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RESEARCH ARTICLE

The role of neuropeptides in regulating ecdysis and reproduction in the hemimetabolous insect *Rhodnius prolixus*

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ABSTRACT

In ecdysozoan animals, moulting entails the production of a new exoskeleton and shedding of the old one during ecdysis. It is induced by a pulse of ecdysone that regulates the expression of different hormonal receptors and activates a peptide-mediated signalling cascade. In Holometabola, the peptidergic cascade regulating ecdysis has been well described. However, very little functional information regarding the neuroendocrine regulation of ecdysis is available for Hemimetabola, which display an incomplete metamorphosis. We use *Rhodnius prolixus* as a convenient experimental model to test two hypotheses: (1) the role of neuropeptides that regulate ecdysis in Holometabola is conserved in hemimetabolous insects; and (2) the neuropeptides regulating ecdysis play a role in the regulation of female reproduction during the adult stage. The RNA interference-mediated reduction of ecdysis triggering hormone (ETH) mRNA levels in fourth-instar nymphs resulted in lethality at the expected time of ecdysis. Unlike in holometabolous insects, knockdown of *eth* and *orcokinin isoform A* (*oka*) did not affect oviposition in adult females, pointing to a different endocrine regulation of ovary maturation. However, *eth* knockdown prevented egg hatching. The blockage of egg hatching appears to be a consequence of embryonic ecdysis failure. Most of the first-instar nymphs hatched from the eggs laid by females injected with dsRNA for eclosion hormone (dsEH), crustacean cardioactive peptide (dsCCAP) and dsOKA died at the expected time of ecdysis, indicating the crucial involvement of these genes in post-embryonic development. No phenotypes were observed upon corazonin (*cz*) knockdown in nymphs or adult females. The results are relevant for evolutionary entomology and could reveal targets for neuropeptide-based pest control tools.

KEY WORDS: Evolution, Metamorphosis, Holometabola, Ametabola, Hormonal regulation, Moulting, Egg hatching, Ecdysis triggering hormone, Eclosion hormone, Crustacean cardioactive peptide, Corazonin, Orcokinin

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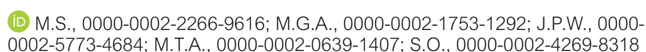
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INTRODUCTION

Insects are a predominant life form on Earth; different species are adapted to every terrestrial ecological niche, from deserts to glaciers (Chown and Nicolson, 2004). This spectacular adaptability is due to their particular developmental, physiological and reproductive strategies. The exoskeleton, for example, is an excellent adaptation to protect insects from desiccation and physical damage but imposes constrictions on post-embryonic development. To grow and develop, insects must shed the old cuticle and emerge as the following instar during ecdysis (Zitnan and Adams, 2012). Moulting involves the synthesis of a new cuticle underneath the old one, the digestion and resorption of most of the old cuticle during pre-ecdysis, and the shedding of the old cuticle during ecdysis. Finally, during post-ecdysis, the cuticle of recently emerged insects becomes dark and rigid (sclerotized) as a result of melanin deposition and protein cross-linking. Failure to complete ecdysis will be deleterious to the individual, making the regulation of this process an excellent target in the search for new insect pest management strategies (Ewer, 2005; White and Ewer, 2014; Žitňan et al., 2003, 2007).

Ecdysis is tightly regulated by the neuroendocrine system, i.e. neuropeptides and their receptors; this regulation has been well studied in species with a complete metamorphosis, called holometabolous insects. The core components of this network are the neuropeptides corazonin (CZ), eclosion hormone (EH), ecdysis triggering hormone (ETH) and crustacean cardioactive peptides (CCAP) (Zitnan and Adams, 2012). The accepted model proposes that a peak of ecdysone concentration in haemolymph controls the expression and release of neuropeptides that regulate ecdysis (Kim et al., 2004, 2006a,b; Kingan et al., 1997; Kruger et al., 2015; Mena et al., 2016; Žitňan et al., 1999, 2003). This model of ecdysis regulation is based on studies performed mainly in *Drosophila melanogaster* (Diptera), *Tribolium castaneum* (Coleoptera), *Manduca sexta* and *Bombyx mori* (Lepidoptera) (Zitnan and Adams, 2012). However, ecdysis regulation has been scarcely studied in Hemimetabola, a group comprising numerous species of economic and sanitary relevance. As Holometabola and Hemimetabola differ in their post-embryonic development, differences in ecdysis regulation can be hypothesized. Whereas some studies indicated conservation in the neuropeptides regulating ecdysis throughout the class Insecta (Lee et al., 2013; Lenaerts et al., 2017; Shi et al., 2022; Verbakel et al., 2021), other reports point to differences in hormonal control of this process in holometabolous and hemimetabolous insects (Silva et al., 2021; Wulff et al., 2017, 2018).

The expression of neuropeptides involved in ecdysis regulation persists in the adult stage when this process does not occur, suggesting an involvement of these neuropeptides in processes such as reproduction. In this sense, an allatotropic role necessary for ovary maturation has been demonstrated for ETH in dipteran species

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(Areiza et al., 2014; Meiselman et al., 2017; Shi et al., 2019). Furthermore, ETH receptors (ETHR) are involved in male courtship behaviour in *D. melanogaster* (Deshpande et al., 2019).

