

Molecular cloning and biological activity of ecdysis-triggering hormones in *Drosophila melanogaster*

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Abstract Ecdysis-triggering hormones (ETH) initiate a defined behavioral sequence leading to shedding of the insect cuticle. We have identified *eth*, a gene encoding peptides with ETH-like structure and biological activity in *Drosophila melanogaster*. The open reading frame contains three putative peptides based on canonical endopeptidase cleavage and amidation sites. Two of the predicted peptides (DrmETH1 and DrmETH2) prepared by chemical synthesis induce premature eclosion upon injection into pharate adults. The promoter region of the gene contains a direct repeat ecdysteroid response element. Identification of *eth* in *Drosophila* provides opportunities for genetic manipulation of endocrine and behavioral events underlying a stereotypic behavior.

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Key words: Ecdysis; Eclosion; Peptide; Hormone; Ecdysis-triggering hormone; Behavior; *Drosophila*

1. Introduction

Peptidergic signaling is an ancestral form of cell–cell communication underlying numerous physiological events including feeding, locomotion, and social behaviors [1–3]. Stereotypic behaviors such as insect ecdysis provide favorable models for understanding how peptidergic signaling cascades evoke complex, sequential motor patterns [4]. The motor patterns characteristic of pre-eclosion and eclosion behaviors [5] are orchestrated by peptidergic cells located both peripherally and within the central nervous system (CNS). In the tobacco hornworm, *Manduca sexta*, at least four peptides are known to be involved in activation of this process: eclosion hormone (EH) [6], ecdysis-triggering hormone (ETH) [4], pre-eclosion triggering hormone (PETH) [5], and crustacean cardioactive peptide (CCAP, also known as CAP2b) [7].

Direct and circumstantial evidence indicate that the eclosion behavior of pharate adult *Drosophila melanogaster* is regulated by neuropeptide and peptide hormone signals similar to those described in *Manduca*. For instance, the gene encoding *Drosophila* EH shows high similarity to its counter-

part in *Manduca* [8], and an EH-cell knockout mutant *Drosophila* shows partial impairment of eclosion behavior [9]. Injection of *Manduca* ETH (MasETH) into *Drosophila* pharate adults induces premature eclosion behavior [9], suggesting that the ETH signaling pathway is functional in *Drosophila*. Finally, the presence of a CCAP-like peptide has been demonstrated by immunohistochemical staining in *Drosophila* [10,11]. The presence of peptides similar to ETH, EH, and CCAP in *Drosophila* leads to the hypothesis that endocrine events leading to ecdysis may be evolutionarily conserved.

We recently identified the gene in *Manduca*, which encodes three peptides: PETH, ETH, and ETH-AP [5]. Here we describe a gene in *Drosophila* encoding three peptides, two of which resemble *Manduca* PETH and ETH. The structure of this gene, referred to here as *eth*, and the biological activity of peptides it encodes show striking similarities to the corresponding elements in *Manduca*. The identification of *eth* in *Drosophila* provides opportunities for the use of genetic tools to investigate the signaling pathways leading to ecdysis.

