

Molecular Characterization and Expression Profiling of Chemosensory Proteins in Male *Eogystia hippophaecolus* (Lepidoptera: Cossidae)¹

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Abstract *Eogystia hippophaecolus* Hua, Chou, Fang et Chen (Lepidoptera: Cossidae) is a notorious carpenterworm pest of sea buckthorn, *Hippophae rhamnoides* L. (Elaeagnaceae). Chemosensory proteins (CSPs) are thought to be responsible for initial biochemical recognition during olfactory perception by the insect. We examined the structure, function, and expression profiles of these proteins in four structures (e.g., antennae, labipalp, legs, and external genitalia) of male adults. Molecular weight, isoelectric point, hydrophilicity and hydrophobicity of proteins, and signal peptide prediction of 18 *E. hippophaecolus* CSPs (EhipCSPs) were investigated via software. Expression profiles in the four male structures were analyzed by fluorescence quantitative real-time polymerase chain reaction. Bioinformatics analysis showed that most EhipCSPs are low-molecular-weight proteins with hydrophobic regions and a high proportion of alpha-helices, consistent with the general characteristics of insect CSPs. Eight EhipCSPs (*EhipCSP2*, *EhipCSP5*, *EhipCSP7*, and *EhipCSP13–17*) were predominantly expressed in the labipalp ($P < 0.01$), and three (*EhipCSP6*, *EhipCSP8*, and *EhipCSP9*) were predominantly expressed in legs ($P < 0.01$). We speculate that these proteins may be related to contact sensations, host recognition, and other functions. Two EhipCSPs (*EhipCSP4* and *EhipCSP11*) were highly expressed in the external genitalia ($P < 0.01$), suggesting that they may be involved in spousal positioning or mating activities. Most EhipCSPs were differentially expressed in the four structures, with wide overall expression, indicating an important role in olfactory recognition in multiple tissues. Our findings establish the foundation for further investigation of EhipCSPs and potential development of nonpesticide control measures.

Key Words *Eogystia hippophaecolus*, CSPs, molecular characterization, tissue distribution

In insects, chemical communication involves sensing of various semiochemicals, and olfaction plays a vital role in processing environmental chemical signals to guide fundamental behaviors, such as searching for hosts, avoiding predators, feeding, and oviposition (Benton 2009, Renou and Guerrero 2000). From the external environment, lipo-soluble odor molecules enter the water-soluble sensillum lymph, and then reach the dendritic membrane of neurons, where they activate receptors on the dendritic membrane, eventually leading to physiological and

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behavioral changes (Leal 2013). It is widely believed that two types of olfactory proteins, odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), are involved in initial biochemical recognition (Leal 2013; Niu et al. 2016, Pelosi 2005, Zhou et al. 2019). Insect CSPs are a class of small (13 kDa, 100–115 amino acids), acidic, water-soluble proteins with four conserved cysteine residues forming two disulfide bonds. The most basic function of CSPs is to dissolve and transport various lipophilic ligands and, thereby, identify a large number of nonvolatile semiochemicals in the environment, while performing other functions including regulating growth, development, and circadian rhythms (Pelosi 2005, Pelosi et al. 2018). While OBPs are mainly present in antennae, CSPs are highly expressed in all olfactory organs and broadly expressed in various tissues throughout the insect body. Moreover, CSPs are characterized by highly conserved sequence motifs, including (a) YTTYKVDN(V/I)(N/D)(L/V)DEIL at the N-terminus, (b) DGKELKXX(I/L)PDAL in the central region, and (c) KYDP at the C-terminus, and most have hydrophobic binding pockets in the interior of the molecule (Jansen et al. 2007, Lartigue et al. 2002, Mosbah et al. 2003, Pelosi 2005, Pelosi et al. 2018, Picone et al. 2001, Wanner et al. 2004).

Eogystia hippophaecolus Hua, Chou, Fang et Chen (Lepidoptera: Cossidae) is a major destructive carpenterworm pest of sea buckthorn, *Hippophae rhamnoides* L. (Elaeagnaceae), an important soil- and water-conservation shrub species distributed in northern and western China (Zhou 2002). The biological and ecological characteristics of this species have been investigated, along with the disaster-causing mechanism (Changkuan et al. 2004, Shi-xiang et al. 2005). Sex pheromones in the female *E. hippophaecolus* have been identified and used to develop specific and efficient artificial sex pheromone traps (Changkuan et al. 2004, Fang et al. 2005, Wang et al. 2014). Additionally, olfactory sensilla have been studied using scanning electron microscopy (Hu et al., 2018a). Based on male and female antennae transcriptome data, Hu et al. (2016, 2018b) examined a series of olfactory-related genes to explore expression and functional characteristics of OBPs (sex pheromone-binding proteins). However, because larvae bore deep into trunks and roots and live a complicated and long life cycle, there are no effective methods for controlling the population density of larvae. In recent years, the olfactory system of insects has been investigated in order to interfere with olfactory recognition and regulate insect pest populations through novel pest control strategies.

