Essential Role of Eclosion Hormone Precursor and Receptor Genes in Desert Locust Ecdysis

Lina Verbakel[1,2], Cynthia Lenaerts[1], Jerom Vranken[1], Elisabeth Marchal[1,3] and Jozef Vanden Broeck[1*]

[1]Molecular Developmental Physiology and Signal Transduction, Biology Department, KU Leuven, Naamsestraat 59 box 2465, B-3000 Leuven, Belgium.

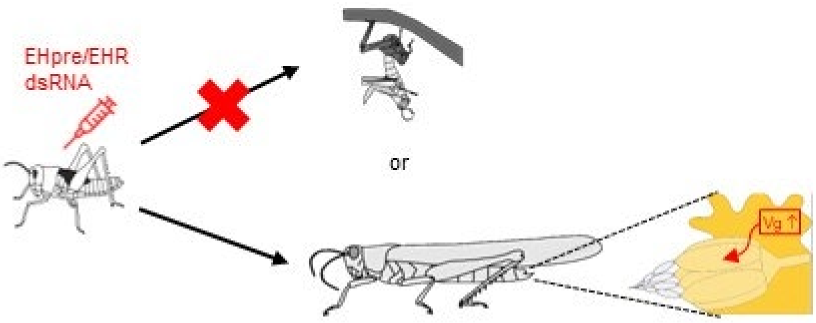
[2]Present address: Nagi Bioscience SA, EPFL Innovation Park (M), Rue des Jordils 1A 1025 Saint-Sulpice, Switzerland.

[3]Present address: Imec, Kapeldreef 75, B-3001 Leuven, Belgium

***Correspondence:** Jozef Vanden Broeck, Molecular Developmental Physiology and Signal Transduction, Biology Department, KU Leuven, Naamsestraat 59 box 2465, B-3000 Leuven, Belgium.

Email: jozef.vandenbroeck@kuleuven.be

Graphical Abstract



Abstract

The process of molting represents a critical phase in the life cycle of arthropods, marking periods of growth and development. Central to this process is the eclosion hormone (EH), a neurohormone that plays a pivotal role in initiating and regulating the complex sequence of events leading to successful molting in holometabolan species. Very little information is available in Hemimetabola, which display a different kind of development characterized by gradual changes. This paper reports on the identification of the two EH precursors and the EH receptor (EHR), a guanylyl cyclase, in a hemimetabolan pest species, the desert locust, *Schistocerca gregaria*. Using qRT-PCR, an in-depth profiling study of *Schgr*-EH-1, -2 and *Schgr*-EHR transcripts was performed. Silencing of *Schgr*-EH-1, -2 and *Schgr*-EHR resulted in lethality at the expected time of ecdysis, thereby showing their crucial role during this process.

Keywords

Hemimetabola, insect, knockdown, molt, neuropeptide, Eclosion Hormone

Highlights

-Identification of *Schgr*-EH and *Schgr*-EHR transcripts in the desert locust

-Expression profiling of *Schgr*-EH and *Schgr*-EHR transcripts in the desert locust

-*Schgr*-EH and *Schgr*-EHR are essential for ecdysis in *Schistocerca gregaria*

-Knockdown led to some nymphs molting into a supernumerary instar

-Knockdown led to some nymphs molting into adults with expedited vitellogenesis

1. Introduction

The success story of insects is in large part due to their exoskeleton, a robust armor that shields them from dehydration and other environmental threats, while also serving as a scaffold for muscle attachment. This exoskeleton, however, necessitates periodic shedding through molting cycles to accommodate growth, culminating in the critical phase of ecdysis, the shedding of the old cuticle. This transition is not merely a physical transformation but the result of a complex interplay of hormonal and neuropeptidergic signals ensuring the seamless progression vital for their survival[1–3].

Central to this orchestration is the Eclosion Hormone (EH), first identified as a pivotal factor in initiating eclosion in various lepidopteran species[4]. Subsequent studies expanded the existing knowledge of EH, revealing its presence and sequence in a range of insects, underscoring its ubiquity and importance across taxa[5–7]. EH's role was shown to be particularly pronounced during pupation in holometabolous species such as *Manduca sexta* and *Drosophila melanogaster*, where it triggers a surge in intracellular cyclic GMP (cGMP) levels[8]. Furthermore, two isoforms of an EH receptor (EHR) belonging to the receptor guanylyl cyclase family have been characterized in the Oriental fruit fly, *Bactrocera dorsalis*; *i.e.*, *Bd*mGC-1 and *Bd*mGC-1β[8,9].

The regulation of ecdysis is highly coordinated and involves not only EH but also other neuropeptides, such as the ecdysis-triggering hormone (ETH), as well as ecdysteroids, the arthropod steroid hormones. Ecdysteroids play a pivotal role in initiating molting cycles and orchestrating the downstream neuropeptide signaling cascade that prepares the insect for ecdysis. In *M. sexta*, ETH is produced by Inka cells, specialized glandular cells that are closely associated with the tracheal system, and acts in tandem with EH to trigger motor patterns required for shedding the old cuticle[1-3]. Binding of EH to its receptor on Inka cells has been shown to stimulate the release of ETH, which then activates central and peripheral neural circuits that drive the suite of ecdysis behaviors[10,11]. These interconnected pathways highlight the involvement of multiple tissues, including the central nervous system (CNS) and the tracheal system, in the regulation of ecdysis.

Despite the clear involvement of EH in the ecdysis of lepidopteran species, such as *Bombyx mori* and *M. sexta*, its role across different insect orders remains complex and varied. In coleopterans, such as *Tribolium castaneum*, EH signaling is crucial for ecdysis[12], and more recent studies in *Drosophila melanogaster* have confirmed that EH also plays a vital role in initiating ecdysis by priming the nervous system to respond to ETH[13]. However, earlier studies suggested a more nuanced role for EH in *D. melanogaster*, with some mutants displaying only minor behavioral deficits[1-3,14]. This variability highlights that, while EH is essential for ecdysis in all studied species, the extent of its functional contribution and its integration with other signaling pathways can differ significantly across insect taxa.

These findings illustrate the multifaceted nature of EH signaling in regulating ecdysis across the class of insects.

The current study extends the exploration of EH's role in ecdysis to the desert locust, *Schistocerca gregaria*, a hemimetabolan (orthopteran) species that stands out for its remarkable population density-dependent phenotypic plasticity and its devastating impact on agriculture caused by huge migrating swarms[15]. Here, we report on the molecular characterization of two EH precursors and a putative EH receptor in this locust species, shedding light on their sequence conservation across hemi- and holometabolan species. In addition, temporal profiling of EH precursor and receptor transcripts was performed at various time points throughout the fifth nymphal instar (N5). Finally, we demonstrate a crucial role for the *S. gregaria* EH signaling system in ecdysis of *S. gregaria* by utilizing RNA interference (RNAi). Our findings not only contribute to the broader understanding of ecdysis regulation but may also have potential implications for controlling locust outbreaks.

2. Materials and Methods

2.1. Insect rearing and tissue sampling

The desert locusts (*S. gregaria*) were reared under crowded conditions at a temperature of 30 ± 1 °C, with a relative humidity between 40-60% and a photoperiod of 14h. For gene expression analysis by qRT-PCR, tissues were sampled from female hoppers that were collected in the fifth nymphal instar and dissected at various time points: immediately after molting (N5D0), two and four days after the molt (N5D2 and N5D4, respectively), at an inter-wing distance of at least 1.8 mm (N5IW, which corresponds to the time of the ecdysteroid peak approximately three days before the adult molt), at the time of the weight peak (N5WP, corresponding approximately to two days before the adult molt), and at the time of the weight drop (N5WD, corresponding approximately to one day before the adult molt). These developmental time points were previously described in detail by Verbakel et al. (2021)[16]. Tissues were collected in 5 independent pools of 3 animals each.

