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Characterization of G-protein coupled receptors from the blackback land crab *Gecarcinus lateralis* Y organ transcriptome over the molt cycle

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Abstract

Background: G-protein coupled receptors (GPCRs) are ancient, ubiquitous, constitute the largest family of transducing cell surface proteins, and are integral to cell communication via an array of ligands/neuropeptides. Molt inhibiting hormone (MIH) is a key neuropeptide that controls growth and reproduction in crustaceans by regulating the molt cycle. It inhibits ecdysone biosynthesis by a pair of endocrine glands (Y-organs; YOs) through binding a yet uncharacterized GPCR, which triggers a signalling cascade, leading to inhibition of the ecdysis sequence. When MIH release stops, ecdysone is synthesized and released to the hemolymph. A peak in ecdysone titer is followed by a molting event. A transcriptome of the blackback land crab *Gecarcinus lateralis* YOs across molt was utilized in this study to curate the list of GPCRs and their expression in order to better assess which GPCRs are involved in the molt process.

Results: Ninety-nine *G. lateralis* putative GPCRs were obtained by screening the YO transcriptome against the Pfam database. Phylogenetic analysis classified 49 as class A (Rhodopsin-like receptor), 35 as class B (Secretin receptor), and 9 as class C (metabotropic glutamate). Further phylogenetic analysis of class A GPCRs identified neuropeptide GPCRs, including those for Allatostatin A, Allatostatin B, Bursicon, CCHamide, FMRFamide, Proctolin, Corazonin, Relaxin, and the biogenic amine Serotonin. Three GPCRs clustered with recently identified putative CHH receptors (CHHRs), and differential expression over the molt cycle suggests that they are associated with ecdysteroidogenesis regulation. Two putative Corazonin receptors showed much higher expression in the YOs compared with all other GPCRs, suggesting an important role in molt regulation.

Conclusions: Molting requires an orchestrated regulation of YO ecdysteroid synthesis by multiple neuropeptides. In this study, we curated a comprehensive list of GPCRs expressed in the YO and followed their expression across the molt cycle. Three putative CHH receptors were identified and could include an MIH receptor whose activation negatively regulates molting. Orthologs of receptors that were found to be involved in molt regulation in insects were also identified, including LGR3 and Corazonin receptor, the latter of which was expressed at much higher level than all other receptors, suggesting a key role in YO regulation.

Keywords: Neuroendocrine signalling pathways, Rhodopsin-like receptors, Secretin-like receptors, Decapod crustaceans, Ecdysis regulation, Crustacean Hyperglycemic Hormone family of neuropeptides, Molting, Ecdysteroids, G protein-coupled receptor

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Background

Crustaceans are a diverse subphylum of arthropods comprising of close to 67,000 species, classified into six classes, which together with their derived insects, comprise the pancrustacea superphylum clade [1]. Malacostraca is the most diverse and species rich class of crustaceans, including the decapod crustaceans, which comprise the more familiar groups of species, such as crabs, crayfishes, lobsters, and shrimps. Crustaceans are adapted to a wide range of conditions, which explains their wide distribution across many ecological niches. Apart from their ecological significance, decapod crustaceans are highly prized in fisheries and aquaculture. In 2015, nearly 7.4 billion tonnes of crustaceans were cultured worldwide, with nearly 90% of total production attributed to coastal regions of Asian countries [2]. Additionally, decapod crustaceans are listed among the worst invasive species globally [3–5]. This great importance necessitates a better understanding of key biological processes of crustaceans in order to address key bottlenecks in the fishery and aquaculture industries to enable it to meet the ever-growing demand in quality protein [6], as well as to devise species-specific treatments for invasive species. One potential application is monosex biotechnology to minimize the species' invasive potential [7]. Growth control is another area of active research.

In crustaceans, like other arthropods, locomotion is facilitated by a rigid exoskeleton. Chitin, the second most abundant carbohydrate in nature following cellulose, is the major component of the exoskeleton, providing a scaffold for cuticular proteins that form links between the chitin fibers, as well as stabilize deposition of minerals that harden the cuticle. The skeletal muscles attach internally to the exoskeleton and contract to enable limb movements [8]. In some crustacean species, such as crabs, the rigid, highly calcified exoskeleton, provides protection against predators [9]. Unlike holometabolous insects (where following larval development there is a complete metamorphic transition through from pupae to adult, for example: flies, mosquitos etc.), crustaceans continually grow after they metamorphose into the juvenile stage. In order to grow and develop following metamorphosis, they must shed their old exoskeleton and produce a new larger one. In this process termed molting (or ecdysis) crustaceans synchronously form a new flexible exoskeleton under the existing one, absorb the minerals from the old exoskeleton, and then emerge from the old exoskeleton and harden the new one [10]. An advantage of continual growth is that crustaceans can increase in size and also regenerate lost limbs.

The molt cycle involves well-defined stages including intermolt, premolt, ecdysis, and postmolt. The longest stage is intermolt, when the animal accumulates organic compounds and stores the energy required for

reproduction and molting. In premolt, the epidermal layer enlarges and starts to separate from the old exoskeleton. It then synthesizes the outer layers of the new exoskeleton, while degrading the inner layers of the old exoskeleton. Ecdysis then takes place, by which the animal emerges from its old exoskeleton. The postmolt stage begins by the rapid uptake of water and minerals (and in some species, minerals are also retrieved from internal storages that dynamically fill prior to ecdysis as pouches outside the stomach, called gastrolithes) and harden the new exoskeleton to complete the molt cycle. During this stage, the animals start to consume food [11]. Molting in arthropods is triggered by a peak in the hemolymph level of derivatives of the steroid hormone ecdysone, which is synthesized in a pair of endocrine glands, known as the Y-organs (YOs), located bilaterally in the cephalothorax. The YOs ecdysteroid production capacity varies over the molt cycle; it is highest in premolt and lowest in postmolt (see review [10]).

In decapod crustaceans, ecdysone is secreted from the YOs into the hemolymph and is carried to target tissues, where it is hydroxylated into the active molt hormone: 20-hydroxyecdysone (20HE) [12–16]. 20HE binds to the ecdysteroid receptor, which then initiates a signaling cascade of transcription factors that alter the molecular program for molting and metamorphosis [17–20]. The synthesis of ecdysteroids by the YO is inhibited by a neuropeptide known as the molt-inhibiting hormone (MIH), produced predominantly by the X-organ (XO) [21, 22]. The XOs are located bilaterally in the eyestalks and are responsible for producing a suite of endocrine factors conveyed to the nearby sinus gland (SG) for storage and regulated release to the hemolymph. These factors include MIH, crustacean hyperglycemic hormone (CHH), vitellogenesis-inhibiting hormone (VIH), mandibular organ-inhibiting hormone (MOIH), and ion transport peptide (ITP) [23]. From a practical point of view, most of the decapod crustaceans produced through aquaculture in farms globally require removal of the eyestalks of broodstock females in order to induce spawning in captivity [24–26]. A better understanding of the mechanism which regulates each process can lead to specific treatments that avoid the use of such an extreme and labor-intensive method.

Our model organism is arguably the most well studied decapod crustacean in the context of molting. One of the earliest scientific studies on *Gecarcinus lateralis* was published in 1952 by Bliss and Welsh [27], describing the XO-SG ultrastructure. Experimental studies by Skinner and later by Chang and Mykles have contributed to a better understanding of the hormonal regulation of molting [10, 11], as well as the development of tools to investigate the molting process. YO assays [28–30] and transcriptomics [31–

[34] have been used to investigate the signaling pathways controlling YO ecdysteroidogenesis. However, one mystery remains, which is the identity of the MIH receptor. Although CHH and MIH share a similar function of inhibiting ecdysteroidogenesis by the YO, the signaling pathways of these two neuropeptides are distinct. A membrane guanylyl cyclase (GC-II) is considered as the receptor activated by CHH, resulting in immediate increase of the intracellular messenger guanosine 3', 5' cyclic monophosphate (cGMP) level. As proposed, an unidentified receptor activated by MIH temporarily increases the cAMP level followed by upregulation of cGMP (see [10, 35] for reviews). This suggests that the MIH receptor is not a GC-II. In 2009, Zmora *et al.* performed binding assays using radio-labeled MIH with YO membranes collected from blue crab juveniles in intermolt stage and hepatopancreas membrane of mature vitellogenic females. MIH was found to bind to both YO and hepatopancreas membranes, but with far higher affinity to the YO, suggesting that the main binding site of MIH is the YO membrane [36].

G-protein coupled receptors (GPCRs) are ancient, ubiquitous, constitute the largest gene family of transducing cell surface proteins, and are integral to cell communication [37–39]. All members of the GPCR gene family contain a domain of seven transmembrane α -helices with three extracellular loops and three intracellular loops [40]. The GPCR gene family is subdivided into three main classes depending on the pharmacological nature of their ligands and sequence similarity [41]. These are rhodopsin-like receptors (class A), secretin-like receptors (class B), and metabotropic-glutamate-receptor-like (class C), which represent about 89%, 7%, and 4%, respectively [42], of the known GPCRs [42]. In insects, most of the neuropeptide-activated receptors are predominantly rhodopsin-like receptors and some are secretin-like receptors [43]. GPCR sequences within these families can share less than 25% identity between species [44], making it difficult to annotate newly identified candidate receptors. More than 1,000 GPCRs have been characterized in *Caenorhabditis elegans* [45], while the number of GPCRs is over 200 in *Drosophila melanogaster* [46], adding another level of complexity presented by the vast variation of GPCR number across ecdysozoans. In crustaceans, there are many efforts to deorphanize neuropeptide receptors, but there are only a few species where a comprehensive list of GPCRs have been identified. Recently, advances in sequencing technologies have facilitated transcriptome-based annotation. In 2014, Nagai *et al.* characterized two GPCRs, BNGR-A2 and A34, as ITP receptors, and A24 as an ITP-like receptor (member of CHHR family) in the silkworm *Bombyx mori*, using *in vitro* binding assays with ITP peptides and 30 GPCRs [47]. Using these ITP and ITP-like receptors from *B. mori* as references for phylogenetic study,

Veenstra identified one contig (Pc-GPCRA9) as an ITP-like receptor based on clustering with BNGR-A24 and three receptors as putative ITP receptors (Pc-GPCRA52, Pc-GPCRA53, Pc-GPCRA63) in the red swamp crayfish *Procambarus clarkii*, based on clustering with BNGR-A34 [48]. A recent study in the eastern spiny lobster *Sagmariasus verreauxi* has also proposed two putative ITP receptors based on phylogenetic alignment to BNGR-A34 and Pc-GPCRA53 [49]. These studies, together with the high similarity shown between insect and decapod neuropeptidomes [50, 51], make it possible to predict the receptors for decapod neuropeptides based on those deorphanized in insects. This study curated a comprehensive list of GPCRs in the *G. lateralis* YO transcriptome across the molt cycle.

