TM1-TM7), as underlined in the figure. Three highly conserved motifs 'DRY', 'npxxy', and fxxxwxp in *Cf5-HT7* is underlined. The n-glycosylation sites are labelled with red circle. The phosphorylation sites are labelled with blue inverted triangle. The palmitoylation sites are marked with green square. The *5-HT7* protein sequences used with the GenBank accession numbers are listed in the order illustrated: *C. formosa* (PQ394082), *Ch. carnea* (XP_044727064.1), *B. mori* (AIZ66402.1), *M. sexta* (AGL46976.1), *P. rapae* (AMQ67549.1), *T. castaneum* (XP_966577.2), *D. melanogaster* (NP_524599.1), *A. aegypti* (AAG49292).

In insects, GPCRs are also critical membrane proteins that transmit environmental and physiological signals, including light, neurotransmitters, and hormones, into the cells (Zhao *et al.*, 2016). The absence of a signal peptide in *Cf5-HT7* suggests that it may not be secreted, but instead function at the cell membrane or within intracellular compartments, where it can mediate local signaling events, as observed in *D. melanogaster* and *Locusta migratoria* (Van Bortle *et al.*, 2015; Veenstra, 2017; Wu *et al.*, 2024). Disordered regions in GPCRs are known to contribute to receptor flexibility, enabling conformational changes that are essential for effective signal transduction (Venkatakrishnan *et al.*, 2014). In the case of the 5-HT7 receptor in *C. formosa*, the disordered region (residues 392–412) may facilitate its interaction with G-proteins or other intracellular signaling molecules, which is critical for the receptor's role in modulating behaviour and physiological processes. The disulfide bridge connecting extracellular loop 1 and the bottom of extracellular loop 2 in *Cf5-HT7* likely plays a key role in maintaining the receptor's structural stability and conformation integrity, as it facilitates the correct folding and function of the protein (Iglesias *et al.*, 2017). Post translational modifications are critical in modulating the function of GPCRs, including the 5-HT receptors. The presence of several posttranslational modifications,

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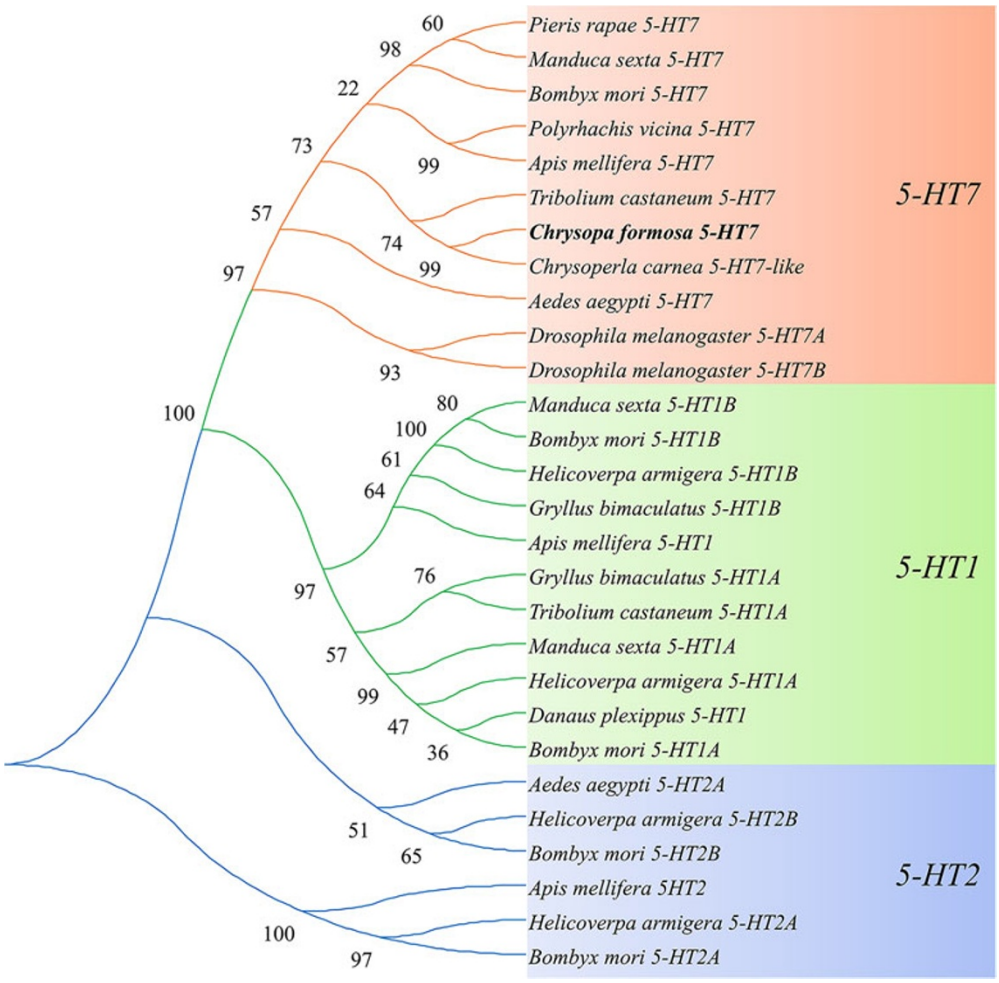