2.2. RNA extraction, cDNA synthesis and qRT-PCR

RNA extraction and subsequent complementary DNA (cDNA) synthesis and quantitative real-time PCR (qRT-PCR) from brain, abdominal ganglia, and trachea tissues were performed as previously described[16]. In brief, lipid rich tissues such as brains and abdominal ganglia were extracted using the Qiagen® RNeasy Lipid Tissue Kit, including the DNase digestion protocol (RNase-Free DNase set, Qiagen), and trachea were extracted using the RNAqueous-Micro Kit (Ambion). Both kits were utilized according to manufacturer's protocol. cDNA was generated from 500 ng extracted RNA using the PrimeScriptTM reagent kit (Takara Bio Inc.) and further diluted ten-fold in sterile water (Milli-Q, Millipore). The transcript levels of the genes of interest were analyzed with qRT-PCR using the Fast

SYBR® Green Master Mix (Applied Biosystems) and oligonucleotide primers (Table S1), following the protocol previously described by Verbakel et al., (2021)[16]. Each primer pair that was utilized for qRT-PCR profiling of the *S. gregaria* EH precursor (*Schgr-EHpre-1* and *-2*) and receptor (*Schgr-EHR*) transcripts (Table S1) was validated as previously described[16]. However, it is important to note that the primers for detecting the *S. gregaria* EH precursor transcripts in this study were designed based on conserved sequence regions and, consequently, do not differentiate between the *Schgr-EHpre-1* and *Schgr-EHpre-2* isoforms. As a result, the expression levels reported represent the combined expression of both (very similar) isoforms. Amplification efficiency and specificity were confirmed by melting curve analysis, agarose gel electrophoresis and sequencing. All qRT-PCR results were calculated according to the comparative ΔΔCt method[17]. GraphPad Prism (GraphPad Software Inc. version 6.3) was used to plot and transform the data using the following equation: Y = log(Y), after which the statistical significance was tested. First normality was determined using the Shapiro-Wilk test. If $n<3$, normality was assumed. Finally, to evaluate the statistical significance, an ANOVA followed by a Dunnett's test or a student's t-test was used. When $n>3$, data were statistically evaluated by the non-parametric Mann-Whitney U test.

2.3. Molecular cloning of *Schgr-EHpre-1*, *Schgr-EHpre-2* and putative *Schgr-EHR*

The complete open reading frames (ORFs) of *Schgr-EHpre-1*, *Schgr-EHpre-2* and putative *Schgr-EHR*, were acquired by tBLASTn analysis using the most recently curated *S. gregaria* transcriptome and genome data[18] (transcript SCHGR_00013205, SCHGR_00000893 and SCHGR_00006115 from locus 104602-168818, 1311805-132056 and 557220-758965 of the seq7654, seq342 and seq7753 contig, respectively). To this end, the amino acid sequence of *M. sexta* EHpre (Genbank accession number: AAA29331.1) was used as a query for both *Schgr-EHpre*, *Schgr-EHpre-1* and *-2*, while the amino acid sequence of *B. dorsalis* GC-1β (Genbank accession number: ACQ90240.1) was used as a query for *Schgr-EHR*. Primers specific to the hit regions are depicted in Table S1. The resulting PCR amplicon sequences were verified by Sanger sequencing (LGC Genomics GmbH).

2.4. RNA interference

The *dsRNA* constructs that target either *Schgr-EHpre-1/-2* or *Schgr-EHR* were prepared according to the previously described approach[16]. In brief, oligonucleotide primers with a T7 promoter overhang (Table S1) were used to generate *dsRNA* constructs and the Ambion's MEGAscript® RNAi Kit, following the procedures described by Verbakel et al. (2021)[16]. The *dsRNA* constructs designed for targeting the *S. gregaria EH* precursor transcripts do not differentiate between the isoforms, as both *dsRNA* contructs target a conserved region common to both *Schgr-EHpre* transcripts. This means that any knockdown effects observed reflect the combined impact on both isoforms. The primers used to construct the different *dsRNA* constructs are listed in Table S1. Each construct was diluted (100 ng/µL) in *S. gregaria* saline ((1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose). The injection schemes were conducted in *S. gregaria* nymphs to elucidate the role of the EH(R) signaling system in ecdysis. Fourth instar nymphs (N4) received a first injection (4µL) at the day of the molt (N4D0). Boost injections were conducted at N4D3, N5D1 and N5D5. Nymphs were injected into the hemocoel with each respective construct or with a combination of *dsEH(pre/R)* constructs. Control locusts were injected with *dsGFP*. To test RNAi knockdown efficiency, tissues of five individual animals were collected at N5D4.

3. Results

3.1. The *Schgr*-EH sequences show sequence similarity to characterized insect EH sequences

The *Schgr-EHpre-1* and *-2* ORF sequences consist of, respectively, 261 and 252 nucleotides encoding prepropeptides of, respectively, 86 and 83 amino acids (Figure 1), with an N-terminal hydrophobic signal peptide of, respectively, 30 and 28 amino acids long, as predicted by SignalP-5.0[19]. A multiple sequence alignment reveals that both mature *Schgr*-EH peptide sequences show a high identity to characterized EH sequences of other insect species, including six highly conserved cysteine residues (Figure 1). The *Schgr*-EH1 sequence is 74.14% and 77.59% similar to *Drome*-EH and *Bacdo*-EH sequences, respectively, and 72.41% and 74.14% similar to *Manse*-EH and *Bommo*-EH sequences, respectively. The similarity between *Schgr*-EH2 sequence and *Drome*-EH, *Bacdo*-EH, *Manse*-EH and *Bommo*-EH sequences is 72.41%, 75.86%, 70.69% and 70,69%, respectively. The *Schgr*-EH1 and EH2 isoforms share a high degree of similarity, 89.28%. Due to this high level of sequence conservation, we were not able to design qRT-PCR primers nor dsRNA constructs that distinguish between the two isoforms, resulting in a combined measurement of their expression levels and effects.

3.2. The putative *Schgr*-EHR shows sequence similarity to the characterized EHRs of *B. dorsalis*

The open reading frame of the putative *Schgr*-EHR encodes a polypeptide of 1432 amino acids (Figure 2). Protein domain prediction identified the putative *Schgr*-EHR as a membrane guanylyl cyclase (mGC) with an extracellular ligand-binding domain (ECD; amino acids 32-492), a hydrophobic transmembrane region (TM; amino acids 488-510), a regulatory pseudokinase-homology domain (PKHD; amino acids 541–828), an adenylyl-/guanylyl cyclase catalytic domain c (CYCc; amino acids 861–1054) and a carboxyl-terminal extension of 378 residues. The alignment (Figure 2) to both *Bacdo*-EHR sequences, *Bd*mGC-1 and *Bd*mGC-1β, reveals the presence of conserved PTFARTXPPDTQ (amino acids 139-150) and AAYLYDAVXLYAXALXXVL (amino acids 350-372) motifs in the ECD, as well as high sequence similarity in the PKHD (58.03%) and CYCc (82.99%) domains. The overall sequence similarity of *Schgr*-EHR with *Bd*mGC-1 and *Bd*mGC-1β is 47.36% and 44.22%, respectively.

3.3. Temporal profiling of *Schgr*-EH precursor and receptor transcripts

In the brain, the levels of the *Schgr-EHpre* transcripts (Figure 3A) showed no significant differences between the timepoints analyzed throughout the fifth instar. Similarly, in the trachea, the expression levels of the putative EH receptor (*Schgr-EHR*) transcript remained consistent across the same timepoints, suggesting stable expression in this tissue during this developmental stage (Figure 3B). By contrast, a significant increase in the *Schgr-EHR* transcript levels was observed in the abdominal ganglia at N5WD compared to earlier timepoints during the intermolt phase, specifically N5D0 and N5D2 (Figure 3C).

3.4. Developmental effects of *Schgr-EH* precursor or receptor depletion

3.4.1. Knockdown efficiency

The knockdown efficiency of the *dsEHpre* (-*a* and -*b*) and *dsEHR* constructs was assessed. The *dsEHpre-a* construct significantly reduced the combined levels of both *Schgr-EHpre-1* and *Schgr-EHpre-2* transcripts in brain tissues dissected at N4D4 and N5D4 by 86 % and 83 %, respectively (Figure S1A, C). Likewise, the *dsEHpre-b* construct significantly reduced the combined levels both *Schgr-EHpre-1* and *Schgr-EHpre-2* transcripts in brain tissues dissected at N4D4 and N5D4 by 86 % and 87 %, respectively. While the *dsEHR* construct did not yet cause a knockdown in the trachea at N4D4, the *Schgr-EHR* transcript levels were significantly reduced by 40 % in the trachea at N5D4 (Figure S1B, D).