Rhodnius prolixus is an excellent insect model for studying development and reproduction (Ons, 2017). It has been used as an experimental model for insect physiology since the pioneering work in the field by Sir Vincent Wigglesworth (1934, 1953, 1939). This species is also of sanitary relevance as a vector of *Trypanosoma cruzi*, the causative agent of Chagas' disease. In the unfed condition, *R. prolixus* remains in a state of arrested development; gorging on a blood meal initiates the physiological and endocrinological events leading to ecdysis in nymphs and reproduction in adults a predictable number of days later. Thus, these events can be precisely timed during experiments and studied accurately. Furthermore, gene silencing through RNA interference (RNAi) is highly effective in this species, long-persistent and vertically transmitted from a treated female to the offspring (Paim et al., 2013; Sterkel and Oliveira, 2017; Sterkel et al., 2019). In this work, we used *Rhodnius prolixus* to test two hypotheses: (1) the role of neuropeptides that regulate ecdysis in Holometabola is conserved in Hemimetabola; and (2) the neuropeptides regulating ecdysis play a role in the regulation of female reproduction during the adult stage. We obtained evidence favouring the first hypothesis; conversely, the role of ecdysis-related neuropeptides as ovary maturation factors was not observed, pointing to a difference in the regulation of reproduction between holometabolous and hemimetabolous insects.

MATERIALS AND METHODS

Insect rearing

A colony of *Rhodnius prolixus* Stål 1859 was maintained in our laboratory under a 12 h light:12 h dark photoperiod at 28°C and 50–60% humidity. Once a month, the insects were fed on chickens, which were housed, cared for, fed and handled following Resolution 1047/2005 (National Council of Scientific and Technical Research, CONICET) regarding the National Reference Ethical Framework for Biomedical Research with Laboratory, Farm and Nature Collected Animals. This framework is in concordance with the standard procedures of the Office of Laboratory Animal Welfare, Department of Health and Human Services, NIH/National Institutes of Health, and the recommendations established in the 2010/63/EU Directive of the European Parliament related to the protection of animals used for scientific purposes. Biosecurity considerations are in agreement with CONICET Resolution 1619/2008, which is in concordance with the WHO Biosecurity Handbook (ISBN 92 4 354 6503).

RNA isolation and cDNA synthesis

Total RNA was extracted from first- and fourth-instar nymphs (whole body) using TRIzol reagent (Ambion) according to the manufacturer's instructions. Following treatment with DNase I (Promega), first-strand cDNA was synthesized using 1 µg total RNA with MMLV Reverse Transcription Kit (Promega) and poly-T primer, according to the manufacturer's instructions.

Quantitative PCR

Forward and reverse quantitative PCR (qPCR) primers were designed in different exons to prevent the amplification of genomic DNA that could be present in the sample despite the treatment with DNase I, except for the *eh* gene, which is composed of one exon. The qPCR primers were also designed to amplify a different region of the gene to that amplified by the primers used for double-stranded RNA (dsRNA) synthesis as the injected dsRNA could be retrotranscribed into cDNA and introduce errors in endogenous mRNA quantification. The only exception was the *eh* gene as it was not possible to design efficient primers outside the region amplified for dsRNA synthesis. All primer pairs used for qPCRs were tested for dimerization, efficiency and amplification of a single product. Only primer pairs with efficiency between 80% and 120% were used (Table 1). The cDNA levels were quantified using FastStart SYBR Green Master Mix (Roche) in an Agilent AriaMx Real-Time PCR instrument (Applied Biosystems). The schedule used for the amplifying reaction was as follows: (i) 95°C for 3 min; (ii) 95°C for 15 s; (iii) 56°C for 15 s; (iv) 72°C for 30 s. Steps ii–iv were repeated for 40 cycles. Controls without a template were included in all batches. *Elongation factor 1* (*ef-1*) and *tubulin* (*tub*) were used as housekeeping genes as they were previously validated as stable genes in *R. prolixus* (Majerowicz et al., 2011; Omondi et al., 2015; Paim et al., 2012). The $2e^{-\Delta CT}$ values obtained for treated and control groups were used to evaluate the relative levels of target mRNAs (Livak and Schmittgen, 2001).

Synthesis of dsRNA

Specific primers for each target gene were designed (Table 1). The primers for RNAi experiments were designed to knock down all isoforms of each gene, given that three isoforms for *ccap* and two isoforms for *cz* genes have been described (Ons et al., 2011). Three isoforms were described for the *orcokinin* (*ok*) gene that encodes different families of peptides (Sterkel et al., 2012), but RNAi primers were designed to knock down only isoform A (*oka*) as only these peptides affect ecdysis in *R. prolixus* (Wulff et al., 2017). A fragment from the *β-lactamase* gene (*β-lac*), absent in the *R. prolixus* genome, was PCR amplified from the pBluescript plasmid and used as a control to assess the putative unspecific effects of dsRNA injections. The designed primers contained the T7 polymerase binding sequence at the 5′ end required for dsRNA synthesis. All PCR products were sequenced (Macrogen, Seoul, Korea) to confirm their identity. Secondary PCR was carried out using 1 µl of the PCR product and a T7 full promoter primer (Table 1). The dsRNAs were *in vitro* transcribed using a T7-RNA polymerase (Promega) according to the manufacturer's instructions. The dsRNAs were precipitated with isopropanol and resuspended in ultrapure sterile water (Milli-Q). The dsRNAs were visualized by agarose gel (1.5% w/v) electrophoresis to verify size, integrity and purity, and quantified from images of the gels using the software ImageJ (National Institutes of Health, Bethesda, MD, USA). The dsRNAs were stored at −20°C until use.

