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The ecdysis triggering hormone signaling in arthropods

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Abstract

Ecdysis triggering hormones (ETH) from peripheral endocrine Inka cells initiate the ecdysis sequence through action on central neurons expressing ETH receptors (ETHR) in model moth and dipteran species. We used various biochemical, molecular and blast search techniques to detect these signaling molecules in representatives of diverse arthropods. Using peptide isolation from tracheal extracts, cDNA cloning or homology search, we identified ETHs in a variety of hemimetabolous and holometabolous insects. Most insects produce two related ETHs, but only a single active peptide was isolated from the cricket and one peptide is encoded by *eth* gene of the honeybee, parasitic wasp and aphid. Immunohistochemical staining with antiserum to *Manduca* PETH revealed Inka cells on tracheal surface of diverse insects. In spite of conserved ETH sequences, comparison of the natural and ETH-induced ecdysis sequence in the honeybee and beetle revealed considerable species-specific differences in pre-ecdysis and ecdysis behaviors. DNA sequences coding for putative ETHR were deduced from available genomes of several hemimetabolous and holometabolous insects. In all examined insects the *ethr* gene encodes two subtypes of the receptor (ETHR-A and ETHR-B). Phylogenic analysis showed that these receptors fall into a family of closely related GPCRs. Here we report for the first time presence of putative ETHs and ETHRs in genomes of other arthropods, the tick (Arachnida) and water flea (Crustacea). Possible source of ETH in ticks was detected in paired cells located in all pedal segments. Our results provide further evidence of structural and functional conservancy of ETH-ETHR signaling.

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The sequence reported in this study for the first time has been deposited in the GeneBank database (Apis ETH: GeneBank accession no. **AB469047**).

Keywords

ecdysis triggering hormone; ETH receptor; Inka cell; ecdysis behavior; insect; tick; crustacean

1. INTRODUCTION

Ecdysis is a common innate behavior necessary for regular shedding of the old cuticle during development of many invertebrates including nematodes and arthropods. The ecdysis sequence is described only in few insects and is usually composed of three phases named pre-ecdysis, ecdysis and post-ecdysis [34]. Each of these behaviors is characterized by specific contractions of skeletal muscles and is under control of peptide hormones and transmitters [35]. The principal humoral factors activating the ecdysis sequence are peptides named pre-ecdysis and ecdysis triggering hormones (ETHs) produced and released by endocrine Inka cells [20,30]. The Inka cell is a major component of the epitracheal gland first described by Ikeda in the silkmoth *Bombyx mori* (1913) [12]. Paired epitracheal glands attached to tracheal trunks near each spiracle have been found in prothoracic and abdominal segments in all representatives of Lepidoptera, Diptera, and some Coleoptera and Hymenoptera. In other Holometabola, including most beetles and bees, and all examined Hemimetabola ETH is liberated during ecdysis from numerous small Inka cells dispersed throughout the tracheal system [33].

ETHs represent a large family of peptide hormones produced by insect Inka cells [35]. Two active peptides named pre-ecdysis triggering hormone (PETH) and ecdysis triggering hormone (ETH) in moths and ETH1 and ETH2 in other insects are usually encoded by *eth* gene. These two peptides have been shown to exhibit different roles and/or potency in activation of the ecdysis sequence in *Manduca sexta* [30,31] and *Drosophila melanogaster* [20,22]. The blood-borne ETHs initiate the ecdysis sequence through direct actions on the central nervous system (CNS) [30,31]. Discovery of the ETH receptor genes in *Drosophila* [13,23] and *Manduca* [14] facilitated identification of target neurons within the CNS. The known *ethr* genes encode two splicing variants (ETHR-A and ETHR-B) of G protein-coupled receptors (GPCR), which are expressed in different sets of central neurons [14,15]. All ETHR-A neurons produce inhibitory and/or excitatory neuropeptides which are released upon ETH action to regulate different phases of the ecdysis sequence [14,15]. The expression and function of ETH and ETHR has been extensively examined only in the moths *Manduca* and *Bombyx* [14,29,31,32], *Drosophila* [22,23,15] and more recently in the beetle *Tribolium castaneum* [3] and the mosquito *Aedes aegypti* [6]. Direct evidence for the presence of ETH-ETHR signaling pathway and its functional analysis is lacking in other insects. In this paper we focus on identification of ETH and ETHR as crucial components for activation of the ecdysis sequence in various insects. The presence of these components in *Ixodes* and *Daphnia* genomes and PETH-like immunoreactivity in paired segmental cells in ticks indicates that similar ecdysis signaling system may be widespread in other arthropods.

2. MATERIALS AND METHODS

2.1. Experimental animals

We used the following insects in this study: the American cockroach *Periplaneta americana* (Blattodea), the cricket *Gryllus* sp. (Ensifera, Orthoptera), the locusts *Locusta migratoria* and *Schistocerca americana* (Caelifera, Orthoptera), the honeybee *Apis mellifera* (Hymenoptera), the mealworm beetle *Tenebrio molitor* and the Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera). Larval stages of *Leptinotarsa* were collected in potato fields and reared at room temperature. Honeybee pharate pupae, pupae and pharate adults were obtained from the laboratory colony of the Institute of Molecular Biology in Bratislava (*Apis mellifera carnica*) and kept at 30°C. Other insects were obtained from our laboratory colonies and reared

at 25 °C until they developed into desired stage. Ticks *Ixodes ricinus* and *Rhipicephalus appendiculatus* were obtained from our laboratory colonies and kept after the blood meal at 25°C in glass vials.

2.2. Peptide isolation and synthesis

For isolation of ETHs, tracheal systems from ~20-30 pharate adults of *Periplaneta*, *Schistocerca* and *Gryllus* were dissected under saline (140 mmol l^{-1} NaCl; 5 mmol l^{-1} KCL; 5 mmol l^{-1} $CaCl_2$; 1 mmol l^{-1} $MgCl_2$; 4 mmol l^{-1} $NaHCO_3$; 5 mmol l^{-1} Hepes; pH 7.2), heated at 90°C for 5 min, homogenized in a tissue grinder and centrifuged at 10 000 g for 10 min. Supernatants were fractionated by reverse-phase high performance liquid chromatography (RP-HPLC) using a Microsorb-MV™ C_4 column, 4.6×250 mm (Rainin Instruments, Woburn, MA, USA) with a linear gradient of acetonitrile (3-50% in 90 min) and constant gradient of 0.1% trifluoracetic acid in water. Isolated fractions were tested by enzyme immunoassays with the rabbit antiserum against PETH (dilution 1:70,000). Conjugates were prepared by coupling synthetic PETH to horseradish peroxidase (HRP; Sigma, St. Louis, MO, USA) with glutaraldehyde [9]. PETH-HRP conjugate was used in dilution 1:4000. Enzyme immunoassays were performed as described [36]. PETH-immunoreactive peptides were sufficiently pure after a single HPLC fractionation. Sequences of these peptides were determined by liquid secondary ion mass spectrometry and MALDI-MAS-MAS. *Apis* ETH, *Tribolium* ETH1, ETH2, and *Drosophila* ETH1 were custom synthesized at the Suntory Institute for Bioorganic Research (Osaka, Japan).

2.3. Molecular biology and bioinformatics

For isolation of total RNA from *Apis*, tracheae from ~20 pharate pupae were dissected and immediately frozen on dry ice and stored at -70°C. Total RNA was prepared from these tracheae using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The first-strand cDNA was prepared from the total RNA using SMART cDNA synthesis kit (BD Biosciences, CA, USA). This cDNA and nucleotide primers designed from predicted partial genomic sequence of *Apis eth* gene were used for rapid amplification of cDNA ends. In the 3′RACE procedure we used ETH-F1 primer (5′-atgaagtgcctgccttcttc) or nested ETH-F2 primer (5′-tcttcctgaaaatcgccaag) and universal 3′SMART primer (5′-tcaacgcagagtacttttttttttt) in PCR (2 min at 94°C and 35 times: 40 sec at 94°C, 40 sec at 55°C, 2 min at 72°C). The 5′RACE was performed by PCR (2 min at 94°C and 35 times: 40 sec at 94°C, 40 sec at 60°C, 2 min at 72°C) in the presence of the adaptor 5′SMART primer (5′-gtatcaacgcagagtacgcg) and ETH-R1 primer (5′-aaagtgttgccacgaccaat) or nested ETH-R2 primer (5′-aggtcttgaaaagggctcgt). PCRs were performed using the DynaZyme™ II DNA Polymerase Kit (Finnzymes, Espoo, Finnland), the cDNA amplicons were purified with the Wizard SV Gel and the PCR Clean Up kit (Promega, Madison, WI, USA), TA ligated into pGEM^R-T Easy vector (Promega) and at least three cDNA amplicon clones were custom sequenced.