3.4.2. Ecdysis defects observed

Administering *dsEHpre-b* and/or *dsEHR* constructs through injections led to most nymphs failing to complete pre-ecdysis or ecdysis successfully. Approximately 22%, 2% and 31% of the *Schgr-EHpre-*, *Schgr-EHR-* and *Schgr-EH(pre/R)* depleted nymphs, respectively, were arrested during pre-ecdysis or ecdysis in the last nymphal-nymphal (N4-N5) molt, while the rest and the control *dsGFP*-injected nymphs successfully molted to the last nymphal instar (Figure 4A). At the nymphal-adult (N5-Ad) molt, another 58%, 78% and 53% of the *Schgr-EHpre-*, *Schgr-EHR-* and *Schgr-EH(pre/R)* depleted nymphs, respectively, were not able to successfully complete ecdysis, whereas the *dsGFP*-injected control nymphs all achieved successful N5-Ad molts (Figure 4A). The *Schgr-EH-*, *Schgr-EHR-* or *Schgr-EH(pre/R)* depleted nymphs attained a pinkish color right before the onset of ecdysis, which was also observed in *dsGFP*-injected control nymphs. However, in contrast to the control nymphs that successfully completed the ecdysis process, the *Schgr-EHpre* and/or *Schgr-EHR* depleted nymphs either darkened and died without completing ecdysis (Figure 4A) or showed developmental deficits with signs of physical weakening, such as reduced mobility and loss of postural control, before succumbing (Figure 4B). These phenotypes were consistently associated with developmental ecdysis deficits (Figure 4A and 4B). The rest of the *Schgr-EHpre-*, *Schgr-EHR-* and *Schgr-EH(pre/R)* depleted nymphs (20%, 20% and 16%, respectively) molted into instantly beige-yellowish colored adult locusts, while the newly ecdysed *dsGFP*-injected control locusts still had a normal, more pinkish appearance. The beige-yellowish *dsEHpre-b* and/or *dsEHR*-injected locusts displayed a similar physical body size and wings as the control fledglings (Figure 4C). It must, however, also be noted that the coloration of the *Schgr-EHpre* and/or *Schgr-EHR* depleted adult locusts was dissimilar from the (bright) yellow coloration observed in mature adult male locusts[20]. It is also worth mentioning that in a few rare cases *EHpre*/*EHR*-depleted nymphs molted into a supernumerary nymphal (N6) instar (Figure 4D).

Adult female locusts that developed from nymphs injected with *dsEHpre* or *dsEHR* already exhibited full vitellogenesis 10 days after the final molt, in contrast to the control group females, which were just initiating this process. Specifically, experimental females from the *Schgr-EHpre*- and *Schgr-EHR* RNAi treatment groups showed average oocyte lengths of 4.4 mm and 4.9 mm, respectively, compared to the average of 2.3 mm in the *dsGFP*-injected control group (Figure 5).

4. Discussion

Consistent with previously characterized EH peptides, the two predicted *Schgr*-EH peptides exhibit a conserved structure, featuring six highly conserved cysteine residues that are likely to form three intramolecular disulphide bonds. These bonds are posited to be crucial for the biological activity of EH[21,22]. Furthermore, computational predictions have identified two EH isoforms in various other insect species, including *Nilaparvata lugens* (the brown planthopper, Hemiptera), *Danaus plexippus* (the monarch butterfly, Lepidoptera), *Diaphorina citri* (the Asian citrus psyllid, Hemiptera), *Locusta migratoria* (the migratory locust, Orthoptera), and *Tribolium castaneum* (the red flour beetle, Coleoptera)[23]. The two putative *EHpre* isoforms have been identified in specific loci in the recently published genome sequence database of *S. gregaria*[18]. Some neuropeptide precursor genes have been shown to have undergone several duplications, including eclosion hormone. Noteworthy is the phylogenetic tree analysis conducted by Veenstra in 2014, which suggested that in some instances, such as in *L. migratoria* and *D. plexippus*, a relatively recent gene duplication event may have occurred, whereas in other species, gene duplication was inferred to have taken place much earlier[22].

One putative *EHR* was found in the *S. gregaria* genome database[18] and it exhibits several common characteristics with both characterized EH receptor isoforms of *B. dorsalis*[8,9]. Specifically, the putative *Schgr*-EHR shows all features of receptor guanylyl cyclases. Sequence alignment revealed two conserved cysteine residues (C_{111} and C_{130}) in their extracellular domain, which will probably form intrachain disulphide bonds that are critical for ligand-binding affinity of receptor guanylyl cyclases[24]. Moreover, the cysteine pairs ($C_{205,206}$ and $C_{212,213}$) are likely to play a role in mGC receptor dimerization through interchain disulphide bonds, as this phenomenon is known to increase ligand-binding affinity of mammalian guanylyl cyclases[25]. In addition, the two proposed conserved extracellular domain motifs might be indicative for crucial amino acid residues in ligand-receptor interaction and/or for structural configurations of the receptor. For example, the AAYLYDAVXLYAXALXXVL motif is hydrophobic, yet also contains some polar and charged amino acids, which is a prerequisite for the ligand-binding domain that often constitutes a hydrophobic-binding pocket that attracts and may bind the ligand through polar interactions[26]. The PTFARTXPPDTQ motif, on the other hand, is proline-rich and might play a role in the configuration of the receptor and influence its cellular signaling[27]. Confirmation of these suggestions would require more detailed structural analysis and mutagenesis studies, which is out of the scope for our research.

Like *Bd*mGC-1 and in contrast to *Bd*mGC-1β, the putative *Schgr*-EHR possesses a long and unique C-terminal tail with an unidentified function[8]. In mammalian sensory organ guanylyl cyclases, the prolonged C-terminal tail is thought to be involved in interactions with cytoplasmic proteins, such as the cytoskeleton[24]. Typically, the amino-terminal region of mammalian guanylyl cyclases contains a heme-binding domain and a carboxyl-terminal catalytic domain, which is also observed in the *Schgr*-EHR sequence. Their activation requires an intermediate messenger, such as nitric oxide (NO) or carbon monoxide (CO), which binds the heme group and may serve as a hub for receptor dimerization[24].

At present, it is unknown if the putative *Schgr*-EHR can indeed be activated by *Schgr*-EH-1 and/or -2, in a similar way as described for both *Bd*mGC-1 and *Bd*mGC-1β[8,9]. However, the presented evidence suggests important parallels between *Schgr*-EHR and the characterized *B. dorsalis* EH receptors, such as the pronounced sequence similarity of *Schgr*-EHR with *Bd*mGC-1[9] and the ecdysis defects that resulted from the functional knockdown.

This study showed that depletion of *Schgr-EHpre-1/-2* and/or *Schgr-EHR* transcripts resulted in severe suppression of (pre-)ecdysis during the incomplete metamorphosis of the vast majority of the experimentally treated locusts. EH has been implicated in the functional control of ecdysis and metamorphosis in multiple holometabolan insect species[1–3]. In addition, EH release from the neurohemal sites has been observed prior to ecdysis in both *M. sexta*[28] and *B. mori*[6,29]. Moreover, exposure of isolated CNS to *Manse*-EH induced the ecdysis motor program in *M. sexta*[2]. In addition, *D. melanogaster eh* null mutants failed to express pre-ecdysis and ecdysis behaviors[13]. However, by contrast, older literature in *D. melanogaster* stated that EH was solely vital for the execution of eclosion[1,2,14]. *Trica-EHpre* depleted *T. castaneum* also displayed severe weakening of pre-ecdysis and complete suppression of ecdysis[12].

