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Research paper

Ecdysis triggering hormone modulates molt behaviour in the redclaw crayfish *Cherax quadricarinatus*, providing a mechanistic evidence for conserved function in molt regulation across Pancrustacea

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ABSTRACT

Molting enables growth and development across ecdysozoa. The molting process is strictly controlled by hormones – ecdysteroids. Ecdysteroidogenesis occurs in the prothoracic glands and stimulated by prothoracicotropic hormone in insects, while it ensues in the Y-organ and regulated by the molt inhibiting hormone in crustaceans. A peak in ecdysteroids in the hemolymph induces a cascade of multiple neuropeptides including Ecdysis Triggering Hormone (ETH) and Corazonin. The role of ETH is well defined in controlling the molt process in insects, but it is yet to be defined in crustaceans. In this study, we investigated the behavioral response of intermolt crayfish to ETH and Corazonin injections as well as the impact of ETH on the molt period using *in vivo* assays. Injection of Corazonin and ETH resulted in a clear and immediate eye twitching response to these two neuropeptides. The Corazonin injection induced eye twitching in slow and asynchronous manner, while ETH injection caused eye twitching in a relatively fast and synchronous way. A single injection of ETH to crayfish resulted in a remarkable prolong molt period, at twice the normal molting cycle, suggesting that ETH plays a key role in controlling the molt cycle in decapod crustaceans. Given the key significance of ETH in molt regulation and its plausible application in pest control, we characterized ETH across the pancrustacean orders. Bioinformatic analysis shows the mature ETH sequence is identical in all studied decapod species. ETH can be classified into specific groups based on the associated motif in each insect order and shows an insect motif –KxxPRx to be conserved in crustaceans.

1. Introduction

Pancrustacea (crustaceans and hexapods) is the most species-rich clade in the Arthropoda phylum, comprising the majority of animal species on earth (Price et al., 2011). Arthropods comprise four major extant groups classified by morphological and ecological characteristics including Hexapoda (e.g. flies, beetles, and grasshoppers), Myriapoda (millipedes and centipedes), the Crustacea (e.g. crabs, shrimp, copepods, and lobsters), and Chelicerata (scorpions, spiders, mites, and ticks). Up until recently, Myriapoda was classified as closely related to Hexapoda based on appendages morphology and terrestrial habitat. This classification was based on shared features like the non-branched appendages (uniramous), which set Myriapoda and Hexapoda apart from the biramous crustaceans (Grimaldi and Engel, 2005). According to the more recently adopted Pancrustacea theory (Hexapoda plus Crustacea), insects are more closely related to Crustacea than Myriapoda (Boore et al., 1998; Friedrich and Tautz, 1995), supported by recent phylogenomic analysis of nuclear protein-coding sequences (Regier et al., 2010), transcriptomic analysis (von Reumont et al., 2012), molecular and morphological phylogenies (Oakley et al., 2013), and molecular and physiology analysis (Tamone and Harrison, 2015). Indeed, many molecular mechanisms were found to be quite similar between crustaceans and insects, including the repertoire of neuropeptides found in both clades, which is highly conserved with a few exceptions (Veenstra, 2016b).

A well-studied process in arthropods which articulates well the conserved neuroendocrine mechanisms, and the exceptions that set hexapods and crustaceans apart, is the process through which arthropods grow. Arthropods possess an exoskeleton which enables limb movement and provides protection against microorganisms (Andersen, 2009), although the rigidity of the exoskeleton also restricts the animal from expanding in size. Hence, in order to grow and develop,

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arthropods must shed their outer exoskeleton, meaning they must form a new one underneath prior to that and once shed, in a process known as molting, they need to harden the new exoskeleton. Molting (also known as ecdysis) is controlled by ecdysteroids – a class of arthropod steroid hormones. Ecdysteroid biosynthesis occurs mainly in the prothoracic glands (PGs) in insects and a pair of molting glands, the Y-organs (YOs), in decapod crustaceans (Covi et al., 2012). The YO regulatory pathways that affect the production and secretion of ecdysteroids are considered parallel to those in the insect PGs with a few key differences including the neuropeptidergic control of the molt process. A brain-derived prothoracicotropic hormone (PTTH) stimulates PGs ecdysteroidogenesis in insects (Gilbert et al., 2002; Kawakami et al., 1990), while molt-inhibiting hormone (MIH) inhibits YO ecdysteroidogenesis in crustaceans via a yet to be identified G protein coupled receptor (GPCR) (Chang and Mykles, 2011; Covi et al., 2009; Mykles, 2011). In the blackback land crab (*Gecarcinus lateralis*), the majority of GPCRs identified in the YOs were found to be up-regulated in the premolt period and down-regulated in the postmolt period. The overall expression of the GPCRs, including those that putatively respond to MIH, were remarkably lower than that of the Corazonin receptors (CrzRs) (Tran et al., 2019). This elevated expression of CrzR in the YOs suggests a role for Corazonin (Crz) in crustacean molting, which could provide another parallel to the mechanism in insects. Crz is a non-apeptide paralog of the gonadotropin-releasing hormone (GnRH) found across many arthropod lineages. Evolutionary study on GnRH receptor and its ligand showed a common evolutionary origin with adipokinetic hormone (AKH), AKH/Crz-related peptide (ACP), and Crz in arthropods. (Hauser and Grimmelikhuijzen, 2014; Zandawala et al., 2018). In some insect lineages, Crz regulates some aspects of molting. For example, in the tobacco hornworm (*Manduca sexta*), the production of ecdysis triggering hormone (ETH) is triggered by Crz (Kim et al., 2004), while in *Tribolium castaneum* Crz is entirely absent (Li et al., 2008). In crustaceans, Crz functions in the pigmentation of the green shore crab *Carcinus maenas* (Alexander et al., 2017), a process which is a manifestation of the molt. In *C. maenas*, elevated expression of CrzRs in the YOs was documented (Alexander et al., 2017), in keeping with the observation made in the closely related species *G. lateralis* (Tran et al., 2019), suggesting a potential conserved role of Crz in molt regulation in decapod crustaceans.

The increased titer of ecdysteroids in the hemolymph at the end of intermolt and begin of premolt stages is followed by a reduced level, due to the uptake of these ecdysteroids by target tissues, where they regulate expression of a vast array of molt-related genes by binding nuclear receptors (Hyde et al., 2019), leading to molting. Unlike Chelicerates, where ponasterone A is the main active ecdysteroid, in the Pancrustacea it is primarily 20-hydroxyecdysone (20E), synthesized from cholesterol by Cytochrome P450 (CYP450) enzymes encoded by a group named the Halloween gene family. While CYP450s are notoriously known for rapidly evolving, the Halloween gene family is a highly-conserved exception across arthropods with the recent addition of the shed genes in decapods (Cummins and Ventura, 2019; Ventura et al., 2017a, 2017b), further supporting the similarity in molecular mechanisms between insects and crustaceans. Prior to ecdysis, many preparatory processes occur sequentially in arthropods. Increasing ecdysteroid titers in the hemolymph stimulate the synthesis of the new cuticle underneath the old cuticle. Cuticle synthesis is preceded by apolysis – the process of separating the old exoskeleton from the underlying epidermal cell layer. Apolysis is triggered by the response of epidermal cells to the ecdysis cassette of hormones, including Crz, ETH, and additional neurohormones (kinin, FMRFamides (Tv1–3), eclosion hormone (EH), or CCAP, MIPs, and bursicon) that surge following the secretion of ETH (Kim et al., 2006b). During apolysis, molting fluid, filled with enzymes required to separate and absorb the old cuticle, fills the space between the old exoskeleton and epidermal layer. This elaborate chain of events, accompanied by vast differential expressions and cellular modifications surging across the animal, is followed by the ecdysis event itself – when the animal detaches and emerges from the old exoskeleton, expands and then hardens the new exoskeleton. The ecdysis process also involves certain behavioral patterns (Chang and Mykles, 2011).