Figure 4. Evolutionary relationship of 5-HT receptors from *C. formosa* and other insects. A phylogenetic tree was constructed based on amino acid sequences of *Cf5-HT7* and 5-HT receptor subtypes from different insect species using the neighbour-joining method with 1,000 bootstraps. The numbers at the forks indicates the bootstrap. The 5-HT receptor proteins, used for phylogenetic analysis, with their corresponding GenBank accession numbers, are listed in table 2.

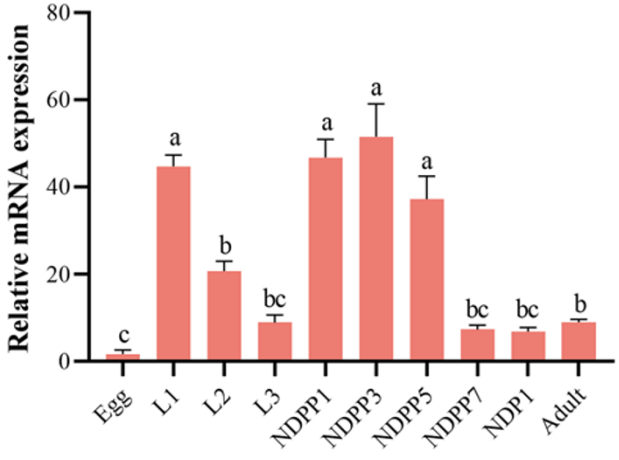


Figure 5. Relative expression levels of *Cf5-HT7* at different normal developmental life stages of *C. formosa*. The mRNA abundance of *Cf5-HT7* in eggs, larval stage (L1, L2 and L3), prepupal stage (NDPP1, 3, 5 and 7), pupae (NDP1) and adults were measured by qRT-PCR. Values are means ± SE (n = 6). Bars with different letters indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Tukey's multiple comparison test.

such as palmitoylation, N-glycosylation and phosphorylation sites in *Cf5-HT7*, is particularly interesting as these modifications are known to affect receptor localization, trafficking, stability and signal transduction (Duhr *et al.*, 2014; Patwardhan *et al.*, 2021; Vuorio *et al.*, 2021).

The MSA and phylogenetic analysis of *Cf5-HT7* indicate that this receptor is evolutionarily conserved. The presence of highly conserved motifs, including 'D-R-Y', 'N-P-x-x-Y', and 'FxxxWxP', suggests their functional significance in receptor activation, ligand binding and signal transduction (Fritze *et al.*, 2003; Venkatakrishnan *et al.*, 2013). These findings are consistent with mechanisms of 5-HT7 receptors described in other insect models (Chen *et al.*, 2023; Pietrantonio *et al.*, 2001; Yang *et al.*, 2019). Phylogenetic analysis further confirms that *Cf5-HT7* belongs to the 5-HT7 receptor family and exhibits a high degree of sequence similarity (89.26%) with the Neuropteran species *Ch. carnea*. The sequence identity between *Cf5-HT7* and 5-HT7 receptors from other insect species, such as *T. castaneum* and *B. mori*, supports previous studies highlighting the functional conservation of 5-HT7 receptors across insect taxa (Rao *et al.*, 2017; Schlenstedt *et al.*, 2006). This high conservation