In contrast to what has been reported in *D. melanogaster* (Diptera)[5] and *Heortia vitessoides* (Lepidoptera)[30], the transcript levels of *Schgr-EHpre-1/-2* did not dramatically increase prior to ecdysis in *S. gregaria* brain tissues, at least not at the measured time points. The transcript levels of the putative *Schgr-EHR* also remained relatively stable in the tracheal tissues, which house the Inka cells, specialized glandular cells responsible for production and secretion of the ecdysis-triggering hormone (ETH)[2], whereas in the abdominal ganglia a significant increase in *Schgr-EHR* transcript levels was observed prior to ecdysis. It has been shown that it is not peripheral, but central EH release that is responsible for EH's role in triggering the ecdysis motor program in *M. sexta*[31]. Moreover, as shown by Kruger et al. (2015), *eh* null mutants (*D. melanogaster*) experienced a diminished responsiveness to ETH in the 27/704 neurons that express crustacean cardioactive peptide (CCAP), which are critical for the downstream activation of the ecdysis motor program[13]. This indicated that, at least in *D. melanogaster*, EH is crucial for priming the nervous system to respond to ETH, with both EH and ETH together setting the stage for the initiation of ecdysis at the end of the molt.

Further exploration is warranted to assess a possible necessity of EH and its receptor during the N4-N5 molt in *S. gregaria*. Despite the initiation of *dsRNA* injections in fourth instar nymphs, a mere 20% of the *Schgr-EHpre* depleted nymphs were arrested at the N4-N5 molt, suggesting partial efficacy. Given that EH is a neuropeptide hormone, it is plausible that it accumulates in secretory vesicles located at the axon terminals of neurohemal organs or within other regions of the nervous system. In *M. sexta*, the expression of EH is constitutive, with the ventromedial (VM) neurons projecting their axons throughout the ventral nerve cord and terminating in neurohemal proctodeal nerves on the hindgut surface. These release sites, connected to the CNS via VM neuron projections, are critical for systemic coordination of ecdysis behaviors [1-3]. Analogously, in adult *D. melanogaster*, VM neurons have been shown to also project to the retro-cerebral complex[5], a major site of neurohemal release. Hence, it is conceivable that EH reserves were not yet sufficiently exhausted (by the RNAi-mediated knockdown) at the onset of N4-N5 ecdysis, possibly contributing to the observed molting outcomes.

Despite the substantial ecdysis-related deficits and nymphal lethality observed in the majority of the population following the depletion of *S. gregaria* EH and/or EHR, a subset of adults managed to emerge, albeit with notable abnormalities. Among these anomalies, the adult females that developed from nymphs injected with *dsEHpre*- and/or *dsEHR* exhibited expedited vitellogenesis and a beige-yellowish hue. A regulatory role in oogenesis for EH has been implicated in the crustacean *Eriocheir sinensis* (the chinese mitten crab)[32]. In addition, a comparative transcriptomic study has shown that EH transcripts are downregulated during ovarian maturation in the crustacean *Litopenaeus vannamei* (the Pacific white shrimp)[33]. Furthermore, the occurrence of supernumerary N6 nymphs, albeit rare, alongside the described phenotype, suggests a potential disruption in juvenile hormone (JH) signaling. JH is crucial for the reproductive physiology in adult locusts[34–36]. Notably, JH also induces the expression of the yellow protein, which is involved in the induction of yellow body color (yellowing) in

S. gregaria[35,37]. Under crowded conditions, yellowing is particularly pronounced in sexually mature male adults, but to a lesser extent also occurs in aged female adults[20,37] . In addition, JH's role in maintaining the developmental *status quo* in juvenile insects, by inhibiting metamorphosis, might provide an explanation for the emergence of supernumerary N6 nymphs.

5. Conclusion

In conclusion, the physiological data reported in this study support the existence of an important regulatory role for EH signaling in the initiation of the ecdysis sequence during the (incomplete) metamorphosis of *S. gregaria* and suggests a possible inhibitory role in ovarian maturation. This finding is imperative as the exact function of peptides in the ecdysteroid-induced neuropeptide cascade differs across species[1,2].

CRediT authorship contribution statement

Conceptualization: L.V., C.L., E.M. and J.V.B.; methodology: L.V., JV; validation: L.V. and J.V.B.; formal analysis: L.V.; investigation: L.V., J.V. and C.L.; data curation: L.V.; writing—original draft preparation: L.V. and J.V.B.; writing—review and editing: all authors; supervision: C.L., E.M. and J.V.B.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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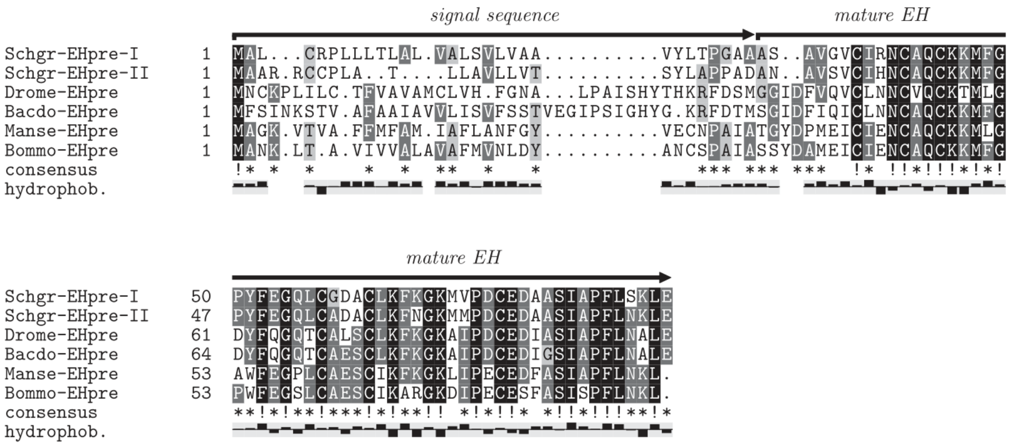
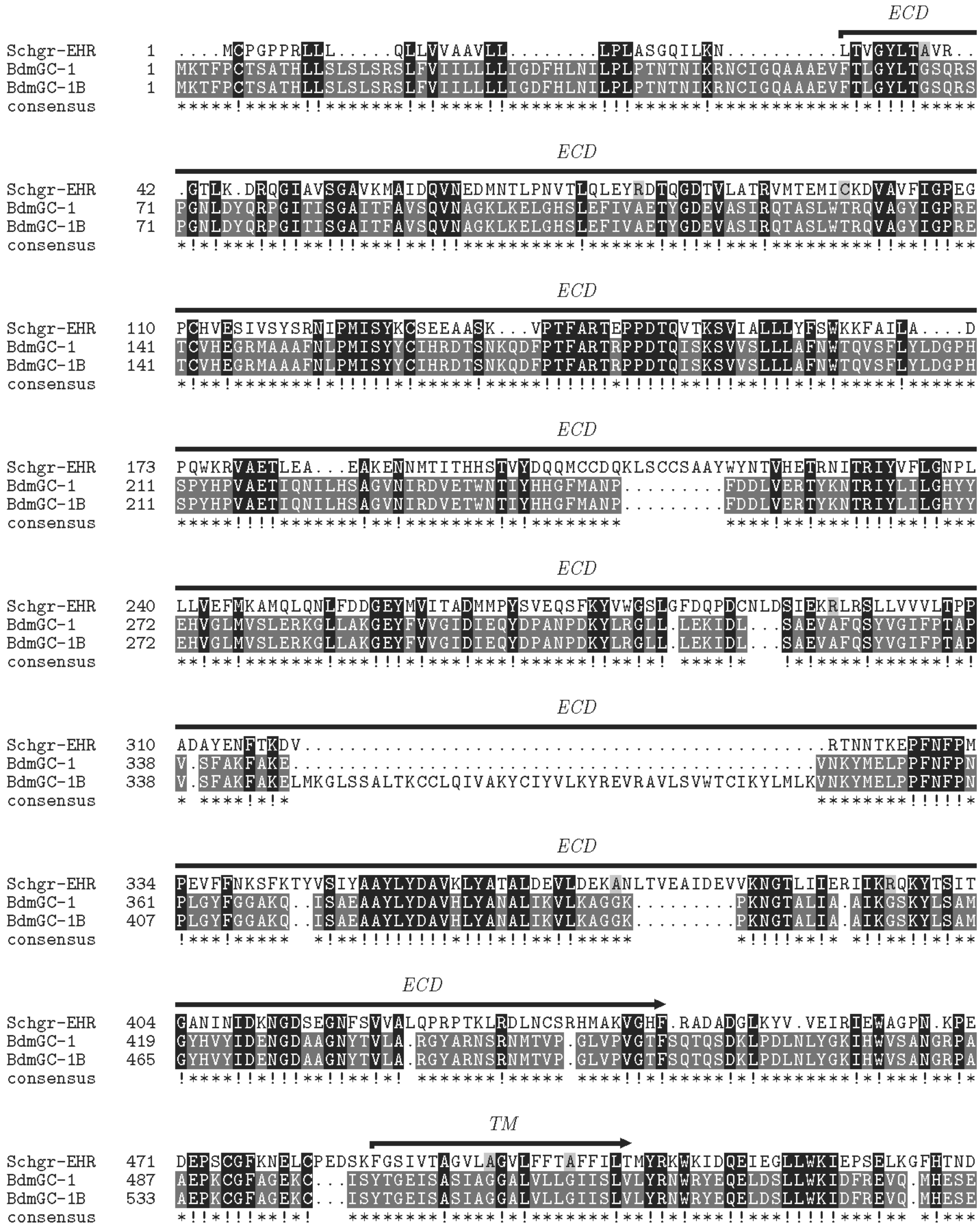
Figures and Figure legends