2. Materials and methods

2.1. Molecular cloning and peptide synthesis

We utilized the MasETH prepropeptide sequence [5] to search for *Drosophila eth* in the Berkeley *Drosophila* Genomic Project database (BDGP) [12]. This search yielded a genomic sequence encoding a peptide similar to MasETH. To show that this gene is expressed, primers designed from this sequence were used to perform reverse transcriptase polymerase chain reactions (RT-PCR) on mRNA isolated from *Drosophila* pharate adults. We used DrmETHf4 (TGTGGAGCAGGAGTCAGGTG), DrmETHf3 (CAGCATGAG-AATCATAACAG), DrmETHf1 (CTGGGCAAGCGAGGCGAG-AA), and DrmETHf2 (TCGCATAGGACGCAGTGAGC) as forward primers, and DrmETHr1 (GAGAGTCTGCTTGAGGTGCT) and DrmETHr2 (CCGCCGTAAAGTTCCTGAT) as reverse primers (Fig. 1). Messenger RNA was isolated from staged animals using a Dynabeads mRNA direct kit (Dynal, Lake Success, NY). First strand cDNA was synthesized by 1 unit of Superscript RT (Gibco BRL Life technologies, Gaithersburg, MD) for 1 h at 37°C with 10 pmol adapter-oligo dT primer (GACTCGAGTCGACATCGA-T+17T) or a sequence specific reverse primer (DrmETHr2). PCR reactions (50 µl volume) were composed of 1×Taq polymerase buffer (Gibco BRL), 1–2 mM MgCl₂, 0.2 mM dNTP, forward and reverse primers (0.2 µM each), 1 unit of Taq polymerase, and 1 µl of the first strand cDNA synthesized by reverse transcription reaction. Following a 2 min denaturation at 94°C, the cDNA was amplified using 27 cycles of 35 s at 94°C, 1 min at 50–55°C, 30–60 s at 72°C, and by 5 cycles with increased extension time of 2 min at 72°C. The PCR products were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI Model 377 automated sequencer. Sequence discrepancies between the genomic sequence appearing in GenBank (GenBank accession number AC005710) and the sequence from RT-PCR in this study (AF170922)

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Abbreviations: ETH, ecdysis-triggering hormone; CNS, central nervous system; EH, eclosion hormone; PETH, pre-eclosion triggering hormone; CCAP, crustacean cardioactive peptide; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; EcRE, ecdysteroid response element

were confirmed in at least two independent clones. The peptides Drm-ETH1 and Drm-ETH2 were synthesized by Research Genetics (Birmingham, AL).

2.2. Animals

The Oregon-R strain of *D. melanogaster* was reared on yeast agar medium at 25°C under constant light conditions. Pupae were collected daily with a wet brush and kept in a petri dish until staging was made. Males and females were separated in the late pupal stage (P13) [13]. Staging in the last few hours before adult eclosion was done according to Kimura and Truman [14]. Pharate adults ~4 h before eclosion onset were used for injections. This time point was determined after observation of morphological changes visible on untreated flies, which differed for males vs. females. For males, this occurred 3 h after appearance of wrinkles on the head ('smooth' to 'smooth/grainy' transition, details in Fig. 2 and Section 3) [14]. For females, this occurred 30 min after the transition from 'smooth' to 'smooth/grainy'. Each fly was observed every 20 min to determine the injection time.

2.3. Bioassays

For observations of the peptide-induced eclosion behavioral sequence, pharate adults were arrayed on a glass slide with double sticky tape at the P14 stage, determined by the presence of green meconium at the end of the dorsal abdomen [13]. The pupal cuticle covering the dorsal part of the head and thorax was removed to facilitate injections and observation. Peptides were dissolved in nanopure water and injected in a volume of 9.6 nl into the thorax of a pharate adult using a nanojector (Drummond Scientific Co., Broo-

mall, PA). The injection needle, made from a broken microelectrode tip, was inserted through the metanotum at an angle of ~30° relative to the body axis and 30° of horizon. Two groups of animals were used as controls: un-injected or distilled water-injected. The un-injected control was a good indicator of accuracy of the staging animals and injection time. Behavior was recorded on videotape using a Sony CCD camera. Drm-ETHs (0.1 or 1 nmol dissolved in 1 µl) were tested in *Manduca* by injection into pharate 5th instar larvae 6–8 h prior to natural eclosion (brown mandible stage).

3. Results

3.1. Identification of a putative eth

A Blast search of the *Drosophila* genomic database using the amino acid sequence of the MasETH prepropeptide (Tblastn search with matrix of blosum62) yielded a sequence with relatively high sequence identity at the amino acid level (16/41, 39%). This sequence is located in the right arm of the second chromosome (60E1-2) covered by the P1 clone DS04983 (GenBank accession number AC005710, Fig. 1A). The P1 clone contains three other genes described as *Distal-less* (*Dll*) [15], origin recognition complex 4 (*ocr4*) [16], and rhythmically expressed gene 5 (*reg-5*) [17].