RNAi

Fourth-instar nymphs and adult *R. prolixus* females were injected in the thorax with 2 µg of dsRNA dissolved in 2 µl of ultrapure water using a 10 µl Hamilton microsyringe. Control insects were injected with 2 µg of β-lac (unspecific) dsRNA. Only mated females that had been fed once during the adult stage were used. Females were injected with dsRNA 21 days after the first blood meal. Fourth-instar nymphs and adult females were fed on chicken 7 days after dsRNA injection. The feeding day was considered day zero post-blood meal (pbm). Fourth-instar nymphs were collected in TRIzol reagent (Ambion) for RNA extraction to check the levels of gene knockdown by qPCR on day 11 pbm, which is the day before the expected moulting in our conditions. A fraction of the first-instar nymphs hatched from the eggs laid by dsRNA-injected females were fed on chickens 7 days after hatching. On that day, starved first-instar nymphs from different groups were collected in TRIzol

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Table 1. Sequences of primers used in this study

Primer name	Sequence ID	Forward sequence	Reverse sequence
EF-1_qPCR	RPRC015041	GATTCCACTGAACCGCCTTA	GCCGGGTTATATCCGATTTT
Tub-α_qPCR	RPRC003672	TGTGCCCAAGGATGTGAACG	CACAGTGGGTGGTTGGTAGTTGAT
CZ_qPCR	KQ034239*	TGCCCTTTACTAGACAGGGG	CTGAAATGTTTGGCCAAAGACA
ETH_qPCR	RPRC014486	GCAGAGATGAGTTCCACGAG	GTGATCCGCTGAACTGTCAC
EH_qPCR	RPRC014242	CCAAGCTTCTTTGTAGGTACAC	CTGATGCACACGCCAATCTG
CCAP_qPCR	gb|GQ888668.1|, gb|HQ264139.1|, rp_asb-51124‡	CTGCAAAAAAGGCTTTATTTTCC	TCCCATAACTTCGCTTCAGAC
OKA_qPCR	RPRC014678	TGCCGCAGACAAACGTAATT	ACAAAACCATCAAAACCGCTTC
β-lac_RNAi		TAATACGACTCACTATAGGGGAACTGGATCTCAACAG	TAATACGACTCACTATAGGGGGATCTTCACCTAGATC
CCAP_RNAi	gb|GQ888668.1|, gb|HQ264139.1| and rp_asb-51124‡	TAATACGACTCACTATAGGGAGATCGGATGAATCTATGGCCACATT	TAATACGACTCACTATAGGGAGATGACTCCTTTTCCTGATGGTC
CZ_RNAi	KQ034239*	TAATACGACTCACTATAGGGAGAAGAGGTAACCGCATGTCAGC	TAATACGACTCACTATAGGGAGAACTGGCAGCGAAGTCAACTC
ETH_RNAi	RPRC014486	TAATACGACTCACTATAGGGAGAAACTGCAAAGGCAAGCGATG	TAATACGACTCACTATAGGGAGAGCGATCTACTCTGGCTCCAC
ETH_RNAi2	RPRC014486	TAATACGACTCACTATAGGGGCTGCTGATGGTAGAAGTGG	TAATACGACTCACTATAGGGTACAGCAATGGAACTTCGCC
EH_RNAi	RPRC014242	TAATACGACTCACTATAGGGAGAGAAGCTGCTGTTGGTGATTC	TAATACGACTCACTATAGGGAGAATGGTCCAATTGAGGCAATG
OKA_RNAi	RPRC014678	TAATACGACTCACTATAGGGGAAGCGGTTTTGATGGTTTTGT	TAATACGACTCACTATAGGGGGGATTCTTTGCATAAATGGTCA
T7 promoter		ATAGAATTCTCTCTAGAAGCTTAATACGACTCACTATAGGG	ATAGAATTCTCTCTAGAAGCTTAATACGACTCACTATAGGG

Sequence ID is as per VectorBase (https://www.vectorbase.org/) or NCBI (https://www.ncbi.nlm.nih.gov/). **Rhodnius prolixus* genomic scaffold number in RproC3 assembly. The transcript is not predicted in VectorBase. ‡Transcript identifier in a transcriptomic database (Ribeiro et al., 2014).

reagent (Ambion) for RNA extraction to check the levels of gene knockdown by qPCR.

Microscopy and histology

Fourth-instar nymphs from dsβ-lac- and dsETH-injected groups were collected on day 12 pbm in Bouin's solution (saturated picric acid, 70%; formol, 25%; and acetic acid, 5%). Insects from the dsβ-lac group had already moulted to the next instar. After 24 h, insects were washed 3 times with 70% ethanol. After washing, insects were kept in 70% ethanol for 6 h. Insects were transferred to 90% ethanol for 12 h, 96% ethanol for 2 h and 96% ethanol:*n*-butyl alcohol (1:1) for 2 h. Finally, insects were transferred to *n*-butyl alcohol for 24 h. *n*-Butyl alcohol was changed 3 times every 24 h. Insects were kept in *n*-butyl alcohol until microscopy was performed.

For microscopic studies, the samples were dehydrated with ethyl alcohol of increasing strength, and butyl acetate was used as an intermediate. A synthetic medium (Histoplast) was used for inclusion, and 4–5 µm cuts were made using an SLEE Mainz CUT 4062 microtome. Histological sections were stained with Hematoxylin–Eosin (H–E) and Toluidine Blue. Microphotographs were taken with a Zeiss Primo Star microscope coupled to a Canon Power Shot G10 digital camera.

Survival, ecdysis, oviposition and eclosion measurements

Fully engorged females were individually isolated in vials and kept at 28°C and 50–60% relative humidity under a 12 h light:12 h dark photoperiod. Survival and the number of eggs laid by each female were recorded daily. The eclosion ratios were calculated as the number of hatched first-instar nymphs/number of eggs laid by the female. Nymph survival and ecdysis were also scored daily.

Statistical analysis

At least two independent experiments were performed for each treatment. Each replica included N=5–15 insects per experimental group. The data from different replicates were combined into a single graph for the design of the figures. The statistics were performed on both each independent experimental replicate and the final data containing the information from the different replicates combined. The P-values indicated in the text are from the data of the independent experiments combined. Statistical analysis and graph designs were performed using Prism 8.0 software (GraphPad Software). A one-way ANOVA was used to evaluate differences in mRNA levels on different days pbm in fourth-instar nymphs. The log-rank (Kaplan–Meier) test was used to evaluate differences in survival rate. A two-way ANOVA was used to evaluate differences in daily rates of oviposition, eclosion of eggs and ecdysis. An unpaired t-test was performed to evaluate differences between the experimental and control groups in relative mRNA levels; $P \leq 0.05$ was considered statistically significant.