In one study, 18 *E. hippophaecolus* CSP (EhipCSP) genes were identified from male and female *E. hippophaecolus* antennae transcriptome data, and phylogenetic relationships between EhipCSPs and homologs in other species were explored (Hu et al., 2016). However, the molecular mechanisms of olfactory action remain unclear. Herein, we analyzed the sequence characteristics of these 18 EhipCSP sequences and performed fluorescence quantitative real-time polymerase chain reaction (qPCR) to examine their expression profiles in male tissues from four olfactory structures (e.g., antennae, legs, external genitalia, and labipalp).

Materials and Methods

Insects and tissue collection. *Eogystia hippophaecolus* were collected from infested *H. rhamnoides* plants in Baishan Forest Farm (N 32°39', E 119°42'),

Jianping County, Liaoning Province, China, during June 2019. Antennae, labipalp, legs, and external genitalia of males were excised, placed in RNAlater (Ambion, Austin, TX), and stored at -80°C until used.

Total RNA was extracted from male and female tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA integrity was monitored by 1.2% agarose gels, and RNA quantity was measured using a NanoDrop 8000 instrument (Thermo, Waltham, MA). Total RNA was then employed as a template for first-strand cDNA synthesis using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan). All products were stored at -20°C .

Sequence analysis of EhipCSPs. Gene sequences were obtained from published transcriptome sequencing (PRJNA328551) (GenBank accession numbers: KX655936–KX655953). Open reading frames (ORFs) and putative amino acid sequences of the 18 EhipCSPs were determined using the online software ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). Based on the amino acid sequences, the online software ExpASy (<http://web.expasy.org/protparam/>) was used to predict the molecular weight, isoelectric point (pI), and hydrophilicity of proteins. Hydrophobicity of proteins was analyzed using BioEdit software (Hall 1999), and signal peptide prediction was executed using SignalP5.0 (<http://www.cbs.dtu.dk/services/SignalP/>). The phylogenetic tree analysis of EhipCSPs with similar CSPs to other insect species were constructed by MEGA 10 software.

Fluorescence qPCR. Primers used for qPCR were designed using online software Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Table 1). The *Eogystia hippophaecolus* β -actin gene served as an internal reference (Hu et al., 2016).

qPCR was performed on a Bio-Rad CFX96 PCR System (Hercules, CA) in 12.5- μl reactions containing 6.25 μl of SYBR Premix Ex Taq II (No. RR820A; TaKaRa), 1 μl of each primer (10 mM), 1 μl of sample cDNA (2.5 ng of RNA), and 4.25 μl of ddH₂O (sterile distilled water). Thermal cycling was performed at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 59°C for 30 s, and melting curve analysis at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Each qPCR experiment was conducted in triplicate with three biological replicates for each transcript. One biological replicate takes approximately 10 insects. Two negative controls lacking cDNA template were included for each reaction. Bio-Rad CFX Manager software (Bio-Rad) was used to normalize expression based on $\Delta\Delta\text{Cq}$ values versus control samples using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001).

Statistical analysis. Relative expression levels were subjected to one-way analysis of variance followed by Tukey's honestly significant difference tests implemented in SPSS Statistics 25.0 (IBM, Armonk, NY). Values are presented as means \pm standard errors (SE).

Results

Analysis of EhipCSP sequences. The molecular characteristics of EhipCSPs are displayed in Table 2. None of the *EhipCSPs* include a complete ORF, and 14 contain signal peptides. EhipCSPs are between 102 and 523 aa in length. Furthermore, the molecular weight of 12 EhipCSPs is ~ 14 kDa, and the pI values range from 3.79 to 9.54.

Table 1. Primers used for fluorescence quantitative real-time polymerase chain reaction and the efficiency of amplification.

Gene Name	Forward Primer	Reverse Primer
Actin	CGACTTCGAACAGGAGATGG	TCGTCTCATGAATGCCACAG
CSP1	ATGTTGGACCAGGGAAAATG	CAAAGTGACGGTCTGGATCA
CSP2	CAGCGACAACCTCAAATGTGC	ATATTGGCGGACGATCTTTG
CSP3	CTGGATAAAGGGTCTTGCACG	TCCTTGTATTGGTTCGTCTGGA
CSP4	CTGCCGAAGAAACCTACAGC	CGCGGGAGTACATTTAGCAC
CSP5	ACATCTGGAGGAAAGGGCAA	AGAGGTGGAAGTGGTTTGGG
CSP6	AGAAGGCGCTGACTTCAAAA	AGGAAGGCCTCGAAAGATTC
CSP7	GCAGTTGGAGAGGTCTCTGG	CCGTATTGTCGGACGATTTT
CSP8	AAAGGTCGTTGTAGCCCAGA	GATCATACTTGGACGCGAGC
CSP9	CCTTGGAAAGGGCAAATGTA	TCCGGGTTCGTACGTCTTTAC
CSP10	TTTGCCTGACGCGATAACTA	AGATTTGAGCTCGGAAGACG
CSP11	CAACAAGAGACTGCTGGGC	TGAGGGTCGTATTTCTCCACC
CSP12	GTATGGTGTCTGATGCTGCG	GTCTACGTCAGCACCACCTA
CSP13	TGTGGAGACCGTTTGTGGTA	CTCGATTGGATGAATTTGTCTG
CSP14	ATTGCGCTGATTTGTGTGTT	TCTGCAATGCTTCTTCGACA
CSP15	CAAACGAACGCATCCTTCT	CAACATTTTTCGGACTACCA
CSP16	TGTTGTTACTATCGCATCGGC	CGTTACGTTGAGTTTCGGTACA
CSP17	TCGACGAAATCCTTGGCAAC	CCCAGGATTCAGGTTTCATTGT
CSP18	TGACTTTGATATACGACCCTTGC	TGTTGCTTCATTACTGCCTTGA