A cDNA with an open reading frame (ORF, GenBank

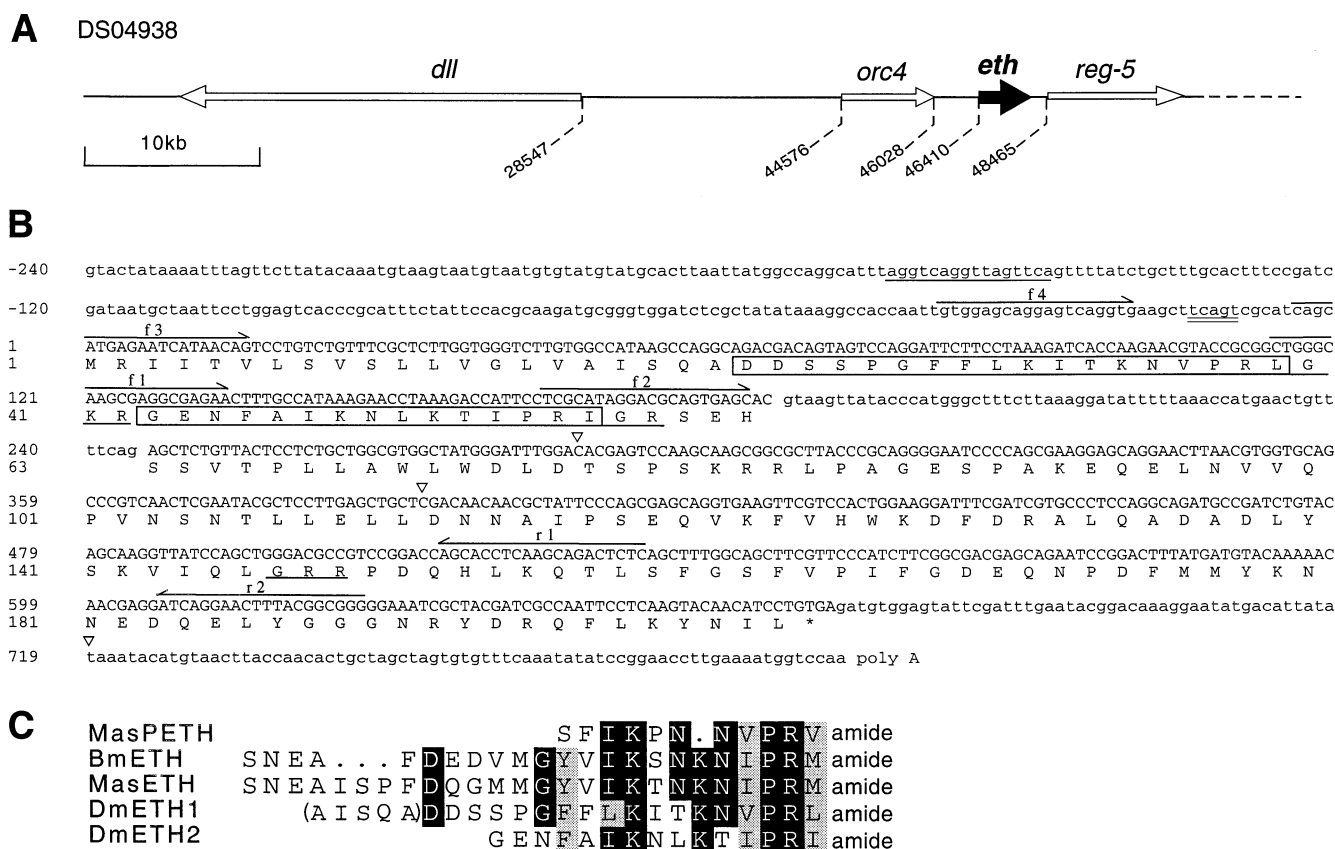


Fig. 1. Sequence and structure of the *eth* of *D. melanogaster*. A: Genomic structure of the *Drosophila* second chromosome right arm 60E1-2 region covered by the P1 clone of DS04983. The clone includes *Distal-less* (*Dll*), origin recognition complex 4 (*ocr4*), DrmETH, and rhythmically expressed gene 5 (*reg-5*). Arrows indicate position and direction of the genes. Numbers are from GenBank accession number AC005710. B: DrmETH sequence and translation of the open reading frame (GenBank accession number AF170922). Boxes depict predictions for the amino acid sequences of DrmETH1 and DrmETH2 and underlined amino acids are the amidation/processing signatures GKR, GR, and GRR. Underlined nucleotides at -163 to -148 in the top line comprise a putative ecdysone response element. Doubly underlined nucleotides at -14 to -10 comprise a putative arthropod capsite. Primers for PCR amplifications and names are indicated by arrows above the nucleotide sequence. Inverted triangles above the nucleotide sequence are for polymorphic sites compared to the AC005710 in GenBank (see details in text). C: Alignment of amino acid sequences of ETH-related peptides from *Drosophila*, *Manduca*, and *Bombyx mori*. Identical and similar amino acids in more than 50% of taxa in the alignment are indicated by black and shaded boxes, respectively.