Results and discussion

GPCRs play a central role in cell signaling as receptors for several transmitters, mediators, hormones, and neuropeptides. In crustaceans, most of the neuropeptides with known receptors act through GPCRs. Although many dozens of neuropeptides have been identified in hundreds of crustacean species over the last decade [48, 52, 53], information about their receptors (GPCRs) in terms of sequence, structure, and function, is very limited. Recent studies on decapod crustaceans formed a foundation for the future discovery of GPCRs. In 2015, Veenstra mined publically-available databases of *P. clarkii* and found several neuropeptide receptors, including those for Ast C, LGR, PDF, DH31, and DH44 [48]. In 2016, Buckley *et al.* computationally identified 86 GPCRs in the eastern spiny lobster *S. verreauxi*, including important neuropeptide receptors [49]. In this study, transcriptomic analysis identified 99 GPCRs in the *G. lateralis* YO. Of these, 71 were annotated either by phylogenetic or domain analysis, which include Ast receptor, Crz receptor, CHHamide receptor, and FMRamide receptor. These outcomes are comparable to previous studies in Decapoda [48, 49]. N-glycosylation motif arrangement analysis of selected receptors was used to confirm the annotation.

In silico transcriptomic analysis identified 299 sequences with seven TM (Table 1). Ninety-nine of the 299 sequences, have the seven TM domain characteristic of GPCRs. Among them, 49 proteins were identified as Rhodopsin-like receptors (designated as GeclatGPCR_A1 to 45). Phylogenetic analysis and blast search using annotated GPCRs from other arthropods enabled annotation of 37 Rhodopsin-like receptors (class A GPCRs; Additional files 1, 2 and Table 2). Thirty-five GPCRs were classified as secretin-like (class B) GPCRs. Of the 35 class B receptors, one was annotated as diuretic hormone 44 (DH44) receptor, one diuretic hormone 31 (DH31) receptor, one parathyroid hormone (PTH) receptor, and three were annotated as putative pigment

Table 1 The numbers of sequences comprising each of the Pfam accessions

Domain name	Pfam accession	Description of domain	Number of unique predicted peptide sequences
7tm_1	PF00001.16	7 transmembrane receptor (rhodopsin family)	108
7tm_2	PF00002.19	7 transmembrane receptor (Secretin family)	54
7tm_3	PF00003.17	7 transmembrane sweet-taste receptor of 3 GCPR	17
7tm_7	PF08395.7	7tm Chemosensory receptor	1
7TM_GPCR_Srsx	PF10320.4	Serpentine type 7TM GPCR chemoreceptor Srsx	31
7TM_GPCR_Srv	PF10323.4	Serpentine type 7TM GPCR chemoreceptor Srv	11
7TM_GPCR_Srw	PF10324.4	Serpentine type 7TM GPCR chemoreceptor Srw	15
7TM_GPCR_Srx	PF10328.4	Serpentine type 7TM GPCR chemoreceptor Srx	16
ABC_tran	PF00005.22	ABC transporter	159
ABC_tran_2	PF12848.2	ABC transporter	9
ABC_transp_aux	PF09822.4	ABC-type uncharacterized transport system	1
Frizzled	PF01534.12	Frizzled/Smoothed family membrane region	10
Na_Ca_ex	PF01699.19	Sodium/calcium exchanger protein	7
GpcrRhopsn4	PF10192.4	Rhodopsin-like GPCR transmembrane domain	4

dispersing factor (PDF) receptors. Nine class C and six class F receptors were inferred based on BlastP results and the Frizzled domain in class F.

A heat-map profile of GPCR expression was generated based on reads mapping to the transcriptome database using a normalized read count (RPKM) [32] in five different molt stages. The RPKM of most GPCRs showed down-regulation at the postmolt stage (67% of the GPCRs; Fig. 1).

It is noteworthy that one GPCR (A37) showed specific expression at the postmolt stage, and one putative corazonin receptor (A6) was not expressed in the intermolt and early premolt stages, but was expressed in the mid and late premolt stages with higher expression in the postmolt stage. All three predicted CHHRs (A9, A10, A12) were expressed throughout the molt cycle except in the postmolt stage. GPCR families known to be involved with molting in arthropods and those that show differential expression in the present study are discussed in further detail below.

Rhodopsin-like receptors (class A)

Allatostatin receptors

Allatostatins (ASTs) are pleiotropic neuropeptides that function as inhibitors of juvenile hormone (JH) production. JH is synthesized in the corpora allata in insects, while its crustacean active analogous compound (the JH precursor, methyl farnesoate; MF) is synthesized in the mandibular organ [54]. JH/MF maintains the appropriate stage and size and prevents metamorphosis [55]. Three types of ASTs have been identified in insects and characterized based on their conserved C-terminal sequences. The first class is FGLamide (Ast A), first discovered in

cockroaches, which includes the conserved C-terminal sequence F-G-Lamide (other cases Y/F-X-F-G-L/Iamide) [56]. The second family of ASTs was isolated in crickets. These are C-terminally amidated peptides with tryptophan in the second and ninth positions, and are designated as the W(X)6Wamide or B-type ASTs [57, 58]. The third family of ASTs was first isolated in 1991 from the brain of the lepidopteran *Manduca sexta* [59]. It is a single 15 amino acid peptide with the nonamidated C-terminal pentapeptide P-I-S-C-F (Ast C). All three classes of peptides have since been identified in crustaceans [60–62].

Ast A regulates metabolism, feeding homeostasis, and energy mobilization by controlling release of glucagon-related adipokinetic hormone (AKH) and *Drosophila* insulin-like peptides (DILPs) [63]. One putative Ast A receptor (AstA_R; Gl-GPCRA1) was identified through phylogenetic analysis. *G. lateralis* Ast A receptor (AstA_R) contains three N-glycosylation motifs at the N-terminus and two N-glycosylation motifs at the C-terminus, while *P. clakii* AstA_R has three N-glycosylation motifs at the N-terminus and one at the C-terminus (Fig. 2a). In *G.lateralis*, the AstA_R was up-regulated during intermolt and premolt stages, and was not expressed at the postmolt stage, which is consistent with the role of Ast A in regulating metabolism and energy.

Ast B is known for its myoinhibitory role and is therefore also referred to as myoinhibitory peptide (MIP) [64]. In *D. melanogaster*, Ast B (also called sex peptide in insects) blocks the receptivity of copulated females and increases food uptake after copulation [65]. Ast B is also known as a signaling molecule for settlement behavior in *Platynereis* larvae [66]. One putative Ast B

Table 2 List of GPCR class A (Rhodopsin-like receptor)

ID	Predicted function	Predicted ligand
GI_GPCR_A1	Allatostatin A receptor	Allatostatin A
GI_GPCR_A2	TRH receptor	TRH
GI_GPCR_A3	ETH receptor	ETH
GI_GPCR_A4	GPA2/GPB5 receptor	GPA2/GPB5
GI_GPCR_A4b	GPA2/GPB5 receptor	GPA2/GPB5
GI_GPCR_A5	Allatostatin B receptor	Allatostatin B
GI_GPCR_A5b	Allatostatin C receptor	Allatostatin C
GI_GPCR_A6	Corazonin receptor	Corazonin
GI_GPCR_A7	Corazonin receptor	Corazonin
GI_GPCR_A8	CCHamide receptor	CCHamide
GI_GPCR_A8b	CCHamide receptor	CCHamide
GI_GPCR_A9	CHH-like receptor	CHH family
GI_GPCR_A10	CHH-like receptor	CHH family
GI_GPCR_A11	FMRFamide receptor	FMRFamide
GI_GPCR_A12	CHH-like receptor	CHH family
GI_GPCR_A13	Proctolin receptor	Proctolin
GI_GPCR_A14	LGR-C1 receptor	Unknown
GI_GPCR_A14b	LGR-C2 receptor	Unknown
GI_GPCR_A15	Unknown receptor	Unknown
GI_GPCR_A16	HPR1 receptor	Unknown
GI_GPCR_A17	HPR1 receptor	Unknown
GI_GPCR_A18	HPR1 receptor	Unknown
GI_GPCR_A19	Bursicon receptor	Bursicon
GI_GPCR_A20	Unknown receptor	Unknown
GI_GPCR_A21	TIE receptor	Unknown
GI_GPCR_A22	Unknown receptor	Unknown
GI_GPCR_A23	Moody receptor	Unknown
GI_GPCR_A24	Unknown receptor	Unknown
GI_GPCR_A25	Unknown receptor	Unknown
GI_GPCR_A26	sNPF receptor	sNPF
GI_GPCR_A27	Unknown receptor	Unknown
GI_GPCR_A28	Unknown receptor	Unknown
GI_GPCR_A29	Unknown receptor	Unknown
GI_GPCR_A30	Serotonin receptor	Serotonin
GI_GPCR_A31	Unknown receptor	Unknown
GI_GPCR_A32	Serotonin receptor	Serotonin
GI_GPCR_A33	Adenosine receptor	Adenosine
GI_GPCR_A34	Octopamine receptor	Octopamine
GI_GPCR_A35	Prostaglandin receptor	Prostaglandin
GI_GPCR_A36	Prostaglandin receptor	Prostaglandin
GI_GPCR_A37	Prostaglandin receptor	Prostaglandin
GI_GPCR_A38	Prostaglandin receptor	Prostaglandin
GI_GPCR_A39	Peropsin receptor	Peropsin
GI_GPCR_A40	FMRFamide receptor	FMRFamide

Table 2 List of GPCR class A (Rhodopsin-like receptor)

ID	Predicted function	Predicted ligand
GI_GPCR_A41	Unknown receptor	Unknown
GI_GPCR_A42	Unknown receptor	Unknown
GI_GPCR_A43	Unknown receptor	Unknown
GI_GPCR_A44	HPR1 receptor	Unknown
GI_GPCR_A45	CCAP receptor	CCAP

receptor (AstB_R; GI-GPCRA5) was identified, which clustered with AstB_R identified in *P. clarkii* (Fig. 2b). GI-GPCRA5 showed the same RPKM expression trend as most other receptors, with the highest level in the intermolt stage and gradually decreasing in expression in premolt stages, with no expression at the postmolt stage (Fig. 3). This expression pattern is opposite to that found in holometabolous insects [11] (also based on expression data in *D. melanogaster* as found in Flybase; Sex peptide receptor (CG16752, FBgn0029768)), where AstB_R expression increases towards the molt and persists in the postmolt, when the animal is not feeding, suggesting a different role for Ast B in crustaceans. Another plausible explanation is that in this study we focused on the expression of AstB_R in the YO, the crustacean analog of the prothoracic gland (PG) in insects, while the AstB_R temporal expression pattern in insects was examined in whole animals.