Analysis of the hydrophilicity and hydrophobicity of EhipCSPs. The grand average (GRAVY) of hydrophobicity scores ranged from -1.020 to 0.688 , and were negative for 17 of the 18 sequences, suggesting they were hydrophilic. From the hydrophobicity analysis (Fig. 1), we concluded that 12 of the 18 EhipCSPs (EhipCSP3–4, EhipCSP7, EhipCSP10, EhipCSP13–18) possessed an obvious hydrophobic region (regions with a positive GRAVY score) (Table 3).

Secondary structure of EhipCSPs. As shown in Table 4, the proportion of α -helices in the secondary structure of EhipCSPs was high (59.3% to 87.8%), while the proportion of β -sheets ranged from 30.7% to 60.0%, and the proportion of random coil structure ranged from 7.8% to 18.0%.

Phylogenetic tree analysis of EhipCSPs. Based on a neighbor-joining tree of CSPs (Fig. 2), we found that EhipCSPs were divided into different groups. *EhipCSP1*, *EhipCSP3*, *EhipCSP5*, *EhipCSP9*, *EhipCSP11*, *EhipCSP12*, *EhipCSP16*, and *EhipCSP17* were monophyletic with the big dipteran (*Drosophila melanogaster*

Table 2. Characteristics of the 18 *Eogystia hippophaecolus* chemosensory proteins (EhipCSPs).

Number	Gene ID	UniGene Length (bp)	Amino Acid Length (aa)	MW (kDa) ^a	pI	Complete ORF	Signal Peptide AA
EhipCSP1	c18130_g1	555	125	14.73598	6.37	N	1–18
EhipCSP2	c24905_g1	745	107	11.97900	9.27	N	1–20
EhipCSP3	c31175_g1	1,159	123	14.42853	6.12	N	1–18
EhipCSP4	c33479_g1	511	122	13.86996	5.23	N	1–16
EhipCSP5	c20987_g1	2,813	523	60.01510	8.90	N	N
EhipCSP6	c18929_g1	1,710	124	14.09817	5.54	N	1–16
EhipCSP7	c13549_g1	851	111	12.54482	9.54	N	1–21
EhipCSP8	c7730_g1	1,011	128	14.71997	7.57	N	1–18
EhipCSP9	c26669_g1	937	144	16.21983	9.05	N	N
EhipCSP10	c10515_g1	2,378	172	20.12057	5.61	N	N
EhipCSP11	c18659_g1	902	123	14.26756	8.55	N	1–19
EhipCSP12	c31194_g3	2,895	102	10.31698	3.79	N	N
EhipCSP13	c22559_g1	660	121	13.79392	8.94	N	1–16
EhipCSP14	c3982_g1	691	130	14.69897	5.33	N	1–18
EhipCSP15	c23394_g1	1,087	125	14.46184	9.26	N	1–19
EhipCSP16	c7482_g1	1,082	127	14.79296	7.58	N	1–18
EhipCSP17	c7703_g1	1,039	127	14.71781	6.09	N	1–18
EhipCSP18	c25262_g1	1,684	121	14.28654	8.22	N	1–17

^a MW indicates molecular weight; pI, isoelectric point; ORF, open reading frame, AA, amino acid, N, not complete ORF/signal peptide nor predicted.

Meigen) and lepidopteran clade. *EhipCSP10* was monophyletic with *SinfCSP21*. *EhipCSP8*, *EhipCSP15*, and *EhipCSP18* were monophyletic with CSPs of *Bombyx mori* Linnaeus and *Papilio xuthus* Linnaeus. *EhipCSP2* and *EhipCSP6* were monophyletic with *P. xuthus*. *EhipCSP7* was monophyletic with *B. mori*. *EhipCSP4* and *EhipCSP13* were monophyletic with many other lepidopteran clade such as *B. mori*, *P. xuthus*, *Agrotis ipsilon* (Rottemberg), and *Sesamia inferens* Walker.