<u>Figure 1</u>:



Multiple sequence alignment of Eclosion Hormone precursors. The amino acid position is indicated at the left. Identical residues between the aligned sequences are highlighted in black and an exclamation mark (!), and conservatively substituted residues in grey and an asterisk (*). Dots indicate gaps that are introduced to maximize similarities in the alignment. Hydrophobicity of the amino acids is indicated by black bars. *Schgr* = *Schistocerca gregaria*, *Drome* = *Drosophila melanogaster* (Q07892.1), *Bacdo* = *Bactrocera dorsalis* (XP 011203182.1), *Manse* = *Manduca sexta* (P11919.2), *Bommo* = *Bombyx mori* (P25331.2).

Figure 2:


ECD
Schgr-EHR 1 ....MCPGPPRLLL.....QLLVVAAVLL........LPLASGQILKN..........LTVGYLTAVR..
BdmGC-1 1 MKTFPCTSATHLLSLSLSRSLFVIILLLLIGDFHLNILPLPTNTNIKRNCIGQAAAEVFTLGYLTGSQRS
BdmGC-1B 1 MKTFPCTSATHLLSLSLSRSLFVIILLLLIGDFHLNILPLPTNTNIKRNCIGQAAAEVFTLGYLTGSQRS
consensus *****!*****!!*******!*!****!!********!!!******!************!*!!!!*****
ECD
Schgr-EHR 42 .GTLK.DRQGIAVSGAVKMAIDQVNEDMNTLPNVTLQLEYRDTQGDTVLATRVMTEMICKDVAVFIGPEG
BdmGC-1 71 PGNLDYQRPGITISGAITFAVSQVNAGKLKELGHSLEFIVAETYGDEVASIRQTASLWTRQVAGYIGPRE
BdmGC-1B 71 PGNLDYQRPGITISGAITFAVSQVNAGKLKELGHSLEFIVAETYGDEVASIRQTASLWTRQVAGYIGPRE
consensus *!*!***!*!!**!!!***!**!!!**********!******!*!!*!***!*********!!**!!!**
ECD
Schgr-EHR 110 PCHVESIVSYSRNIPMISYKCSEEAASK...VPTFARTEPPDTQVTKSVIALLLYFSWKKFAILA....D
BdmGC-1 141 TCVHEGRMAAAFNLPMISYYCIHRDTSNKQDFPTFARTRPPDTQISKSVVSLLLAFNWTQVSFLYLDGPH
BdmGC-1B 141 TCVHEGRMAAAFNLPMISYYCIHRDTSNKQDFPTFARTRPPDTQISKSVVSLLLAFNWTQVSFLYLDGPH
consensus *!**!*******!*!!!!!*!*****!*****!!!!!!*!!!!!**!!!**!!!*!*!*****!******
ECD
Schgr-EHR 173 PQWKRVAETLEA...EAKENNMTITHHSTVYDQQMCCDQKLSCCSAAYWYNTVHETRNITRIYVFLGNPL
BdmGC-1 211 SPYHPVAETIQNILHSAGVNIRDVETWNTIYHHGFMANP.........FDDLVERTYKNTRIYLILGHYY
BdmGC-1B 211 SPYHPVAETIQNILHSAGVNIRDVETWNTIYHHGFMANP.........FDDLVERTYKNTRIYLILGHYY
consensus *****!!!!*******!**!********!*!******** ****!**!***!!!!**!!***
ECD
Schgr-EHR 240 LLVEFMKAMQLQNLFDDGEYMVITADMMPYSVEQSFKYVWGSLGFDQPDCNLDSIEKRLRSLLVVVLTPP
BdmGC-1 272 EHVGLMVSLERKGLLAKGEYFVVGIDIEQYDPANPDKYLRGLL.LEKIDL...SAEVAFQSYVGIFPTAP
BdmGC-1B 272 EHVGLMVSLERKGLLAKGEYFVVGIDIEQYDPANPDKYLRGLL.LEKIDL...SAEVAFQSYVGIFPTAP
consensus **!**!*******!***!!!*!***!***!******!!**!*! ****!* !*!****!******!*!
ECD
Schgr-EHR 310 ADAYENFTKDV..RTNNTKEPFNFPM
BdmGC-1 338 V.SFAKFAKE..VNKYMELPPFNFPN
BdmGC-1B 338 V.SFAKFAKELMKGLSSALTKCCLQIVAKYCIYVLKYREVRAVLSVWTCIKYLMLKVNKYMELPPFNFPN
consensus * ****!*!* ********!!!!!*
ECD
Schgr-EHR 334 PEVFFNKSFKTYVSIYAAYLYDAVKLYATALDEVLDEKANLTVEAIDEVVKNGTLIIERIIKRQKYTSIT
BdmGC-1 361 PLGYFGGAKQ..ISAEAAYLYDAVHLYANALIKVLKAGGK.........PKNGTALIA.AIKGSKYLSAM
BdmGC-1B 407 PLGYFGGAKQ..ISAEAAYLYDAVHLYANALIKVLKAGGK.........PKNGTALIA.AIKGSKYLSAM
consensus !***!***** *!**!!!!!!!!*!!!*!!**!!***** *!!!!**!* *!!**!!*!**
ECD
Schgr-EHR 404 GANINIDKNGDSEGNFSVVALQPRPTKLRDLNCSRHMAKVGHF.RADADGLKYV.VEIRIEWAGPN.KPE
BdmGC-1 419 GYHVYIDENGDAAGNYTVLA.RGYARNSRNMTVP.GLVPVGTFSQTQSDKLPDLNLYGKIHWVSANGRPA
BdmGC-1B 465 GYHVYIDENGDAAGNYTVLA.RGYARNSRNMTVP.GLVPVGTFSQTQSDKLPDLNLYGKIHWVSANGRPA
consensus !****!!*!!!**!!**!*! *******!***** ****!!*!*****!*!********!*!***!**!*
TM
Schgr-EHR 471 DEPSCGFKNELCPEDSKFGSIVTAGVLAGVLFFTAFFILTMYRKWKIDQEIEGLLWKIEPSELKGFHTND
BdmGC-1 487 AEPKCGFAGEKC...ISYTGEISASIAGGALVLLGIISLVLYRNWRYEQELDSLLWKIDFREVQ.MHESE
BdmGC-1B 533 AEPKCGFAGEKC...ISYTGEISASIAGGALVLLGIISLVLYRNWRYEQELDSLLWKIDFREVQ.MHESE
consensus *!!*!!!**!*! ********!****!*!*******!**!!*!***!!***!!!!!***!** *!***

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                                      PKHD
Schgr-EHR    541  IVSSPSKMS.....LVSATSFESKCGPQ.......WFATVGQYRNLRVRVKELKFSKKKDINRDIMKEMR
BdmGC-1      553  KEQSSQKQTRSTHPLIRTSQVSLSSNPDADFRYTTIFTPIGLYKNQLYAIKKVR.KKSVDITREMKKELK
BdmGC-1B     599  KEQSSQKQTRSTHPLIRTSQVSLSSNPDADFRYTTIFTPIGLYKNQLYAIKKVR.KKSVDITREMKKELK
consensus         ***!**!*******!***********!*********!***!*!*!*****!*** *!**!!*!***!!**