RESULTS

To test the hypothesis of a functional conservation of the neuropeptides regulating ecdysis between insects with different post-development strategies, the levels of CZ, ETH and EH mRNAs were measured in fourth-instar nymphs at different time points during the moulting cycle. Transcription of the three neuropeptide precursors could be detected throughout the study period (days 4, 6, 8, 10 and 12 pbm). All the transcripts were upregulated on day 12 pbm, just before the expected ecdysis (n=7) (Fig. 1). Additionally, ETH and EH mRNA levels were also upregulated on day 10 pbm (n=5) and slightly on day 6 pbm (n=5), coinciding

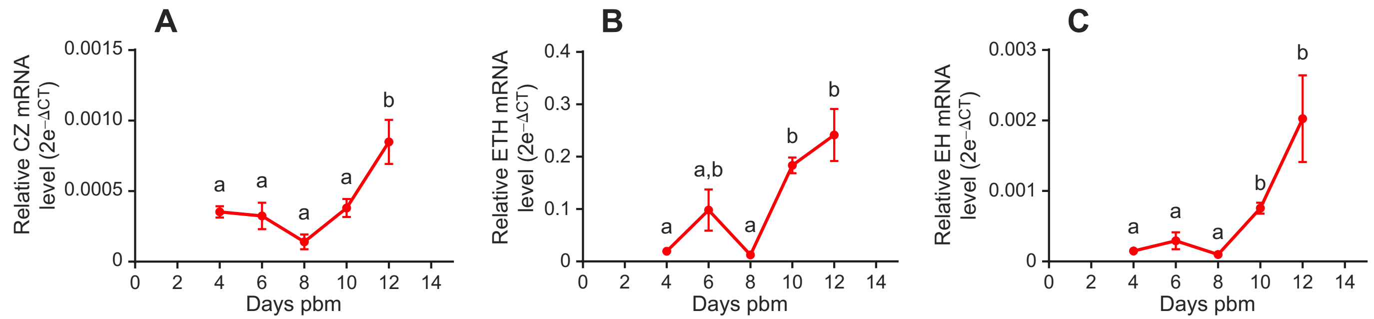

Fig. 1. mRNA levels of corazonin (CZ), ecdysis triggering hormone (ETH) and eclosion hormone (EH) in fourth-instar *Rhodnius prolixus* nymphs during the moulting cycle. (A) CZ, (B) ETH and (C) EH mRNA levels assessed by qPCR; *elongation factor 1* (*ef-1*) and *tubulin* (*tub*) were used as housekeeping genes. Data from two independent experiments were combined into a single graph and plotted as the mean±s.e.m. Note that the ecdysone peak occurs on day 6 post-blood meal (pbm) (Wulff et al., 2017), whereas ecdysis occurred on day 14±4 pbm. Different letters indicate significant differences (one-way ANOVA: P<0.05; n=5 on day 4, 6, 8 and 10 pbm and n=7 on day 12 pbm).

with the ecdysone peak in haemolymph (Wulff et al., 2017). The increase in mRNA levels on day 6 was more evident for the ETH precursor. This neuropeptide presented higher mRNA levels than CZ and EH at all time points evaluated (Fig. 1).

The role of these neuropeptides in ecdysis was then explored through RNAi-mediated gene silencing. Compared with controls on day 11 pbm, dsRNA treatments significantly reduced the transcript levels of ETH (96.44%, P<0.01, n=10), CZ (86.44%, P<0.0001, n=6) and EH (69.12%, P=0.0448, n=6) in fourth-instar nymphs (Fig. S1). None of the experimental groups showed phenotypic differences with controls until the expected ecdysis period (Fig. 2). At that time (day 14±4 pbm), most of the insects belonging to the control group (n=45) moulted to the fifth instar, as did dsCZ-injected (n=14) and dsEH-injected insects (n=25) (Fig. 2A). However, those insects treated with dsETH did not moult (P<0.0001, n=61). Instead, at the expected time of ecdysis, they stopped any movement and died 1–2 days later (Fig. 2A,B). Even though *eth*-silenced individuals did not shed the fourth-instar cuticle, they presented the fifth-instar cuticle hardened and tanned, as revealed by gently removing the upper cuticle with entomological forceps (Fig. S2). Histological sections confirmed that apolysis and partial degradation of the cuticle occurred in the arrested specimens, pointing to a defect in ecdysis itself but not in other moulting-related events (Fig. 2C,D).