Tissue-dependent distributions of EhipCSPs. We investigated the expression patterns of the 18 EhipCSPs in chemosensory tissues from antennae, legs, external genitalia, and labipalp in males using qPCR (Fig. 3). The results suggest that two EhipCSPs (*EhipCSP12* and *EhipCSP18*) were expressed mostly in antennae ($P < 0.01$). Three EhipCSPs (*EhipCSP6*, *EhipCSP8*, and *EhipCSP9*) were predom-

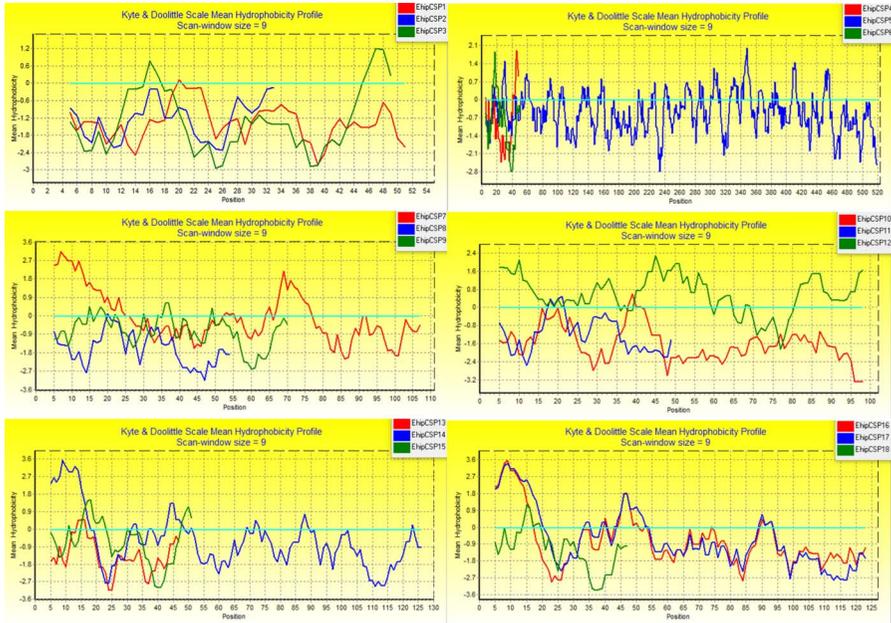


Fig. 1. Hydrophobicity of 18 *Eogystia hippophaecolus* chemosensory proteins (EhipCSPs).

inantly expressed in legs. Expression levels of two EhipCSPs (*EhipCSP4* and *EhipCSP11*) were higher in the external genitalia. Intriguingly, high expression of 8 EhipCSPs (*EhipCSP2*, *EhipCSP5*, *EhipCSP7*, *EhipCSP13–17*) was observed in the labipalp. Expression of *EhipCSP5* was extremely high in labipalp (nearly 4,000 times higher than in controls). Furthermore, *EhipCSP1* was expressed at high levels in both legs and labipalp. *EhipCSP3* and *EhipCSP4* were expressed highly in legs and external genitalia, of which *EhipCSP4* was highly expressed in the external genitalia. Similar to *EhipCSP5*, *EhipCSP10* was expressed at significantly higher levels in antennae and legs (Tables 5, 6).

Discussion

Eogystia hippophaecolus is a destructive pest of sea buckthorn that causes huge ecological and economic damage (Shi-xiang et al. 2005, Zhou 2002). The ability to precisely locate the correct host plant is essential for the survival of phytophagous insects because it mitigates feeding, oviposition, and predator avoidance. Therefore, attractants developed from host volatiles are widely applied to control pest populations by interfering with olfactory recognition. An understanding of the olfactory mechanisms can establish the foundation for the development of new attractants or repellents. CSPs are key proteins that perform crucial functions in insect olfactory recognition (Pelosi et al. 2018). In the present study, we performed molecular characterization of 18 EhipCSPs and found that most of the

Table 3. Grand average of hydrophobicity (GRAVY) scores for the 18 *Eogystia hippophaecolus* chemosensory proteins (EhipCSPs).

Gene Name	GRAVY Score
EhipCSP1	-0.564
EhipCSP2	-0.255
EhipCSP3	-0.620
EhipCSP4	-0.279
EhipCSP5	-0.524
EhipCSP6	-0.378
EhipCSP7	-0.054
EhipCSP8	-0.715
EhipCSP9	-0.311
EhipCSP10	-1.020
EhipCSP11	-0.409
EhipCSP12	0.688
EhipCSP13	-0.543
EhipCSP14	-0.518
EhipCSP15	-0.342
EhipCSP16	-0.641
EhipCSP17	-0.582
EhipCSP18	-0.464

primary and secondary structures corresponded to the general characteristics of insect CSPs, suggesting that they may perform typical CSP functions (Jansen et al. 2007, Lartigue et al. 2002, Mosbah et al. 2003, Picimbon et al. 2000). The neighbor-joining phylogenetic analysis of EhipCSPs demonstrates that they were significantly divided into different groups, which may suggest EhipCSPs could have various functions.