Nucleotide and protein sequences were analyzed by free bioinformatics tools on the JustBio web site (http://www.justbio.com/, Clustal W 1.8 Aligner, Translator and Primer3), by the neural network promoter prediction software (Berkeley Drosophila Genome Project web site; Reese, 2001), by the transmembrane domain prediction software (TMHMM 2.0.; Krogh et al., 2001) and by the signal peptide prediction software (SignalP 3.0; [7]). Putative and recently annotated genes for ETH and ETHR were determined by BLAST (tblastn, blastp) at the NCBI web site. The data matrix of aligned sequences (Clustal W) was analyzed using parsimony in PAUP* software version 4.0b10 (Swofford, 2002), with a TBR heuristic search of 10,000 replicates and the option 'save multiple trees' activated. Node support was measured using non-parametric bootstrapping [8] using 1,000 pseudoreplicates of 50 random additions. The phylogenic tree of ETHR was constructed by a minimum evolution method in MEGA4.

Phylogenies of each conceptually translated ETHR sequences were based on the sequences aligned using CLUSTAL W. The aligned sequences were trimmed for conserved region of transmembrane domains 1 to 7 or for the coding region for alternatively spliced exons (subtype A and B) depending on the purpose of analysis. Trees were constructed in MEGA4 software by using neighbor-joining or minimum evolution method with 500 bootstrapping.

2.4. Immunohistochemistry

To describe Inka cells in different insects we used wholemount immunofluorescent procedure described by [33]. Briefly, tracheae from pharate stages of *Locusta, Periplaneta, Apis, Tenebrio* and *Leptinotarsa* were dissected under the saline described above. Pharate nymphs and pharate adults of ticks *Ixodes ricinus* and *Rhipicephalus appendiculatus* were cut and opened along lateral sides with spring scissors under the same saline and the gut and fat body were carefully removed. Insect tracheal system and cleaned tick bodies were fixed at 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight, washed with PBS-0.3% Triton X-100 (PBST), pre-absorbed with 5% normal goat serum and incubated in the rabbit PETH antiserum for 2 days [31]. Bound antibody was visualized with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen/Molecular Probes). Tissues were then washed with PBST and mounted in glycerin containing DAPI (2 μg ml^{-1}; Sigma, St. Louis, MO, USA). Stained cells were observed under fluorescent microscope (Nikon Eclipse 600) using a triple band pass filter and photographed with a Nikon Coolpix 990 digital camera (Nikon, Tokyo, Japan).

2.5. Behavioral observations

To determine role(s) of newly identified ETHs we compared the natural and peptide-induced eclosion sequence in pharate adults of *Apis* and *Tenebrio*. Pharate adults of *Apis* execute the eclosion sequence in honeycomb cells. For observation of natural and peptide-induced behaviors one wall of each honeycomb cell was removed. Pharate adults of *Tenebrio* were placed on a filtration paper in groups of 5-10 for determination of developmental markers under stereomicroscope. Synthetic ETHs were dissolved in distilled water and injected into abdomens of these animals 3-12 hr before natural initiation of ecdysis. Distilled water was injected as a negative control. The time of initiation, duration and specific patterns of induced behavioral sequences were observed under stereomicroscope. The latency and duration of each behavioral phase were measured with a stopwatch.

3. RESULTS

3.1. Isolation and characterization of ETHs from orthopteroid insects

We used enzyme immunoassay with PETH antiserum to isolate ETHs in RP-HPLC-fractionated tracheal extracts dissected from 20-30 pharate adults of the cockroach *Periplaneta americana*, the locust *Schistocerca americana* and the cricket *Gryllus sp*. A single fractionation resulted in isolation of two ETHs in the cockroach and locust and one substance in the cricket. Their amino acid sequences were determined by liquid secondary ion mass spectrometry and MALDI-MAS-MAS (Fig. 1A). The *Schistocerca* ETH1 and ETH2 are identical with those encoded by the cDNA precursor of *Locusta* [5]. Comparison of ETHs identified in these orthopteroid insects with other isolated or deduced sequences revealed that -FFLKASKSVPRI-NH_2 sequence motif is highly conserved in all hemimetabolous insects as well as in hymenopterans, most dipterans and *Tribolium* ETH2 (Fig. 1B).

3.2. Structure of the *eth* gene and its precursor in the honeybee

We blasted the *Drosophila* ETH pre-propeptide sequence against the honeybee genome database to identify the *eth* gene. The resulting match in the genomic contig (GeneBank

accession no. **NW_001253067.1**) contains ETH sequence, followed by amidation and restriction site, and another related peptide with no restriction site in an open reading frame (ORF).

This DNA sequence served for design of the specific nucleotide primers. Subsequent RACE-PCRs resulted in the identification of 5′and 3′ cDNA fragments with 143 bp overlaps. The entire transcript (618 bp) contains an ORF starting with ATG at bp 39 and ending with TAA at bp 507. The deduced 156 amino acid pre-propeptide starts with a signal peptide (34 amino acids) followed by a single copy of ETH and associated peptide (AP). The ETH and AP sequences are separated by G-R residues indicating the presence of amidation and cleavage sites (Fig. 2).

In silico screening for the corresponding genomic sequence revealed that *Apis eth* gene is split into 6 exons located on the 13th chromosome. All introns contained canonical splicing acceptor (GT) and donor (AG) sites. The transcription start of the very short exon 1 (bp 1 to 23) was recognized by the promoter prediction software analysis (Score 0.88; 99% accuracy of prediction).

3.3. *In silico* identification of ETHs from insects and other arthropods

We compared newly identified *Apis* ETH pre-propeptide sequence with other ETH precursors in the genomic sequences of other arthropods (Table 1). *In silico* screen revealed recently annotated *eth* genes in the mosquitoes *Aedes aegypti* [6], *Anophles gambiae* [33], the beetle *Tribolium castaneum* [3] and the parasitic wasp *Nasonia vitripennis* (automated annotation), but also detected sequences encoding ETHs in the aphid *Acyrthosiphon pisum*, the lice *Pediculus humanus*, the mosquito *Culex quinquefasciatus*, the tsetse fly *Glossina morsitans*, eleven *Drosophila* species, the tick *Ixodes scapularis* and the water flea *Daphnia pulex* (Fig 1B).

All identified or anotated ETH precursors displayed moderate to high similarities with *Apis* ETH precursor sequence (Fig 3). As expected, the predicted precursor protein (GeneBank accession no. NP_001136107) from *Nasonia* encodes only a single ETH and showed the highest sequence similarity with *Apis* ETH precursor. *Apis* ETH was 88 % identical and 94% similar with *Nasonia* ETH, whereas *Apis* AP was 46 % identical and 73% similar with the *Nasonia* AP. In the aphid *Acyrthosiphon* cDNA sequence (GeneBank accession no. EX621344) we again predicted a single ETH flanked by usual processing and amidation sites (Fig 3). Other precursors containing ETHs almost identical with Drome-ETH1 and ETH2 were identified in conceptually translated hypothetical proteins from eleven *Drosophila* species (Table 1, Fig. 1). Some of the newly identified ETHs were synthesized and injected into pharate stages of *Apis* and *Tenebrio* to confirm their biological activity and determine eclosion behavior in these species (see below).

We also identified the cDNA sequence from *Daphnia* (wFleaBase accession no. WFes0080416) encoding the first putative ETH precursor in a representative of Crustacea (Fig 3). Similar to the insect ETH precursors the putative *Daphnia* ETH pre-propeptide consists of signal sequence followed immediately by two ETHs and associated peptide sequence. A fragment of genomic DNA encoding putative ETH was found in the *Ixodes* database (Table 1, Fig. 1), but the entire precursor sequence could not be determined.

3.4. The eclosion sequence in *Apis*

The following morphological markers proved useful to determine developmental stage of pharate adults before eclosion. Dark pigmentation appears in eyes (-70 h) and cuticle of the head capsule (-48 h), thorax (-30 h), legs (-24 h) and the entire pharate adult (-12 h).

Pharate adults of *Apis* executed the eclosion sequence in honeycomb cells. Natural pre-eclosion started ~2-2.5 h before eclosion onset and was characterized by antennal movements, short contractions of proboscis up and down, rhythmic opening and closing of mandibles, movements of the head capsule sideways and soft tarsal movements. The initial pre-eclosion movements were weak and occasionally interrupted by quiet periods, but after ~1-1.5 h contractions of all appendages became gradually stronger and regular, while tarsal movements develop to robust crossing and scratching of entire legs. This regular and obvious pre-eclosion behavior usually lasts for ~1 hr and is followed by a quiet period for ~20-25 min. Pharate adults then switch to eclosion behavior characterized by anteriorly directed peristaltic contractions of abdomen and grooming-like movements of legs. Peristaltic contractions result in rupture of the old cuticle along the dorsal thorax and abdomen, while strong grooming-like movements of legs result in pealing of the old pupal cuticle from the head and all appendages. Eclosing animals utilize sticky walls of honeycomb cells for support and successful shedding of the old cuticle (n=10). Pharate adults removed from cells showed the eclosion behavior for several hours but failed to shed the pupal cuticle (n=8).