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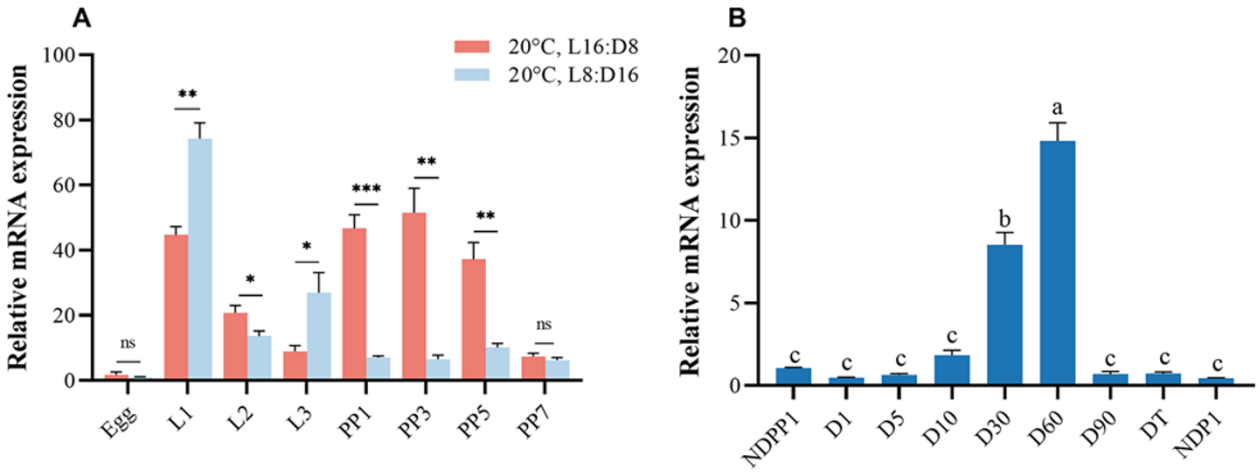


Figure 6. Expression pattern of *Cf5-HT7* throughout diapause development of *C. formosa*. Changes in the mRNA expression levels of *Cf5-HT7* in the eggs, larvae (L1, L2, and L3) and prepupae (PP1, PP3, PP5, and PP7) of *C. formosa* destined for diapause, as compared to their nondiapause counterparts (A). Asterisks (*) represent significant differences between the diapause-destined group and nondiapause group (Student's *t*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). The relative expressions of *Cf5-HT7* in nondiapausing prepupae (NDPP1), diapausing prepupae (enter diapause for 1, 5, 10, 30, 60, and 90 days), post-diapausing pupae (DT) and nondiapausing pupae (NDP) (B). Different letters indicate significant differences (one-way ANOVA followed by Tukey's multiple comparations, $p < 0.05$). All the values are expressed as means ± SE based on six independent biological replicates.

suggests that 5-HT7 receptor plays a critical and conserved role in insect physiological processes. The close phylogenetic relationship between the 5-HT7 receptor and 5-HT1A receptors observed in this study highlights the complexity of serotonergic signaling pathways in insects. It suggests a potential evolutionary trade-off between receptor subtypes that balance diverse physiological needs. However, the sequence of the third intracellular loop varies considerably across different insect species (fig. 2), which may reflect functional and evolutionary adaptations to specific physiological or behavioural demands. These results indicate that the 5-HT7 receptor evolved within a conserved functional framework across diverse insect orders, further emphasising its physiological importance.