ETH was previously shown to activate and execute the ecdysis behavior sequence in insects (Zitnan et al., 2007). In *Bactrocera dorsalis*, ETH functions by regulating ecdysis in larvae through binding to ETH receptor A (ETHR-A) (Shi et al., 2017), but it also plays an essential role in reproduction regulation through binding to ETH receptor B (ETHR-B) (Shi et al., 2019). In *D. melanogaster, B. dorsalis, and M. sexta,* a single *eth* gene was found, encoding for 2 mature ETHs following post-translational cleavage (Park et al., 1999; Shi et al., 2017; Zitnan et al., 1999). More recently, a single *eth* gene was identified in several decapod crustaceans (Oliphant et al., 2018). ETHs are produced and released from the endocrine Inka cells of the epitracheal glands in insects (Kingan and Adams, 2000). It is not yet clear whether decapod crustacean ETH is produced primarily by gill epithelia, which are homologous to the epitracheal system in insects. In *C. maenas,* ETH is expressed mostly in the central nervous system (CNS), although the expression in gills was not assessed (Oliphant et al., 2018). It is important to note that there is no solid evidence for conserved functionality of ETH in crustaceans to date, although the elevated ETH expression towards the postmolt in *C. maenas* (Oliphant et al., 2018) is consistent with ETH regulating post-ecdysis behavior.

Successful molting requires the coordinated expression of multiple genes which trigger an orchestrated secretion of many hormone molecules in a cyclic way during molting. Prior to the commencement of the ecdysis behavior sequence, the rising of ecdysteroid titer in the hemolymph induces the expression of the ecdysone receptor (EcR) and activates ETH production and secretion. It also increases the sensitivity to ETH by promoting expression of the ETH receptor (ETHR) and EH in the CNS (Kim et al., 2006a; Kim et al., 2006b; Kingan et al., 1997; Zitnan et al., 1999). In insects, after ETH is released from the Inka cells, ecdysteroid titer drops to a baseline level which induces the secretion of EH from the CNS. EH acts on the epitracheal endocrine system as an ETH releasing factor, and ETH, in turn, facilitates EH secretion. In insects, only one EH encoding gene was identified, while in several decapod crustaceans there are two EH encoding genes (Oliphant et al., 2018). Both EH and ETH form a positive feedback loop which leads to a high titer of both neuropeptides in the hemolymph (Kingan et al., 1997; Zitnan et al., 2002). Elevating the level of ETH and EH in the hemolymph provides a clear ecdysis signal by directly acting on the CNS to trigger the neuropeptidergic signaling cascade including kinin, FMRFa from neurosecretory cells (Tv1-3), crustacean cardioactive peptide, and bursicon (Kim et al., 2006b). These peptides, together with ETH and EH activate the ecdysis motor program which drives the whole process of ecdysis behavior (pre-ecdysis I, pre-ecdysis II, ecdysis, and post-ecdysis) (Gammie and Truman, 1999; Kim et al., 2006a; Kim et al., 2006b).

ETHs are a conserved family of peptide hormones in both hemimetabolous and holometabolous insects. Most insects produce two ETH neuropeptides and an associated ETH (A-ETH, the remaining protein sequence after cleavage of the signal peptide and mature ETH), all three components encoded by the single *eth* gene and are formed following a series of cleavages. Some insects such as honeybees, parasitic wasps and aphids produce only a single ETH (Roller et al., 2010). In Lepidoptera, most species produce two ETH peptides named pre-ecdysis triggering hormone (PETH) and ETH. PETH and ETH were first identified in *M. sexta* (Zitnan et al., 1999), and later in the silk moth *Bombyx mori* (Zitnan, 2003; Zitnan et al., 2002), and the beet armyworm *Spodoptera exigua* (Llopis-Gimenez et al., 2018). PETH was distinct from ETH group because it contains no KxxKxxPRx motif which is common in the ETH group. PETH was hypothesized to induce pre-ecdysis I, while ETH initiates pre-ecdysis II and post-ecdysis behaviors. Injection of ETH alone into *M. sex*ta induced all three ecdysis behaviors leaving the actual function of the PETH yet to be defined (Park et al., 2002; Zitrian et al., 1996). In other insects, both ETH1 and ETH2 contain the conserved

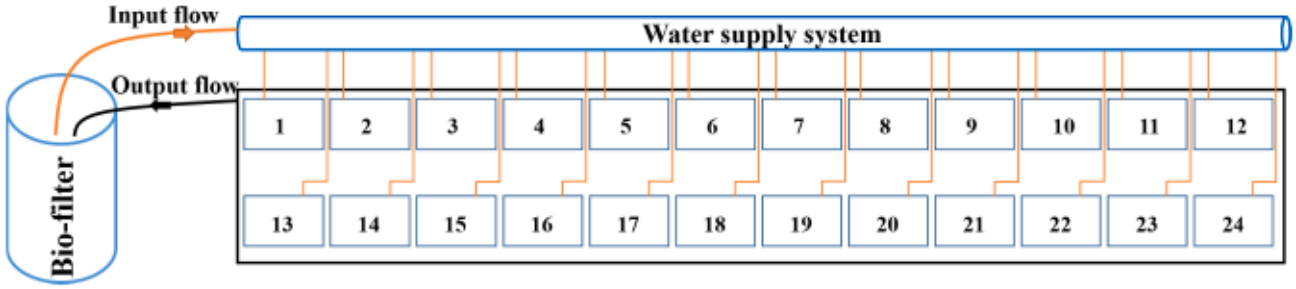


Fig. 1. Experimental system design. Water flowing into all 24 containers (orange) was pumped from the biofilter. Water flowing out from the fenestrated containers was collected by a tray which drained the water back to the biofilter by gravity (black).

sequence motif -KxxKxxPRx (Roller et al., 2010). Injection of these two peptides into the larvae, pupae and adult mosquitos (*Aedes aegypti*) activated the ecdysis behavior (Dai and Adams, 2009). In *D. melanogaster*, ETH1 activity is 10-fold higher than that of ETH2 (Park et al., 2003). ETH1 injection into *eth* gene deletion animals rescues the entire ecdysis behavior sequence and enables ecdysis, while ETH2 elicits tracheal dynamics and ecdysis at massive concentration without pre-ecdysis behavior (Park et al., 2002).

In decapod crustaceans, ETH was identified through many transcriptomic databases, which are becoming increasingly available in recent years (He et al., 2012; Li et al., 2012; Suwansa-Ard et al., 2015; Veenstra, 2015; Ventura et al., 2014; Zhang et al., 2019), however, ETH role in crustacean molting was not functionally proven. In a bioinformatic study, Decapod crustacean ETHs were described in an intensive data mining study (Veenstra, 2016b) using insect ETHs as a template. In all decapod species, only one *eth* gene was identified with varying number of putative transcripts, all giving rise to the same mature ETH. The examined species included: *Scylla paramamosain, Litopenaeus vannamei* (2 *eth* variants in each), *Eriocheir sinensis, Homarus americanus, Procambarus clarkii, Macrobranchium rosenbergii* and *C. maenas* (1 *eth* variant in each, (Veenstra, 2016b)). In another study, 3 putative *eth* variants were identified in *C. maenas* (Oliphant et al., 2018).