Injection of *Apis* ETH (5 and 10 pmol) into pharate adults ~12 h prior to natural eclosion induced premature pre-eclosion in 9-13 min (N=12). The pre-eclosion behavior sequence lasted for 32-45 min and was followed by a quiet period for 10-15 min. Most pharate adults (9 out of 12) progressed to eclosion behavior which lasted for ~15-40 min, but only three adults completely emerged from the old pupal cuticle after prolonged eclosion movements (30-40 min). Remaining animals (n=6) shed the old cuticle only partially confirming that synthetic ETH induced the premature eclosion behavior. Animals injected with ETH (1 pmol) or saline showed no specific response. *Drosophila* ETH1 (5 and 10 pmol) induced similar behaviors in honeybee pharate adults (n=9), except the latency from injection to initiation of pre-eclosion was longer (12-17 min) and only 3 out of 9 animals progressed to eclosion behavior.

3.5. The eclosion sequence in *Tenebrio*

Pharate adults displayed following markers before natural eclosion. Yellow pigmentation of eyes, mandibles and antennae appeared 2 days and turned dark brown 1 day before expected eclosion (-48 h and -24 h, respectively). The entire cuticle turned brown ~12–16 h before eclosion and the old pupal cuticle starts to shrink between all abdominal segments ~3-3.5 h before eclosion.

Natural pre-eclosion-like movements started about 7–8 h before eclosion and were characterized by up and down twitches of the entire abdomen. This behavior begun with irregular series of 3-60 twitches; each twitch contraction lasted ~0.5 sec and intervals between twitches were ~1 sec. These series of twitches were interrupted by quiet pauses lasting from several sec to 3 min. Pre-eclosion contractions were gradually intensified and more regular, developing to continuous abdominal twitches ~3-4 h before eclosion (~50-65 twitches per min). At about -2 h contractions were even stronger and include head and thorax movements up and down towards strong abdominal twitches. At this phase animals occasionally opened and close mandibles, stretched and relax claws and all tarsi. The pre-eclosion movements result in separation of the old and new cuticle, which can be observed at the tip of adult abdomen and appendages at -2 h. Before initiation of eclosion all legs were stretched laterally. The eclosion behavior was initiated by a strong abdominal rotation followed by anteriorly directed peristaltic movements combined with 3-7 occasional robust abdominal rotations (each rotation lasts for 1-2 s). These movements and rotations resulted in rupture of the old cuticle along the dorsal body midline and shedding of the old cuticle on head and thorax followed by legs and abdomen. The eclosion behavior lasted for 16-18 min from the first abdominal rotation to adult emergence.

Premature pre-eclosion behavior was induced by injection of *Tribolium* ETH1 (5 and 10 pmol) at ~12 h or 3-4 h before natural eclosion (n=20). Injected ETH1 induced in 19 out of 20 animals pre-eclosion (up and down twitches) in 5-12 min. This behavior ceased after 40-70 min and animals did not progress to the eclosion phase. Injection of ETH1 (1 pmol) induced premature pre-eclosion in 4 out of 7 animals, whereas pharate adults injected with 0.5 pmol (n=6) or saline (n=5) showed no response.

Injection of *Tribolium* ETH2 (5 and 10 pmol) into pharate adults (n=13) at -3–4 h induced the complete eclosion sequence. After ~5 min latency all tested animals displayed pre-eclosion movements lasting for ~30 min and then switched to eclosion behavior. After 18–23 min all animals successfully eclosed. Injection of synthetic ETH2 at −12 h triggers pre-eclosion (n=10) and 8 animals progressed into prolonged eclosion behavior. Four of them eclosed in 20-27 min, but remaining four pharate adults displayed peristaltic movements and ~20 abdominal rotations for 30–60 min, but they failed to rupture and shed the old cuticle. Four out of 6 animals injected at -3–4 h with ETH2 (1 pmol) showed pre-eclosion behavior in 8-12 min. This behavior lasted for 30-55 min and ceased without progression to eclosion behavior. Animals injected with ETH2 (0.5 pmol) and saline injected controls showed no responses.

3.6. Variability of Inka cells in different insects

To confirm that newly identified ETHs in tracheal extracts of various insects are produced by Inka cells, we used immunohistochemical staining with antiserum against *Manduca* PETH recognizing C-termini of all ETHs (PRXamide; X = I, L, V, M). In all the examined insects the antiserum reacted with Inka cells on tracheal surface within one day prior to ecdysis and no other structures were stained. Numerous and small Inka cells with short cytoplasmic processes were individually scattered on the surface of broad and narrow tracheae in pharate nymphs and pharate adults of the cockroach *Periplaneta* (Figs. 4A,B). Similar Inka cells were observed in pharate nymphs and pharate adults of the locust *Locusta*, but they were more oval with very short cytoplasmic processes (Figs. 4C,D). In both species these cells may rarely occur in couples. In pharate adults of the honey bee *Apis* a large number of small oval Inka cells was located around narrow segmental tracheal branches connecting the main longitudinal trachea with each spiracle (Fig. 4E).

We also stained Inka cells in two beetle species (*Tenebrio* and *Leptinotarsa*). The former beetle was used for functional examination of *Tribolium* ETHs. Morphology and number of Inka cells was strikingly different in *Tenebrio* (closely related to *Tribolium*) compared to the unrelated Colorado potato beetle *Leptinotarsa*. In *Tenebrio* pharate adults a group of 4-6 or 1-2 middle-sized cells with broad cytoplasmic processes were located on broad tracheae near each prothoracic or each abdominal spiracle, respectively (Fig. 4F). In addition, numerous small oval immunoreactive cells were observed mostly on narrow tracheal branches (not shown). On the other hand, *Leptinotarsa* contained nine pairs of large oval and multinucleated Inka cells (Figs. 4G,H). These cells were individually and segmentally distributed in tracheal bushes attached to the prothoracic and each abdominal spiracle.

Blast search in the genome of the tick *Ixodes scapularis* led to identification of a peptide showing high sequence similarity with insect ETHs (Fig. 1). Therefore, we used the PETH antiserum to examine possible source of this peptide on the tracheal system or in the periphery of two available tick species - *Ixodes ricinus* and *Rhipicephalus appendiculatus*. No immunoreactive cells were observed on tracheal surface of pharate 2nd instar larvae and pharate adults, but oval paired cells were detected on lateral sides of each pedal segment in pharate larvae of both species (Figs. 5A,B). Since these peripheral cells with putative endocrine function have not been described before, we named them “pedal endocrine cells”. Although these cells show strong PETH-immunoreactivity, it is not clear that they represent Inka cell homologues in ticks.

3.7. *In silico* identification of ETH receptors

Important parts of the ETH signaling are two splicing variants of its receptor (ETHR-A and ETHR-B) differentially expressed in the CNS of *Drosophila* and *Manduca* [14,15]. We searched sequence databases to find related proteins in *Apis* and other insects. Gene encoding *Apis* ETHR-A composed of 421 aa was predicted by automated computational analysis (Table 2). In the corresponding genomic region (GeneBank accession no. **NW_001253200**) we deduced an alternative 3-prime exon (bps 149234-149956) encoding the subtype B specific sequence composed of 464 aa. The entire coding region of *Apis ethr* is split into 8 exons of which the last two are subtype-specific (Fig. 6).

Both subtypes of *Apis* ETHR contain seven putative transmembrane domains (Fig. 7) and share N-terminal sequences up to the 4th transmembrane region domain as described in *Drosophila* and *Manduca* ETHRs. The BLAST search revealed that *Apis* ETHRs are similar to already characterized ETHRs as well as to GPCRs recently inferred from genomic sequences (Table 2).

The first Hemimetabola ETHRs have been recently predicted in the aphid *Acyrthosiphon* (Table 2). However, the inferred aphid ETHR-A and ETHR-B sequences were incomplete and did not contain all transmembrane domains. Therefore we manually predicted the aphid ETHRs using *Drosophila* ETHR-A as a BLAST probe. We deduced one ETHR common exon, as well as subtype A and B specific exons located in a single genomic contig (**NW_001918139.1**). An additional upstream common exon was found in the contig **NW_001924517.1**. Our predicted *Acyrthosiphon* ETHRs still lack N′ and C′ termini, but contain all 7 transmembrane domains and display striking similarities with all known ETHRs (Fig. 7). Using similar BLAST based approach we predicted ETHRs in the lice *Pediculus*, the bug *Rhodnius*, the mosquito *Culex* and more interestingly, in the water flea *Daphnia* and the tick *Ixodes* (Table 2, Supplement 1). Similar to the insect ETHRs, two hypothetical *Daphnia* ETHRs contain a common N-terminal part and subtype-specific C-terminal part. On the other hand, two different genes encoding GPCRs were predicted as putative ETHR-A and ETHR-B in *Ixodes*. Screening for ETHR-related sequences in other invertebrate databases revealed two putative 7-transmembrane receptors, which have been annotated by automated computational analysis in the purple urchin (*Strongylocentrotus purpuratus*; Echinodermata) (Table 2). These putative GPCRs displayed only weak sequence similarity with conserved domains of insect ETHRs (see Fig 7 for conserved domains) and therefore were not used for subsequent cladistic analysis.