In the current study, the expression profile of *Cf5-HT7* across various developmental stages of *C. formosa* showed significant variability, underscoring its crucial role in regulating growth, development and other physiological processes. Notably, the significant upregulation of *Cf5-HT7* during the first instar larval stage suggests its involvement in active feeding and rapid growth early in development. Similarly, high expression of the 5-HT7 receptor in the Malpighian tubes and guts of *M. separata* larvae highlights its role in digestion and metabolism (Chen *et al.*, 2023). Ganguly *et al.* (2020) demonstrated that knockdown of 5-HT7 receptor expression in mushroom body neurons impaired olfactory appetitive learning in *D. melanogaster* larvae, emphasising the receptor's essential role in mediating olfactory neural circuits. The 5-HT7 receptor is also crucial for larval locomotion in Drosophila (Moncalvo and Campos, 2009; Silva *et al.*, 2014). Furthermore, serotonin interacts with the 5-HT7 receptor in the salivary ligands of *Calliphora vicina*, promoting saliva secretion, which likely facilitates feeding (Röser *et al.*, 2012). Collectively, these studies support our findings, suggesting that the 5-HT7 receptor plays a vital role in various aspects of larval behaviour, growth and physiology. The observed decrease in *Cf5-HT7* expression during later larval stages, followed by a dramatic increase during the prepupal stage, suggests its involvement in transition processes such as growth and developmental metamorphosis. As seen in *A. aegypti*, deletion of the *5-HT7A* receptor resulted in larval and pupal abnormalities, reduced motor activity, increased mortality, delayed stimulus response, and lower egg hatching rates, demonstrating its critical role in development, metamorphosis, and physiological functions (Li *et al.*, 2022). Interestingly, the high expression of *Cf5-HT7* during the prepupal stage of *C. formosa* suggests a functional role in initiating the metamorphosis from prepupa to pupa. This is consistent with findings in *D. melanogaster*, where serotonin signaling through the 5-HT7 receptor modulates nutrient-dependent ecdysteroid biosynthesis, controlling developmental timing (Shimada-Niwa and Niwa, 2014). In their study, inhibiting the 5-HT7 receptor delayed pupariation, reduced ecdysteroid levels, and downregulated steroidogenic gene expression in *D. melanogaster*. cAMP levels in the prothoracic gland also decreased upon 5-HT7 knockdown, linking serotonin to hormonal synthesis. These results establish serotonin signaling as a critical mediator connecting environmental nutrition to endocrine timing, ensuring adaptive developmental transitions through neuronal modulation of steroid hormone production. Additionally, the slightly higher expression of the *5-HT7* receptor in the pupae of the ant *P. vicina* may suggest its role in modulating adult formation (Yang *et al.*, 2019). Previous studies have also shown that *5-HT7* is involved in the normal courtship and mating behaviours of *Drosophila* adults (Luo *et al.*, 2012), and higher expression of *5-HT7* is also observed in adults of *Harmonia axyridis, P. vicina* and *M. separata* (Chen *et al.*, 2023; Yang *et al.*, 2019; Zhang *et al.*, 2023). However, the transcriptional level of *Cf5-HT7* in newly emerged adults is much lower than that in the larval and prepupal stages of *C. formosa*, suggesting that the role of this receptor may vary across different insect species.

The significant changes in *Cf5-HT7* expression of *C. formosa* larvae in response to diapause-inducing conditions (short day-length and low temperature) highlight the receptor's role in the physiological adaptations necessary to cope with altered environmental conditions (Ullah *et al.*, 2024). Diapause is triggered by environmental stimuli well before the onset of developmental arrest, and insects destined for diapause typically undergo physiological changes during the prediapause preparatory period. These