In the context of the Pancrustacea theory, the overall machinery that regulates molt in crustaceans and hexapods is quite conserved, including a well conserved repertoire of neuropeptides in both lineages (Veenstra, 2016b). Interestingly, Crz is known to induce the release of ETH from the Inka cells in insect. While the Inka cells were not identified in Malacostraca, the high expression of Crz receptors in the YO of *G. lateralis* (Tran et al., 2019) and the differential expression of ETH in the CNS across the molt cycle in *C. maenas* (Oliphant et al., 2018), suggest a conserved functionality of both Crz and ETH in regulating the ecdysis and post ecdysis behavior sequence in Malacostraca, as it was observed in insects. To test this, we identified the Crz and ETH from the crayfish (*Cherax quadricarinatus*) and performed an *in vivo* assay, where we injected synthesized Crz and ETH into crayfish and recorded the molt interval to evaluate the effect of the neuropeptides on the molting period. We also observed a clear behavioral response immediately after neuropeptide administration, suggested to be involved in the ecdysis behavior. Protein sequence analysis of ETH across Pancrustacea lineages was also carried out, providing the conserved mature protein motifs in each order.

2. Materials and methods

2.1. Animals

Australian red claw crayfish (*Cherax quadricarinatus*) males and females were purchased from a local farm (Freshwater Australian Crayfish Traders, Tarome, Queensland). Crayfish weighing 1–4 g, clean from parasites and with intact appendages were selected for the experiments and placed in separate compartments (as detailed below). The crayfish were fed once per day in the morning ~2–4% wet body weight with commercially available tropical fish pellet (32% protein, 18% fat). Over the first 2 weeks, the weak and dead animals were replaced with healthy ones, and molt interval was recorded from the third week. Individuals were randomly assigned per treatment.

2.2. Apparatus

Each animal was housed in a separate fenestrated plastic container (20 cm L × 15 cm W × 10 cm H) with water level at 9 cm. Containers were covered with lids to prevent crayfish escaping. Three 15 mm diameter pebble rocks were placed in each tank, submerging a piece of 3 cm × 7 cm shade cloth at the bottom of each tank, serving as shelter to reduce stress. All containers were placed on a holding tray draining the water flowing out of all containers to a gravity fed biofilter (300 L circular tank). The biofilter contained plastic biofilter media, an air pump, and a 1 kW heater with a thermostat set at 25 °C. Water from the biofilter was supplied back to the tray system through a submersible pump at a flow rate of 100 L per hour. Each container was fitted with a 3 mm diameter tube and a small valve supplying water a constant rate of ~3 L per hour (Fig. 1). The system was located at the University of the Sunshine Coast temperature regulated aquaculture room (air temperature 24 °C ± 2) adjacent to a shaded window to provide natural light (~12:12 L:D). Dissolved oxygen (> 9 ppm), temperature (24 °C ± 2), pH (7.2 – 7.5), and NO_3^- (less than15 ppm) in the containers were checked on a weekly basis throughout the experiment. About 10–15% of water was exchanged every week using temperature equilibrated, dechlorinated tap water.

2.3. Hormones and hormone administration

Crz and ETH peptides (Table 1) were synthetically produced by Pepmic (Jiangsu, China). Synthetic Crz (p-QTFQYSRGWTN-amide) and ETH (DAGHFFAETPKHLPRI-amide) were dissolved in 1 ml of ultrapure

Table 1
Behavioral response following neuropeptides injection.

Peptide / eye twitch effect	Crz (20 nM per g of animal)	ETH (10 nM per g of animal)	Water injected control	Uninjected control
Eye twitches per 5 min	16 ± 0.8	69.4 ± 5.9	0.4 ± 0.2	0.2 ± 0.2
Duration	> 1 h	30–60 mins	NA	NA
Synchronized	No, 1 eye delayed	Yes	Yes	Yes
Eye twitch speed	A few seconds	Fraction of a second	Fraction of a second	Fraction of a second

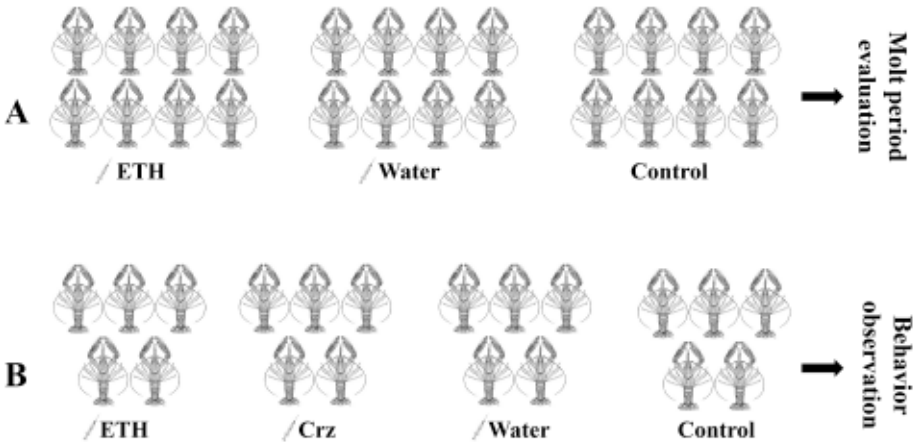


Fig. 2. Experimental design to assess molt interval (A) and behavior response (B) of *C. quadricarinatus* to a single injection of Crz and ETH. A) Three groups of crayfish (n = 8 per group) were injected with either ETH, water, or no injection, followed by daily monitoring of molts. B) Four groups of crayfish (n = 5 per group) were injected with either ETH, Crz, water or no injection, followed by behavioral response assessment.

water to make a stock solution of 4 mg/ml. Individuals were injected with the peptides (or equivalent water volume, or not injected as two control groups) 15 ± 1 days post molt. Since the molt interval in our system was on average 30 days, administering the peptides 15 days post molt ensured the majority of treated individuals are in the intermolt period. ETH and Crz were administrated at 10 nM and 20 nM per g of animal, respectively. Molarity was calibrated to animal weights. The syringe was inserted laterally through the first abdominal segment and held in place for 5 s following injection, and the animal was kept upside down for another 5 s to ensure peptides were dispersed through the hemolymph. After injection, the animals were immediately placed back in their holding containers. The molt observation was recorded daily for 90 days to assess the long-term effect of ETH on the molt interval, animals were injected once with either ETH, water or no injection (n = 8 per group). To assess the short-term behavioral response to ETH or Crz, individuals were injected with either ETH, Crz, water or no injection (n = 5 per group) followed by behavioral observation (Fig. 2).

2.4. Behavioral observations

Crayfish behavior was determined by direct observation over the first hour post injection and revalidated from recorded videos. A camera (iPhone 6) was set directly above the containers, recording the differences in eyes twitching and leg movement behavior. The eye twitching analysis was based on four different parameters *(1)* number of eye twitching per minute, *(2)* speed of eye twitching, *(3)* bilateral synchrony between eyes when twitching, *(4)* duration of eye twitching.

2.5. Statistical analysis

The effect of ETH injection on molt interval and molt increment were evaluated with SPSS version 24. One-way ANOVA was used to compare the differences between the groups. Molt intervals (1st interval, 2nd interval, delta) was set as a dependent variable and the treatments (ETH, water, negative control) as a fixed factor. In the behavioral response observation, the number of eye twitching was set as a dependent variable and the treatment (ETH, Crz, water, negative control) was set as a fixed factor. Post-hoc LDS and Tukey test were performed for both tests to determine the significance of difference between treatments. Probability of less than 5% ($P < 0.05$) was considered as statistically significant.

2.6. ETH conservation analysis study

Arthropods ETH encoding sequences were obtained from NCBI and Uniprot databases. Protein sequences and their accession numbers can be found in Supplementary file 1. To validate the search, neuropeptide sequences were submitted to NeuroPred (Southey et al., 2006) to determine the cleavage sites, followed by signalP 5.0 (Almagro Armenteros et al., 2019). Only neuropeptide sequences with a signal peptide and cleavage sites were selected for analysis. The Illustrator for Biological Sequences (IBS) tool v1.0.3 (Liu et al., 2015) was employed to draw schematics of ETH. ETH conservation was assessed by multiple sequence alignment of the mature ETH peptide sequences using MUSCLE tools implemented in MEGA7 with UPGMB method (Kumar et al., 2016).