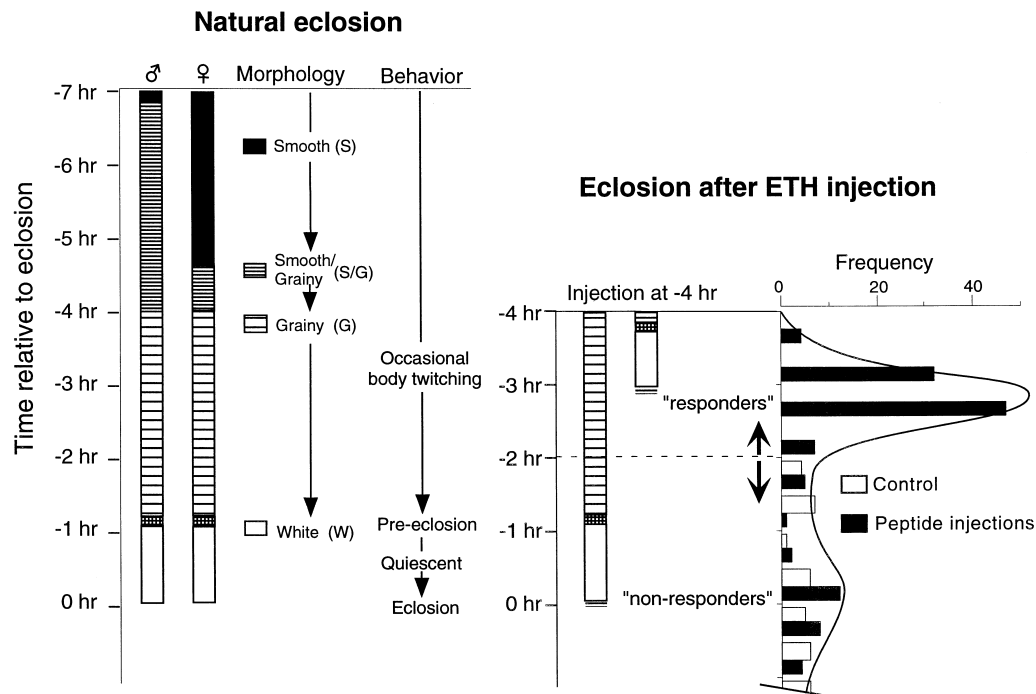


Fig. 2. Time course of 'natural eclosion' (left) and ETH-induced eclosion (right) in pharate adult *Drosophila*. Left: The time course of natural eclosion, which differs for males and females, is depicted according to morphology; i.e. 'Smooth' (black), 'Smooth/Grainy' (thin-hatched), 'Grainy' (wide-hatched), and 'White' (white) and according to behavior. The nomenclature for morphological markers follows that of Kimura and Truman [14]. The timing of events relative to eclosion is based on observations made in this study. Right: ETH injections made at about -4 h (relative to eclosion onset at 0 h) result in induction of premature pre-eclosion and eclosion behaviors in 'responders'. 'Non-responders' include saline-injected and non-injected flies. A histogram plotted at 30 min intervals shows a bimodal frequency distribution of eclosion time following injections of DrmETH1, DrmETH2, DrmETH1-AIS, and MasETH (peptide injections; black bars) or saline (controls; open bars). The solid line depicts a theoretical reconstruction of the bimodal distribution for two normal distributions: 'responders' ($1\text{ h }4\text{ min} \pm 18\text{ min}$, $n=105$; mean \pm S.D.) and 'non-responders' ($4\text{ h }7\text{ min} \pm 1\text{ h }8\text{ min}$, $n=90$). The observation was well fit to the theoretical bimodal distribution in a chi-square test for goodness of fit ($\chi^2=11.8$, $df=8$ in 1 h interval histogram). The dotted line at 1 h 56 min after injection is for $P=0.05$ of normal distribution for un-injected controls and this was used as the criterion for designating either 'responders' or 'non-responders' following peptide injection (see details in Fig. 3 and text).