Knowledge of the expression patterns of CSPs in different insect tissues could help to clarify their physiological functions. Compared with OBPs, which occur primarily in antennae, the distribution of CSPs is ubiquitous (Gong et al. 2007, Gu et al. 2011, Yao et al., 2016). Herein, the distribution of EhipCSPs differed among the four tested tissues of male *E. hippophaecolus*, consistent with previous research with *B. mori* (Gong et al. 2007), *Spodoptera exigua* (Hübner) (Gong et al. 2012), *Mamestra brassicae* (L.) (Nagnan-Le Meillour et al. 2000), *Heliothis armigera* (Hübner) (Tian-Tao et al. 2011), and *S. inferens* (Zhang et al. 2013). This implies that broad expression is a general feature of these proteins, and CSPs may be involved in olfactory functions in diverse tissues (Hu et al. 2018a). Furthermore,

Table 4. Secondary structure analysis of 18 *Eogystia hippophaecolus* chemosensory proteins (EhipCSPs).

Protein Name	H (%) ^a	E (%)	T (%)
EhipCSP1	72.8	39.2	16.8
EhipCSP2	78.5	48.6	13.1
EhipCSP3	87.8	41.5	14.6
EhipCSP4	77.0	35.2	15.6
EhipCSP5	59.3	39.6	15.3
EhipCSP6	78.2	58.1	14.5
EhipCSP7	70.3	46.8	9.9
EhipCSP8	83.6	26.6	18.0
EhipCSP9	68.1	44.4	12.5
EhipCSP10	64.0	48.8	14.5
EhipCSP11	77.2	48.8	14.6
EhipCSP12	64.7	50.0	7.8
EhipCSP13	70.2	47.1	14.9
EhipCSP14	77.7	30.8	16.2
EhipCSP15	71.2	60.0	16.0
EhipCSP16	70.1	39.4	15.7
EhipCSP17	70.9	30.7	14.2
EhipCSP18	74.4	46.3	13.2

^a H indicates α -helices; E, β -sheets; T, random coil structure.

analysis of the types and distributions of sensilla in six olfactory tissues in male *E. hippophaecolus* revealed that triconoid sensilla, basiconica sensilla, and coeloconica sensilla mainly perform olfactory chemical recognition (Hu et al. 2018b). In the present work, many of the EhipCSPs were expressed at high levels in the labipalp and legs, which mainly contain triconoid and chaetica sensilla, implying that EhipCSPs may be associated with chaetica sensilla, but this requires further investigations with techniques such as in situ hybridization.

EhipCSPs were expressed predominantly in external genitalia, which may help to solubilize hydrophobic semiochemicals such as pheromone compounds, and assist their controlled release into the environment (Pelosi et al. 2018), thereby contributing to spousal positioning and mating activities. Binding experiments with radioactively labeled pheromone showed that CSPMbraA is localized in both antennae and pheromone gland extracts, and both tissues displayed high affinity for ligands, suggesting that these proteins may be involved in transporting hydrophobic molecules through aqueous media. A similar expression pattern was also reported