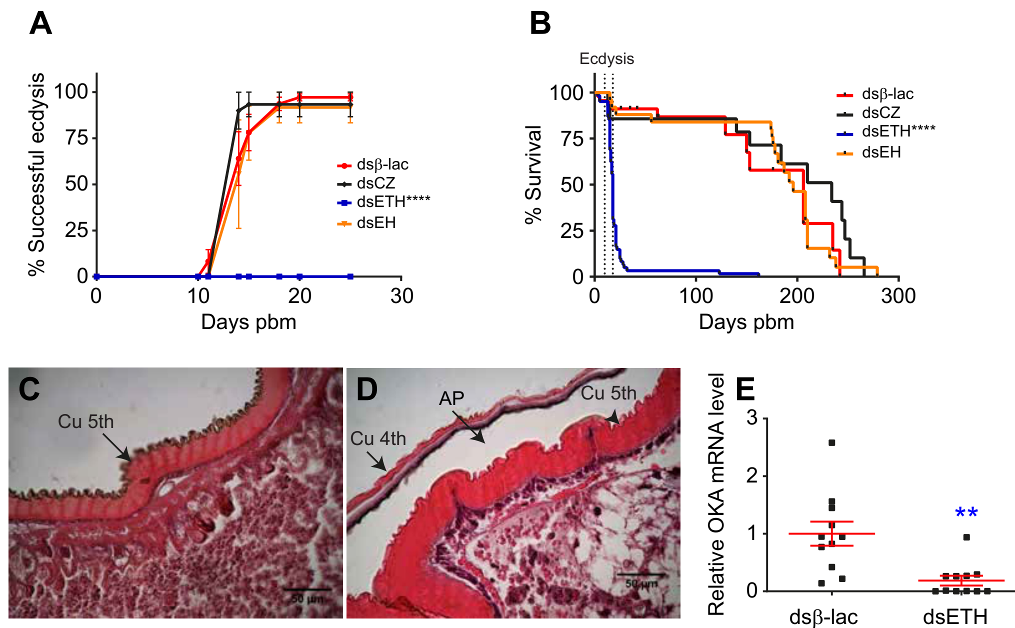

Fig. 2. Effects of *cz*, *eth* and *eh* knockdown in fourth-instar nymphs. (A) Percentage of successful ecdysis on different days pbm following treatment with dsβ-lac (n=45), dsCZ (n=14), dsETH (n=61) and dsEH (n=25). A two-way ANOVA was used to evaluate differences. (B) Fourth/fifth-instar nymph survival following dsβ-lac, dsCZ, dsETH and dsEH treatment. Data are shown as Kaplan–Meier curves (log-rank test). Dotted vertical lines indicate the period when ecdysis occurred in controls. (C) Cross-section of the cuticle from a just emerged control fifth-instar nymph. (D) Cross-section of the cuticle from a dsETH-injected fourth-instar nymph during the arrested ecdysis. Magnification ×400. AP, apolysis space; Cu 4th, old cuticle from the fourth-instar individual; Cu 5th, new cuticle from the fifth-instar individual. (E) Orcokinin A (OKA) mRNA levels in control and dsETH-treated fourth-instar nymphs 11 days pbm assessed by qPCR; *elongation factor 1* (*ef-1*) and *tubulin* (*tub*) were used as housekeeping genes (n=11 for dsβ-lac and dsETH). All $2e^{-\Delta CT}$ values were divided by the average of the $2e^{-\Delta CT}$ values in the control group. The data from three, four and five independent experiments were combined into a single graph for dsCZ, dsEH and dsETH, respectively, and plotted as the mean±s.e.m. An unpaired t-test was performed to evaluate differences between the experimental and control groups in relative mRNA levels. **P=0.001, ****P<0.0001.

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As the phenotype observed upon dsETH treatment was similar to that previously observed following silencing of *oka* (Wulff et al., 2017), we measured the mRNA levels of OKA transcript in dsETH-treated and control insects on day 11 pbm. We observed a significant reduction in the mRNA level of OKA in insects belonging to the dsETH group (Fig. 2E; P=0.001, n=11). We ruled out off-target effects by injecting dsRNA covering different regions of the ETH transcript, obtaining similar results in both lethality and phenotype during ecdysis and in the downregulation of the OKA transcript.

To test our second hypothesis, we evaluated the possible role of the neuropeptides involved in regulating the ecdysis process in Holometabola and Hemimetabola (ETH, CZ, EH, CCAP, OKA) in *R. prolixus* reproductive fitness by RNAi-mediated gene silencing. Female survival was not affected in dsETH (n=25), dsCZ (n=13), dsCCAP (n=17) or dsOKA (n=7) groups; a small but significant increase in survival (resistance to starvation, P=0.044, n=15) was observed in females injected with dsEH in comparison with the control group (n=26) (Fig. 3A,B). Egg production was not affected in any experimental group (Fig. 3C,D). However, egg hatching was dramatically reduced in dsETH-treated animals (P<0.0001, n=25; Fig. 3E,F). We carefully opened several of the unhatched eggs laid by dsETH-injected females to evaluate whether they were embryonated or showed possible defects in the development of the embryos. The eggs were opened on day 12 after being laid (the day when control nymphs had hatched). Interestingly, we observed completely formed pro-nymphs without any evident defect in development (Fig. S3), indicating that the impairment in egg hatching was not due to problems in fertilization or embryonic development. Off-target effects were ruled out by injecting dsRNA covering different regions of the ETH mRNA, achieving identical phenotypes.

The qPCR determinations revealed reduced levels of target mRNAs in the first-instar nymphs hatched from females treated with dsCZ (87.9%), dsEH (46.5%), dsCCAP (97.6%) and dsOKA (80%) (Fig. S4). We fed first-instar nymphs and evaluated the effects on survival and ecdysis. Unexpectedly, unlike fourth-instar nymphs, 83% of the first-instar nymphs silenced for *eh* expression died during the expected ecdysis period without performing ecdysis (n=58, P<0.0001). Similar results were observed for *oka*-silenced (88.9% mortality, n=18; P<0.0001) and *ccap*-silenced nymphs (86.8% mortality, n=38, P<0.0001; Fig. 4A,B). As observed in fourth-instar nymphs, most of the control (n=55) and *cz*-silenced first-instar nymphs moulted normally to the second instar, even though the survival of the latter group was reduced (n=38, P=0.0077; Fig. 4A,B). Unfed nymphs (which do not start the moulting process) did not show differences in survival rates among the different experimental groups (Fig. 4C), with the only exception being *eh*-silenced nymphs (n=145), which presented reduced