accession number AF170922) encoding 203 amino acids was found by RT-PCR using primers designed from the predicted *eth* in the DS04983 clone (Fig. 1A,B). Using RACE, we identified the 3' end of the mRNA, including the stop codon and 120 bp of untranslated sequence before the poly A tail. The 5' end of the transcript was deduced by the absence of PCR product from RT-PCR using the f3 primer located just before the arthropod capsite signature [18], and by the presence of product from RT-PCR using the f4 primer that covers the predicted methionine translation initiation site. A 58 bp intron interrupts the ORF with a gt-ag intron donor and acceptor consensus sequence.

Conceptual translation of *Drosophila eth* revealed a signal sequence and three canonical processing and amidation sites: GKR, GR, and GRR (Fig. 1B) [19]. The putative processing motifs and algorithm searching for signal peptide processing sites [20] predicted three mature peptides named tentatively as DrmETH1 (amino acids 22–40), DrmETH2 (43–58), and DrmETH-associated peptide (DrmETH-AP, 61–146, Fig. 1B). Only DrmETH1 and DrmETH2 showed sequence similarity to MasPETH and MasETH (Fig. 1B,C). An alternative DrmETH1 sequence begins at the N-terminus with Ala-Ile-Ser (AIS, amino acid numbers 17–19 in Fig. 1B) [20], which matches positions 4–7 in the MasETH sequence (Fig. 1C). This peptide is here designated 'DrmETH1-AIS' (amino acid 17–40). The amino acid sequence of DrmETH-AP is highly

divergent from the ETH-like peptides as well as from Mas-ETH-AP [5].

There is a putative ecdysteroid response element (EcRE) upstream of the ORF, defined by an imperfect direct repeat of AGGTCA separated by GGTT between -163 and -148 (AGGTCAGGTTAGGTCA, Fig. 1B) [21]. The EcRE is followed by an arthropod-capsite consensus TCAGT [18].

Three nucleotide polymorphic sites were found by RT-PCR in this study compared to the genomic sequence reported for the P1 clone DS04983. These are non-synonymous polymorphisms A/C (DS04938/RT-PCR) at position 291 resulting in Glu (GAA)/Asp (GAC) at amino acid number 76; a synonymous polymorphism T/C at position 391 in Leu at number 111; and G/T at position 719 in the 3' untranslated region. The polymorphic sites were confirmed in at least two independent RT-PCR experiments.