Ast C or PISCF-type Ast was originally described as an unknown neuropeptide in *M. sexta* [67]. Its analog was then identified in several insect species, e.g. *D. melanogaster* [68], *Tribolium castaneum* [69], and later in the decapods *P. clarkii* [48] and *S. vereauxi* [49]. Ast C inhibits JH biosynthesis in *M. sexta*, *Helicoverpa zea* and *Aedes aegypti* [70]. It also functions as an immunosuppressive factor that prevents immunopathology or reduces unnecessary metabolic costs following microbial exposure [71]. One putative Ast C receptor (AstC_R; GI_GPCRA5b) was predicted through phylogenetic analysis (Fig. 4). The RPKM expression of GI-GPCRA5b remained at a low level throughout the molt cycle, and could not be detected at early premolt, mid premolt, and postmolt stages.

Corazonin receptors

Corazonin (Crz) was initially discovered as a strong cardioaccelerator in the American cockroach *Periplaneta americana* [72] and later in other insect species [73]. It is also found in decapod crustaceans [74–76]. Crz has various functions in different species. In locusts, Crz participates in the pigmentation process [77], while it is also recognized as a cardioaccelerator in the American

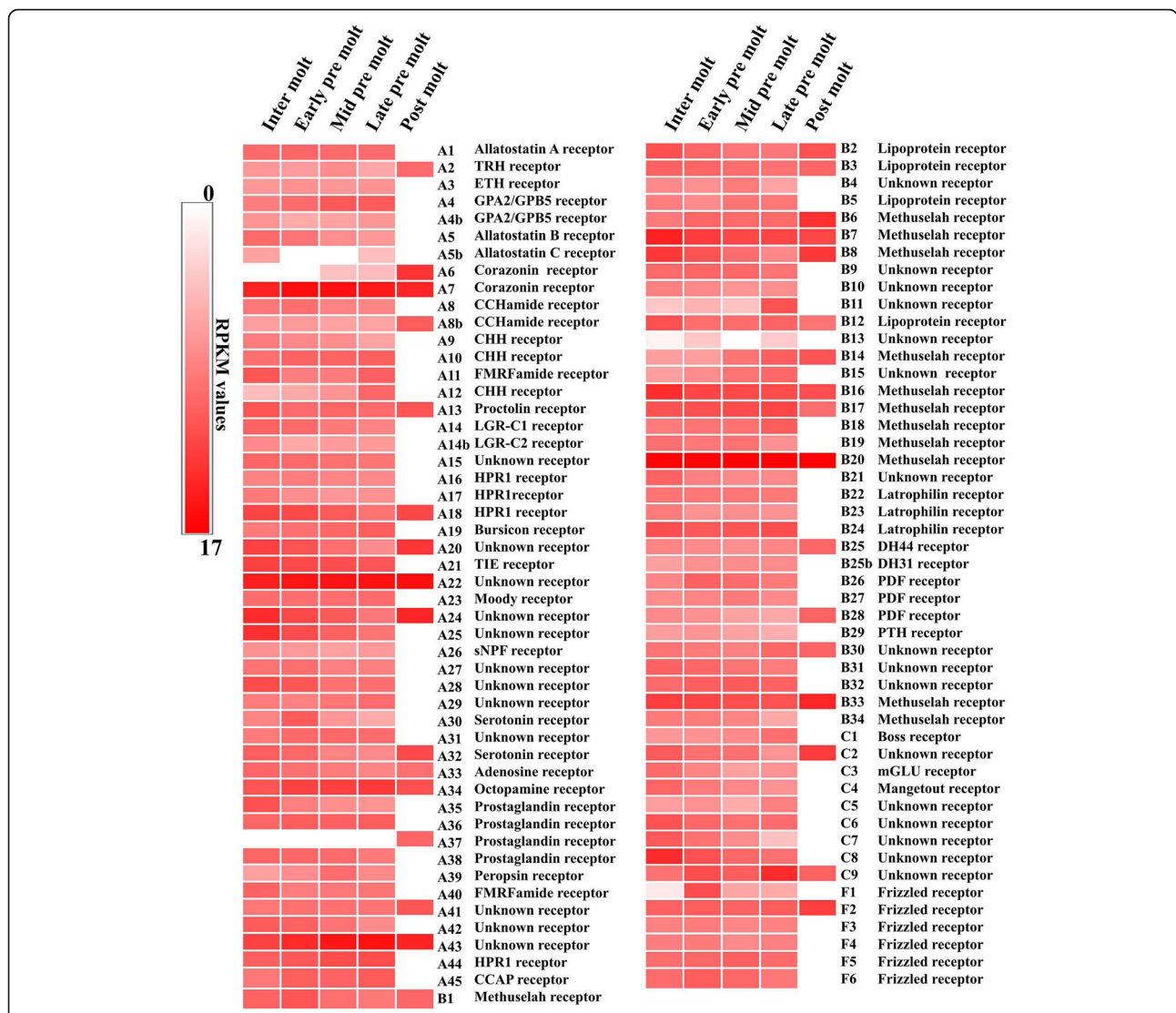


Fig. 1 Gene expression (RPKM) heat map of GPCRs in different molt stages. Clustered by gene expression profile in a transcriptome dataset based on 5 different stages. Scores are coloured on a log₂ scale with the red maximum and white minimum. Putative GPCR receptors are predicted based on a phylogenetic study and domain analysis

cockroach [78]. In addition, Crz indirectly affects ecdysis in *M. sexta* [79], serving as an ecdysis initiator [80]. Although Crz receptors are well conserved in amino acid sequence across species, a number of isoforms have been discovered in insects, crustaceans, and ticks [78].

Two putative Crz receptors were predicted in our study. GI-GPCRA6 and GI-GPCRA7 are clustered into the Crz receptor clade, sharing similar amino acid distribution in the seven TM domain with three N-glycosylation motifs at the N-terminus and one N-glycosylation motif at the C-terminus (Fig. 5a). Motif analysis indicates that both Crz receptors have identical amino acid sequences in the transmembrane domain, and a pairwise alignment shows high similarity in their

sequences (72%). This suggests that both receptors are closely-related isoforms. GPCRA6 (Crz_R1) and A7 (Crz_R2) showed much higher expression in the YO (more than 10 times), compared with all other putative GPCRs. The expression pattern of these two putative receptors showed different trends. Crz_R1 showed no expression in the intermolt and early premolt stages, peaking at the postmolt stage. This expression suggests a role of Crz_R1 in postmolt. This is consistent with Alexander *et al.* (2017) who report that, in *C. maenas*, Crz_R1 (*C. maenas* clustered to GI_GPCRA6 (Fig. 5a)) is highly expressed in the YO, but it has little effect on ecdysteroid biosynthesis, except a modest stimulation in early postmolt [76]. While Crz_R1 peaked in

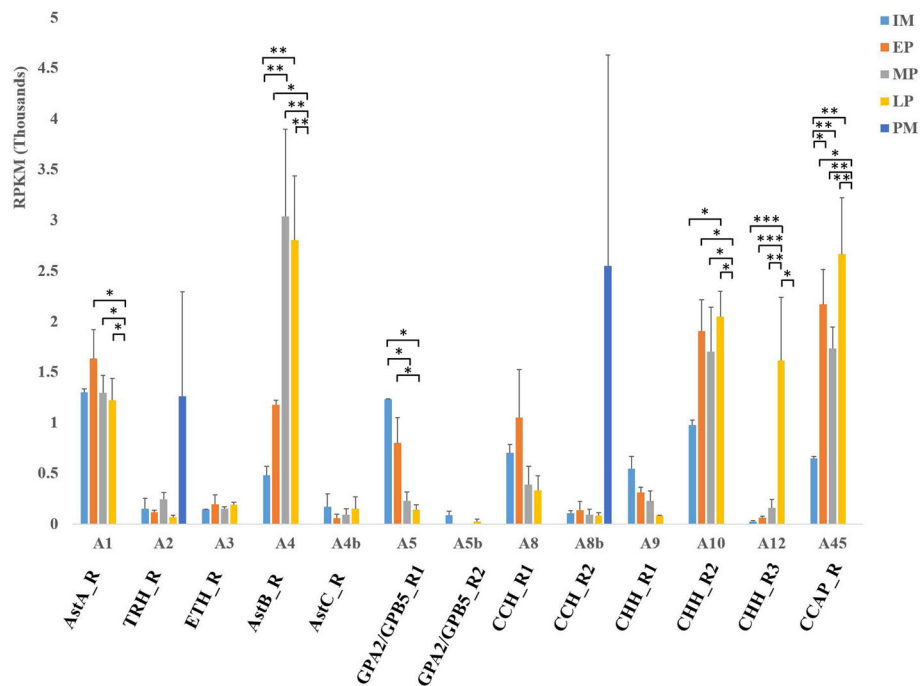


Fig. 2 Statistical analysis of transcript RPKM expression. Molt-related GPCRs in *G. lateralis* YO were differentially expressed in five different stages of molt cycle ($P < 0.05$ and $FDR < 0.05$, highlighted in green in Additional file 2). Abbreviations: IM, intermolt stage; EP, early premolt stage; MP, mid premolt stage; LP, late premolt stage; and PM, postmolt stage. Significance level is marked as: * = $P < 5E^{-02}$, ** = $P < 5E^{-04}$, *** = $P < 5E^{-06}$

expression in the postmolt, Crz_R2 showed high expression in intermolt, peaked in early premolt, and decreased towards the postmolt stage (Fig. 5b). Crz initiates ecdysis-triggering hormone (ETH) production in ‘inka’ cells in insects. ETH, in turn, triggers a signal cascade that leads to ecdysis. The elevated expression of the Crz receptors in the YOs reflects the key role of Crz receptors in molt regulation. Further studies are warranted to clarify the function of Crz_R2 in relation to the molt cycle in crustaceans.