In the phylogenic analysis of entire ETHRs (transmembrane domains 1 to7) all insect ETHRs were grouped into a monophyletic clade together with the predicted GPCRs from *Daphnia* and *Ixodes*. As expected, related insect taxa were grouped together (e.g. hemimetabolous insects, moths, dipterans and hymenopterans). Note strong support for insect, *Daphnia* and *Ixodes* ETHRs when related pyrokinin and CAPA receptors are used as the outgroup. The entire clade containing all Arthropoda ETHRs is apparently related to the outgroup of *Drosophila* receptors for pyrokinins and CAPA (Fig. 8).

Phylogenic analysis of subtype-specific sequences (transmembrane domains 4 to 7) clustered ETHR-A and ETHR-B into clearly separated groups (Fig. 9). This suggests that subtype-specific sequences of all insects are closely related and distinct ETHR-A and ETHR-B exons probably originated before the diversification of insects into different families.

4. DISCUSSION

4.1. Structural similarity of ETHs and their receptors

After the first identification of ETH in *Manduca* [30], related peptides have been isolated from epitracheal gland extracts, cloned from the cDNA or deduced *in silico* from available genomes

and databases of several species belonging to diverse insect orders - Orthoptera, Coleoptera, Hymenoptera, Lepidoptera and Diptera [2,5,11,20,31,32,33,35]. So far, the locust *Locusta* is a single representative of Hemimetabola in which two almost identical sequences of ETHs have been deduced from the whole body cDNA library. Presence of the fully processed amidated peptides was confirmed in tracheal extracts using the nanoflow liquid chromatography and MSMS fragmentation analysis [5]. However, it was not clear if related peptides are produced by Inka cells of other hemimetabolous insects. Therefore, we isolated and identified ETHs from tracheal extracts of other three hemimetabolous orthopteroid insects, the cockroach *Periplaneta*, the cricket *Gryllus* sp. and the locust *Schistocerca*, and deduced closely related peptides from putative *eth* genes of the aphid *Acyrthosiphon* and the lice *Pediculus*. Recent progress in insect genomics has resulted in prediction of ETHs from several holometabolous insects including *Apis* [11], *Tribolium* [2,35], and mosquitoes *Anopheles* [33] and *Aedes* [6]. In this study we cloned *Apis* cDNA encoding ETH and predicted closely related peptides in the mosquito *Culex quinquefasciatus*, tsetse fly *Glossina palpalis* and numerous *Drosophila* spp. (Table 1). Primary structures of these peptides are highly conserved especially at their C-termini.

ETH are known to act via two splicing variants of GPCRs differentially expressed in the CNS of *Drosophila* and *Manduca* [14,15]. Orthologs of two ETHR subtypes have been recently identified and characterized in several holometabolous insects including *Tribolium*, *Bombyx* and *Aedes* [3,6,29]. In this study we inferred both subtypes of ETHR from genomic sequences of *Rhodnius*, *Acyrthosiphon*, *Pediculus*, *Apis*, *Anopheles* and *Culex*. These data demonstrate conserved structures and organization of ETHRs in all examined representatives of Hemimetabola and Holometabola.

We also identified orthologs of *eth* and *ethr* genes in other arthropods including the tick *Ixodes* (Arachnida) and water flea *Daphnia* (Crustacea) indicating that ETH-ETHR signaling is widespread in ecdysing animals. Curiously, two proteins structurally related to ETHRs have been predicted from the genome of the urchin *Strongylogaster* (Echinodermata) (Table 2). Possible role(s) of ETHR-like proteins in the non-ecdysing deuterostomian invertebrate are elusive, but suggest functional modification of these GPCRs during evolution. Functional shift of ecdysis-signaling molecules in primitive deuterostomians is also indicated by bursicon-like proteins deduced from the sea urchin genome. This heterodimeric neurohormone required for plasticization and hardening of the cuticle in insect post-ecdysis processes is missing in more advanced deuterostomians [27].

ETHs belong to a group of insect -PRXamide peptides evolutionary related to the vertebrate neuromedin U [21]. In our analysis receptors for selected *Drosophila* -PRXamide neuropeptides (CAPA and pyrokinins) constitute a clade well separated from a clade of all mentioned ETHRs. These data provide further evidence for ligand-receptor co-evolution [21] in insects and other invertebrates.

4.2. Redundant function of ETHs in most insects

Most insects produce two active ETHs encoded by a single *eth* gene [5,20,31,32,33,35]. Inka cells of *Manduca* and *Drosophila* produce shorter and longer peptide hormones which exert different behavioral responses. Shorter peptides (*Manduca* PETH and *Drosophila* ETH2) elicit only a subset of behaviors, whereas longer peptides (*Manduca* ETH and *Drosophila* ETH1) induce the entire ecdysis sequence [22,31]. Therefore, shorter peptides seem to be redundant. *Bombyx* PETH (11 aa) and ETH (23 aa) also differ considerably in length, but their potency to induce the entire ecdysis sequence in all stages is very similar [32]. Recent studies also showed that *Aedes* ETH1 and ETH2 are equally potent in inducing the ecdysis sequence of pharate larvae, pupae and adults of this mosquito species [6].

In this report we show that *Apis* cDNA encodes a single ETH located immediately after the signal sequence and predicted *eth* genes from the wasp *Nasonia* and aphid *Acyrthosiphon* also encode a single ETH. *Apis* ETH elicits the entire eclosion sequence in pharate adults of honeybees, similarly as longer ETHs elicit complete ecdysis sequences in different stages of *Manduca* and *Drosophila* [22,31]. This clearly indicates that the longer ETH can substitute role(s) of the second shorter peptide and two similar peptides do not seem to be important for binding to different receptor subtypes and regulation of distinct functions. Instead, duplication of ETHs may serve for rapid increase of their concentration in the hemolymph and faster activation of target cells.

Alternative splicing of the ETHR precursor results in production of ETHR-A and ETHR-B subtype each expressed in different networks of central neurons producing multiple neuropeptides in *Manduca* and *Drosophila* [14,15]. Ca^{2+} imaging showed that ETH1 application to the isolated CNS of *Drosophila* results in subsequent activation of these peptidergic ETHR-A networks corresponding to pre-ecdysis, ecdysis and post-ecdysis [15]. Specific roles of various neuropeptides in ETHR neurons were further supported by electrophysiological experiments in *Manduca*. For example, a mixture of kinins and diuretic hormones produced by ETHR-A neurons control pre-ecdysis I, while CCAP and myoinhibitory peptides regulate ecdysis behavior [14]. Molecular approaches in *Drosophila* and *Tenebrio* showed that targeted killing of specific peptidergic ETHR neurons or suppressed expression of certain peptides or their receptors results in specific behavioral deficits corresponding to expected functions of each peptide and its receptor [3,15,19]. Curiously, beetles with suppressed ETHR-B expression display no apparent behavioral phenotype suggesting different roles of each ETHR subtype in the ecdysis sequence [3] Since the structure and organization of all functionally characterized and putative ETHR-A and ETHR-B are conserved in all examined invertebrates (except the tick) they probably play different roles in activation or inhibition of pre-ecdysis, ecdysis and post-ecdysis processes. We can speculate that *Ixodes* ETHR-A and ETHR-B encoded by two different genes also control different functions during ecdysis processes. These observations also support previous conclusions and show that a single ETH is sufficient for activation of both receptor subtypes and subsequent execution of the entire ecdysis sequence.

4.3. Comparison of ETH action during the ecdysis sequence in various insects

The role of ETH in ecdysis sequence has been described in detail only in few insects including two moths [30,31,32], *Drosophila* [15,18,22], *Tribolium* [3] and *Aedes* [6]. The ecdysis sequence was also described in the cricket *Teleogryllus oceanicus* [4], the locust *Schistocerca gregaria* [10] and saturniid moths [28], but ETH role in these species has not been examined. Since we identified ETHs in *Apis* and *Tribolium*, we wanted to confirm their biological activity in the honeybee and *Tenebrio*, a beetle species closely related to *Tribolium*. Previously published observations and our recent data show that all studied insects display species- and stage-specific differences in larval, pupal and adult ecdysis/eclosion behavioral sequences. The pre-ecdysis and pre-eclosion motor programs appear to be the most diverse and each examined species displays a very characteristic set of movements for loosening and splitting of the old cuticle. For example; pre-eclosion behavior of *Tenebrio* is characterized by up and down twitches of the entire body, whereas pharate adults of *Apis* exhibit various movements of head and body appendages. Pre-ecdysis is followed immediately by ecdysis, but adult eclosion is delayed in some species by a quiescent period, e.g. in *Drosophila* [18] and *Bombyx* [32]. Pharate adults of *Apis* also exhibit this quiescent period for ~15-20 min while *Tenebrio* switch from pre-eclosion to eclosion behavior immediately.