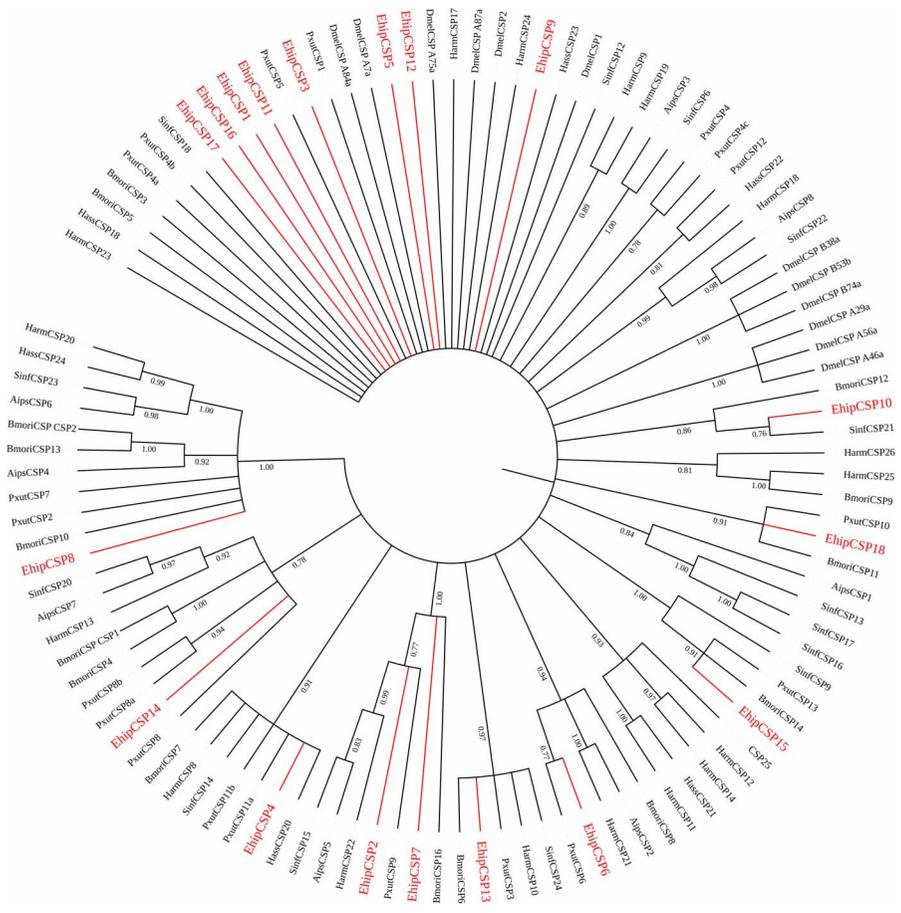


Fig. 2. The neighbor-joining phylogenetic analysis of chemosensory proteins (CSPs) of *Eogystia hippophaecolus* (*EhipCSP*, red) was performed with reference CSPs of *Bombyx mori* (*BmorCSP*), *Helicoverpa armigera* (*HarmCSP*), *Heliothis assulta* (*HassCSP*), *Seamia inferens* (*SinfCSP*), *Papilio xuthus* (*PxutCSP*), *Agrotis ipsilon* (*AipsCSP*), and *Drosophila melanogaster* (*DmelCSP*, Diptera). The stability of the nodes was assessed by bootstrap analysis with 1,000 replications, and only bootstrap values ≥ 0.75 are shown at the corresponding nodes.

for *S. inferens* (Zhang et al. 2013), *M. brassicae* (Jacquin-Joly et al. 2001), *Cnaphalocrocis medinalis* Guenée (Liu et al. 2015), and *Athetis lepigone* (Mschler) (Zhang et al. 2017). We speculate that *EhipCSPs* in external genitalia may perform a dual role in receiving and emitting the same chemical message.

Furthermore, many *EhipCSPs* displayed biased expression in legs and labipalp, exemplified by *EhipCSP5*, which was expressed at levels 4,000 times higher in labipalp than in controls, consistent with research with *Choristoneura fumiferana*

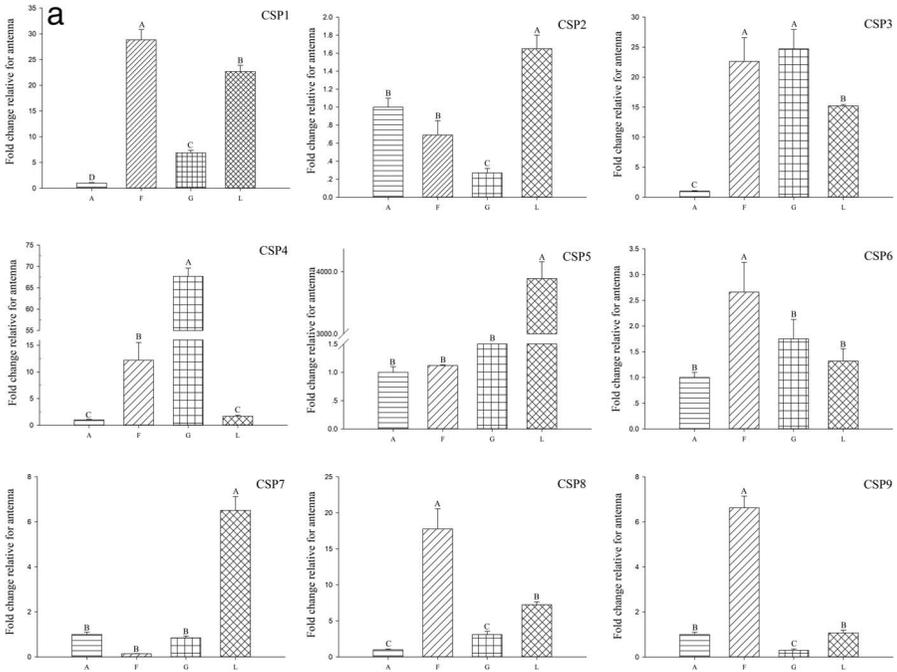


Fig. 3. Transcript levels of the 18 CSPs in four tissues in male *Egystia hippophaecolus* (A indicates antennae; L, legs; G, external genitals; La, labipalp). β -actin served as an internal reference gene for normalizing target gene expression (Hu et al., 2016). Standard errors are represented by error bars, different lowercase letters (a, b, c, d) above bars denote significant differences at $P < 0.05$, and different capital letters (A, B, C, D) above bars denote significant differences at $P < 0.01$.