3. Results

3.1. ETH injection prolongs the molt interval

The effect of ETH on molt interval was assessed by monitoring three consecutive molt cycles. In the first measured interval (between the first molt and the second molt), all individuals were observed without injection and the average molt interval for all treatments-allocated individuals (ETH, water and control) were 29.5 ± 1.75, 23.5 ± 2.71 and 25.7 ± 2.18 days, respectively (Fig. 3). One-way ANOVA analysis showed that there was no statistically significant difference between the three groups (Supplementary file 2a). Based on the first molt interval and the fact that it took longer than 16 days for each individual to molt, at the second molt interval (between the second molt and the third molt) the ETH group-allocated individuals were injected a single dose of ETH at day 14 following the second molt. In the second interval, both water injected and uninjected control groups showed similar molt intervals (31.8 ± 4.07 and 31.7 ± 1.63 days), while the ETH group took more than twice longer than the control and water groups

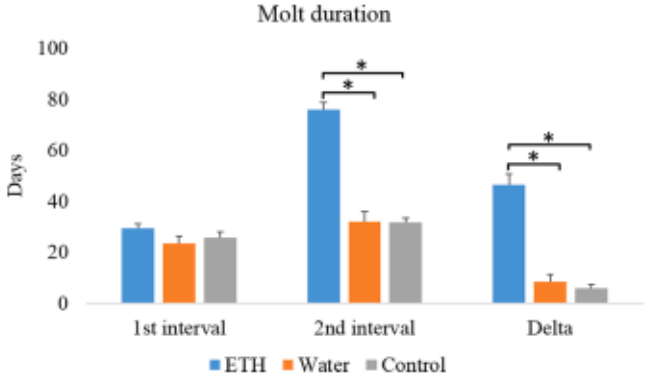


Fig. 3. Effect of ETH on molt interval. Molt interval of three different treatments (ETH colored in blue, water colored in orange, and control colored in grey) was calculated by subtracting between two molting times, the delta was calculated by subtracting between two intervals. Asterisks show significant difference between two groups tested by Post-hoc test (Tukey HSD and LSD). Error bars indicate the standard error of the mean. N = 8/treatment group.

(76 ± 2.73 days; Fig. 3). Subtracting the first molt interval from the second molt interval (referred to as 'delta' in Fig. 3), shows positive values in all three groups, meaning that the second molt interval was longer than the first molt interval in all groups. Yet, the delta was much higher in the ETH injected group (46.5 ± 4.16 days), than the control (6 ± 1.18 days) and water (8.38 ± 2.87 days) groups (Fig. 3). Statistical analysis using one-way ANOVA, followed by Post-hoc test (Tukey HSD and LSD) showed significant differences between ETH treatment and the water and control groups (Supplementary file 2b, 2c).

3.2. ETH and Crz induce a clear behavioral response

Four groups of five individuals each were set up for behavioral observation. Three groups were injected with ETH (10 nM/per g of animal), Crz (20 nM/per g of animal) and water control (20 µl). The fourth group served as an untreated control. The individuals injected with ETH and Crz showed a clear response of eye twitching which was distinctively different between treatments and different to the water and control group individuals. The eye twitching, with the distinctive frequency, was observed immediately after injection. The recorded data for over 5 min following the first eye twitching (observed within 2 min following injection) was obtained and used for calculations. ETH injection showed very high eye twitching frequency (69.4 ± 5.9 twitches per 5 min) while the average eye twitching frequency of Crz-injected individuals was less than one-fourth of ETH-injected individuals (16 ± 0.8 twitches per 5 min). In both water injected and control group individuals, eye twitching frequency was very low (0.4 ± 0.22 for water and 0.2 ± 0.18 for control; Fig. 4).

Besides the eye twitching frequency, the eye twitching duration, bilateral synchronization, and speed of twitching were also different between the treatments. Crz -induced eye twitching sustained for more than an hour until the animals returned to a normal twitching behavior. ETH-induced eye twitching sustained for 30 min to 1 h. Crz -injected individuals twitched their eyes much slower than ETH-injected individuals. Crz induces eye twitching with eyes retracted underneath the rostrum for a few seconds before releasing back to normal position, while it took a fraction of second for the one cycle of eye twitching with ETH-injected individuals, similar to the occasional eye twitching of the control individuals. Additionally, while ETH-injected individuals retracted their eyes synchronously (similar to the occasional eye twitching of the control individuals), Crz -injected individuals retracted their eyes asynchronously, with one eye retracted by a split of a second delay after the other. All measured characteristics of the eye twitching are summarised in Table 1. A Supplementary Video is provided, showing an ETH-injected and a Crz -injected as well as the control individuals (Supplementary Video).

The leg movement was observed at the same time with eye twitching observation. The Crz injected individuals showed a normal leg movement. The ETH injected individuals showed increased activity and moved around the container. The three pairs of walking legs (1–3) reached to their carapace and eyestalks in a grooming-like behavior. These behavior were observed in all tested animals 20–25 min following injection, and lasted for several minutes with a decrease in frequency.

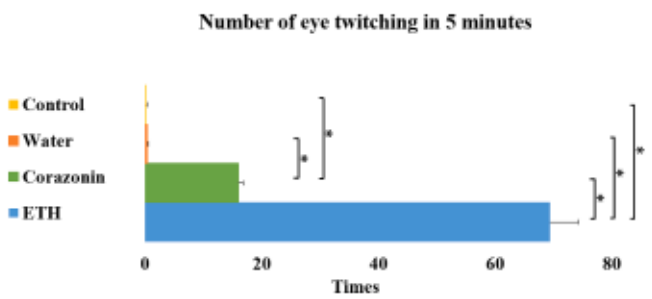


Fig. 4. Eye twitching response to ETH and Crz. The number of eye twitching was recorded over 5 min following the first eye twitching after injection with water (orange), Crz (green), ETH (blue) and the control without injection (yellow). * shows significant differences between the groups, tested by Post-hoc test (Tukey HSD and LSD). Error bars indicate the standard error of the mean.

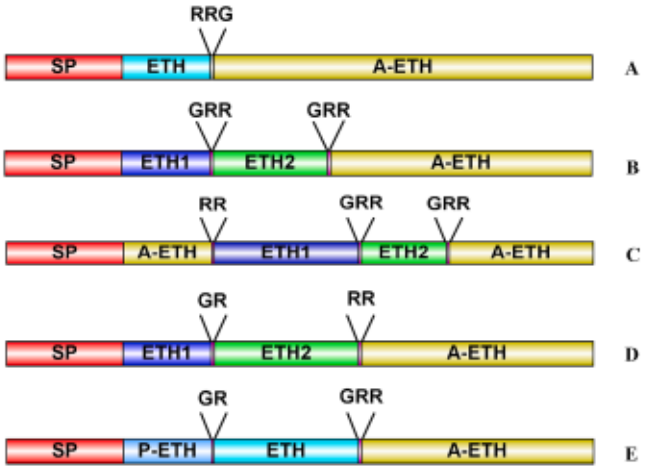


Fig. 5. The schematic diagrams showing the organization of different components of the eth gene in 5 different orders within arthropod phylum. A) Decapod, B) Diptera, C) Hemiptera, D) Hymenoptera, and E) Lepidoptera. The eth gene includes a signal peptide (SP) which is colored in red, a cleavage site and an amidated residue colored in pink, an alternative mature peptide ETH (ETH1 colored in blue, ETH2 colored in green, PETH colored in light blue, ETH colored in cyan) and associated ETH colored in yellow.