Behavioral sequences of most studied animals show some common features. Ecdysis and eclosion behaviors involve in most insects peristaltic abdominal movements necessary for

complete shedding of the old cuticle. However, abdominal rotations or grooming-like leg movements have been observed along peristaltic eclosion contractions in *Apis* and *Tenebrio*, respectively. This suggests that ETHs act via different networks of central neurons in various insects and these networks may be modified during metamorphosis to control shedding of new adult organs.

In summary, our data provide evidence that Inka cells, ETHs and their receptors occur in a wide range of insects. In addition, we have shown here for the first time that peptides closely related to ETHs and ETHRs occur in representatives of ticks and crustaceans. Although there is remarkable variability in number and morphology of Inka cells, as well as in behavioral displays during the ecdysis/eclosion sequences, our data indicate that the conserved ETH-ETHR signaling may be widespread in arthropods and perhaps in other invertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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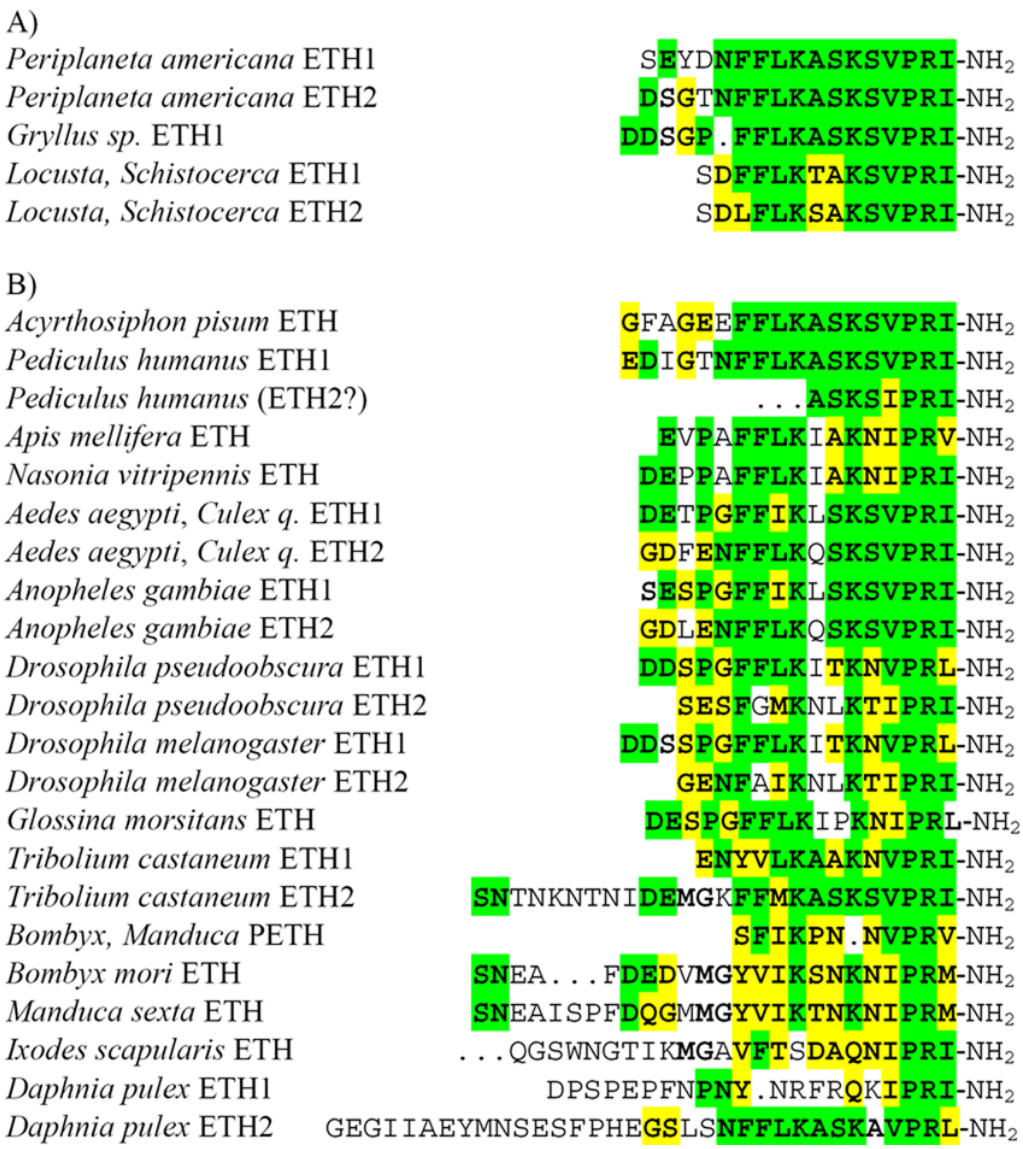

Fig. 1.

Comparison of identified and characterized ETHs with those predicted from cDNA clones or genomes of diverse insects, the water flea and the tick.

A) Biochemically identified amino acid sequences of ETHs from orthopteroid insects.

B) Predicted ETH sequences from cDNA clones or genomes of the hemi- and holometabolous insects, the water flea and the tick show high homology with identified and functionally characterized ETHs from the beetle *T. castaneum*, the moths *B. mori* and *M. sexta*, the mosquito *A. aegypti* and the fruit fly *D. melanogaster*. Sequence identities and similarities are indicated by bold letters highlighted by green and yellow, respectively.

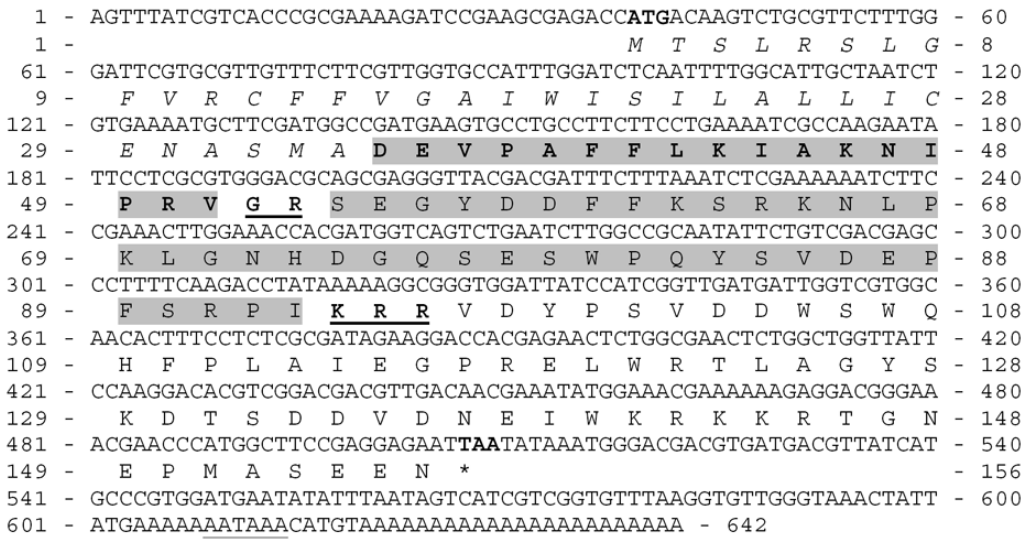

Fig. 2.

The entire cDNA encoding ETH in *Apis mellifera*. The predicted signal peptide is in italics, a single copy of ETH is depicted in bold and highlighted grey and putative non-amidated associated peptide is highlighted grey. The amidation and processing sites between peptides are bold and underlined. The putative translation start and stop codons are bold and polyadenylation signal is underlined in the DNA sequence.

Fig. 3.

Identified or predicted ETH precursors in insects and crustacean. Predicted signal peptides are italicized, ETH sequences are bold and highlighted grey, C-terminal amidation sites are underlined and consensual proteolytic cleavage sites flanking active peptides are bold.