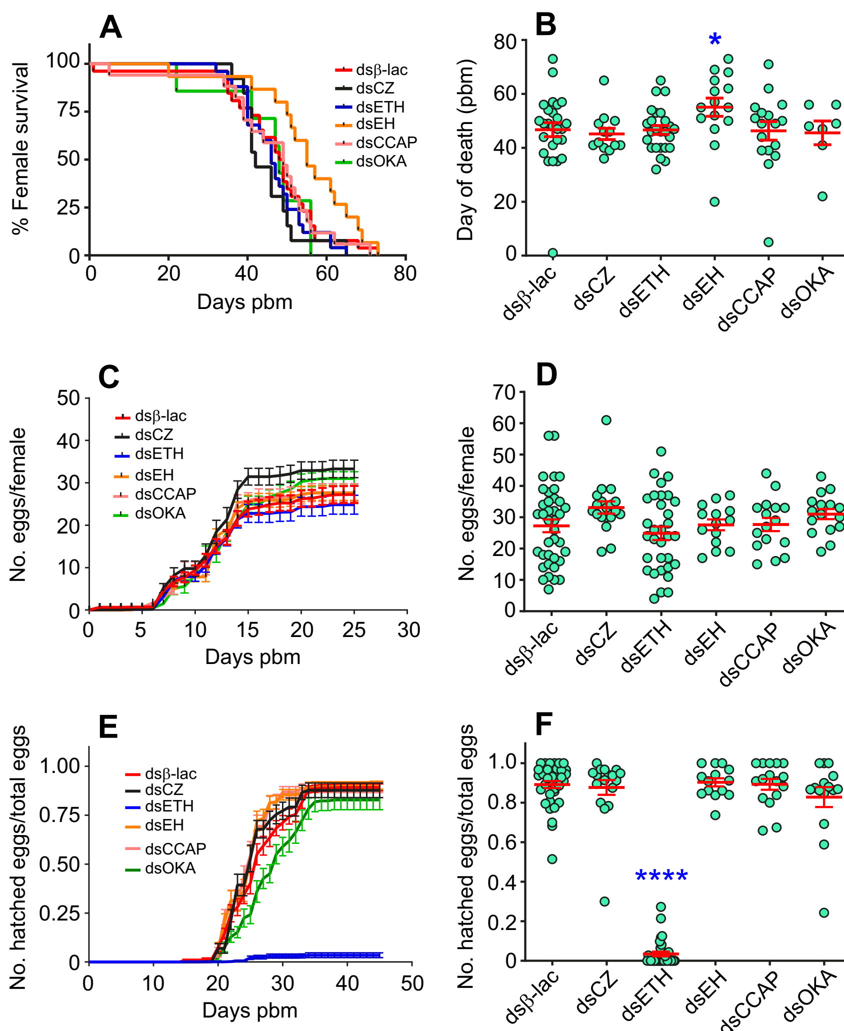

Fig. 3. Effects of *cz*, *eth*, *eh*, *ccap* and *oka* knockdown in females. (A) Kaplan–Meier curves (log-rank test) of female survival following dsβ-lac, dsCZ, dsETH, dsEH, dsCCAP and dsOKA treatment. (B) Day of death (pbm) for females injected with different dsRNAs. (C) Cumulative number of eggs laid daily by *R. prolixus* females. A two-way ANOVA was used to evaluate differences. (D) Total number of eggs laid by *R. prolixus* females by 25 days pbm. (E) Proportion of eggs hatched. A two-way ANOVA was used to evaluate differences. (F) Proportion of eggs hatched by 45 days pbm. Data are plotted as the mean ±s.e.m. *P=0.044, ****P<0.0001.

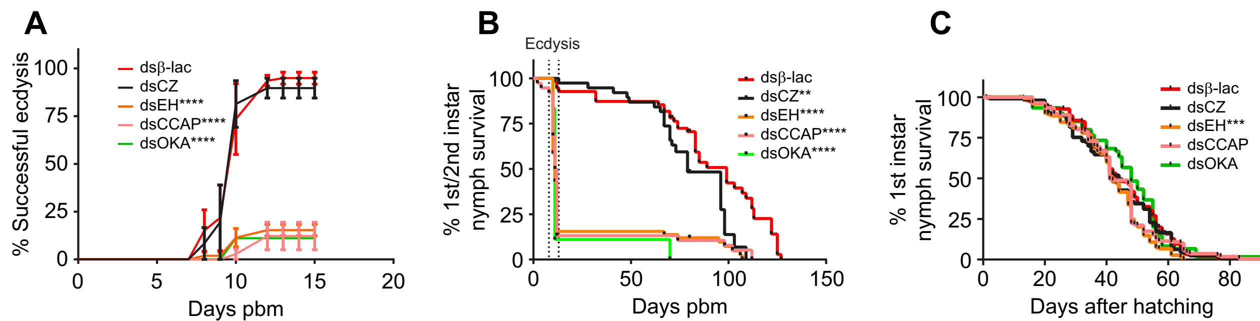

Fig. 4. Effects of *cz*, *eh*, *ccap* and *oka* knockdown in females on offspring. (A) Percentage of successful ecdysis in first-instar nymphs on different days pbm (mean±s.e.m.). A two-way ANOVA was used to evaluate differences. (B) First/second-instar nymph survival. Data are shown as Kaplan–Meier curves (log-rank test; *n*=55 for dsβ-lac, *n*=38 for dsCZ, *n*=38 for dsCCAP, *n*=18 for dsOKA and *n*=25 for dsEH). Dotted vertical lines indicate the period when ecdysis occurred in controls. (C) Unfed first-instar nymph survival. Data are shown as Kaplan–Meier curves (log-rank test; *n*=252 for dsβ-lac, *n*=203 for dsCZ, *n*=163 for dsCCAP, *n*=53 for dsOKA and *n*=145 for dsEH). ***P*=0.0077, ****P*=0.0003, *****P*<0.0001.

survival compared with controls (*n*=252, *P*=0.0003). This indicates that the reduction in survival was due to failures during ecdysis in the blood-fed nymphs. Recording the moulting rate for the dsETH group was not possible given the almost null rate of egg hatching.