3.3. ETH conservation across Pancrustacea

3.3.1. ETH in decapod crustaceans

ETH sequences from 8 different decapod species including crabs *Eriocheir sinensis, G. lateralis, C. maenas and Scylla paramamosain*, freshwater prawn *Macrobrachium rosenbergii*, marine prawn (shrimp) *Litopenaeus vannamei*, freshwater crayfish *Procambarus clarkii* and lobster *Homarus americanus* were used for the analysis. All examined decapods had one conserved mature ETH, comprising an identical mature peptide of 16 aa. Decapod ETHs had variable signal peptides (19 – 29 aa), followed by the conserved mature ETH, followed by the A-ETH, which is separated from the mature ETH by a conserved cleavage site (RRG) (Fig. 5A). Blast search against *Cherax destructor* database from NCBI using ETH mature peptide as a template revealed Cd_ETH transcript which possess the same mature protein sequence (Fig. 6A).

3.3.2. ETH in Diptera

Two mature ETHs encoded by a single *eth* gene were found in Diptera. ETH1 is located right after the signal peptide, followed by ETH2 (separated by a RR cleavage site), and the tail A-ETH (also separated from the ETH2 by a GRR cleavage site) (Fig. 5B). The mature ETH1 possess 17 or 18 aa in length while mature ETH2 comprise 15–19 aas. Both ETHs contain a conserved insect motif –KxxPRx (Fig. 6C & D). Blast search against NCBI nr database using mature ETH1 as a query identified ETHs in *Aedes albopictus, Anopheles gambiae and Anopheles sinensis* (Table 2). ETH1 contains four conserved amino acid (–PGFFx) which is unique motif within arthropods and serves as a signature to distinguish Diptera ETH1 from other insect orders.

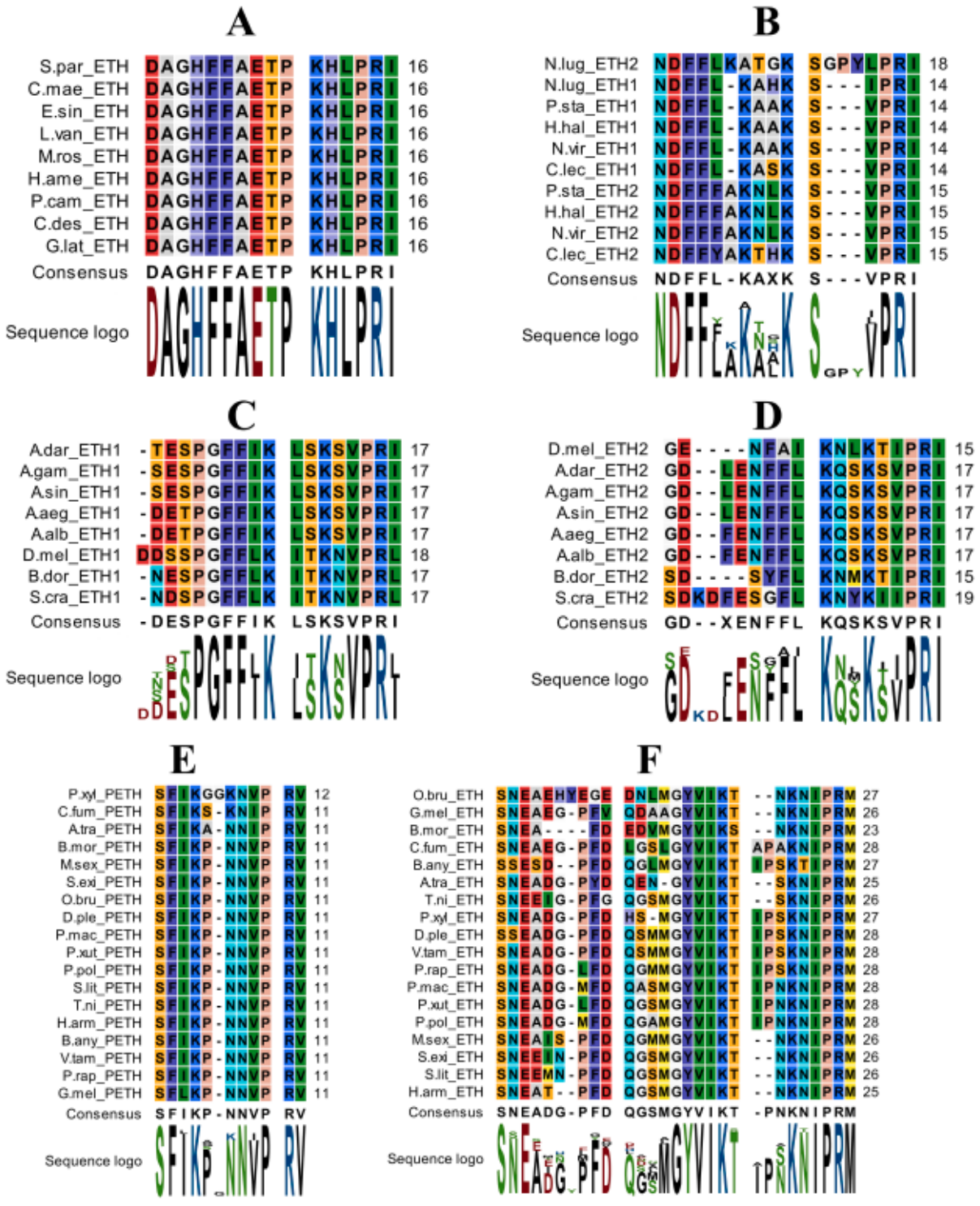
3.3.3. ETH in Hemiptera

In Hemiptera, ETH neuropeptides were identified in two species, *Nilaparvata lugens* and *Nezara viridula*, using Blast search against insect databases (Lavore et al., 2018; Tanaka et al., 2014). Two type of ETHs were found to be encoded by an *eth* gene. A typical structure of the *eth*

gene is found in this order, including a signal peptide (22–28 aa) and the A-ETH, separated from the mature ETH1 (14 aa) by an RR-cleavage site. The ETH2 (15–18 aa) is located right after ETH1 and before terminal A-ETH, separated by two RR cleavage sites (Fig. 5C). Both ETHs possess high sequence similarity and contain the same motif NDFFxxKxxKxPRI (Fig. 6B). Similar to Diptera, Hemiptera ETHs contain an insect motif –KxxKxPRx and NDFFx motif at the C-terminus which could serve as a fingerprint to identify the ETHs in this order. In *Plautia stali, Halyomorpha halys, Nezara viridula*, ETH1 was repeated and located right after ETH2 (Supplementary file 1). Blast search against NCBI database discovered ETH neuropeptides in *Cimex lectularius* which show the same motif as other Hemiptera.