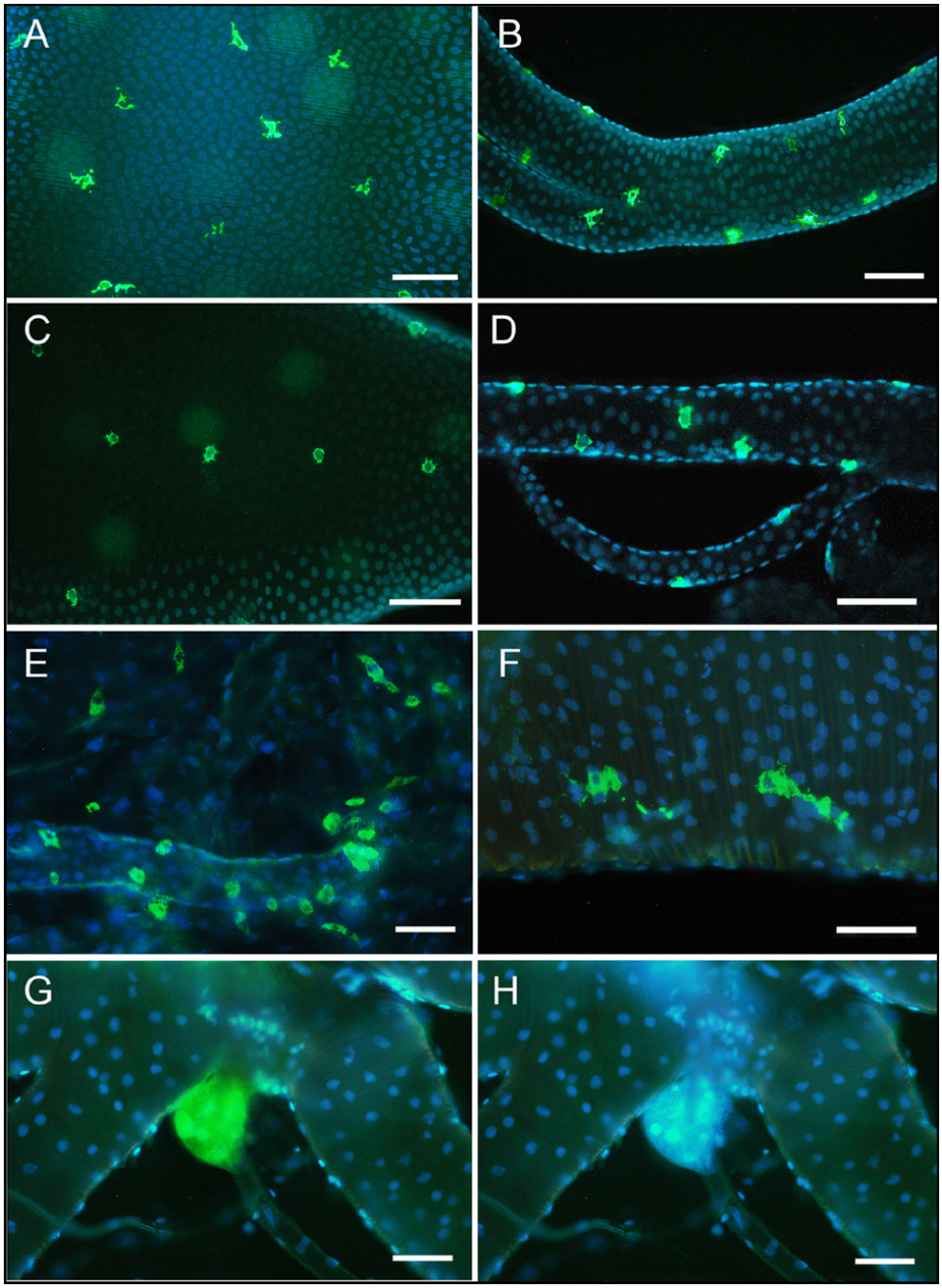

Fig. 4.

PETH-immunoreactive Inka cells in pharate stages of diverse insect species.

(A, B) Numerous small Inka cells (green color) scattered on surface of the broad segmental trachea (A) and narrow tracheal branch (B) in pharate nymph of the cockroach *Periplaneta.* (C, D) Similar cells are located on tracheal surface in pharate nymph of the locust *Locusta*. (E) A large number of small Inka cells is concentrated on narrow tracheal branch near each spiracle of pharate adult of the honeybee, *Apis*. (F) Paired Inka cells with prominent cytoplasmic processes in pharate adult of the beetle *Tenebrio*. (G, H) The beetle *Leptinotarsa* contains single large oval Inka cells attached to tracheal bush near each spiracle. DAPI nuclear staining (blue color) revealed multiple nuclei in these cells (H). Scale bars, 100 μm.

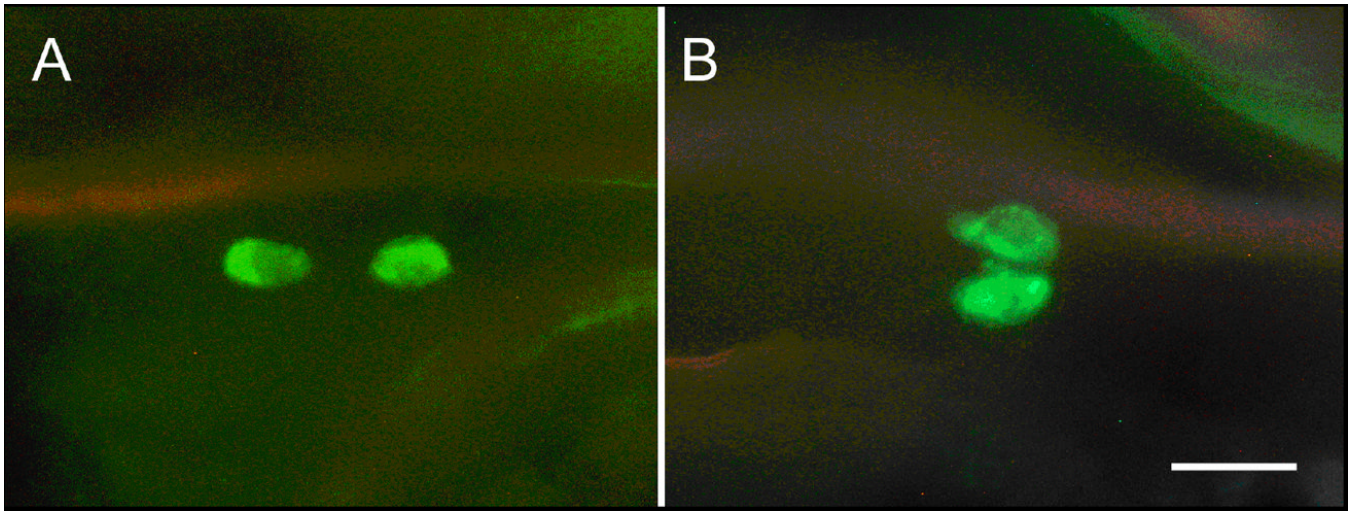

Fig. 5.
PETH-immunoreactive peripheral cells in pharate nymphs of the ticks *Ixodes ricinus* (A) and *Rhipicephalus appendiculatus* (B). These paired cells are located on lateral sides of each pedal segment. Scale bar, 5 μm.

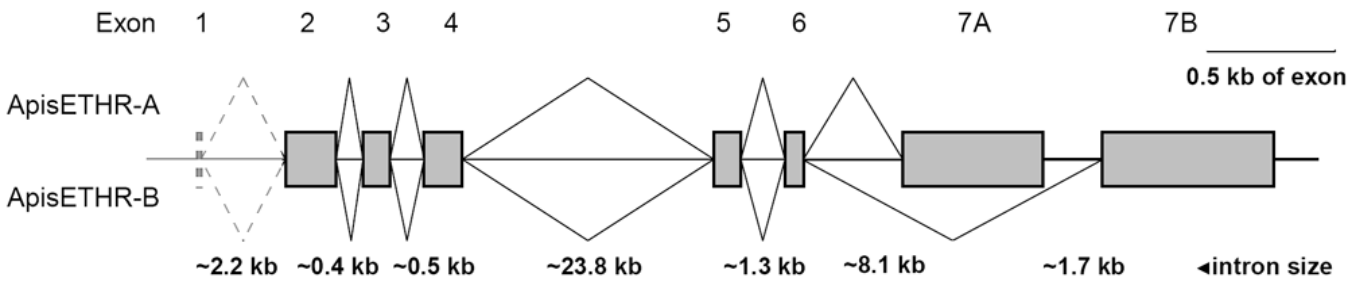

Fig. 6.
Organization of the predicted *Apis-ethr*. The putative *Apis* ETHR encodes two receptor subtypes produced by alternative splicing of mutually exclusive exons 7A and 7B.