CCHamide receptor

CCHamide is an invertebrate neuropeptide that was initially designated as ‘synthetic peptide, CCM’ [81]. Roller and colleagues assigned it with the new name CCHamide (CCHa) based on two conserved cysteines and an amidated histidine residue at the C terminus [82]. A comprehensive study found two CCHamide neuropeptides (CCHamide-1, CCHamide-2) in eleven insect species [53]. In *D. melanogaster*, CCHamide-2 is mainly located in endocrine cells in the gut, where the cells

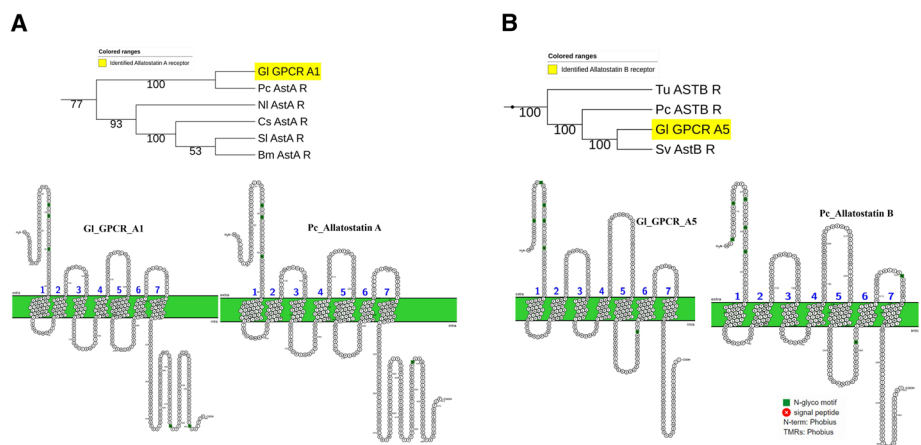
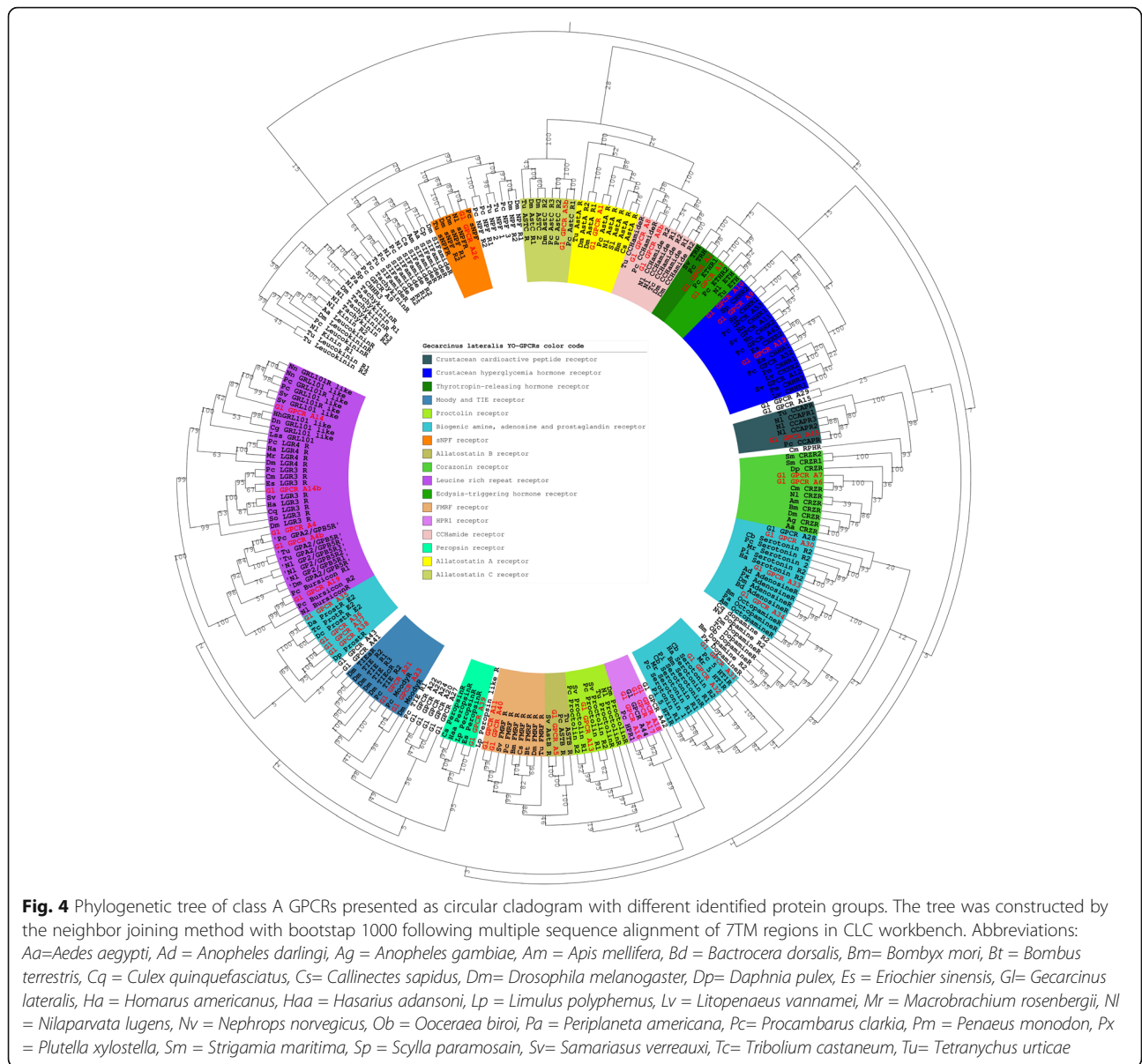


Fig. 3 Pruned tree of Ast receptors and their amino acid sequence arrangement. **a** *G. lateralis* Ast A receptors in comparison with *P. clarkii* Ast A receptor and **b** *G. lateralis* Ast B receptors in comparison with *P. clarkii* Ast B receptor



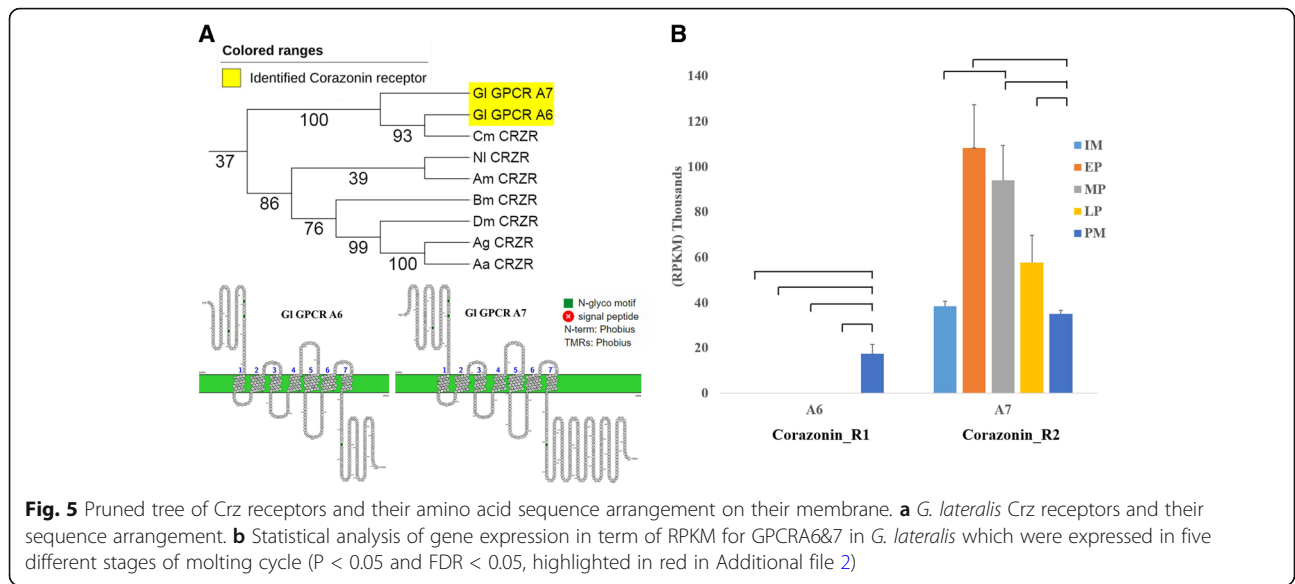
sense the quality of food and signal for the transport of CCHamide-2 to the brain, where it binds to a CCHamide-2 receptor and alters feeding behavior [83].

Two putative CCHamide receptors were identified (CCH_R1; GI-GPCRA8, and CHH_R2; GI-GPCRA8b), which were clustered with *P. clarkii* CCHamide receptor in the phylogenetic analysis (Fig. 6a). CCH_R2 contained a partial sequence with an incomplete 7-TM domain. Comparison of the 7-TM domain distribution across the membrane between CCH_R1 and CCH_R2 showed one N-glycosylation motif in common at the second extracellular loop of both CCHRs. CCH_R1 showed high expression in all four molt stages except postmolt (Fig. 3). The rapid increase from the intermolt stage to early pre-molt stage, followed by a drop in expression at the mid

and late premolt stages, and no expression at the post-molt stage also implicates CCHamide receptor in early premolt. CCH_R2 was expressed at low levels from the intermolt to late premolt stage, and peaked in expression at the postmolt stage (Fig. 3). The SEM of RPKM expression at postmolt stage of CCH_R2 was relatively high because of high variation between replicates. The great difference in RPKM expression between postmolt stage to other stages suggests an important role in the postmolt stage.