                                      PKHD
Schgr-EHR    599  ILRDLRHDNVNSFIGACIEPMRILLITDYCAKGSLYDIVENEDIKLDMMFIASLVFDLIKGMIYLHKSAL
BdmGC-1      622  LLRDTRHDNICAFIGACTDPPNICIITEYCTRGSLKDILENEDVKLDNMFIASMVADIIRGVIYLHESPI
BdmGC-1B     668  LLRDTRHDNICAFIGACTDPPNICIITEYCTRGSLKDILENEDVKLDNMFIASMVADIIRGVIYLHESPI
consensus         *!!!*!!!!***!!!!!**!**!**!!*!!**!!!*!!*!!!!*!!!*!!!!!*!*!*!*!*!!!!!*!**

                                      PKHD
Schgr-EHR    669  GCHGNLKSSNCVVTSRWVLQITDFGLHELRQCAEGDSIGEHQHYRSC...FWKAPELLRDDTA..IGGTQ
BdmGC-1      692  RFHGSLCTSNCLVDSRWVVKLTDFGLFAFKKGMEDNSTEVPNMTVKCTKLLYRAPELLRLGAASLVQGTQ
BdmGC-1B     738  RFHGSLCTSNCLVDSRWVVKLTDFGLFAFKKGMEDNSTEVPNMTVKCTKLLYRAPELLRLGAASLVQGTQ
consensus         **!!*!**!!!*!*!!!!***!!!!!*******!**!*********!******!!!!!!***!****!!!

                                      PKHD
Schgr-EHR    734  KGDVYAFAIILYEIIGRRGPYGGCPYEPKEIVELVKRVPSEGEKPFRPDLAVLDESDIDCTDYIVKCIED
BdmGC-1      762  KGDSYSFGIVLYEIHVRHGPFGETGLTPMQCLQKVLQ.PQDHLYPYRPSLQPLE.TSFDCVREILR...E
BdmGC-1B     808  KGDSYSFGIVLYEIHVRHGPFGETGLTPMQCLQKVLQ.PQDHLYPYRPSLQPLE.TSFDCVREILR...E
consensus         !!!*!*!*!*!!!!**!*!!*!*****!******!** !*****!*!!*!**!* ***!!***!**   *

                        PKHD                                          CYCc
Schgr-EHR    804  CWEENPEHRPDFPTIRNRLRKMREGMHSNIMDQMMEIMEKHANNLEYLVSERTRLLFEEKRKTEDLLHRM
BdmGC-1      827  CWSERPEERPDFKTIRAKLRPLRKGMKPNIFDNMMAMMEKYANNLEALVDERTDQLQEEKKKTDALLLEM
BdmGC-1B     873  CWSERPEERPDFKTIRAKLRPLRKGMKPNIFDNMMAMMEKYANNLEALVDERTDQLQEEKKKTDALLLEM
consensus         !!*!*!!*!!!!*!!!**!!**!*!!**!!*!*!!**!!*!!!!!*!!*!!!**!*!!!*!!**!!**!

                                      CYCc
Schgr-EHR    874  LPAPVAERLTKGFGVEPESFDLVTIYFSDIVGFTAMSAESTPLQVVNFLNDLYTLFDRIIRGYDVYKVET
BdmGC-1      897  LPRPVAEQLKKGNKVDPENYEQVSIYFSDIVGFTAMSAESTPLQVVDFLNDLYTCFDSIIGHYDVYKVET
BdmGC-1B     943  LPRPVAEQLKKGNKVDPENYEQVSIYFSDIVGFTAMSAESTPLQVVDFLNDLYTCFDSIIGHYDVYKVET
consensus         !!*!!!!*!*!!**!*!!****!*!!!!!!!!!!!!!!!!!!!!!!!*!!!!!!!*!!*!!**!!!!!!!

                                      CYCc
Schgr-EHR    944  IGDAYMVVSGLPLRNGDRHAGEIASMSLELLEAVKHHQIAHRPNDVLKLRIGVHTGPVVAGVVGLTMPRY
BdmGC-1      967  IGDAYMVVSGLPIRNGDLHAAEIASMSLHLLSAVSEFKIRHRPTNKLLLRIGIHTGPVCAGVVGLKMPRY
BdmGC-1B    1013  IGDAYMVVSGLPIRNGDLHAAEIASMSLHLLSAVSEFKIRHRPTNKLLLRIGIHTGPVCAGVVGLKMPRY
consensus         !!!!!!!!!!!!*!!!!*!!*!!!!!!!*!!*!!****!*!!!***!*!!!!*!!!!!*!!!!!!*!!!!

                               CYCc
Schgr-EHR   1014  CLFGDTVNTASRMESNGEPLKIHISMQCHEALEKLGGYIIEERGMVYMKGKGEVRTFWLVGATDKAIQKR
BdmGC-1     1037  CLFGDTVNTASRMESSGVPLKIHCSQQCRELLEKLGGYHYQERGLISMKGKGDQRTYWLLGEDEDARNKR
BdmGC-1B    1083  CLFGDTVNTASRMESSGVPLKIHCSQQCRELLEKLGGYHYQERGLISMKGKGDQRTYWLLGEDEDARNKR
consensus         !!!!!!!!!!!!!!!*!*!!!!!*!*!!*!*!!!!!!!***!!!***!!!!!**!!*!!*!****!**!!

Schgr-EHR   1084  EVDLTELPPLFCRPRRSPKMNTDSRQPSICGGFGPGSRRQSSIPRENSLDVESASTLRAFAVCNAANNSS
BdmGC-1     1107  NYER............SQRRGSRALNKFI..........QGTIKQNNQQQITSA................
BdmGC-1B    1153  NYER............SQRRGSRALNKFI..........QGTIKQNNQQQITSA................
consensus         ****            !***********!          !**!***!*****!!

Schgr-EHR   1154  PVANHSQRSGSKRAELSPLYINDVSESRTTLS.ASALESQSVLSVEDCNENCVNKTSINQSQRALSTVET
BdmGC-1     1139  .VVDYGIRSSLKNKGMP..........RNSLTRSSSLESPKKLRFATGSLLEH...HRHHSDEALLEVIT
BdmGC-1B    1185  .VVDYGIRSSLKNKGMP..........RNSLTRSSSLEFF..............................
consensus          !*****!!**!*****          !**!***!*!!*   *                 *  **  * *
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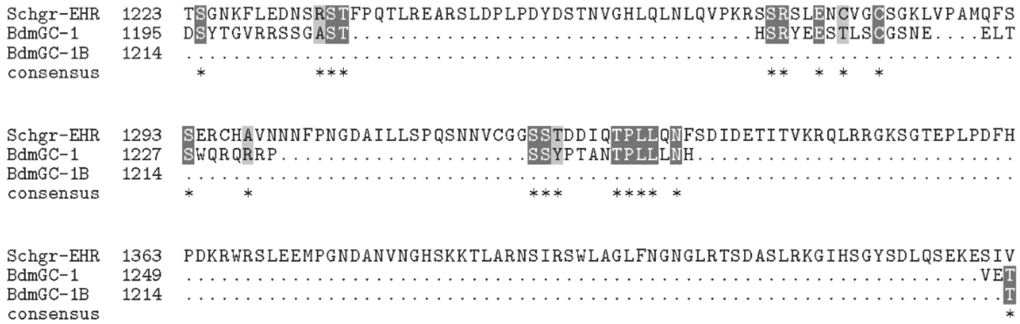
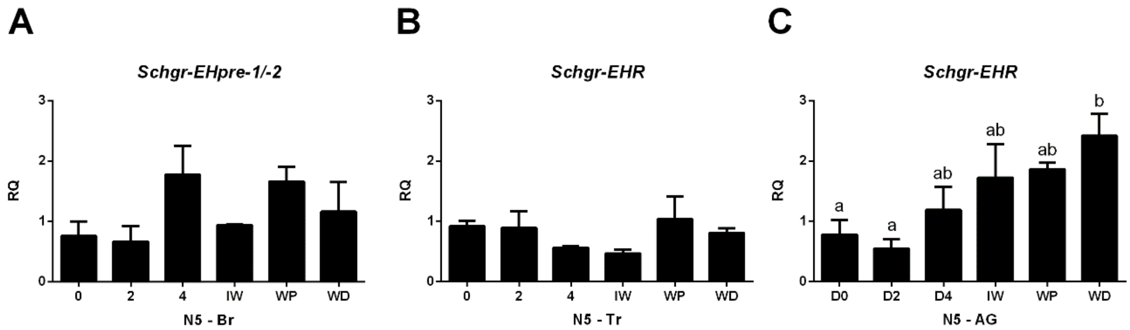