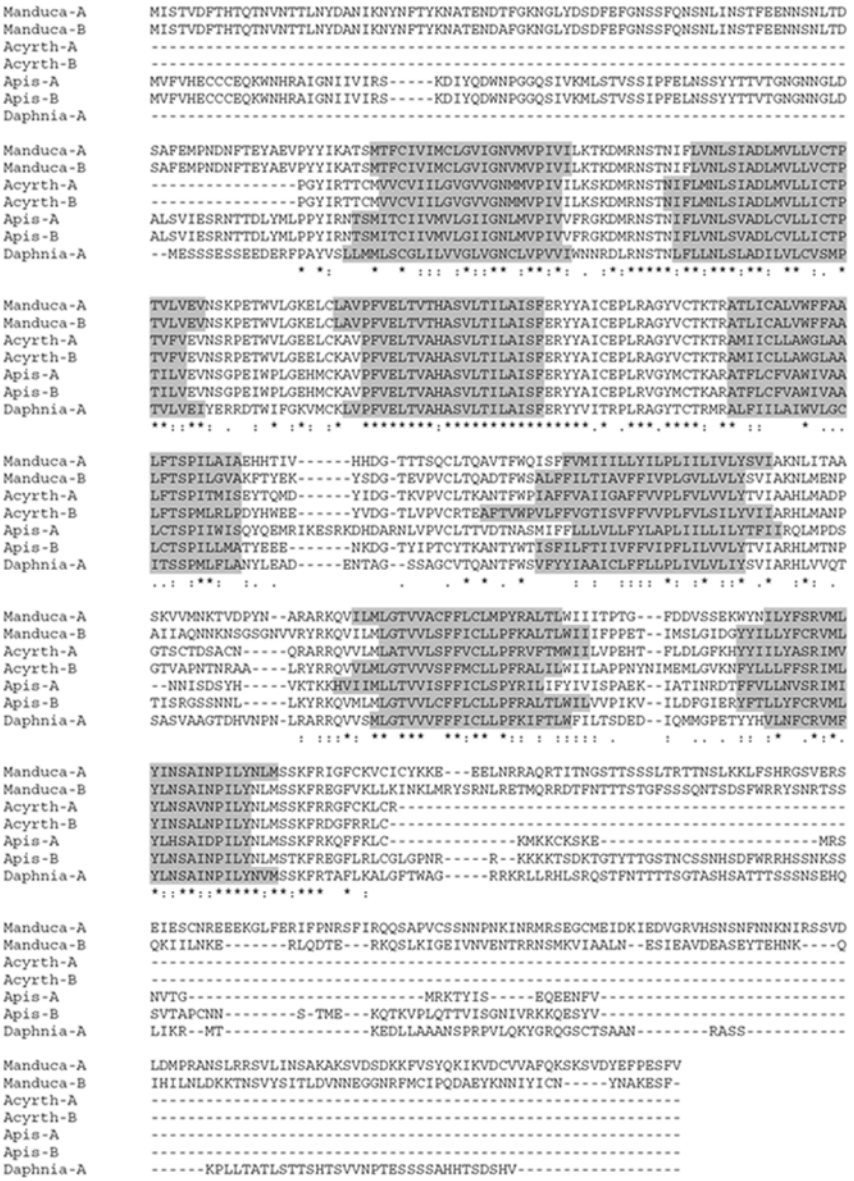

Fig. 7.

Amino acid sequence alignment (CLUSTAL W 1.8) of the predicted honeybee *Apis* ETHR-A and -B, the pea aphid *Acyrthosiphon* ETHR-A and-B, the water flea *Daphnia* ETHR-A and the characterized moth *Manduca* ETHR-A and -B. Sequences of *Acyrthosiphon* ETHRs are incomplete. Seven predicted transmembrane domains are grey highlighted. Sequence identities are indicated by asterisks, gaps by dashes and similarities are denoted by periods and colons.

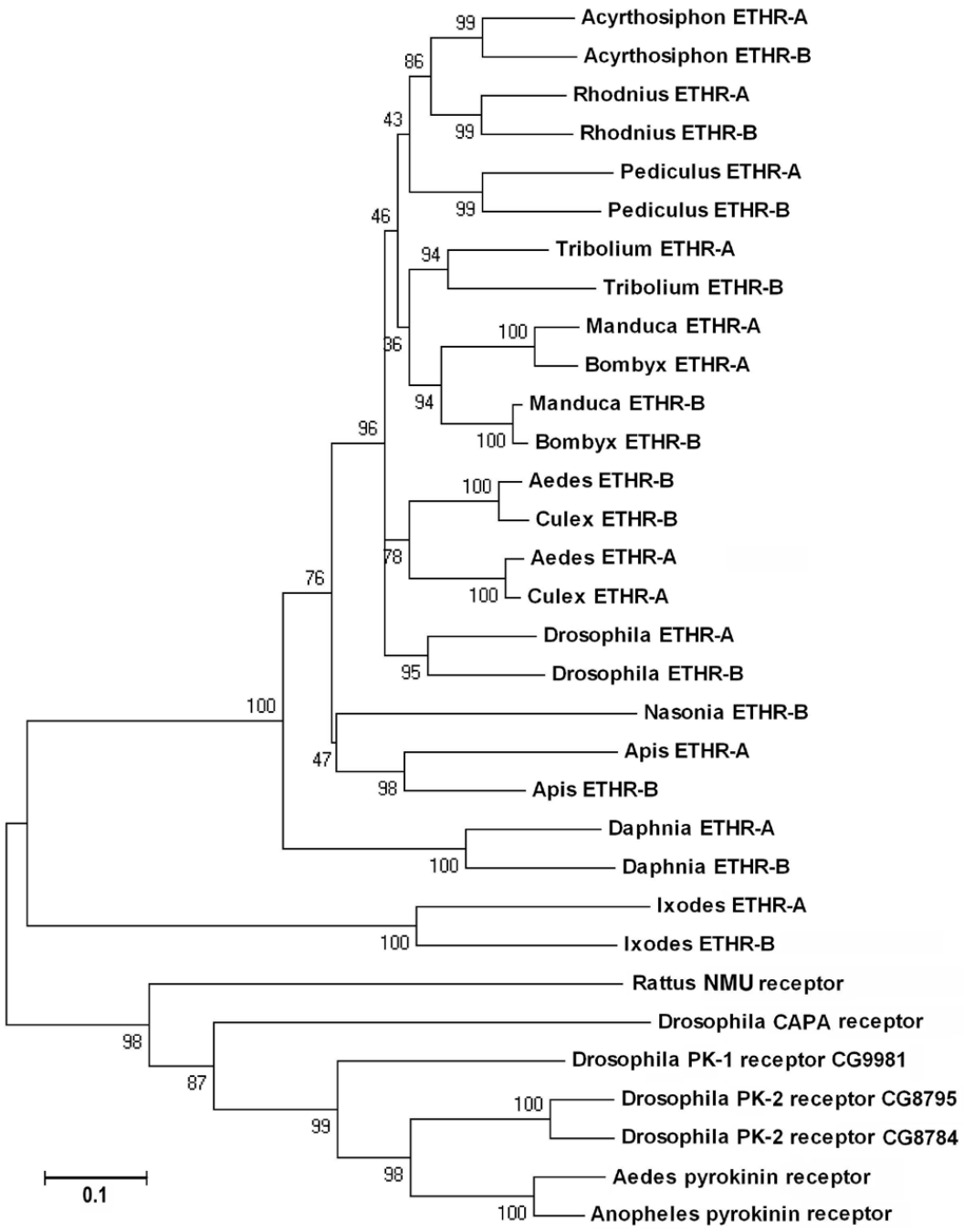

Fig. 8.

The phylogenic relationship of ETHRs and related GPCRs using all transmembrane domains 1 to 7. Note that all insect ETHRs are grouped with predicted *Daphnia* and *Ixodes* ETHRs when related pyrokinin receptors are used as the outgroup. The tree was constructed by a minimum neighbor-joining method (Gaps/Missing Data-Parwise deletion) in MEGA4. Numbers are for percent supports in 500 bootstrapping. PK-1, pyrokinin-1; PK-2, pyrokinin-2; NMU, neuromedin U-25.