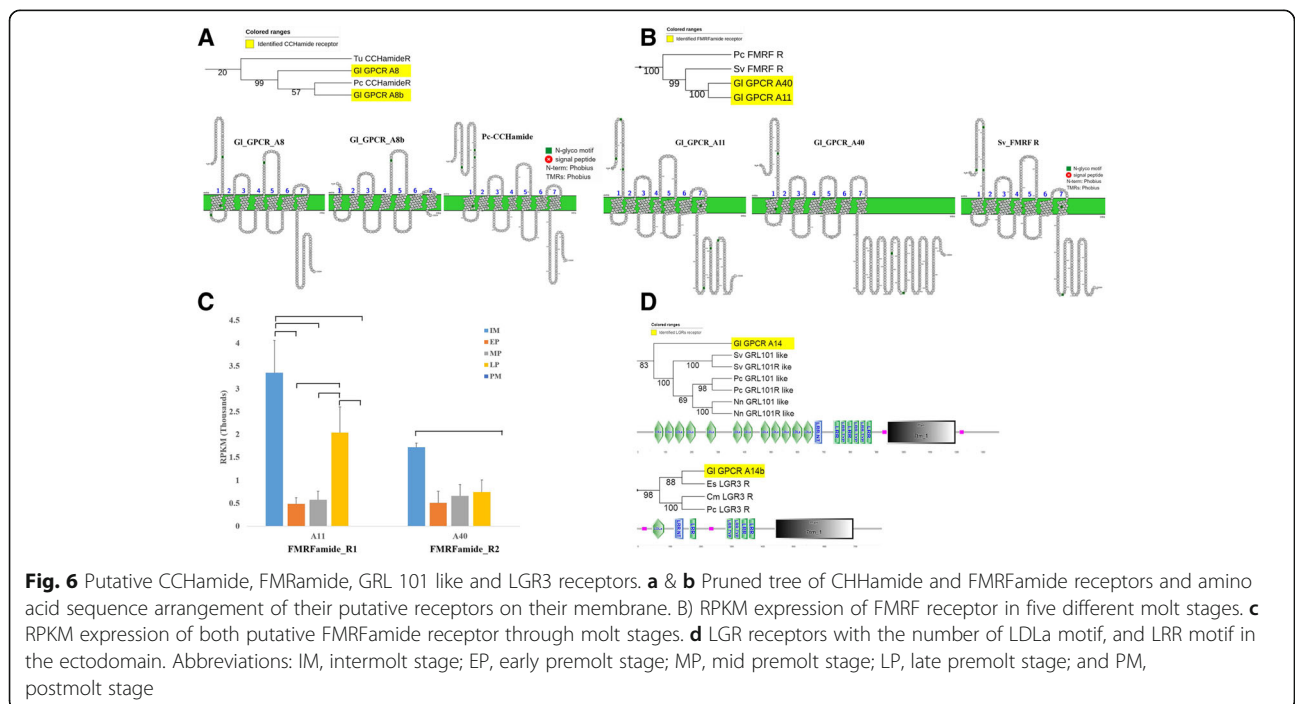
Crustacean cardioactive peptide receptors

Crustacean cardioactive peptide (CCAP) is produced in the pericardial organ of the shore crab *Carcinus maenas*, where it accelerates heart contraction [84]. It was later



found in the pericardial organ of *Homarus americanus* and *Cancer productus* [85, 86]. CCAP also plays a role in stress response and biosynthesis adaptation in decapod crustaceans [87]. In *Macrobrachium nipponense*, CCAP is among six key neuropeptides found to be involved in reproduction regulation [88]. Although the specific name of CCAP applies to crustaceans, it also stimulates the heartbeat in insect species, such as *P. americana*, *D. melanogaster*, *Baculum extradentatum*, and *Locusta migratoria* [89]. CCAP also functions in

ecdysis in several insect species. CCAP initiates the ecdysis motor program in *M. sexta* [90] and regulates timing of ecdysis behavior in *D. melanogaster* [91]. One putative CCAP receptor (CCAP_R) was identified that clusters with *P. clarkii* CCAP_R in the phylogenetic tree (Fig. 4). RPKM expression analysis showed a similar expression to that of other receptors, with low expression at the intermolt stage and higher expression throughout the premolt stages, followed by no expression at the postmolt stage (Fig. 3).



CHH/CHH-like receptor

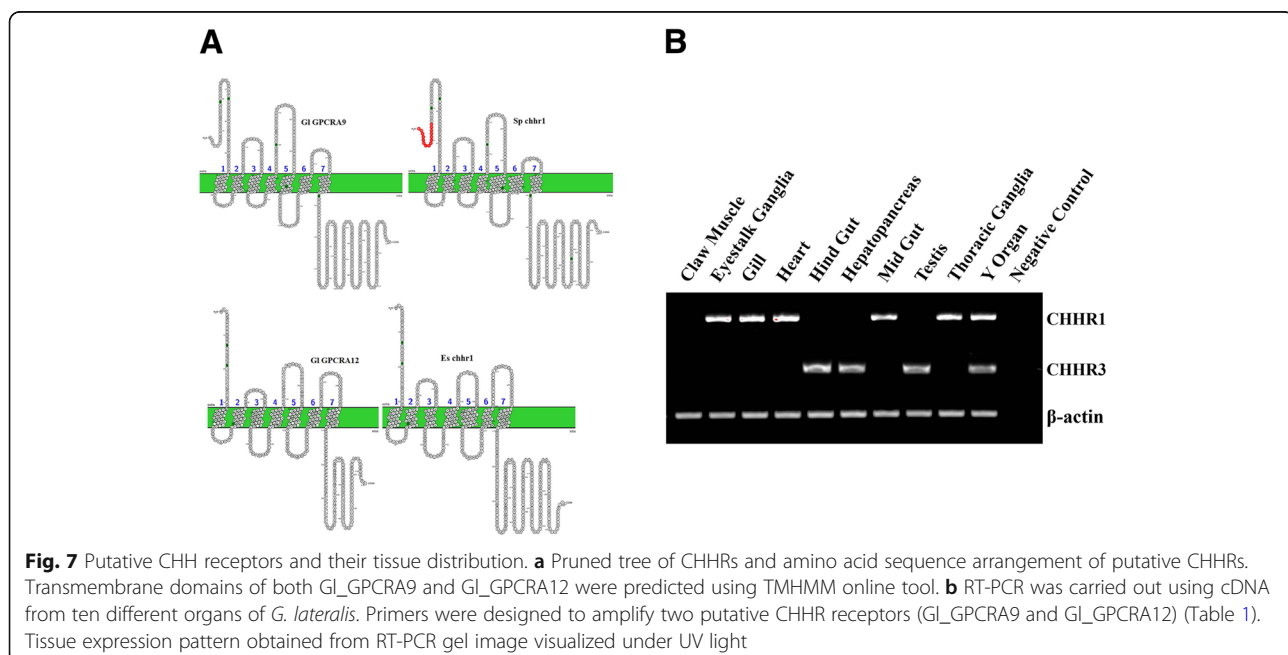
MIH is a neuropeptide that controls molting in decapod crustaceans. The identification of its receptor has been the focus of crustacean endocrinological research for decades. In 2014, Nagai *et al.* identified two putative GPCRs, BNGR-A2 and A34, as ITP receptors, and A24 as an ITP-like receptor (member of CHHR family) in the silk moth *B. mori*. This led to the identification of CHH-like family receptors in crustaceans. Veenstra identified three CHH-like receptors clustered with BNGR-34 in *P. clarkii*, and two putative CHH-like receptors have also been found in *S. verreauxi* [46].

Three putative GPCRs (GI-GPCRA9, GI-GPCRA10, GI-GPCRA12) were identified as CHH-like receptors, as these sequences clustered into the CHHR clade (Fig. 7a). Further analysis of the transmembrane domains of these three proteins with TMHMM indicated that GI-GPCRA9 and GI-GPCRA10 contain a complete 7-TM domain, while GI-GPCRA10 contains 6 transmembrane helices (data not shown). This could be explained by incomplete recovery of the sequence from the assembled contigs. GI-GPCRA9 and GI-GPCRA10 were expressed in all molt stages, while the expression of GI-GPCRA12 decreased in late pre-molt and postmolt stages. Two CHHRs (GI-GPCRA9 and GI-GPCRA12) were examined using RT-PCR expressed in the YOs. Notably, both receptors were expressed not only in YOs, but they were also expressed in other tissues (Fig. 7b). Zmora *et al.* conducted MIH binding assays in the blue swimmer crab *Callinectes sapidus* [36]. This study showed that MIH

bound predominantly to membranes extracted from the YOs but to a lesser extent also to membranes extracted from the hepatopancreas [36]. Based on this result, the spatial expression pattern of the putative CHHRs identified in this study cannot determine which receptor might be binding MIH and therefore receptor activation assays are required.

Ecdysis-triggering hormone receptors

Ecdysteroids are responsible for initiating the molting process, and are synthesised and released from the PGs in insects or from the YOs in crustaceans [10]. In insects, ecdysteroids trigger the production of ETH in 'inka' cells, a specialized group of endocrine cells scattered across the epithelial cells of the insect tracheae [92]. ETH production causes a surge in Ecdysis Hormone (EH) secretion, which leads to the release of CCAP and upregulation of cGMP [93]. ETH travels to the central nervous system (CNS) where it stimulates the sensitivity of the CNS to ETH by promoting the expression of the ETH receptor (ETHR). In *M. sexta*, two alternatively spliced ETHRs (ETHR-A and ETHR-B) are encoded by *ethr* gene and expressed in discrete central neurons [94]. Previous studies indicated ETH is a key regulator that initiates ecdysis in insects [reviewed in [95]]. In *D. melanogaster*, the *eth* gene encodes two neuropeptides designated ETH1 and ETH2. Injection of ETH1 into pharate pupae strongly induces proecdysis within 1-3 minutes, followed by ecdysis, while injection ETH2 has no effect [96]. One putative ETHR was identified



and clustered with *P. clarkii* ETH_R1. ETHR was expressed at a similar level of RPKM through the molt cycle except the postmolt stage, where no expression was detected (Fig. 3).