3.2. The eclosion behavioral sequence of *Drosophila*

Morphological markers and sequential behaviors leading to eclosion in *Drosophila* have been described previously [9,14], but we observed slight differences in the temporal pattern of the eclosion behavior in our experimental conditions (Fig. 2). Pre-eclosion, initiated about 1 h prior to eclosion onset (-1 h), is composed of three distinct events: head inflation, head tracheal filling, and ptilinum extension. We used head inflation as a marker for initiation of pre-eclosion behavior. Head in-

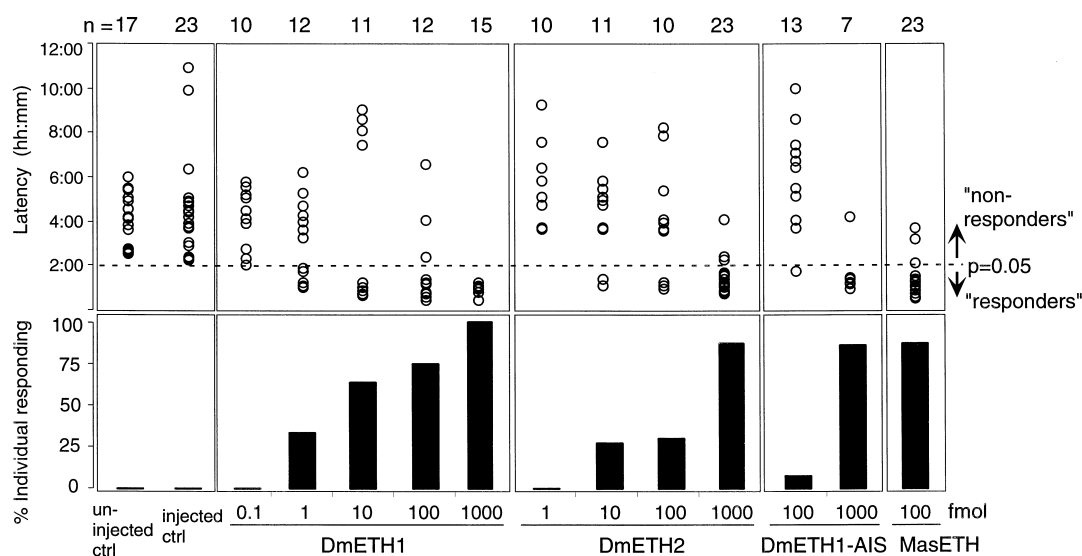


Fig. 3. Dose-response relationships for ETH peptides injected into pharate adult *Drosophila*. Latency in the upper panel indicates the time from treatment to eclosion onset in individually injected flies (see details in Section 2). Sample numbers are shown on the top (n). The dotted line, indicating $P=0.05$ for un-injected controls assuming a normal distribution, was used for criterion distinguishing 'responders' from 'non-responders' (Fig. 2). The lower panel depicts the percent of individuals responding to various doses of ETH peptides.

flation lasts ~ 8 min and coincides with pulsatile dorso-ventral contractions of the first abdominal tergum at 3–10 s intervals. Those insects which performed pre-eclosion entered a quiescent state until the onset of eclosion behavior. In four out of 17 individuals (24%), eclosion occurred without pre-eclosion behavior.

Eclosion behavior is composed of four consecutive events occurring within a 3 min time interval: (1) a short, strong head inflation which appears to be the same as that observed in pre-eclosion, (2) forward head thrusts, (3) bilateral, alternating contractions of the thorax, and (4) strong peristaltic abdominal contractions which push the body forward. We scored forward head thrusts as indicative of eclosion onset.

3.3. DrmETHs cause premature onset of the eclosion behavioral sequence

We found that injection of DrmETH1, DrmETH2, DrmETH1-AIS, and MasETH into pharate adults induced premature eclosion behavior in *Drosophila*. Within groups of water-injected controls, a small but significant number of individuals showed delays in initiation of eclosion that exceeded un-injected controls. This appeared to be a consequence of trauma caused by impalement with the injection needle. For this reason, increased mean and variance were observed for latency to onset of eclosion in injected controls ($4:33 \pm 2:11$, h:mm, mean \pm S.D.), vs. un-injected controls ($4:07 \pm 1:08$ min, $P < 0.001$ in F test of injected control vs. un-injected control).