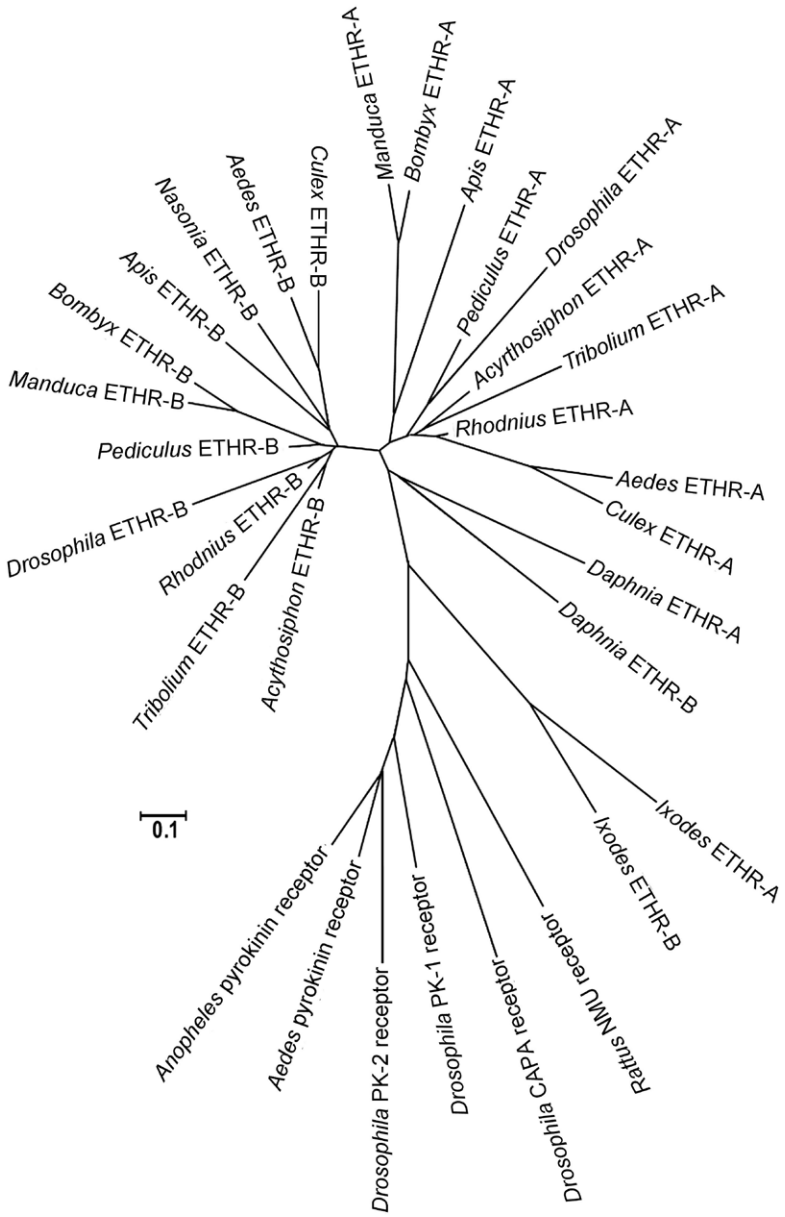

Fig. 9.

The phylogenic relationship of the alternatively spliced exons encoding a subtype A and B regions of the ETHR and related GPCRs (transmembrane domains 4 to 7).

ETHR-A- and ETHR-B-specific sequences form two apparently separate groups in all insects. Note that branches for alternatively spliced exons (A and B) are dated before the diversification of insect taxa. The tree was constructed by a minimum evolution method (Gaps/Missing Data-Parwise deletion) in MEGA4.

Table 1

List of identifed or predicted ETHs and their precursors in arthropods

Species	Evidence	GeneBank accession no. *DNA*/Protein	Reference
Periplaneta americana	Peptide		This study
Locusta migratoria	Peptide, mRNA	***CO822155***	[5]
Schistocerca americana	Peptide		This study
Gryllus sp.	Peptide		This study
Acyrthosiphon pisum	mRNA	***EX621344***	This study
Pediculus humanus	Predicted gDNA	match in gb|***AAZO01005618.1***	This study
Aedes aegypti	Peptide, mRNA	**ABI93272**	[6]
Anopheles gambiae	mRNA		[24,33]
Culex quinquefasciatus	Predicted gDNA	match in gb|***AAWU01006874.1***	This study
Drosophila ananassae	Predicted gDNA	**XP_001960394**	This study
D. erecta	Predicted gDNA	**XP_001976675**	This study
D. grimshawi	Predicted gDNA	**XP_001986745**	This study
D. melanogaster	mRNA	**AAF14282**	[20,21]
D. mojavensis	Predicted gDNA	**XP_002004620**	This study
D. persimilis	Predicted gDNA	**XP_002018415**	This study
D. pseudobscura	Predicted gDNA	**XP_001361212**	This study
D. sechellia	Predicted gDNA	**XP_002043132**	This study
D. simulans	Predicted gDNA	**XP_002083035**	This study
D. virilis	Predicted gDNA	**XP_002048870**	This study
D. willistoni	Predicted gDNA	**XP_002062955**	This study
D. yakuba	Predicted gDNA	**XP_002092896**	This study
Glossina morsitans	Predicted gDNA	match in *Tfly_23-t4364c16.p1k*[a]	This study
Apis mellifera	mRNA	**BAG80963**	This study
Nasonia vitripennis	mRNA	**NP_001136107**	Comp annotation
Tribolium castaneum	Peptide, mRNA		[2,3,17]
Manduca sexta	Peptide, mRNA	**AAD45613**	[30,31]
Bombyx mori	Peptide, mRNA		[1,31,32]
Daphnia pulex	mRNA	*WFes0080416*[b]	This study
Ixodes scapularis	Predicted gDNA	match in **gnl|ti|1309379169**	This study

Abbreviations: Comp annotation - peptide sequence annotated by automated computational genome analysis; mRNA - identified whole or partial gene transcript; Peptide - amino acid sequence of biochemically identified peptides; Predicted gDNA – whole or partial prepro-peptide predicted from the genomic DNA.

[a] These sequence data were obtained from the Wellcome Trust Sanger Institute and can be obtained by the *Glossina morsitans* Blast server.

[b] These sequence data were produced by the US Department of Energy Joint Genome Institute in collaboration with the *Daphnia* Genomics Consortium and are available in the wFleaBase web site. Other sequences can be retrieved via Entrez, The Life Science Search Engine at NCBI web site.

Table 2

Identified and predicted GPCRs in arthropods showing homology with characterized *Drosophila* ETHR-A. Sequence *Drosophila* ETHR-A was used for prediction of closely related genomic DNA (gDNA) encoding amino acid sequences in putative exons of common N-terminal and/or subtype specific sequences.

Protein and species	GeneBank accession no.	Score (bits)	Expect Value	Citation
ETHR-A *Acyrthosiphon pisum*	**XP_001944756**[a]	403	5e-117	This study
ETHR-B *Acyrthosiphon pisum*	**XP_001944816**[a]	416	8e-121	This study
ETHR-A *Pediculus humanus*	Predicted gDNA	319	7e-92	This study
ETHR-B *Pediculus humanus*	**EEB17194**	362	1e-104	This study
ETHR-A *Rhodnius prolixus*	Predicted gDNA	434	2e-126	This study
ETHR-B *Rhodnius prolixus*	Predicted gDNA	410	2e-119	This study
ETHR-A *Aedes aegypti*	**ABI93273**	471	3e-137	[6]
ETHR-B *Aedes aegypti*	**ABI93274**	449	1e-130	[6]
ETHR-A *Anopheles gambiae*	Predicted gDNA			This study
ETHR-B *Anopheles gambiae*	Predicted gDNA			This study
ETHR-A *Culex quinquefasciatus*	**XP_001845191**[a]	458	2e-133	This study
ETHR-B *Culex quinquefasciatus*	**XP_001845190**[a]	437	3e-127	This study
ETHR-A *Drosophila melanogaster*	**NP_650960**		100	[13,15,23]
ETHR-B *Drosophila melanogaster*	**NP_996255**	457	4e-133	[13,15,23]
ETHR-A *Nasonia vitripennis*	Not detected			
ETHR-B *Nasonia vitripennis*	**XP_001606566**	343	7e-99	Automated annotation
ETHR-A *Apis mellifera*	**XP_001120367**	309	1e-88	Automated annotation
ETHR-B *Apis mellifera*	Predicted gDNA	414	3e-120	This study
ETHR-A *Tribolium castaneum*	**ABN79653**	413	7e-120	[3]
ETHR-B *Tribolium castaneum*	**ABN79654**	416	6e-121	[3]
ETHR-A *Manduca sexta*	**AAX19163**	398	3e-115	[14]
ETHR-B *Manduca sexta*	**AAX19164**	440	5e-128	[14]
ETHR-A *Bombyx mori*	**AB330426**	387	5e-112	[29]
ETHR-B *Bombyx mori*	**AB330427**	427	4e-124	[29]
ETHR-A *Daphnia pulex*	NCBI_GNO_62774[b]	315	2e-90	Automated annotation
ETHR-B *Daphnia pulex*	Predicted gDNA	285	2e-81	This study
ETHR-A *Ixodes scapularis*	**XP_002399688**	100	5e-26	This study
ETHR-B *Ixodes scapularis*	**XP_002434887**	83.6; 50.8	1e-20; 1e-10	This study
ETHR-A *Strongylocentrotus purpuratus*	**XP_786973**	129	2e-34	Automated annotation
ETHR-B *Strongylocentrotus purpuratus*	**XP_781583**	137; 89	1e-36; 2e-22	Automated annotation

[a] Referenced hypothetical proteins predicted by automated computational analysis contain only part of ETHR related sequence. The functional ETHRs containing all transmembrane domains were predicted manually in this study.

[b] *Daphnia* ETHRs are based on sequence data produced by the US Department of Energy Joint Genome Institute in collaboration with the *Daphnia* Genomics Consortium (wFleaBase web site). Other sequences can be retrieved via Entrez, The Life Science Search Engine at NCBI web site.