(Clemens) (Wanner et al. 2004) and *Heortia vitessoides* Moore (Picimbon et al. 2001) showing higher expression in labipalp and legs, suggesting that it may be related to contact sensation and host recognition via a gustatory role. Studies on *P. xuthus* showed that a large number of CSPs are present in female tarsi, indicating potentially important roles in chemoreception as transporters of ligands in oviposition behavior (Ozaki et al. 2008). However, CSPs are expressed in legs, indicating non-chemosensory functions (Cheng et al. 2015, Cheng et al. 2019, Pelosi 2005, Pelosi et al. 2018, Picone et al. 2001, Qiao et al. 2013). RNA interference-based gene silencing of *S. exigua* CSPs (SexiCSPs) resulted in high mortality and oviposition inhibition, suggesting that CSPs influence the survival and reproduction of *S. exigua* (Gong et al. 2012). Also, expression levels of CSP9 in *Solenopsis invicta* Buren are highest at the end of the third instar, and silencing of this gene by RNA interference affects fatty acid biosynthesis and other metabolic pathways, and prevents cuticle development and ecdysis, suggesting that Si-CSP9 may be involved in the network that contributes to the development of third-instar

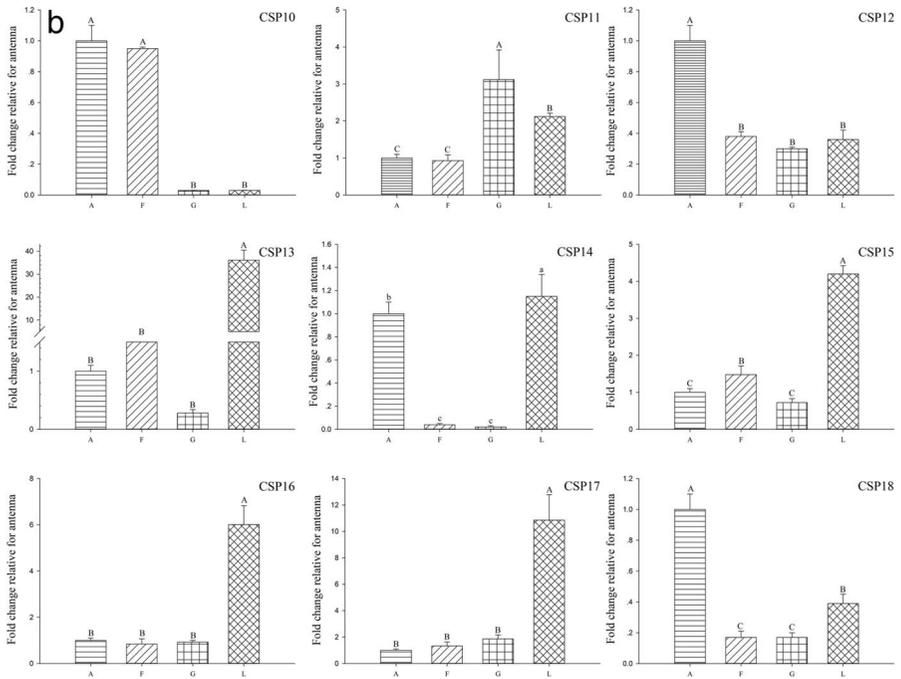


Fig. 3. Continued.

larvae (Cheng et al. 2015). In a study on the cockroach *Periplaneta americana* L., expression of a CSP was increased dramatically during limb regeneration, indicating that CSPs may contribute to other physiological processes beyond chemosensory functions (Nomura et al. 1992).

Furthermore, evidence indicates that expression of insect CSPs can exhibit distinct patterns during development. In a study on *Clostera restituta* (Walker), expression of CresCSP3 reached a peak at 3–4 d after eclosion in males and at 4–5 d in females (Hui et al. 2018). Analysis of SexiCSP expression in larval and pupal stages revealed relatively strong expression in pupae, which suggests a possible chemosensory role in pupae and/or a simple correlation with the onset of development of chemosensory tissues during pupation (Gong et al. 2012). Similar phenomena were also reported for *Heliothis virescens* Fabricius (Picimbon et al. 2001) and *B. mori* (Gong et al. 2007) in which expression levels of CSPs varied significantly during different developmental stages. There are also reports that CSPs differentially regulated over insecticide exposure in the study of *B. mori* (Xuan et al. 2015) and *Bemisia tabaci* (Gennadius) (Liu et al. 2014, 2016). Those studies led to the hypothesis that CSPs might be involved in physiological processes beyond chemosensory functions, such as host localization, mate allocation, development, and insecticide resistance. In conclusion, our findings provide a foundation for further structural and functional research, could support the screening of potential attractants or repellents, and may assist the development of novel pest control strategies.

Table 5. ANOVA tests statistic of 18 *Eogystia hippophaecolus* chemosensory protein (CSP) expression profiles.