Responses to injected peptides fit a bimodal distribution of 'responders' vs. 'non-responders'. Increasing concentrations of peptides did not produce a dose-dependent shortening of the latency (Fig. 2) [22]. The mean latency time of all 'responders' for eclosion was 64 ± 18 min (mean \pm S.D.), which was not significantly different at each dose. We therefore assessed the relative potency of each peptide by measuring the frequency (percent) of 'responders' at each dose of injected peptide (Fig. 3). Individuals showing latency to eclosion of less than 1 h 56 min, which is $P=0.05$ for the normal distribution of latency

time in un-injected controls (dashed line in Fig. 3), were counted as 'responders'; otherwise they were counted as 'non-responders'.

Fig. 3 shows that the lowest effective dose for DrmETH1 is between 0.1 and 1 fmol, and for DrmETH2 between 1 and 10 fmol (0.1–1 nM and 1–10 nM, respectively, assuming a 1 μ l blood volume for *Drosophila* [23]). DrmETH1-AIS, which includes an additional five amino acids at the N-terminus of DrmETH1 (Fig. 1C), also is active at relatively high doses (1000 fmol), but is clearly less effective than DrmETH1 (Fig. 3).

Pre-eclosion indicated by head inflation occurs in a majority of flies (76%, 67%, and 54% in un-injected control, 'responders', and 'non-responders', respectively). Injections of ETH peptides induced head inflation (16 ± 11 min, mean \pm S.D., in 'responders', Fig. 2) and altered its pattern. Contractions of the first abdominal tergum became stronger with reduced duration (3–5 min, $n=6$) as compared with un-injected and water-injected controls (8 min, $n=8$). The time between head inflation and head thrusting for eclosion showed a slight, but significant reduction in 'responders' (51 ± 17 min) compared with un-injected controls (59 ± 13 min, $P < 0.05$ in t -test).

In order to test whether a particular ETH peptide induces either head inflation (pre-eclosion) or eclosion behavior, we performed an independence test of contingency table [24]. The frequency of 'presence of head inflation event' is not significantly different in all combinatory comparisons including the comparison between DrmETH1 and DrmETH2. Thus, we conclude that all peptides accelerate both head inflation and onset of eclosion behavior, even though head inflation did not occur in all experimental animals.

3.4. Cross-reactivity of ETH peptides between *D. melanogaster* and *Manduca*

Injections of MasETH into *Drosophila* pharate adults induced the eclosion behavioral sequence at doses of 100 fmol as previously reported [9]. However, injections of MasPETH,

which induces pre-ecdysis I in *Manduca* larvae, produced no effect in *Drosophila* pharate adults at doses up to 1 nmol ($n = 6$).

Injection of DrmETH1 into *Manduca* pharate larvae (100 pmol, $n = 11$) induced pre-ecdysis I and II characterized by dorso-ventral and postero-ventral contractions, but no proleg retractions were observed. None of the animals initiated ecdysis. DrmETH2 produced no effect at doses up to 100 and 1000 pmol ($n = 5$ and 16, respectively).

4. Discussion

We have identified *eth*, a gene encoding peptides with ecdysis-triggering hormone activity in *Drosophila*. The ORF for *eth* encodes 203 amino acids, including three putative amidation and basic processing sites common in many neuropeptide genes [19]. DrmETH1 and DrmETH2 show significant sequence identity with PETH and ETH from *Manduca*, particularly at their C-termini. Even though the processing site between the signal peptide and DrmETH1 is not yet demonstrated experimentally, results from (1) algorithm searching for signal peptide processing sites [20] and (2) bioassays showing that DrmETH1 is significantly more active than DrmETH1-AIS, together support the hypothesis that processing occurs at amino acid 21 for the first mature peptide. The third peptide (amino acids 61–146), named here DrmETH-AP, shows no sequence homology to other known neuropeptides found in extensive literature and computer database searches.