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changes include increased food intake, enhanced digestive efficiency, and the diversion of nutrients from somatic growth to storage (Hahn and Denlinger, 2011). The elevated expression of *Cf5-HT7* in the first and third instar larvae is likely associated with feeding and digestion. Although we did not measure *Cf5-HT7* level in the digestive system directly, the high expression of *5-HT7* in the midgut of *H. axyridis, Gryllus bimaculatus* and *M. separata* suggests a conserved role for this receptor in digestion and nutrition metabolism (Chen *et al.*, 2023; Watanabe *et al.*, 2011; Zhang *et al.*, 2023). The third instar larvae of *C. formosa* is particularly sensitive to reduced day lengths, and the higher *Cf5-HT7* expression observed may be involved in regulating neuronal sensitivity and circadian rhythms (Li *et al.*, 2018). Previous studies have shown that serotonin signaling regulates the circadian pacemakers in *G. bimaculatus* (Saifullah and Tomioka, 2002), and the 5-HT7 receptor is known to play a significant role in regulating sleep and circadian rhythms in animals (Sprouse *et al.*, 2004). The function of 5-HT7 receptor in modulating circadian systems in insects warrants further investigation. A decline in *Cf5-HT7* expression following the initiation of diapause, followed by the gradual increase during the maintenance phase, aligns with the known role of serotonin in modulating the neurophysiological changes associated with diapause in insects (Denlinger, 2022a). For example, serotonin levels in *Chymomyza costata* significantly rise during diapause (Kostal *et al.*, 1999). In the diapausing pupae of *Pieris brassicae*, 5-HT levels are maintained at a high level, potentially inhibiting brain metabolism and endocrine activity to sustain diapause (Isabel *et al.*, 2001). The 5-HTB receptor may induce or maintain diapause in *A. pernyi* pupae by preventing the synthesis or release of PTTH, and interference with 5-HTB can prematurely terminate diapause (Wang *et al.*, 2013). However, Matsumoto and Takeda (2002) found that both 5-HT and ecdysteroid levels increase significantly during diapause termination in *A. pernyi* pupae, suggesting that 5-HT may promote the release of ecdysteroids, facilitating diapause exit. In this study, the dramatic increase in *Cf5-HT7* expression in diapausing prepupae at 60 days coincides with the onset of diapause termination in *C. formosa* (Li *et al.*, 2018). We speculate that the 5-HT7 receptor plays an important role in the termination of diapause in *C. formosa*, particularly in regulating the timing of ecdysteriod biosynthesis. As shown in *D. melanogaster*, serotonin signaling through the 5-HT7 receptor regulates the timing of ecdysteriod synthesis in the PG and determines the transition from larva to pupa (Shimada-Niwa and Niwa, 2014). Experiments currently underway seek to explore biological role of serotonin and its receptors in regulating the diapause process of *C. formosa*.

Conclusion

In conclusion, this study provides valuable insights into the molecular characteristics and functional dynamics of the *Cf5-HT7* receptor in *C. formosa*. The receptor's conserved structural features and its phylogenetic relationships with other insect species highlight its significance in serotonin signaling. The expression profiles demonstrate that the 5-HT7 receptor plays a crucial role in regulating developmental and physiological processes, particularly in response to environmental cues during diapause. These findings suggest potential strategies for manipulating insect populations, either by promoting or delaying development or diapause through targeted modulation of serotonin signaling pathways.

In the context of beneficial insects such as *C. formosa*, targeting the 5-HT7 receptor with RNA interference or small molecule agonists could delay or accelerate diapause termination, offering precise control over the diapause period. Such strategies would enhance the storage and rearing of natural enemies, improving their availability for biological control programs. Moreover, the ability to modulate diapause or development could be applied to pest management. Targeting the 5-HT7 receptor in pests could delay or prevent diapause, disrupt key developmental transitions, and thereby reduce pest survival. Compounds targeting the 5-HT7 receptor could be used as environmentally friendly insecticides or growth regulators, enhancing the ability to manage pest populations with higher precision and efficiency.

Future research should aim to elucidate the molecular mechanisms by which *Cf5-HT7* influences diapause and development. Exploring the interactions between *Cf5-HT7* and other signaling pathways, including hormonal and environmental factors, will be crucial. Investigating the role of serotonin and *5-HT7* in other life stages, such as reproductive behaviour and stress responses, will also broaden our understanding of its biological functions, paving the way for more effective natural enemy mass production or pest control strategies.

Acknowledgements. We thank Dr. Linhao Ge and Mingxuan Jia, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, for their assistance in helping us analyse protein structure by using AlphaFold 2. This research was financed by the National Natural Science Foundation of China (32372629) and National Key R&D Program of China (2023YFE0123000, 2023YFD1400600).

Conflicts of interest. The authors declare no conflict of interest.

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https://doi.org/10.1017/S0007485325000148 Published online by Cambridge University Press