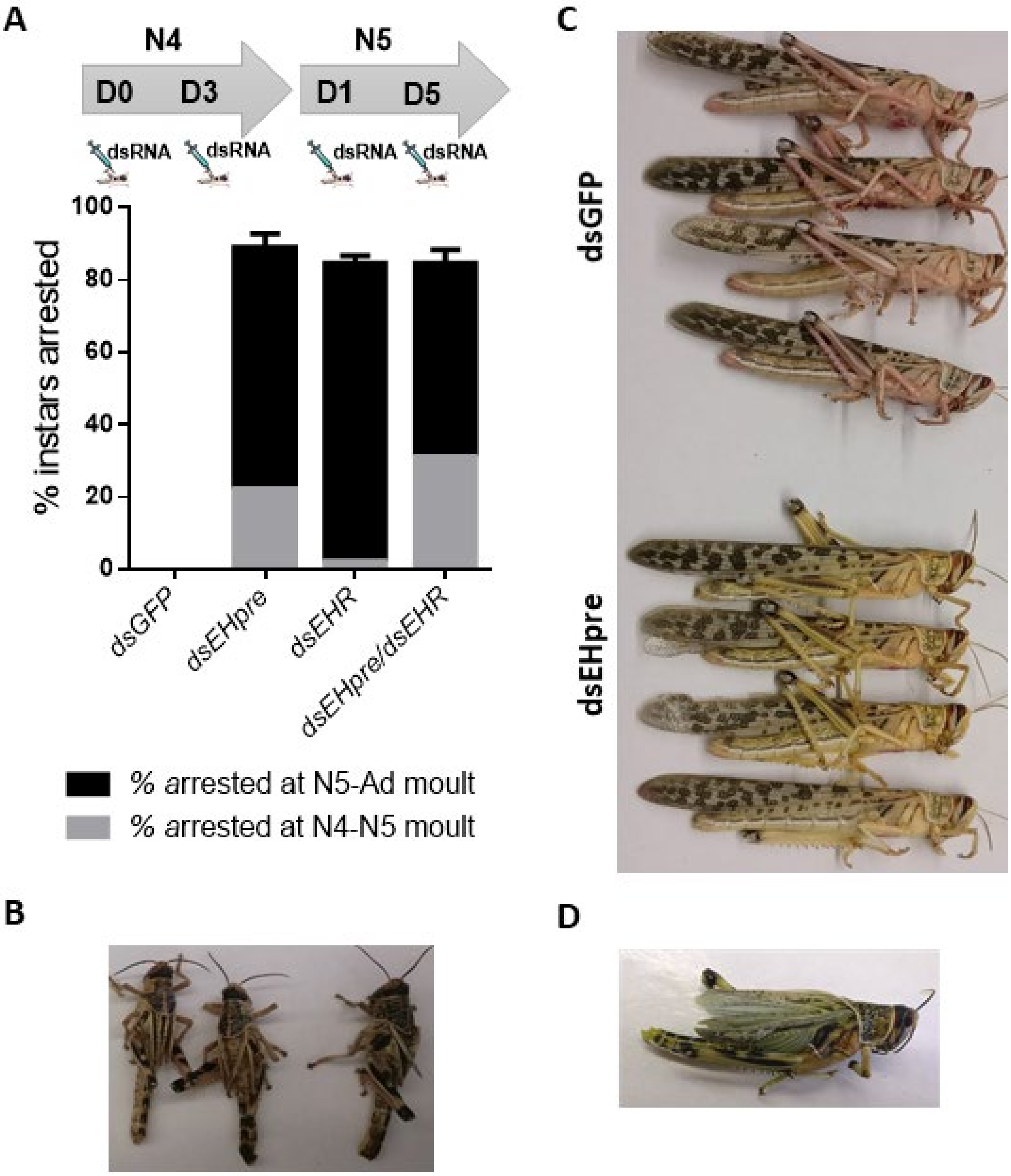
Sequence alignment of the putative *Schgr*-EHR with the functionally characterized *Bacdo*-EHR. The amino acid position is indicated at the left. Identical residues between the aligned sequences are highlighted in black and an exclamation mark (!), and conservatively substituted residues in grey and an asterisk (*). Dots indicate gaps that are introduced to maximize similarities in the alignment. Abbreviations: *Schgr* = *Schistocerca gregaria*, *Bd*mGC-1(B) = *Bactrocera dorsalis* membrane guanylyl cyclase 1(β) (AY125819.1 and ACQ90240.1), ECD = extracellular ligand-binding domain, TM = transmembrane segment, PKHD = Pseudokinase homology domain, CYCc = Adenylyl-/ guanylyl cyclase, catalytic domain type c.

Figure 3:



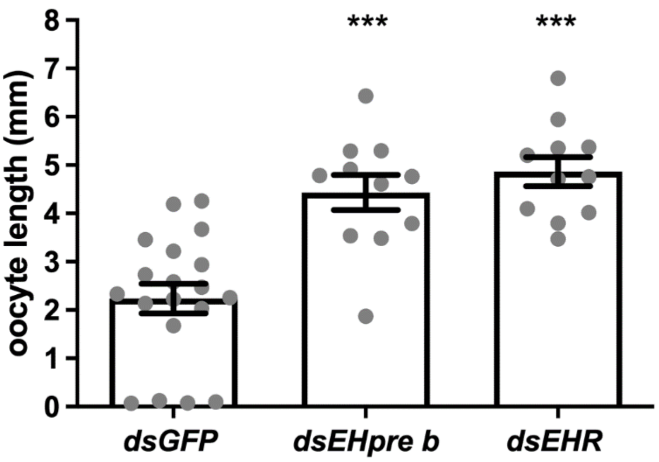
Temporal expression profile of *Schgr-EHpre-1/-2* and *Schgr-EHR* in the fifth nymphal instar (N5) of *S. gregaria* nymphs. Using qRT-PCR, (**A**) the relative transcript levels of *Schgr-EHpre-1/-2* were measured in the brain and the relative *Schgr-EHR* transcript levels were measured, respectively, in (**B**) trachea and (**C**) abdominal ganglia, at different time points during N5. The data represent means ± S.E.M. of five independent pools of three nymphs, run in duplicate and normalized to the expression of reference genes: *Schgr-EHpre* transcript levels were normalized to *RP-49* and *GAPDH* transcript levels, while *Schgr-EHR* transcript levels were normalized to *β-Actin*, *CG13220* and *GAPDH* transcript levels. Significant differences were calculated by One-way ANOVA, followed by a Dunnett's Test. Abbreviations: *Schgr = Schistocerca gregaria*, *EHpre* = *Eclosion hormone precursor*, *EHR* = *Eclosion hormone receptor*, IW = inter-wing distance of at least 1.8 mm, WP = weight peak, WD = weight decrease, RQ = relative quantity.

Figure 4:



Phenotypic effects of *dsEHpre*and/or *dsEHR* injections in *Schistocerca gregaria*. A) Percentage of nymphs arrested at the last nymphal-nymphal (N4-N5) and at the nymphal-adult (N5-Ad) molt. Phenotypes: B) Nymphs arrested at ecdysis, C) Yellowed coloration in *dsEHpre*-injected adults at AdD1, D) Supernumerary sixth instar nymph.