Gene	Degree of Freedom, df	F Statistic	P Value	
CSP1	Between groups	2	585.249	0.000
	Within group	23		
CSP2	Between groups	2	56.005	0.000
	Within group	23		
CSP3	Between groups	2	81.027	0.000
	Within group	23		
CSP4	Between groups	2	894.605	0.000
	Within group	23		
CSP5	Between groups	2	32196.156	0.000
	Within group	23		
CSP6	Between groups	2	11.640	0.000
	Within group	2		
CSP7	Between groups	2	27.042	0.002
	Within group	23		
CSP8	Between groups	2	104.968	0.000
	Within group	23		
CSP9	Between groups	2	392.300	0.000
	Within group	23		
CSP10	Between groups	2	12.218	0.000
	Within group	23		
CSP11	Between groups	2	30.878	0.000
	Within group	23		
CSP12	Between groups	2	270.423	0.000
	Within group	23		
CSP13	Between groups	2	200.835	0.000
	Within group	23		
CSP14	Between groups	2	244.424	0.000
	Within group	23		
CSP15	Between groups	2	302.363	0.000
	Within group	23		

Table 5. Continued.

Gene	Degree of Freedom, df		F Statistic	P Value
CSP16	Between groups	2	91.761	0.000
	Within group	23		
CSP17	Between groups	2	76.548	0.000
	Within group	23		
CSP18	Between groups	2	324.883	0.000
	Within group	23		

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Table 6. Tukey's honestly significant difference tests after analysis of variance tests ($P < 0.01$).

Gene		Group Means				Dq ^a	Standard Errors
		Group1	Group2	Group3	Group4		
CSP1	A ^a	1.000				2.286	0.117
	L				28.836		
	G		6.876				
	La			22.692			
	Sig.	1.000	1.000	1.000	1.000		
CSP2	A		1.000			3.000	0.117
	L		0.690				
	G	0.271					
	La			1.645			
	Sig.	1.000	0.126	1.000			
CSP3	A	1.000				2.182	0.117
	L			24.897			
	G			23.024			
	La		15.213				
	Sig.	1.000	1.000	0.263			

Table 6. Continued.

Gene		Group Means				Dq ^a	Standard Errors
		Group1	Group2	Group3	Group4		
CSP4	A	1.000				2.400	0.117
	L		12.205				3.323
	G			67.679			1.950
	La	1.677					0.194
	Sig.	0.963	1.000	1.000			
CSP5	A	1.000				359.750	0.117
	L	1.120					0.014
	G	1.500					0.165
	La		3890.390				269.502
	Sig.	0.951	1.000				
CSP6	A	1.000				3.000	0.117
	L		2.664				0.587
	G	1.614					0.382
	La	1.323					0.246
	Sig.	0.102	1.000				
CSP7	A	1.000	1.000			2.000	0.117
	L	0.141					0.012
	G	0.848	0.848				0.085
	La		6.509				1.818
	Sig.	0.783	0.011				
CSP8	A	1.000				2.286	0.117
	L			17.767			2.825
	G	3.105					0.443
	La		7.241				0.431
	Sig.	0.328	0.039	1.000			
CSP9	A	1.000				2.000	0.117
	L		6.626				0.526
	G			0.337			0.061
	La	1.069					0.123
	Sig.	0.155	1.000	1.000			

Table 6. Continued.

Gene		Group Means				Dq ^a	Standard Errors
		Group1	Group2	Group3	Group4		
CSP10	A	1.000				2.182	0.117
	L	0.950					0.013
	G		0.025				0.001
	La		0.030				0.001
	Sig.	1.000	0.917				
CSP11	A	1.000				2.400	0.117
	L	0.980					0.154
	G		3.125				0.807
	La			2.116			0.090
	Sig.	0.039	1.000	1.000			
CSP12	A		1.000			2.667	0.117
	L	0.361					0.035
	G	0.299					0.012
	La	0.339					0.069
	Sig.	0.017	1.000				
CSP13	A	1.000				2.667	0.117
	L	1.249					0.810
	G	0.281					0.068
	La		36.077				4.371
	Sig.	0.913	1.000				
CSP14	A		1.000			2.526	0.117
	L	0.043					0.011
	G	0.020					0.013
	La		1.151				0.196
	Sig.	0.983	0.167				
CSP15	A	1.000				2.400	0.117
	L		1.602				0.232
	G	0.775					0.114
	La			4.085			0.220
	Sig.	0.104	1.000	1.000			

Table 6. Continued.

Gene		Group Means				Dq ^a	Standard Errors
		Group1	Group2	Group3	Group4		
CSP16	A	1.000				2.182	0.117
	L	0.838					0.237
	G	0.933					0.076
	La		6.013				0.822
	Sig.	0.968	1.000				
CSP17	A	1.000				2.667	0.117
	L	1.330					0.304
	G	1.989					0.356
	La		9.813				1.941
	Sig.	0.135	1.000				
CSP18	A			1.000		2.182	0.117
	L	0.192					0.047
	G	0.196					0.030
	La		0.354				0.062
	Sig.	0.991	1.000	1.000			

^a Dq, harmonic mean sample size; CSP, chemosensory protein, A, antennae; L, legs; G, external genitalia; La, labialpalp; Sig., significance level. Boldface values indicate significant differences between gene expression ($P < 0.01$).

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