DISCUSSION

The ancestral insect orders have an ametabolous post-embryonic development strategy, where the juvenile stages look like the adults, except for the presence of wings and mature genitalia (Truman and Riddiford, 1999). In ametabolous species, ecdysis and growth still occur during the adult stage (Truman and Riddiford, 2019). The evolution of insect metamorphosis boosted their success in radiation, colonizing almost all the environments on Earth. Hemimetabola emerged earlier during insect evolution, whereas the Holometabola is a monophyletic group that evolved from a hemimetabolous ancestor (Misof et al., 2014). Ecdysis is critical in insect post-embryonic development as the final stage of moulting and/or metamorphosis. The hormonal regulation of ecdysis is well studied in holometabolous models but not in ametabolous or hemimetabolous ones, leaving gaps in the understanding of insect evolution. To contribute to filling these gaps, we evaluated the neuropeptides involved in ecdysis regulation in Holometabola in the hemimetabolous insect *R. prolixus*, to test the hypothesis of a conserved role of neuropeptides that regulate ecdysis between holometabolous and hemimetabolous insects.

Even though mRNAs coding for CZ, ETH and EH were detected at every time point analysed throughout the fourth-instar moulting cycle, they were significantly upregulated 1–2 days before ecdysis. ETH mRNA levels were also upregulated on day 6 pbm, coinciding with the ecdysone peak in haemolymph (Wulff et al., 2017). The correlation between ecdysone peak and upregulation of ETH expression was also observed in Holometabola and the hemimetabolous *Schistocerca gregaria* (Lenaerts et al., 2017; Zitnan and Adams, 2012). Silencing of *eth* or *ethr* in different holometabolous insects resulted in lethality at the expected time of ecdysis (Arakane et al., 2008; Diao et al., 2016; Shi et al., 2017), as well as in *S. gregaria* (Lenaerts et al., 2017). Our results reinforce the evidence indicating that the role of ETH in the control of ecdysis is conserved across the class Insecta. Those specimens that failed to moult exhibited the duplicated cuticle phenotype characteristic of interrupted ecdysis (Wulff et al., 2017). Histological sections showed that they could perform other events of the moulting process (apolysis, digestion of the old cuticle and synthesis of the new one). However, they failed to shed the exuvia and died.

The similarity in the phenotypes obtained in fourth-instar nymphs upon gene silencing suggested that a functional relationship between ETH and OKA could exist. Indeed, we found that dsETH-treated insects had reduced levels of OKA transcripts on day 11 pbm. In our previous work, we demonstrated that the silencing of *oka* negatively affected the expression of ETH on day 6 but not on day 11 pbm (Wulff et al., 2018). Altogether, the results could suggest positive feedback between OKA and ETH to act on the regulation of ecdysis in *R. prolixus*. Interestingly, a recent survey demonstrated that the expression profiles of ecdysis-related neuropeptides correlate positively with each other in a wide range of species (Zieger et al., 2021); the results presented here confirm this tendency for *R. prolixus*. Given that the downregulation of OKA expression does not affect ecdysis in *D. melanogaster* (Silva et al., 2021) or *Blattella germanica* (Ons et al., 2015), positive feedback seems not to be conserved among insects. Further experiments with species from different orders will be interesting to infer the role of OKA signalling throughout the evolution of ecdysis regulation.

Neuropeptides regulating ecdysis are expressed in insects during the adult stage when ecdysis does not occur. This striking fact led to the second hypothesis of our study: the neuropeptides regulating ecdysis play a role in female reproduction. Indeed, the gonadotropic role of ETH has recently been demonstrated for dipteran species (Areiza et al., 2014; Meiselman et al., 2017). ETH signalling deficit in *D. melanogaster* reduced egg production, ovary size and juvenile hormone levels (Meiselman et al., 2018). In parallel, *oka* silencing affects vitellogenesis and ovary maturation in the cockroach *B. germanica* (Ons et al., 2015) and egg production in *D. melanogaster* (Silva et al., 2021). However, we did not observe differences in egg production either in *eth*-silenced insects or in the animals expressing diminished levels of EH, OKA, CZ or CCAP, pointing to a rejection of the second hypothesis and suggesting a different endocrine regulation of ovary maturation in *R. prolixus*. The allatotropic role of ETH reported in holometabolous females (Areiza et al., 2014; Meiselman et al., 2017; Shi et al., 2019) may not be conserved in *R. prolixus*, given that failure in ovary development was demonstrated in this species when a deficiency in juvenile hormone signalling was induced (Villalobos-Sambucaro et al., 2015). Interestingly, one of the adaptive advantages of complete metamorphosis is that juveniles and adults occupy different environments, avoiding competition for resources. We speculate that because of this fundamental difference in physiology between juveniles and adults, the same neuropeptides could also be adapted to play radically different roles in the two stages. As the

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lifestyle and physiology in hemimetabolous insects, such as *R. prolixus*, is similar in nymphs and adults, this could be a constraint on adapting hormones to different functions through their life cycle.

The hatching rate of the eggs was significantly affected in dsETH-treated females, although completely developed nymphs were observed in the eggs upon manual dissection, indicating that embryo development proceeded correctly until the late stages of embryogenesis. In hemipterans and other hemimetabolous organisms, shedding of the embryonic cuticle occurs during egg hatching (Truman and Riddiford, 2019). This process is called pronymphal ecdysis, which requires a coordinated activity. The role of neuropeptides in regulating pronymphal ecdysis has not been studied to date, even though a significant upregulation of ecdysis-related neuropeptides occurs during egg hatching in Hexapoda species with direct development (Zieger et al., 2021). Our results indicate that the insects died inside the egg during the pronymphal ecdysis when *eth* was silenced, suggesting that ETH is also involved in regulating this process.