FMRFamide/ FMRFamide-like receptor

FMRFamides are widely distributed neuropeptides with four signature amino acids at their C terminus (F-M-R-F) [97]. Many isoforms with variations of this signature tetrapeptide occur, which therefore classified as FMRFa-like peptides (FLPs) [98]. The first FLP was identified in *D. melanogaster* by cDNA cloning [99]. More FLPs have since been identified in insects using mass spectrometry [100, 101]. The FLP family comprises several neuropeptides that include sulfakinin, neuropeptide F (NPF), short neuropeptide F (sNPF), and myosuppressin [102–104]. FMRFamides are gut and heart contraction factors that also control digestive processes [105, 106]. Their role also extends to the ecdysis process by activating FMRFamide neurons during premolt in *D. melanogaster* [96] through direct innervation of the PG [107, 108].

Two *G. lateralis* putative FMRFamide receptors clustered with *P. clarkii* and *S. verreauxi* FMRFamide receptors (FMRF_R1; Gl_GPCRA11 and FMRF_R2; Gl_GPCRA40). Motif analysis of the *S. verreauxi* and *G. lateralis* FMRFamide receptors showed high similarity between FMRF_R1 and FMRFamide receptor of *S. verreauxi*. In particular, there were three N-glycosylation motifs at the N-terminus and one N-glycosylation motif at the seven TM domain. The only difference was at the C-terminus where two additional N-glycosylation motifs were predicted in *G. lateralis* (Fig. 6b). Motif analysis of FMRF_R2 showed distinct organization of one N-glycosylation motif at the N-terminus, two at the C-terminus, and one at the first TM domain. RPKM expression analysis showed a similar trend in both FMRFamide receptors, in which they were expressed high levels in the intermolt stage, slightly decreased in the next two molt stages, followed by an increase in the late premolt, and no expression in the postmolt stage (Fig. 6c). A small neuropeptide F (sNPF) receptor is also predicted based on the phylogenetic analysis (Gl-GPCRA26), where it clustered with the *P. clarkii* sNPF receptor (Fig. 4).

Leucine-rich repeats containing GPCRs

Leucine-rich repeats-containing GPCRs (LGRs) belong to the rhodopsin-like GPCR family. LGRs have, in addition to the GPCR-conserved 7-TM domain, multiple repeats of leucine-rich regions (LRRs) and low density lipoprotein (LDL) motifs for hormone binding. LGRs are classified into three types (A, B, and C) based on the number of LRRs, number of LDL

motifs, and the structure of the hinge region [109]. Type A LGRs contain 7–9 LRRs and a long hinge region in their ectodomain, while type B LGRs typically have about twice the number of LRRs (16–18 LRR) and a shorter hinge region. In *D. melanogaster*, LGR1 (Type A LGR) is activated by GPA2/GPB5 neuropeptide, a heterodimer formed by GPA2 and GPB5 [110], and LGR2 (Type B LGR) is activated by bursicon [111]. Bursicon is a heterodimeric protein, consisting of α and β subunits. In *C. maenas*, Bursicon is co-localized with CCAP, both being released from neurons in the CNS. Bursicon plays a key role in cuticle hardening during post molt [112, 113]. Bursicon is also involved in reproduction by increasing vitellogenin and stimulating ovarian development in female shrimp, *Penaeus monodon* [114]. The number of LRRs in type C is similar to type A, but the hinge region is quite short and the LDL motifs are N-terminal to the LRRs. The Type C LGRs are subdivided into two subgroups: C1, which contains only one LDL motif and two cysteines in their hinge region and C2, which has five, six, ten or twelve LDLs N-terminal to the LRRs and four cysteines in their hinge region [109].

Five putative LGRs were predicted in the phylogenetic analysis. Gl_GPCRA4 and Gl_GPCRA19 clustered with the Bursicon receptor (type B LGR) and Gl_GPCRA4b clustered with the GP2/GP5 receptor (type A LGR). Another LGR identified (Gl-GPCRA14) clustered with GRL 101-like. Like other GRL 101-like receptors, Gl-GPCRA14 consists of 11 LDLs and 7 LRRs in its ectodomain, which defines it as type C2-like LGR (Fig. 6d). Gl-GPCRA14b clustered into the LGR3 clade, belonging to type C1 LGR, as it contains 1 LDL and 6 LRRs in its ectodomain. In *D. melanogaster*, LGR3 is activated by Dilp8, a member of the insulin-like neuropeptide family [115]. LGR3 activation induces nitric oxide synthase production in the PG in response to Dilp8, which is elevated in injured imaginal discs [116, 117]. The increased nitric oxide synthase activity reduces ecdysone synthesis by the PG, which coordinates molting with the growth of the regenerating imaginal discs [118]. The identification of LGR3 in *G. lateralis* YOs suggests a similar function for this receptor in delaying the molt by damaged or lost limbs [11, 119, 120]. Autotomy of a regenerating limb in early premolt suspends molting until a secondary limb regenerate differentiates and grows to replace the lost regenerate (108). Secondary limb regenerates produce a peptide-like factor, designated limb autotomy factor - proedysis (LAF_{pro}) (11), that delays molting by lowering hemolymph ecdysteroid titer (109). Given that several insulin-like peptides were recently identified in decapods [121, 122], it is possible that LAF_{pro} functions as the Dilp8 ortholog.

Thyrotropin-releasing hormone/ Thyrotropin-releasing hormone like receptors

In mammals, thyrotropin-releasing hormone (TRH) is a hypothalamic releasing factor that is synthesized mainly in the hypothalamus. Upon release TRH stimulates the release of thyroid-stimulating hormone and prolactin by the pituitary. TRH is also produced in peripheral tissues and the nervous system [123]. TRHs have a tripeptide Glu-His-Pro in their sequences and stimulate thyroid-stimulating hormone (TSH) biosynthesis [124]. In vertebrates, two TRH receptor (TRHR) isoforms are classified as type 1 (TRH-R1) and type 2 (TRH-R2). These two receptors belong to the rhodopsin/ β -adrenergic receptor-like family of GPCRs and share up to 50% similarity in their amino acid sequences [125]. In arthropods, TRH-like receptors were identified both in insects (*Nilaparvata lugens*, *Rhodnius prolixus* [126]) and a crustacean *S. verreauxi* [49] by phylogenetic analysis. One putative *G. lateralis* TRHR clustered with *P. clarkii* and *S. verreauxi* TRHRs.

Biogenic amine, adenosine, and prostaglandin receptors

Biogenic amines are neuroactive molecules involved in synaptic transmission in the nervous system [127]. This group includes serotonin, dopamine, and octopamine. Serotonin (5-HT) increases blood glucose, while dopamine decreases blood glucose in hemolymph in several crustacean species [128]. Dopamine has a hyperglycemic effect in intact *P. clarkii* [129] and *Macrobrachium malcolmsonii* [130], but it has no effect on bilaterally eyestalk-ablated individuals. In insects, serotonergic neurons innervate the PG and control ecdysterodogenesis [131]. Autocrine signaling through the β 3-octopamine receptor is essential for PTTH and insulin-like peptide stimulation of ecdysteroidogenesis in the *Drosophila* PG [132].

Three putative receptors clustered in the biogenic amine clade. Gl_GPCRA30 and Gl_GPCRA32 clustered with serotonin receptor 1 and serotonin receptor 2, respectively. Gl_GPCRA34 clustered with the octopamine receptor clade. One adenosine (Gl_GPCRA33) and four prostaglandin (Gl-GPCRA35-A38) receptors were also identified (Fig. 4). These results are comparable with previous studies in decapods [48, 49]. The expression of Gl_GPCR32-34 did not change significantly between stages in the YOs, while Gl_GPCR31, Gl_GPCR35-36, and Gl_GPCR38 show no expression in the postmolt stage.

Immune-related GPCRs

Crustaceans have an innate immune system to protect them from pathogenic bacteria and viruses. This immune system relies on the recognition of pathogen membrane proteins using pattern recognition proteins

and defense using lectins, antimicrobial peptides, and clotting/melanization mechanisms [133]. A recent study on immune-related genes of *P. clarkii* activated by *Aeromonas hydrophila* infection identified a putative GPCR that is similar to HPR1 (protein receptor in hepatopancreas 1) [39]. Three putative receptors (Gl-GPCRA16, A17, A18, and A44) clustered with *P. clarkii* HPR1 in the phylogenetic analysis (Fig. 4). It is yet to be determined if they have a direct role in the innate immunity.