3.3.4. ETH in Hymenoptera

The Hymenoptera *eth* gene produces two ETH neuropeptides. The *eth* gene structure comprises of a signal peptide (22–40 aa) followed by an ETH1 domain (18 or 20 aa) which contains a first cleavage site (GR). ETH2 (18–41 aa) is located next to the ETH1 domain, and separated from the last A-ETH domain by a second cleavage site (RR) (Fig. 5D). ETH1 possesses an insect motif KxxKxPRx and a very conserved



(caption on next page)

Fig. 6. Multiple sequence alignment of different Arthropod orders using mature ETH neuropeptide sequences. The colors of residues were followed by Rasmol color scheme (Sayle, 1995). The consensus sequence was determined by common aa across species. The sequence logo expression represents the approximate percentage of each aa in the sequence. The number at the end indicates the number of aa of the sequence *A)* Decapods ETH; S.par – *Scylla paramamosain*, C.mae – *Carcinus maenas*, E.sin – *Eriocheir sinensis*, L.van – *Litopenaeus vannamei*, M.ros – *Macrobrachium rosenbergii*, H.ame – *Homarus americanus*, P.cam – *Procambarus clarkii*, C.des – *Cherax destructor*, G.lat – *Gecarcinus lateralis*. *B)* Hemiptera ETH; N.lug – *Nilaparvata lugens*, P.sta – *Plautia stali*, *H.hal* – *Halyomorpha halys*, *N.vir* – *Nezara viridula*, *C.lec* – *Cimex lectularius*. *C)* Diptera ETH1 *and D)* Diptera ETH2. D.mel – *Drosophila melanogaster*, B.dor – *Bactrocera dorsalis*, A.dar – *Anopheles darlingi*, S.cra – *Sarcophaga crassipalpis*, A.aeg – *Aedes aegypti*, A.alb – *Aedes albopictus*, A.gam – *Anopheles gambiae*, A.sin – *Anopheles sinensis*. *E)* Lepidoptera PETH and *F)* Lepidoptera ETH. B.mor – *Bombyx mori*, M.sex – *Manduca sexta*, S.exi – *Spodoptera exigua*, C.fum – *Choristoneura fumiferana*, O.bru – *Operophtera brumata*, D.ple – *Danaus plexippus*, P.mac – *Papilio machaon*, P.xut – *Papilio xuthus*, P.pol – *Papilio polytes*, S.lit – *Spodoptera litura*, T.ni – *Trichoplusia ni*, H.arm – *Helicoverpa armigera*, B.any – *Bicyclus anynana*, V.tam – *Vanessa tameamea*, P.rap – *Pieris rapae*, G.mel – *Galleria mellonella*, A.tra – *Amyelois transitella*, P.xyl – *Plutella xylostella*. *G)* Hymenoptera ETH1 and *H)* Hymenoptera ETH2. A.cer – *Apis cerana*, A.mel – *Apis mellifera*, N.vit – *Nasonia vitripennis*, E.mex – *Eufriesea mexicana*, H.lab – *Habropoda laboriosa*, D.nov – *Dufourea novaeangliae*, B.imp – *Bombus impatiens*, M.qua – *Melipona quadrifasciata*, M.rot – *Megachile rotundata*, B.ter – *Bombus terrestris*, A.dor – *Apis dorsata*, A.flo – *Apis florea*, P.dom – *Polistes dominula*, L.nig – *Lasius niger*, D.all – *Diachasma alloeum*, F.ari – *Fopius arisanus*. *I)* Tardigrades, Centipedes and Chelicerates ETH. D.pul – *Daphnia pulex*, S.mar – *Strigamia maritima*, H.duj – *Hypsibius dujardini*, R.var – *Ramazzottius varieornatus*, M.mar – *Strigamia maritima*, P.cit – *Panonychus citri*, D.far – *Dermatophagoides farina*, S.mim – *Stegodyphus mimosarum*, P.pse – *Pardosa pseudoannulata*, P.tep – *Parasteatoda tepidariorum*, A.gen – *Acanthoscurria geniculate*.

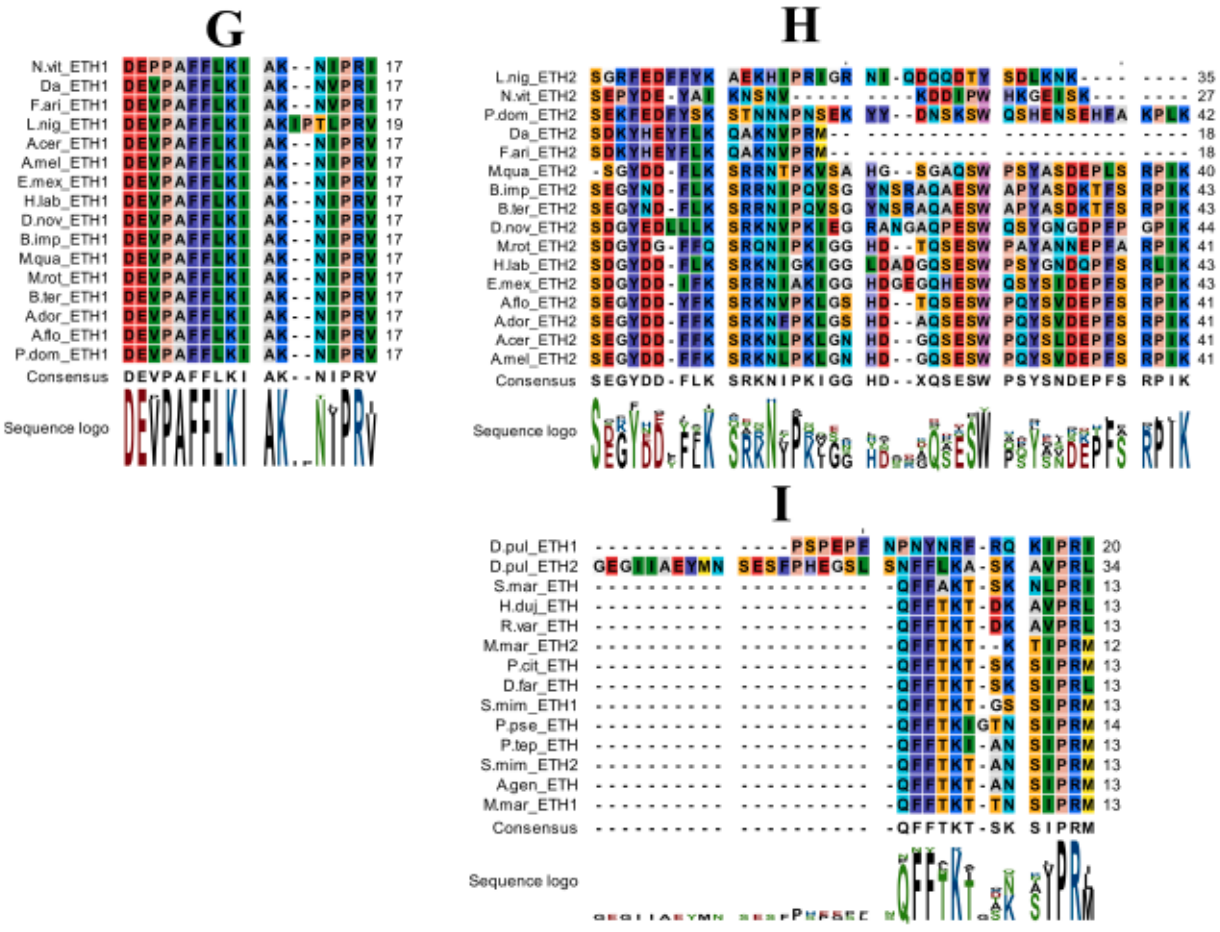


Fig. 6. (*continued*)

sequence ADEVPAFFL- at the C-terminus (Fig. 6G). Unlike ETH1, Hymenoptera ETH2 varies in length and aa sequence without any conserved motifs.

3.3.5. ETH in Lepidoptera

The Lepidoptera *eth* gene encodes three neuropeptides including PETH, ETH and A-ETH. The structure of the *eth* gene comprises of a signal peptide (21–36 aa) at the N-terminus which separates from the next PETH domain (14–15 aa) by the first cleavage site (SS, GS, CS or AS) (Supplementary file 1). Downstream to the PETH sequence is an ETH domain separating from the PETH by a second cleavage site (GR). The A-ETH is located right after the ETH and disjointed by a third cleavage site (GRR) (Fig. 5E). The PETH comprises of an 11–12 aa, out of which 8 aa appear to be conserved among species; SFxK- motif is the recognition site of this peptide besides the conserved C-terminus motif –NxPRV (Fig. 6E). By using these conserved motifs, Blast search identified PETH in many species within the Lepidoptera (Table 2). The ETH neuropeptide is much longer than the PETH, which contains 25–28 aa. The N-terminus of ETH is quite variable, while the C-terminus contains two conserved regions including the insect motif –KxxKxPRx and a fingerprint motif –GYVI.