Figure 5:



Effect of *Schgr-EHpre* or *Schgr-EHR* depletion on oocyte size at 10-day-after eclosion. Significant differences (p<0.05, p<0.1 and p<0.001) are indicated by asterisks (*, ** and ****, respectively) and were calculated by One-way ANOVA, followed by a Dunnett's Test.}

Supplementary Figure

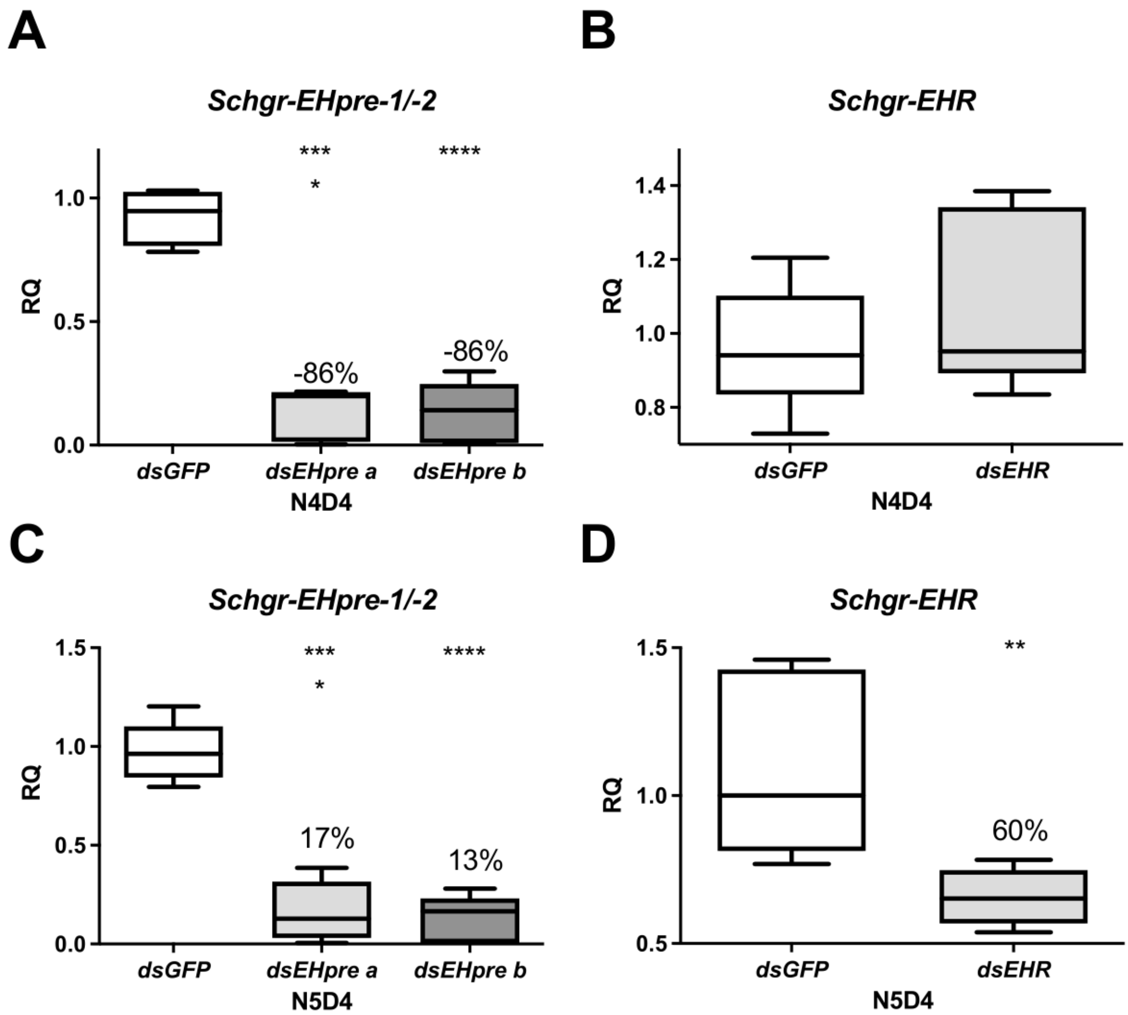


Figure S1:

Relative quantities of *Schgr-EHpre-1/-2* and *Schgr-EHR* transcripts in brain and trachea, respectively, as measured in *S. gregaria* nymphs at N4D4 and N5D4. *dsGFP* and *dsEHpre* or *dsEHR* were injected at N4D0. Boost injections were administered at N4D4, N5D1 and N5D5. A) RNAi-mediated knockdown caused by administering *dsEHpre-a* or *-b* constructs on *Schgr-EHpre-1/-2* transcript levels at N4D4. B) RNAi-mediated knockdown caused by administering *dsEHR* on *Schgr-EHR* transcript levels at N4D4. C) RNAi-mediated knockdown caused by administering *dsEHpre-a* or *-b* constructs on *Schgr-EHpre-1/-2* transcript levels at N5D4. D) RNAi-mediated knockdown efficiency caused by administering *dsEHR* on *Schgr-EHR* transcript levels at N5D4. Significant differences ($p<0.01$ and $p<0.0001$) are indicated by asterisks (** and ****, respectively) and were calculated by One-way ANOVA, followed by a Dunnett's Test.

Supplementary Table

Table S1: Oligonucleotide sequences for primers used in cloning and sequence analysis of *Schgr-EHpre-1*, *Schgr-EHpre-2* and *Schgr-EHR*, for primers used for dsRNA construct creation and for primers used in qRT-PCR

cloning	**Forward primer**	**Reverse primer**
Schgr-EHpre-1	CGGCGATCCTCTCCTGTAGT	GCAGACGGACTTTATTGAGCC
Schgr-EHpre-1_nested	GTAGTAGACATGGCCCTCTGC	CGGCTACTCGAGTTTGCTGA
Schgr-EHpre-2	CCACCAGCACCACTTCACTA	CACGAGGGAGAGCAGGTAAC
Schgr-EHpre-2_nested	CTCTCTTCACACAGAGATGGCCG	CTACTCGAGCTTGTTGAGGAGGG
Schgr-EHR	GTTCCAGACTGTAGCGCT	CATTCACCTGCAGATGGTGT
Schgr-EHR_nested	TCCACCATCCAGACAGTAGAC	GTTAAGATACTACACTACCTATTGGT
dsRNA	**Forward primer**	**Reverse primer**
dsEHR	TAATACGACTCACTATAGGGAGAAGTTGTGCTAACCCCACCTG	TAATACGACTCACTATAGGGAGATTTGCTCCTGTGATGCTCGT
dsEHpre-a	TAATACGACTCACTATAGGGAGAATGGCCCTCTGCCGTCC	TAATACGACTCACTATAGGGAGAACGGCCCGACATCTTCTTA
dsEHpre-b	TAATACGACTCACTATAGGGAGAATGGCCGCCCGCCGCTG	TAATACGACTCACTATAGGGAGAGCGTTGGCGTCCGCTGGCG
dsGFP	TAATACGACTCACTATAGGGAGAAAGGTGATGCTACATACGGAA	TAATACGACTCACTATAGGGAGAATCCCAGCAGCAGTTACAAAC
qRT-PCR	**Forward primer**	**Reverse primer**
Schgr-EHpre-1	TGGTGGCAGCAGTCTACCT	ACAGCTGGCCCTCGAAATAC
Schgr-EHpre-2	AGCGTCTGCATTCACAACTG	CTCGAGCTTGTTGAGGAAGG
Schgr-EHR-1	TTGCATGAGCTTAGGCAGTG	TGGAGGATGCCCTTATGAAC
Schgr-EHR-2	ACAACCTATTCCGCGAACCT	GTGCCTGAAGACAACCTTTGA

Schgr-RP49	CGCTACAAGAAGCTTATGAGGTCAT	CCTACGGCGCACTTCTGTTG
Schgr-β-Actin	AATTACCATTGGTAACGAGCGATT	TGCTTCCATACCCAGGAATGA
Schgr-GAPDH	GTCTGATGACAACAGTGCAT	GTCCATCACGCCACAACTTTC
Schgr-CG13220	TGTTCAGTTTTGGCTGTTCTGA	ACTGTTCTCCGGCAGAATGC

Abbreviations : *Schgr* = *Schistocerca gregaria*, *EHpre* = Eclosion hormone precursor, *EHR* = Eclosion hormone receptor, RP49 = ribosomal protein 49, GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.