Secretin-like family

Class B1

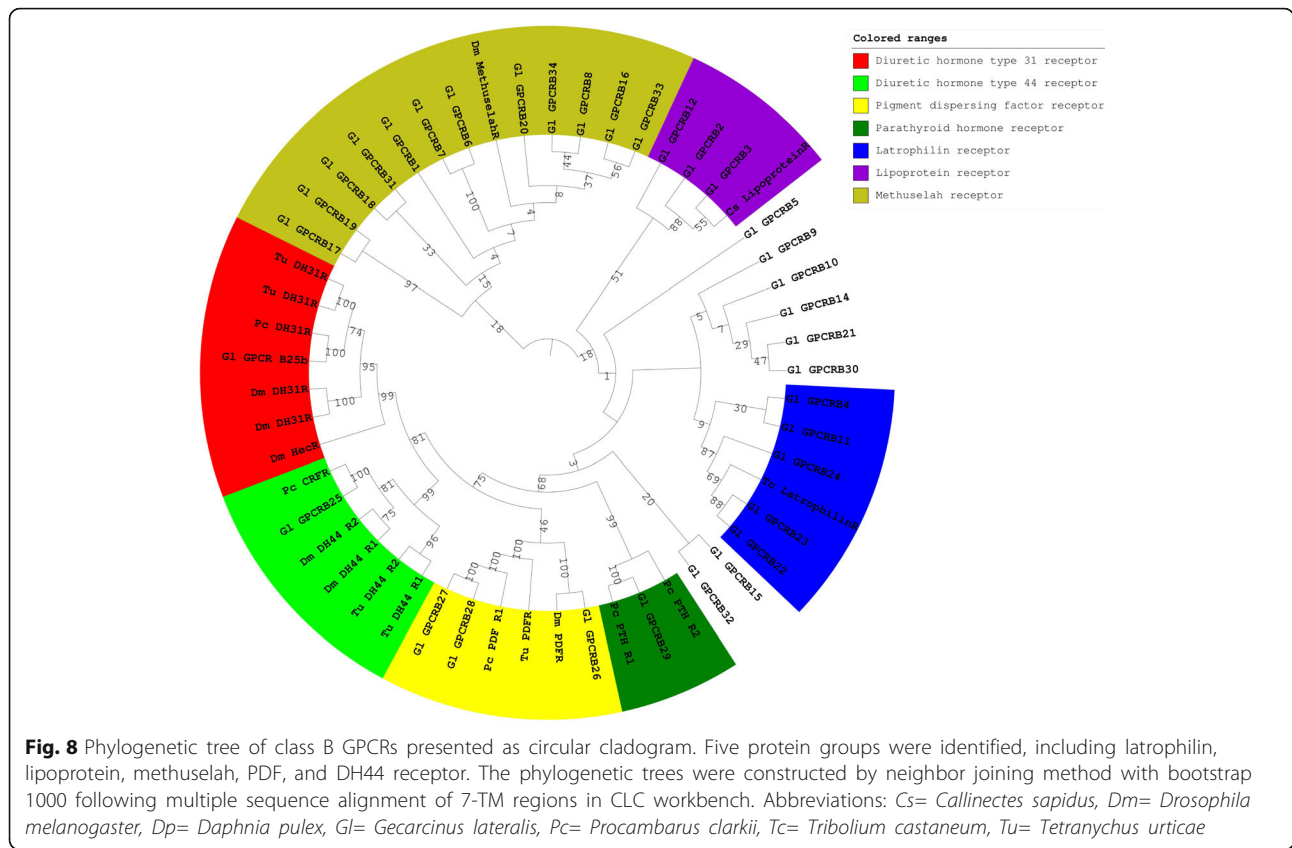
Diuretic hormones (DH) regulate water balance in arthropods [134]. There are three primary insect DHs: corticotropin-releasing factor (CRF)-related peptides, calcitonin (CT)-like peptides, and the insect kinins [135]. CRF is structurally related to mammalian corticotropin, and is called Drome-DH31 in *D. melanogaster* [136]. DH31 was recently identified in several tissues of the green shore crab *C. maenas* and its function in rhythmic coordination was established [137]. CT peptide is structurally related to mammalian calcitonin-like peptide and is called Drome-DH44 in *D. melanogaster* [138]. One putative diuretic hormone type 44 (DH44) receptor (Gl-GPCRB25) and one diuretic hormone type 31 (DH31) receptor (Gl-GPCRB25b), were identified by phylogenetic analysis (Fig. 8).

Pigment dispersing factor (PDF) is a neuropeptide produced by the XO in crustaceans, and is found across invertebrates. It has a variety of functions, including pigment dispersal in chromatophore cells [139] and regulation of locomotion behavior and egg-laying [140]. In *B. mori*, PDF binds to the BNGR-B2 receptor and stimulates the ecdysone biosynthesis in the PGs [141]. Decapod PDF receptors have been identified based on similarity with a gene from *D. melanogaster* (CG13758), including those in *P. clarkii* and *M. rosenbergii* [48, 142]. Three putative PDF/PDF-like receptors were identified in our study (Gl-GPCRB26-A28).

Parathyroid hormone (PTH) belongs to the parathyroid hormone family, which includes PTH, PTH-related peptide (PTHrP), and tuberoinfundibular peptide (PTH2). In vertebrates, the PTH family regulates the calcium titer in serum, affecting most organs [143]. One putative PTH receptor (PTHR) in the *G. lateralis* YOs transcriptome clustered with *P. clarkii* PTHR in the phylogenetic tree (Fig. 8).

Class B2

The class B2 of the secretin-like receptor family includes the calcium-independent receptor, brain-specific angiogenesis inhibitor, starry night receptor, latrophilin receptor, HE6 receptor, and homologs of the vertebrate adhesion receptor [144]. Class B2 members share a



structural similarity in the form of a long extracellular N-terminus in the ectodomain, which consists of cleavage and binding sites, such as proteolytic site (GPS) and epidermal growth factor (EGF) domains [145]. Five putative latrophilin receptors (Gl-GPCRB4, B11, B22-24) and two lipoprotein receptors (Gl-GPCRB2, B3 and B12) were identified in the *G. lateralis* YO transcriptome (Fig. 8). The expression did not change significantly between stages in the YOs.

Class B3 (Methuselah-type receptors)

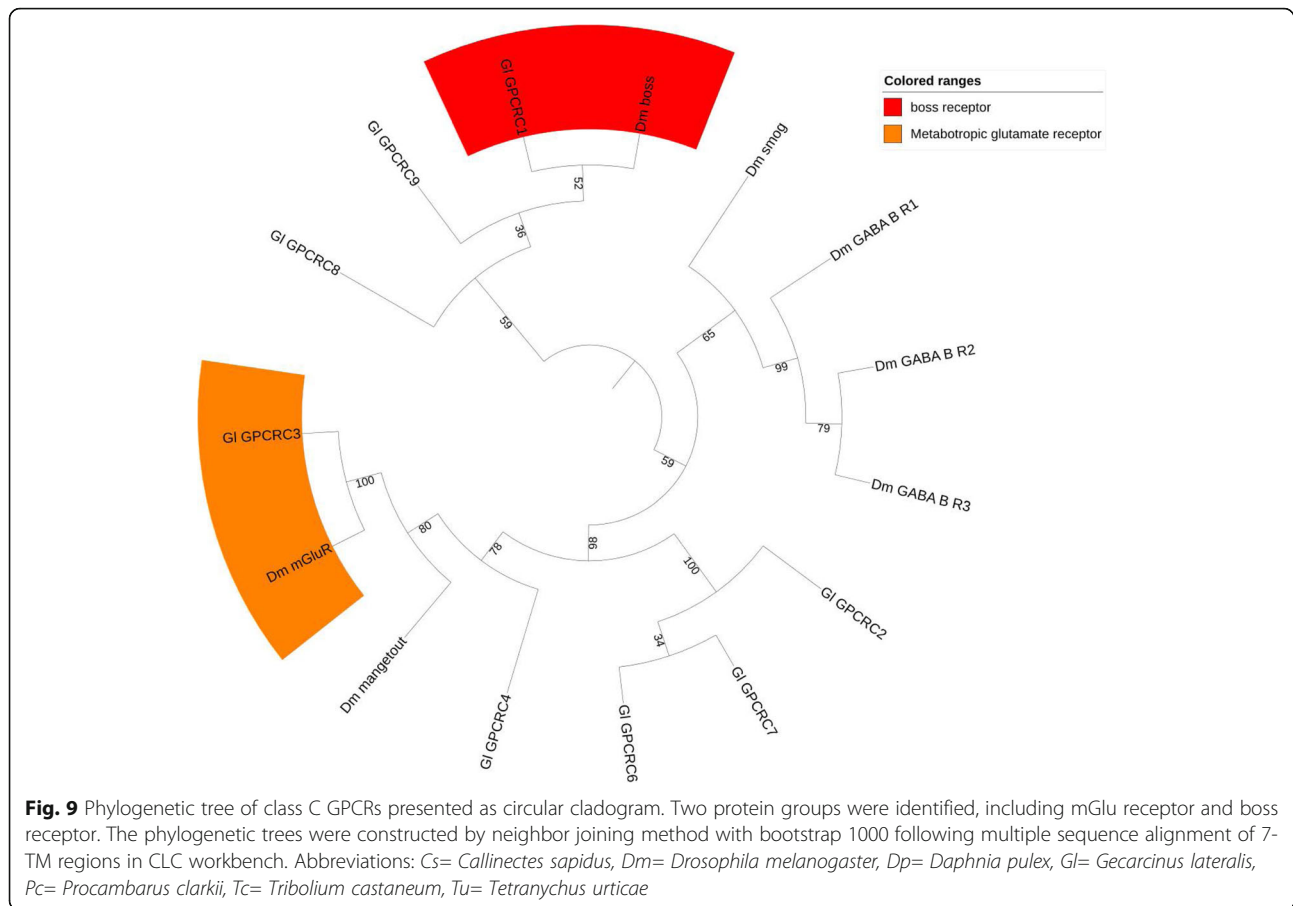
Methuselah/Methuselah-like was originally identified in *D. melanogaster* [146], and named after the methuselah gene. Most methuselah receptors contain conserved cysteine residues and glycosylation sites [147, 148]. This subfamily comprises 15 paralogs based on the similarity in their ectodomain, as well as a 7-TM domain [149]. Methuselah/Methuselah-like are found in several insects, such as *T. castaneum* and *B. mori* [150, 151]. They are involved in stress response and are associated with extended lifespan in *D. melanogaster* [146]. Mth2 in *T. castaneum* also plays a role in heat resistance and eclosion [152]. Twelve variants of the putative methuselah (Mth) receptor in *G. lateralis* clustered with *D. melanogaster* Mth receptor (Gl-GPCRB1, B6-8, B17-20, B31, and B33-34) (Fig. 8).

Class C

Class C GPCRs are classified based on their sequence phylogeny and conservation in the 7-TM domain [114]. These receptors possess a large (hundreds of residues) N-terminal extracellular domain [115]. Class C GPCRs include metabotropic glutamate (mGlu), γ -aminobutyric acid (GABA), Ca²⁺-sensing (CaS), sweet and amino acid taste, pheromone, and odorant receptors in fish, as well as several orphan receptors [114]. One putative mGlu receptor (Gl-GPCRC3) and one putative boss receptor (Gl-GPCRC1) were identified, as they clustered with *D. melanogaster* mGlu and boss receptors, respectively (Fig. 9).

Notable omissions

Several GPCRs that are conserved across arthropods were not identified in the *G. lateralis* YO transcriptome, perhaps due to high divergence of these GPCRs between crustaceans and insects, or simply because they are not expressed in the YO. These include NPF, CNMamide, and Tachykinin receptors. A Blastp search against nr database at NCBI showed Gl_GPCRA24 as an NPF receptor but with low identity (maximum of 23%) and marginal E-value (higher than 1.0E⁻⁷). With the rapid expanding availability of transcriptomes and genomes of decapod species, the spatial-temporal expression pattern and genomic context of these receptors is expected to be elucidated.