3.3.6. ETH in Myriapods, Chelicerates, and tardigrades

The structure of the *eth* gene of chelicerates, myriapods and tardigrades comprises a signal peptide (11–29 aa) at the N-terminus followed by ETH domain (12–14 aa). The ETH domain is separated from

Table 2
List of identified and predicted ETH in 4 orders of insect and decapod crustacean.

Order	Species	Accession no.	Reference
Decapoda	*Scylla paramamosain*		(Veenstra, 2016b)
	Carcinus maenas		(Oliphant et al., 2018; Veenstra, 2016b)
	Eriocheir sinensis		(Veenstra, 2016b)
	Litopenaeus vannamei		(Veenstra, 2016b)
	Macrobrachium rosenbergii		(Veenstra, 2016b)
	Homarus americanus		(Veenstra, 2016b)
	Procambarus clarkii		(Veenstra, 2016b)
	Cherax destructor		This study
	Gecarcinus lateralis		This study
Diptera	*Drosophila melanogaster*	AAF47275.1	(Adams, 2000)
	Bactrocera dorsalis	AVA17335.1	(Shi et al., 2017)
	Anopheles darlingi	ETN66573.1	(Marinotti et al., 2013)
	Sarcophaga crassipalpis	BAQ19554.1	Unpublished
	Aedes aegypti	ABI93272.1	(Dai and Adams, 2009)
	Aedes albopictus	XP_019563397.1	This study
	Anopheles gambiae	XP_308702.3	This study
	Anopheles sinensis	KFB50454.1	This study
Hemiptera	*Nilaparvata lugens*	BAO00949.1	(Tanaka et al., 2014)
	Plautia stali	BAV78804.1	Unpublished
	Halyomorpha halys	XP_014275716.1	This study
	Nezara viridula	AZK31339.1	(Lavore et al., 2018)
	Cimex lectularius	XP_014240257.1	This study
Hymenoptera	*Apis cerana*	PBC31252.1	(Diao et al., 2018)
	Apis mellifera	NP_001136079.1	(Roller et al., 2010)
	Nasonia vitripennis	NP_001136107.1	(Roller et al., 2010)
	Eufriesea Mexicana	OAD61141.1	Unpublished
	Habropoda laboriosa	XP_017788897.1	This study
	Dufourea novaeangliae	XP_015433603.1	This study
	Bombus impatiens	XP_012248764.1	This study
	Melipona quadrifasciata	KOX72530.1	This study
	Megachile rotundata	XP_012143027.1	This study
	Apis dorsata	XP_006609679.1	This study
	Apis florea	XP_003692656.1	This study
	Polistes dominula	XP_015177012.1	This study
	Diachasma alloeum	XP_015115374.1	This study
	Fopius arisanus	XP_011310218.1	This study
Lepidoptera	*Bombyx mori*		(Adams and Zitnan, 1997; Zitnan et al., 2002)
	Manduca sexta	AAD45613.1	(Zitnan et al., 1999; Zitrian et al., 1996)
	Spodoptera exigua	AXY04252.1	(Llopis-Gimenez et al., 2018)
	Choristoneura fumiferana	AGA00107.1	(P et al., 2016)
	Operophtera brumata	KOB69386.1	(Derks et al., 2015)
	Danaus plexippus	A0A212FCU4	Unpublished
	Papilio machaon	XP_014363994.1	This study
	Papilio Xuthus	XP_013176125.1	This study
	Papilio polytes	XP_013136705.1	This study
	Spodoptera litura	XP_022817257.1	This study
	Trichoplusia ni	XP_026742015.1	This study
	Helicoverpa armigera	XP_021186791.1	This study
	Bicyclus anynana	XP_023943925.1	This study
	Vanessa tameamea	XP_026490568.1	This study
	Pieris rapae	XP_022127396.1	This study
	Galleria mellonella	XP_026756357.1	This study
	Amyelois transitella	XP_013198446.1	This study
	Plutella xylostella	XP_011565063.1	This study
Centipede	*Strigamia maritima*		(Chipman et al., 2014)
Chelicerate	*Panonychus citri*	AZL90163.1	(Zhu et al., 2019)
	Stegodyphus mimosarum		(Veenstra, 2016a)
	Parasteatoda tepidariorum		(Veenstra, 2016a)
	Acanthoscurria geniculata		(Veenstra, 2016a)
	Mesobuthus martensii		(Veenstra, 2016a)
	Dermatophagoides farina		(Veenstra, 2016a)
	Strigamia maritima		(Veenstra, 2016a)
	Pardosa pseudoannulata		(Yu et al., 2020)
Tardigrades	*Hypsibius dujardini*		(Koziol, 2018)
	Ramazzottius varieornatus		(Koziol, 2018)
Cladocera	*Daphnia pulex*		(Dircksen et al., 2011)

the A-ETH domain by a cleavage site (RR) (Supplementary file 1). Most examined chelicerates had one *eth* except for *Stegodyphus mimosarum* and *Mesobuthus martensii* where two *eth* genes were identified. The motif QFFxKx at the N-terminus was conserved across all studied chelicerates, myriapods (*Strigamia maritima*) and tardigrades (*Hypsibius dujardini, Ramazzottius varieornatus*), while the motif KxxPRx was found in the myriapods, tardigrades, and some of the chelicerates species (Fig. 6I). ETHs of *Daphnia pulex* were included as the outgroup which showed a distinct pattern in term of sequence length and motif.

4. Discussion

In most studied insects, the increase in ETH hemolymph concentration was found to trigger the release of other neuropeptides (CCAP, EH, Bursicon), initiating a synchronous ecdysis behavior sequence (Diao et al., 2017; Nassel and Winther, 2010). The EH and ETH together trigger the ecdysis motor and are naturally secreted to the hemolymph at a late premolt stage, following the earlier onset of the ecdysone cassette (Hyde et al., 2019). By this stage, apolysis has already occurred, the animal has formed a new cuticle underneath and is ready to shed its old exoskeleton, then expand and harden the new one. In our study, a single injection of ETH into crayfish during the intermolt stage triggered an immediate, observable ecdysis behavior response, and considerably delayed the molt. The significant delay in molt can be explained by the fact that the ETH injection simulated the ecdysis behavioral process in intermolt animals, well before its normal cycle. The old exoskeleton was not detached, new cuticle was not formed underneath, and the animals were not ready for ecdysis. The immediate observed behavioral response suggests that ETH triggered the ecdysis behavioral response which pushed the animals to the next stage. In fact, they are unable to molt and repeat the molt cycle again, which therefore delayed the molt twice as long as it takes for a normal molting cycle. The exceptionally delayed molt due to a single ETH injection out of sync opens the question of what is it that follows the induced ecdysis behavior response that caused this dramatic delay. The recorded phenotypic behavior only lasted for less than 1 hr, still, the molt was delayed by ~1 month.

The eye twitching in *C. quadricarinatus* is naturally occurring across all molt stages at very low frequency, with a bit higher frequency observed at late premolt, peaking at the postmolt right after molting. The eye twitching behavioral response following ETH and Crz injections are different and suggest a link between these two hormones in crayfish. While the ETH injection induced rapid, bilaterally-synchronous eye twitching (similar to the infrequent eye twitching occasionally observed in the control individuals), the Crz injection induced prolonged, bilaterally-asynchronous eye twitching. Nevertheless, both Crz and ETH

induce eye twitching suggesting both peptides are involved in the same pathway, in keeping with findings in insects showing that Crz induces ETH production (Kim et al., 2004).