The role of CZ in insects is less well clarified when compared with that of ETH. Different reports suggest the involvement of CZ in the regulation of feeding, nutrient sensing, nutritional and oxidative stress, ethanol-related behaviour and metabolism, sperm transfer and copulation, fecundity and the gregarious-associated pigmentation of the cuticle in locusts (reviewed by Zandawala et al., 2018). In Lepidoptera, CZ initiates the ecdysis sequence by stimulating the release of ETH from Inka cells (Kim et al., 2004), even though this role was not observed in *D.* melanogaster (Zitnan and Adams, 2012). Given that in *T. castaneum* and other coleopterans the CZ hormonal system seems to be absent, it was proposed that the effect of CZ on ecdysis initiation would be specific to Lepidoptera (Arakane et al., 2008). However, CZ has been implicated in the larval–pupal transition in *Bactrocera dorsalis* (Diptera: Tephritidae) via the regulation of ETH expression in Inka cells (Hou et al., 2017). The results presented here indicate that CZ function is not required for ecdysis in *R. prolixus*. Likewise, no significant effects on ecdysis were observed when the CZ receptor gene was silenced in *R. prolixus* fourth-instar nymphs (Hamoudi et al., 2016).

Injection of EH into lepidopteran species (*M. sexta* and *B. mori*) caused premature ecdysis (Copenhaver and Truman, 1982; Fugo et al., 1983), whereas *eh* silencing in *T. castaneum* suppressed ecdysis (Arakane et al., 2008). These results suggest that EH is necessary and sufficient to induce ecdysis in insects. However, in *D. melanogaster*, EH ablation in ventral medial (VM) neurons caused minor defects in larval ecdysis (Clark, 2004; McNabb et al., 1997). More recently, Krüger et al. (2015) isolated null *eh* mutants that died at the time of ecdysis. This apparent contradiction was recently solved with the identification of a broader expression of EH, previously supposed to be restricted to VM neurons (Scott et al., 2020). The authors identified EH expression in neurons other than the VM at the adult stage and in somatic tissues, mainly tracheae, in all *D. melanogaster* instars. When EH expression was suppressed in larvae, 100% of the animals died with signals of failure in cuticle shedding. By contrast, nearly 50% of the animals were able to moult to the adult stage when EH expression was restricted to the period of pupal development (Scott et al., 2020). Likewise, we obtained 83% lethality at the expected ecdysis time when *eh* was silenced in *R. prolixus* during the first instar, but no observable phenotype when fourth-instar nymphs were injected with dsEH. Zieger et al. (2021) reported a higher expression of ecdysis-related neuropeptides in the early instar of direct development of Hexapoda compared with the late juvenile stage. Altogether, the recent evidence (Scott et al., 2020; Zieger et al., 2021) suggests that younger instars could be more sensitive to EH signalling than later stages. The accepted model of neuroendocrine regulation of ecdysis in Holometabola proposes that the primary regulator of EH secretion is ETH (Zitnan and Adams, 2012). However, recent results revealed EH-expressing cells that do not express ETH receptors, suggesting an alternative regulatory mechanism (Scott et al., 2020). The phenotypes we obtained upon *eth* and *eh* silencing were different in fourth-instar nymphs, suggesting that this could also be the case in *R. prolixus*. Finally, in agreement with previous observations in *R. prolixus* fourth-instar nymphs (Lee et al., 2013), the silencing of *ccap* transcription led to ecdysis deficits in a significant percentage of first-instar animals.

Despite the enormous differences in post-embryonic development between Holometabola and Hemimetabola, our results suggest high functional conservation in the neuropeptides that regulate ecdysis. The only exception found to date is OKA, which seems to be essential for successful ecdysis in *R. prolixus* (present results; Wulff et al., 2017, 2018) but not in *D. melanogaster* (Silva et al., 2021). However, the involvement of OKA in ecdysis could be restricted to Hemiptera or Heteroptera, given that this phenotype was not observed in *B. germanica* (Ons et al., 2015). Differently from holometabolous species (Meiselman et al., 2017; Meiselman et al., 2018; Ons et al., 2015), neither *eth* nor *oka* silencing affected ovary development or oviposition in *R. prolixus*, but *eth* knockdown prevented egg hatching, probably by affecting pro-nymph ecdysis. Further studies on Ametabola and other Hemimetabola orders, particularly those closely related to Holometabola in the phylogeny, will be interesting to clarify the evolution of hormonal regulation of ecdysis and achieve a better understanding of how insects evolved and adapted to occupy almost every environment on Earth.

The information provided here is of interest both for evolutionary studies on the regulation of ecdysis in insects and for research into new targets for pest insect management strategies that would be safe for vertebrates and other non-ecdysozoans. In addition to its relevance for entomological experimentation, RNAi has been proposed as a tool for new-generation insecticides that would be species specific and environmentally sustainable (Christiaens et al., 2020; Vogel et al., 2019). The phenotypes observed in survival and reproduction upon *eth* silencing indicate that it could be an excellent target for controlling triatomine vectors.

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Competing interests
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Author contributions
Conceptualization: M.S., M.V., S.O.; Validation: M.S., M.V., M.G.A.; Formal analysis: M.S., J.P.W.; Investigation: M.S., M.V., M.G.A., J.P.W., M.d.S., P.M.T., M.T.A.; Resources: S.O.; Writing - original draft: M.S., S.O.; Writing - review & editing: M.S., M.V., M.G.A., J.P.W., S.O., M.T.A.; Visualization: M.S.; Supervision: M.S., S.O.; Project administration: M.S., S.O.; Funding acquisition: M.S., S.O.

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