Conclusions

Bioinformatic analysis of RNA-Seq data provides a comprehensive and cost-effective method to catalog and annotate sequences and discover novel sequences (31). Using this approach, we identified contigs encoding 99 GPCRs in the YO transcriptome of *G. lateralis* (Fig. 1). The GPCRs were distributed between the three known GPCR classes. Seventy-two contigs were assigned to 17 identified GPCR groups based on ligand binding specificity and structural motifs (Fig. 4); 27 (27%) remained unidentified (Fig. 1). These data suggest that the YO has at least the potential for responding to a large number and diversity of ligands. Moreover, most were differentially expressed over the molt cycle (Figs. 1, 2, 5, and 6), suggesting that the sensitivity of the YO to these ligands is molt stage-specific. A striking example is the gene expression pattern of the repressed YO in postmolt animals. The repressed YO is characterized by low global gene expression (33). It is hypothesized that this low transcriptional activity prevents YO activation until the synthesis of the new exoskeleton is completed and the animal is in the intermolt stage (33). The majority (67%) of the GPCR contigs follow this pattern of higher levels in intermolt and premolt stages and low levels in postmolt (Fig. 1). However, 33

contigs were expressed at high or their highest levels in postmolt and one in particular, a progesterone receptor (A37), was only expressed in postmolt (Fig. 1), suggesting that these genes are involved in maintaining the repressed state and/or are involved in preparing the YO to transition to the basal state in the intermolt stage (33).

The analysis identified several GPCRs expressed in the YO that are of special interest. The proposed model for MIH signaling pathway consists of a transient cAMP/Ca²⁺-dependent triggering phase and a prolonged NO/cGMP-dependent summation phase, which inhibits YO ecdysteroid secretion between MIH pulses (10, 14, 28). The identity of the MIH receptor has remained elusive, but it is assumed that it is a GPCR, the activation of which initiates the triggering phase. Three contigs, designated CHH_1 (A9), CHH_2 (A10), and CHH_3 (A12), were identified as receptors for the CHH neuropeptide family, which includes MIH, CHH, and ILP (13, 14, 22), and therefore are candidates for the MIH receptor (Figs. 1, 7). CHH_2 was expressed at high levels in the YOs from intermolt and premolt animals (Fig. 2), making it the leading candidate. However, conclusive identification of the MIH receptor awaits a functional assay that shows MIH activation of MIH receptor candidates expressed in a

heterologous reporting system [15, 153]. The identification of FMRFamide, serotonin, and octopamine receptors raise the possibility that the YO is controlled by direct innervation. To our knowledge, innervation of the YO has yet to be demonstrated. However, the insect PG receives neuronal projections that directly control ecdysteroidogenesis [154]. FMRFamides and FMRFamide-related peptides bind to the myosuppressin receptor and inhibit ecdysteroidogenesis by lowering cAMP production [107, 108]. Serotonergic neurons innervating the *Drosophila* PG stimulate ecdysteroidogenic activity [131]. GABA and dopamine have indirect effects on the *P. americana* PG, as they have an inhibitory and excitatory effect, respectively, on the activity of the PG nerve [155]. The identification of an octopamine receptor suggests that octopamine has a direct effect on the YO. However, to our knowledge, there are no published studies determining the effects of octopamine on YO ecdysteroid secretion. A Crz receptor was identified in the YO transcriptome that may be involved with molt regulation (Fig. 5) [76]. Finally, LGR3 may provide a parallel pathway in decapods and dipterans for the coordination of molting and tissue regeneration. LGR3 in the YO may mediate the suspension of molt by LAF_{pro} that is released by secondary limb regenerates, much like Dilp8, released from damaged imaginal discs, inhibits the PG. Transcriptomics has revealed that neuro-peptide control of the YO is becoming more complex.

Methods

Transcriptome analysis

The transcriptome as well as FASTQ of *G. lateralis* YOs were obtained from a previous study [34]. In brief, the animals were collected from the Dominican Republic, shipped by air to Colorado, and maintained as described [29]. Animals were immobilized by severing the brain before removing sections of the carapace containing the YO. Animals were frozen at -20 °C. The animals were induced to molt using multiple leg autotomy (8 walking legs) and the YOs from 2-3 individuals were then collected from animals at the same molt stage. Five different molt stages including intermolt (IM - stage C₄), early premolt (EP - stage D₀), mid premolt (MP - stage D₁), late premolt (LP - stage D₂) and postmolt (PM), were collected and sequenced in triplicates (a total of 15 libraries). The transcriptomic data was screened for the longest open reading frames (ORFs) using transdecoder (version 5.0), generating a fasta file with the amino acid sequences. The amino acid sequences were scanned against the PfamA database (version 27.0) using hidden Markov models (HMMs) to identify the seven TM families. Sequences with seven TM profile were extracted (represented in Table 1). Transmembrane HMM (TMHMM) scan was then applied in parallel on the *G. lateralis* YOs ORF file to find the predicted helices. Two lists of PFAM HMM and TMHMM search outputs were cross-referenced to remove

duplicates. The seven TM sequences were then categorized into GPCR subclasses comprising rhodopsin-like (class A), secretin and adhesion (class B), metabotropic glutamate (class C) based on Pfam. The references list were created using annotated sequences from previous studies [49, 156], and the BLAST analysis of GPCR protein sequences from YO against protein sequences from the NCBI non-redundant (nr) database with *Arthropoda* organism filter, and protein sequences blasted against the FlyBase database. The reference list was entered into TMMHMM [157] using default parameters to obtain the seven TM domain of each protein sequence.

The Reads Per Kilobase of transcript, per Million mapped reads (RPKM) were obtained by mapping sequence reads against YOs database using the RNA-seq tool of CLC Genomics Workbench (CLC Bio, version 10.0) with default parameters. The RPKM was calculated as follows:

$$\text{RPKM} = \frac{\text{Total exon reads}}{\text{mapped reads(millions)} \times \text{exon length (KB)}}$$

The RPKM values of proteins relating to molt cycle were then imported into CLC for statistical analysis to determine whether there were significant differences in RPKM between different molt stages. The Empirical analysis of DGE was performed to compare RPKM between molt stages with the probability distribution less than 5% ($P < 0.05$). To avoid false positive result, the P values were then corrected using false discovery rate (FDR < 0.05).

Phylogenetic and functional study of GPCR families

To further annotate the *G. lateralis* GPCRs for phylogenetic analysis, the seven TM domains of all GPCRs were extracted and compiled with the reference list. Multiple sequence alignment was carried out using MUSCLE tools implemented in CLC Genomics Workbench (CLC Bio, version 10.0). The sequence alignment file was used to generate a phylogenetic tree with CLC Genomics Workbench (Neighbor-joining phylogeny with 1,000 bootstraps). The lists of GPCRs used for phylogeny are given in Additional file 1.

Tissue specific expression of predicted CHHR by RT-PCR

Tissues were harvested from three adult intermolt *G. lateralis* males. A competitive ELISA assay was performed to confirm that the animals were in intermolt (stage C₄) by measuring hemolymph ecdysteroid titer (19.0 ± 2.3 pg/ μl , n = 7). The samples were stored in RNA later at -20 °C until extraction. Total RNA was extracted from 10 tissues comprising claw muscle (CM), eyestalk ganglia (ESG), gill (G), heart (H), hindgut (HG), hepatopancreas (HP), midgut (MG), testis (T), thoracic ganglia (TG), and Y-organs (YOs). Trizol® Reagent (Invitrogen), was used to extract total RNA according to manufacturer's instruction and quantified using a ND-2000 (NanoDrop

Table 3 Set of primers used for RT-PCR

Identifier	Forward primers	Reverse primers
GI-GPCRA9	ggccaacagagtgtgattgcaa	gtgacgtcgggtgtagatcc
GI-GPCRA12	tgactacaactccacggagac agc	gtacacgtgcatcttgtccacctc
Beta-actin	ctgacacactccaccatgt	tcatagatggggacgggtgtg

Technologies, DE, U.S). The extractions were stored at -80 °C for RT-PCR experiment. Primers were designed using Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) and synthesized by Sigma–Aldrich company (Table 3). The stored RNA was then converted into cDNA by reverse-transcription reaction in which 1 µg RNA of each sample was used as templates, using Tetro cDNA synthesis kit (Bioline, Australia) following manufacturer's instructions. PCR was performed using Mytaq Red kit (Bioline, Australia), complemented with 1 µl of cDNA as the template, 0.8 nM of forward and reverse primers and up to 20 µl DNase-free water. Touchdown PCR was set up as follows: 94 °C for 3 min, followed by 5 cycles of 94 °C for 30 s, annealing at 62 °C for 30 s and gradually decrease 1 °C in each cycle, elongation at 72 °C for 45s. Another 35 cycles included denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The amplicons were then loaded on 1 % agarose gel stained with ethidium bromide, in TBE buffer and electrophoresed at 90 volts for 60 min and visualized under UV light.

Additional files

Additional file 1: Table S1. List of curated GPCRs using phylogenetic study and Blast search analysis. GPCRs subgroup classification was defined using Pfam database and reference list. (XLSX 159 kb)

Additional file 2: Table S2. Statistical analysis of defined GPCRs using empirical analysis of DGE and false discovery rate method. (XLSX 80 kb)

Abbreviations

20HE: 20-hydroxyecdysone; 20HE: 20-hydroxyecdysone; CHHRs: Crustacean hyperglycemic hormone receptors; CHHRs: Crustacean hyperglycemic hormone receptors; GC-II: Guanylyl cyclase II; GC-II: Guanylyl cyclase II; GPCRs: G-protein coupled receptors; ITP: Ion transport peptide; ITP: Ion transport peptide; MIH: Molt inhibiting hormone; MIH: Molt inhibiting hormone; MOIH: Mandibular organ-inhibiting hormone; MOIH: Mandibular organ-inhibiting hormone; SG: Sinus gland; SG: Sinus gland; VIH: Vitellogenesis-inhibiting hormone; VIH: Vitellogenesis-inhibiting hormone; XO: X-organ; XO: X-organ; YO: Y-organs; YO: Y-organs; GPCRs: G-protein coupled receptors

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Availability of data and materials

All data generated in this study is available as Additional files.

Authors' contributions

NT and TV designed the study and performed the analysis. NT wrote the initial draft of the manuscript and AE, DLM, and TV assisted with the writing and provided proofreading and editing of the manuscript. All authors have read and approved the manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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