In our observation, non-treated crayfishes were less likely to move their legs across their carapace during intermolt and premolt. However, they used their front three pairs of walking legs to touch their carapace and eyestalk several times during ecdysis (~15 mins) and after ecdysis occurred. The ETH injected individuals showed the same leg movement behavior that is typical of ecdysis, while in intermolt. This response was observed in all examined animals synchronized with the eye twitching, both are behaviors observed during ecdysis of non-treated crayfishes, strongly supporting our conclusion that ETH is directly involved in ecdysis behavior in *C. quadricarinatus*.

Decapod crustaceans ETH was previously discovered by using insect ETH through a phylogenetic study (Veenstra, 2016b). It contains an insect ETH motif –KxxPRx at the C-terminus of the mature peptide. However, the decapod crustacean ETH appears to have diverged from the insect homolog, as it possesses an extremely conserved and unique sequence in the mature peptide, found across all studied decapod species (Fig. 6A). This conservation suggests a functional similarity in decapod species. Unlike most studied insects, which produce two different mature ETHs, only one ETH was found in decapod crustaceans. Interestingly, blast search against nr database in NCBI, using the ETH sequence as a template, showed no hit for any myriapod species. Given the Pancrustacea theory, placing Hexapoda more closely related to Crustacea than Myriapoda, with the open question of which insect lineages derived from which crustacean lineages, it would be prudent to test what is the ETH conservation in cephalocarida, branchiopoda and remipedia and find whether these groups produce one or two mature ETHs. Given the uniquely conserved functionality of ETH in molt regulation across the Pancrustacea, these further studies would facilitate identifying evolutionary linkages among orders within the phylum Arthropoda. The eye twitching induced by ETH implies a conserved function for ETH between decapods and insects, regulating the ecdysis behavior sequence. In insects, the edysis behavior sequence is accompanied by a series of body contractions (Kim et al., 2006b; Zitnan et al., 2002). In decapods, as we observed in *C. quadricarinatus* that the animals twitch their eyes more frequently in late premolt and postmolt than the intermolt. The animals also use their walking legs to touch their head and eyes right before and after ecdysis. This behavior is perhaps a postmolt grooming behavior. The eye twitching behavior could occur at the same time as body contraction, but in *C. quadricarinatus*, the muscle movement under the exoskeleton cannot be observed. The eye twitching in response to ETH and Crz injection could reflect a part of the body contraction during ecdysis sequence behavior. Overall, this study shows for the first time in crustaceans, a conserved function of ETH with insects in regulating the ecdysis response behavior which was studied thus far only in insects. Given the significant delay in molt due to a single exposure to the mature ETH peptide, a thorough investigation of the properties of ETH across the Pancrustacea is warranted. As a first line of investigation, this study mapped the conserved motifs found in the mature ETH peptides of each insect order.

Diptera is one of the largest orders in the insect sub-phylum which comprise approximately 125,000 species. The majority of species in this order have two-wings, unlike the four-winged species in the Lepidoptera and Hemiptera orders. The role of ETH1 and ETH2 in Diptera is still unresolved. Sequence alignment within this order reveals that ETH1 is more conserved than ETH2 (Fig. 6C & D). Studies in *D. melanogaster* and *B. dorsalis* indicate that ETH1 expression is compulsory for inducing ecdysis behavior, while ETH2 is not. The ETH1 elicits stronger response than ETH2 at lower concentrations (Park et al., 2003; Shi et al., 2017). However, both ETHs activated ETHR and trigger ecdysis behavior in *A. aegypti* at pupal stage in the absence of pre-ecdysis behavior, although the effect of both ETHs on triggering ecdysis behavior is slightly different (Dai and Adams, 2009). Both *A. aegypti* ETHs share the same motif FFxKxxKxxPRx, while ETH1 and ETH2 in both *D. melanogaster* and *B. dorsalis* share only the insect motif –KxxKxxPRx. The similarity in sequence motif could explain the similar in function of ETH across species. Further research to better elucidate the structure–function relationship between ETHs in these species is needed. Hemiptera ETH1 and ETH2 share a high sequence similarity and both retain the insect conserved motif. In *Plautia stali, Halyomorpha halys, Nezara viridula*, the ETH1 domain is duplicated and is located right before, as well as right after ETH2 domain (Supplementary file 1). The expression of the *eth* gene in these species therefore could lead to twice as much ETH1 produced compared with ETH2. In Hymenoptera, ETH2 appears to be very different from the other ETH2 in other insect orders. The insect motif as well as recognition site for Hymenoptera were unidentified in its sequence. It can therefore be argued that there is only one ETH in *Hymenoptera* (ETH1 rather than ETH2). However, the *eth* gene structure contains two cleavage sites which is the same for all species within *Hymenoptera*. Moreover, most *Hymenoptera* species share a conserved C-terminus and N-terminus regions in the sequence we refer to as ETH2. Moreover, most of insect possess two isoforms of ETH, it is therefore high probability to have ETH2 in *Hymenoptera.* In Lepidoptera, The PETH is quite different from any other ETH peptide in insect. It possesses a very conserved sequence which contains no insect motif (–KxxKxPRx), suggesting a unique role in ecdysis behavior. In *M. sexta*, PETH injection 6–8 h before ecdysis evoke initial response which is defined as pre-ecdysis I behavior but the animals did not progress to ecdysis, while ETH injection induce later response which is named pre-ecdysis II and ecdysis behavior (Zitnan et al., 1999). In *B. mori*, injection of either PETH or ETH induce the full ecdysis sequence through the action on the CNS. However, PETH Injection 20–24 h before ecdysis evoke only pre-ecdysis behavior while injection 10–15 h before ecdysis induce the entire ecdysis sequence (Zitnan et al., 2002).

The mature ETHs were found in one *eth* gene in insects, decapods and *Daphnia pulex*. In chelicerates, some species had two ETH-encoding genes, which differs from the pancrustacea subphylum (Supplementary file 1). Multiple alignment analysis showed that many chelicerates had no KxxPRx motif which was present in all examined insects and decapods. Besides, QFFxKx was found in the N-terminus of all studied species of chelicerates, myriapods, and tardigrades, suggesting a close relationship between myriapods and chelicerates, apart from insects and crustaceans.

Insecta and Crustacea share a high similarity in their neuropeptidomes. Most neuropeptides are present across all studied lineages of both subphyla, except for allatotropin which is absent in decapod crustaceans (Roller et al., 2010; Veenstra, 2016b). The conserved role of ETH as an ecdysis triggering factor across Hexapods, shows functional conservation in decapods as well. While the function seems to be highly conserved, the difference in ETH secretion source (Inka cells in the trachea system of insects and the CNS in decapods), perhaps illustrates a key adaptation to living on land.

In conclusion, this study indicates that a single injection of ETH significantly delays the molt cycle in *C. quandricarinatus*. Further research is required to see if similar molt delay can be achieved in insects, with the effort to protect crops from pest insects. We therefore provide in this study a comprehensive identification of the conserved ETH motifs in each order which could serve as reference for developing a species-specific approach to control insect molting.

5. Conclusions

ETH is involved in regulating several crucial biological processes in Pancrustaceans, including molting and reproduction. Clarifying the mechanism of how ETH function is triggered and controlled will enable a better understanding of molting and reproduction in this sub-phylum. A single injection of Crz or ETH caused a quick response of eyestalk twitching of Crayfish, *C. quandricarinatus*. Whether the behavior response to Crz and ETH is similar to other decapod species remains to be explored. Crz is known to induce the ETH production in insects. In our

study, the behavior response is similar between these two neuropeptides suggesting a linked signaling pathway. Future research to explore the mechanistic understanding underlying this physiological response is warranted.

CRediT authorship contribution statement

Tran Minh Nhut: Visualization, Writing - original draft. **Donald L. Mykles:** Funding acquisition, Supervision, Writing - review & editing. **Abigail Elizur:** Supervision, Writing - review & editing. **Tomer Ventura:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

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