ETH-like peptides including DrmETH1 and DrmETH2 are well aligned with 33–82% identity (Fig. 1C). The amidated C-termini of these peptides have the consensus sequence –PRX-amide ($X = I, L, M, \text{ or } V$; Fig. 1C). Several insect and mollusk neuropeptides show identity with these C-terminal sequences [25] but with high divergence in overall sequence. Injection of other –PRXa-containing peptides such as leucopyrokinin and PBAN did not induce any ecdysis-related behaviors in pharate larvae of *Manduca* (Zitnan, unpublished), while low doses of DrmETH1 elicit robust pre-ecdysis behavior in the larval *Manduca*. Low doses of DrmETHs trigger premature eclosion in pharate adult *Drosophila*, as does the homologous peptide MasETH. Thus, only a subset of –PRXa-like peptides are able to trigger different phases of ecdysis behavioral sequence. These findings suggest that DrmETHs constitute ecdysis-triggering hormones homologous to ETHs previously described in two lepidopteran insects [4,5,26].

A putative DR-4 EcRE, consisting of an imperfect direct repeat of AGGTCA [21] in the upstream promoter of the *eth*, implies its expression could be under the control of steroid hormones, such as ecdysteroids. Indeed, expression of *eth* in *Manduca*, which contains a similar EcRE, is regulated by fluctuating ecdysteroid levels preceding each ecdysis [5]. Preliminary experiments indicate that the binding of the ecdysteroid receptor heterodimer (EcR and USP) to this EcRE occurs (Fillipov and Gill, unpublished).

Genes encoding multiple peptides are a common occurrence. Such peptides may have distinct functions as in the case of PBAN and diapause hormone [27]. In other cases, multiple peptides derived from one gene (e.g. FMRFamide) may have apparently redundant [28] or divergent actions, depending on the bioassay chosen [29]. We have found that DrmETH1, DrmETH2, and MasETH, function similarly in

pharate adult *Drosophila*, triggering the entire eclosion behavioral sequence consisting of head inflation (pre-eclosion) and head thrusting (eclosion). Thus, while it is tempting to speculate that DrmETH1 and DrmETH2 function as redundant triggers in adult eclosion of *Drosophila*, this would be in sharp contrast to the system described for *Manduca* larval ecdysis, where MasPETH and MasETH have distinct functions in the behavioral sequence leading to ecdysis. MasPETH induces pre-ecdysis I, whereas MasETH induces pre-ecdysis II and ecdysis behavior [5].

We demonstrated here that *Drosophila* and *Manduca* ETHs are reciprocally active in each species, providing further evidence that the endocrine control of ecdysis is conserved across a considerable evolutionary distance. However, drawing parallels between the two systems at this point is difficult. MasPETH has no effect in *Drosophila*, and DrmETH2 is inactive in *Manduca*. Furthermore, while MasETH triggers the entire behavioral sequence in *Drosophila*, DrmETH1 induces only pre-ecdysis I and II in *Manduca*. We emphasize that the present analysis deals with adult eclosion, whereas our previous studies in *Manduca* involved ecdysis of pharate larvae. Therefore, the differences observed may result from different life-stage physiologies. Further detailed analyses of *Drosophila* pre-ecdysis and ecdysis behaviors in larvae, pupae and adults are under way and may yet reveal distinct physiological actions of DrmETHs.

Our understanding of the specific endocrine events preceding *Drosophila* eclosion is at an early stage. However, a conceptual framework for ecdysis in *Manduca* has recently been elaborated in a series of papers [4–7,30] and serves as a starting point for similar model building in *Drosophila*. Pre-ecdysis and ecdysis in *Manduca* as they are presently understood involve four peptides: EH, PETH, ETH, and CCAP. Positive feedback between EH released from the CNS and ETH released from epitracheal glands leads to cGMP elevation in CCAP-containing neurons and consequent release of the CCAP within the CNS to initiate ecdysis. It is not yet clear whether this model can also explain the activation of eclosion behavior in *Drosophila*, since cGMP is not elevated in neurons producing CCAP [10] and a significant percentage of EH-cell knockout flies are able to undergo ecdysis [9].

Physiological studies using large lepidopteran insects have advanced our understanding of ecdysis behavior at cellular and molecular levels. Complementary studies in the *Drosophila* system using genetic tools promise to provide further progress to our understanding. Our discovery of the *Drosophila eth* provides a new tool for such studies.

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